Non-invasive assessment of leaflet deformation and mechanical properties in heart valve tissue engineering

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voor Angelique
&
mijn pa en ma

Contents

Summary

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Tissue-engineered heart valves are a promising alternative for mechanical and bioprosthetic heart valve replacements. Despite their relative success, current valvular implants are made of non-living material and, therefore, do not have the ability to grow, adapt or remodel in response to a change in the valvular environment. This especially has a negative effect on the treatment of congenital valve defects in adolescent and pediatric patients. Heart valve tissue engineering seeks to overcome these limitations by creating functional, autologous and living heart valves. The in-vitro formation of aortic heart valves has proven to be a significant engineering challenge. Although the mechanical properties of tissue-engineered valves may be sufficient, they may need improvement. When compared with native aortic valves, cultured valves are relatively stiff and less anisotropic in the physiological strain range.

Mechanical stimulation of the developing tissue in a bioreactor system is reported to enhance tissue formation and quality, and is widely used in cardiovascular tissue engineering. More particular, inducing strains in the cultured tissue by load application appears to be an important enhancer of tissue development. However, optimal conditioning protocols in heart valve tissue engineering have not been identified, yet. Documented bioreactor systems have been unable to sufficiently control the load to induce predefined or controlled deformations to the cultured heart valves. In addition, the mechanical properties of the engineered valves have been generally examined by sacrificing the heart valves at the end-stage of tissue culture to perform traditional single or multi-axial tensile experiments. To investigate the mechanical behavior of the heart valves during culture as a non-invasive quality check, it is also desired to assess the mechanical properties non-destructively in real-time.

During tissue culture the mechanical properties of the heart valves change with time. To subject the valve to a predefined deformation pattern via the application of a pressure difference over the valve, a feedback controlled bioreactor system is needed. The objective of this thesis is to develop a bioreactor system in which induced heart valve leaflet deformations are measured and controlled and resulting mechanical properties are assessed during culture, non-invasively and non-destructively.

For this purpose, an inverse experimental-numerical approach was developed to measure volumetric and local heart valve leaflet deformations during culture. Volumetric deformation was defined as the amount of fluid displaced by the deformed heart valve leaflets in a stented configuration in response to the valvular pressure difference. This volume was measured non-invasively using a flow sensor. A computational model was employed to relate volumetric deformation to local tissue strains in various regions of the leaflets; e.g. belly and commissures.

Consecutively, the inverse experimental-numerical approach was further developed and applied to assess the mechanical properties of the tissue-engineered

heart valves. A range of increasing pressure differences was applied and the corresponding induced volumetric deformations of the engineered heart valve leaflets were measured during culture. The correlation between the pressure difference and deformation data served as input for the estimation of mechanical properties using the computational model. To validate the method, six heart valves were cultured, and the estimated mechanical properties were in good agreement with uniaxial tensile test data. In addition, the diastolic functionality of the heart valve leaflets was assessed in the bioreactor by studying the deformation and leakage of the cultured heart valves under physiological aortic diastolic pressure differences.

As a second step towards a controlled bioreactor system, the above described deformation assessment method was extended by addition of a feedback control mechanism. The resulting technique enabled both measurement and control of the heart valve deformation in real-time. Functionality of this approach was demonstrated in two tissue engineering experiments in which a total of eight heart valves was cultured by application of two different deformation protocols. Results indicated a good correlation between the measured and the prescribed deformation values in both experiments. In addition, the cultured heart valves showed mechanical properties in the range of previous tissue engineering studies. However, no significant differences in mechanical properties were found between the valves cultured by the dissimilar protocols.

Tissue analyses provide a broader understanding of the development of engineered heart valves during culture. In addition to the mechanical and functional evaluations, analyzing tissue composition on a microscopic and macroscopic level may gain further insight into the relation between mechanical conditioning and tissue development. Consequently, the tissue of the engineered heart valves was analyzed qualitatively; macroscopic appearance and histology, and quantitatively; biochemical assays. The 14 heart valves cultured in four independent experiments all showed a dense, homogeneous tissue with a smooth surface. Tissue composition was comparable to previously performed cardiovascular tissue engineering studies. Collagen type I and III were demonstrated throughout the tissue, as well as striated structure fragments of elastin, both of which are the main structural matrix components of natural heart valves.

The next step towards preclinical application of the controlled bioreactor system was to optimize the system to culture tissue-engineered heart valves suitable for minimal invasive implantation. First, implantable stented heart valves were successfully cultured in the bioreactor system. Thereafter, the bioreactor system was employed to create ovine tissue-engineered heart valves suitable for animal studies. Complete realization of the bioreactor functionality was demonstrated for one of the three cultured ovine heart valves. The presence of leakage along the heart valves was still an issue in the other valves and needs further investigation.

Finally, a further advanced bioreactor design was addressed, which extends the revised bioreactor with systolic flow capabilities. This bioreactor system would allow the independent application of both controlled strain-based and physiological flow-based loads to tissue-engineered heart valves.

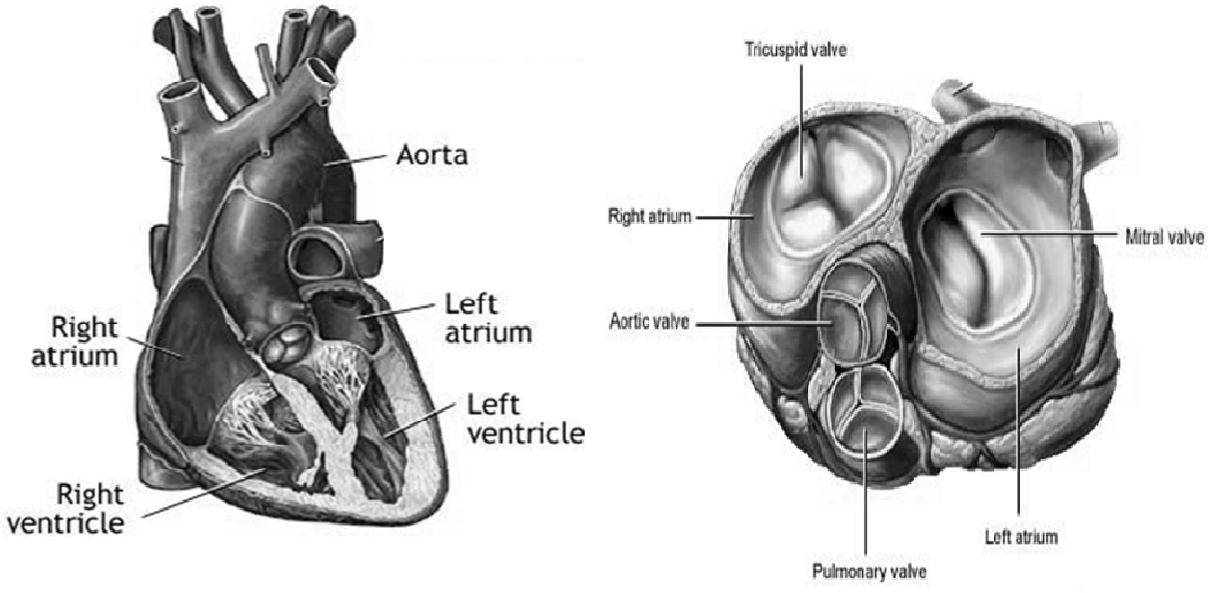
Chapter 1

Introduction

1.1 The aortic valve

1.1.1 Anatomy

The heart (Fig. 1.1a) is one of the most important organs in the human body. It works as a pump that transports blood to the organs, tissues, and cells of the body. Blood delivers oxygen and nutrients to every cell and removes the carbon dioxide and waste products excreted by those cells. The heart has four heart valves; the aortic valve, the pulmonary valve, the mitral valve and the tricuspid valve (Fig. 1.1b).



a. **b.**

Fig. 1.1: *Schematics of the human heart and its four heart valves. (a) Cross-section of the heart, anterior view, with the tricuspid valve situated between the right atrium and the right ventricle, and the mitral valve positioned between the left atrium and the left ventricle. In addition, the pulmonary valve is located between the right ventricle and the pulmonary artery while the aortic valve controls the blood flow between the left ventricle and the aorta. (b) Cross-section of the heart, top view, showing the four heart valves in closed configuration. (adapted from www.urac.org)*

The aortic valve is situated between the left ventricle and the aorta, and is composed of three strong, thin leaflets which are semilunar in shape. The leaflets are passive structures of soft tissue, each with a thickened area in the middle, called the nodules of Arantius. The part of the leaflet surface in contact with the other leaflets in closed configuration is called the coaptation area. At the fixed edge the leaflets are attached to the aortic root, and the attachment points of two adjacent leaflets to the aortic root are called the commissures. The leaflets are attached to and supported by a ring of tough fibrous tissue called the annulus. The annulus helps to provide support and maintain the proper shape of the valve. Directly downstream of the leaflets, the

aorta bulges out into the aortic sinuses. The coronary arteries, springing from two of the three sinuses, provide blood supply to the heart muscle itself.

1.1.2 Function

There are two main phases in the cardiac cycle; the systolic and the diastolic phase (Fig. 1.2). During the systolic phase, the left ventricle contracts which results in a rise in pressure. When the pressure in the left ventricle exceeds the pressure in the aorta, the aortic valve opens and blood flows from the left ventricle into the aorta. During the diastolic phase, the left ventricle relaxes and pressure drops. The aortic valve closes and prevents the blood from flowing back into the left ventricle. Passive filling and contraction of the left atrium refills the left ventricle before the next systolic phase. During the cardiac cycle, the aortic heart valve has to withstand the highest load during the cardiac cycle of all heart valves. The pressure difference over the aortic valve during the diastolic phase is around 80 mmHg (~11 kPa) for a healthy adult person in rest.

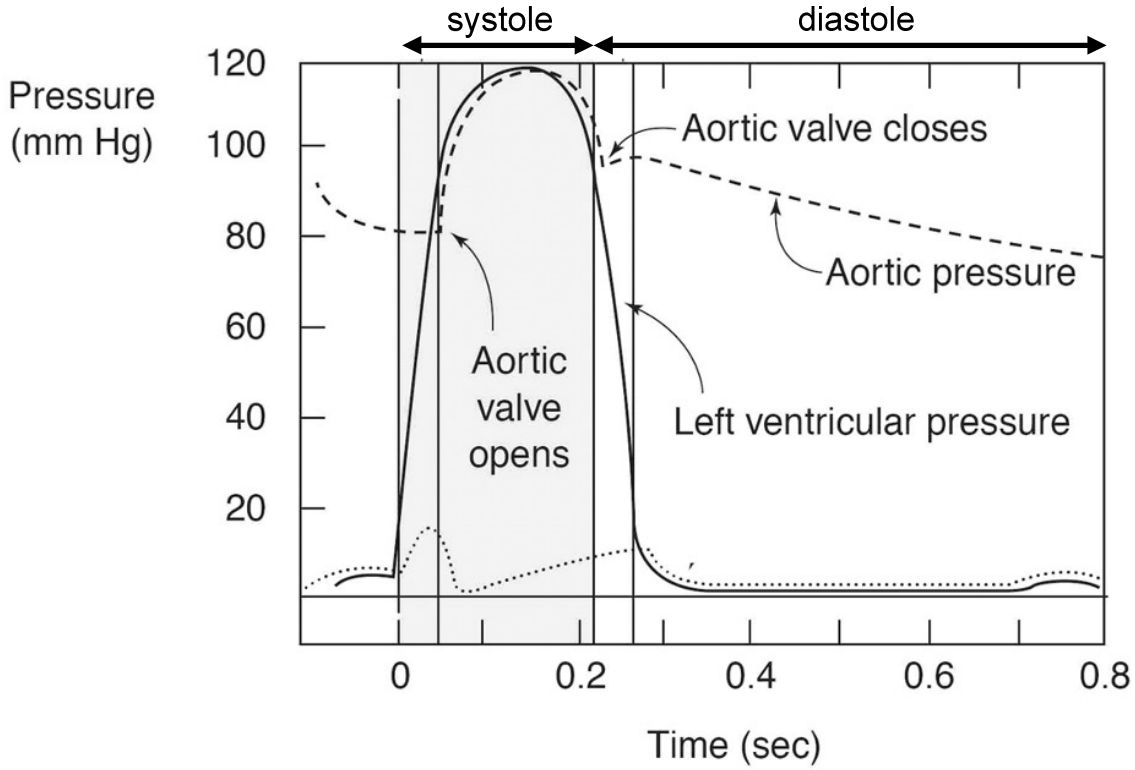


Fig. 1.2: *Aortic and left ventricular pressures during the systolic and diastolic phase of the cardiac cycle. The left ventricle contracts during systole and as a result the left ventricular pressure raises until the aortic valve opens. Blood flows out of the ventricle into the aorta and the left ventricular pressure decreases. At the end of systole, the left ventricle has emptied and the aortic valve closes. The pressure in the left ventricle drops further and diastole starts. During diastole, the left ventricle relaxes and starts to fill with blood again, indicating the start of a new cardiac cycle (adapted from Guyton and Hall, 2006).*

1.2 Tissue structure and composition

In the aortic heart valve two main cell types are present; valvular interstitial cells and endothelial cells, where the valvular interstitial cells are the most prevalent type in the valve. Within the valve leaflet, a heterogenic population of interstitial cells resides, made up of fibroblasts, smooth muscle cells, and myofibroblasts, which have characteristics of both fibroblasts and smooth muscle cells. Interstitial cells are responsible for the synthesis and remodeling of the extracellular matrix (ECM) (Mulholland & Gotlieb, 1996; Taylor et al., 2003). In contrast, the endothelial cells form a single layer of cells lining the heart valve leaflet surface. They provide a protective, non-thrombogenic layer, and play an important role in many physiological functions, including leaflet surface permeability.

Transversely through the aortic valve leaflets, from aorta to ventricle, three distinct layers are distinguished in the aortic valve; the fibrosa, spongiosa and ventricularis (Fig. 1.3). The movement of the aortic valve leaflets during the cardiac cycle depends on the contribution of these three layers. Due to the mobility and ability to compress and shear, the three-layered structure assures low flexural rigidity, essential to allow proper valve opening and non-obstructed passage of blood into the aorta. In addition, it guarantees high tensile strength necessary to withstand transvalvular pressures (Sacks et al., 1998; Langdon et al., 1999).

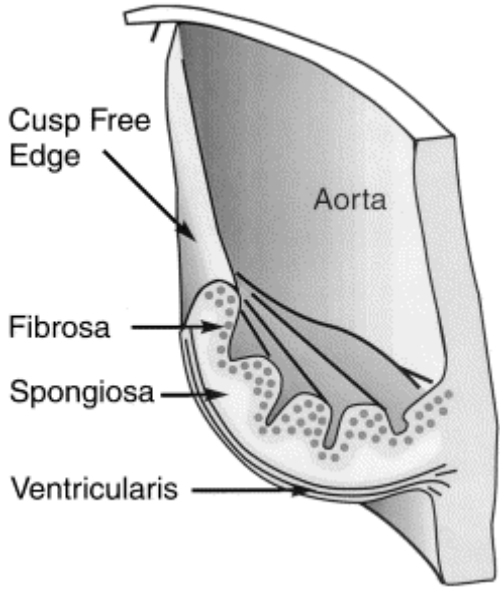


Fig. 1.3: *Schematic cross-section of an aortic valve leaflet; the three distinct layers, fibrosa, spongiosa and ventricularis are shown (adapted from Mulholland and Gotlieb, 1996).*

The fibrosa is predominantly composed of a dense network of collagen fibers, macroscopically crimped and organized into large bundles which are predominantly oriented in circumferential direction. The directionality of the collagen network results

in anisotropic mechanical properties, making the leaflets considerably stiffer in the circumferential direction than the radial. The collagen network in the fibrosa is the strongest and stiffest portion of the leaflet (Sacks et al., 1997; Schoen & Levy, 1999). Therefore, the fibrosa is considered to be the main load bearing layer of the leaflet, and prevents excessive stretching (Thubrikar et al., 1986). The spongiosa is composed of loosely arranged collagen and has a high content of glycosaminoglycans (GAGs). It absorbs water and forms a shock and shear absorbing layer due to the high viscosity and the low compressibility of the GAGs. The ventricularis mainly consists of collagen with radially aligned elastin fibers. Elastin is responsible for generating a preload in the ventricularis. This preload restores the contracted leaflet to its original configuration between loading cycles (Vesely et al., 1998).

Collagen and elastin fibers are arranged perpendicularly to each other in the valve leaflet layers, and cooperate during valve motion. In the first part of the diastolic phase, heart valve leaflets are strained and elastin fibers bear a fraction of the load while collagen fibers extend and uncrimp. Later in diastole, collagen fibers are unfolded and completely take over the load (Schoen et al., 1997). In systole, contraction of the elastin fibers leads to crimping of the collagen fibers, after which the preloaded leaflet configuration is restored.

1.3 Heart valve disease and replacement

Heart valve diseases occur in all four heart valves, but most frequently affect the aortic valve being responsible for high mortality rates (Schoen & Levy, 1999). There are two main processes that can affect the aortic valve. First, aortic stenosis is a disorder in which the valve opening becomes narrowed, damaged or scarred (stiff), obstructing the blood flow out of the ventricles. The heart muscle has to contract with increased force to be able to pump the blood through such a stenotic valve. Common causes of aortic stenosis include congenital diseases, rheumatic fever and degenerative calcification. Second, aortic insufficiency, also called aortic regurgitation, is a condition in which the aortic valve is leaking, causing blood to flow in reverse direction during ventricular diastole, so from the aorta into the left ventricle. It can be present due to abnormalities of either the aortic valve or the aortic root. The most common causes of aortic regurgitation include dilation of the aorta, rheumatic fever, and infective endocarditis. Aortic stenosis and aortic insufficiency frequently co-exist. Either situation can result in an enlarged left ventricle and eventually heart failure (Chikwe et al., 2006).

The most common treatment for a malfunctioning aortic valve due to stenosis or insufficiency is replacement of the valve. In 2006, almost 300,000 cardiac valves were replaced worldwide by either a mechanical valve or bioprosthetic valve (Friedewald et al., 2007). Mechanical valves are generally composed of carbon, or occasionally metal alloys, and are designed to outlast the patient. Although mechanical valves are long-lasting, readily available and generally only one operation is needed per patient's lifetime, there is an increased risk of thrombus forming with mechanical valves. As a

result, mechanical valve recipients must take anti-coagulant drugs for the rest of their lives making the patient more susceptible to often dangerous bleedings (Vongpatanasin et al., 1996).

Bioprosthetic heart valves are usually made from human or animal tissues. They can be divided into autografts; the patient's own pulmonary valve, homografts; preserved donor aortic heart valves, and xenografts; treated porcine or bovine valvular or pericardial tissue. Bioprosthetic valves wear out faster than mechanical valves; some 10-20% of homograft and 30% of xenograft valve replacements fail within 10-15 years after implantation and require replacement (Hammermeister et al., 2000; O'Brien et al., 2001). This process is age-dependent; bioprosthetic valves last longer in the elderly population because the haemodynamic demands on the valve are less. However, bioprosthetic valves are less thrombogenic than mechanical valves and anticoagulation therapy is not required. In addition, their haemodynamic performance is relatively good. Availability of these heart valves varies depending on the type of valve. Xenografts are abundantly available, but homografts are more difficult to obtain. Of all bioprosthetic valves, a homograft is technically more demanding to implant, but is preferred to a xenograft due to its longer lifespan and reduced incidence of prosthetic endocarditis (O'Brien et al., 2001).

Current heart valve replacements have shown their value, but are still associated with disadvantages that limit their success. The most important shortcoming of all prosthetic heart valves is that they originally consist of non-living material and, therefore, do not have the ability to grow, adapt or respond to a change in the tissue's environment. This especially has a negative effect on the treatment of congenital defects in adolescent and pediatric patients. Since currently available valve prostheses cannot grow along with these patients, repeated replacement operations are necessary to replace their valve prostheses with a larger one, leading to exponentially increasing morbidity and mortality rates (Mayer, 1995).

1.4 Heart valve tissue engineering

Tissue engineering is an interdisciplinary field which applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function (Langer & Vacanti, 1993; Winterswijk & Nout, 2007). To overcome the limitations of the conventional heart valve prostheses, heart valve tissue engineering is focused on the in-vitro fabrication of functional, living heart valve implants. The generation of autologous heart valves, as investigated in this thesis, is based on three main stages.

First, donor cells are harvested from the patient and expanded in-vitro. The two cell types often used for tissue engineering are myofibroblasts and endothelial cells (Schnell et al., 2001; Mol et al., 2006; Schmidt et al., 2007a; Boerboom et al., 2008). Myofibroblasts synthesize extracellular matrix elements, and endothelial cells form a monolayer having non-thrombogenic and mechanotransducing properties. Second, a heart valve shaped scaffold or cell-carrier is created, which is porous and

biodegradable. The autologous cells are seeded onto this scaffold, which provides a temporary support matrix until the extracellular matrix produced by the seeded cells has sufficient mechanical strength of its own. Third, the seeded scaffold is positioned in a bioreactor system in which biological as well as mechanical stimuli are applied to the cells to promote cell proliferation and production of extracellular matrix components. Thus, tissue formation is initiated and eventually results, after a couple of weeks, in a completely autologous, new, functional and living heart valve which can be implanted into the patient (Fig. 1.4).

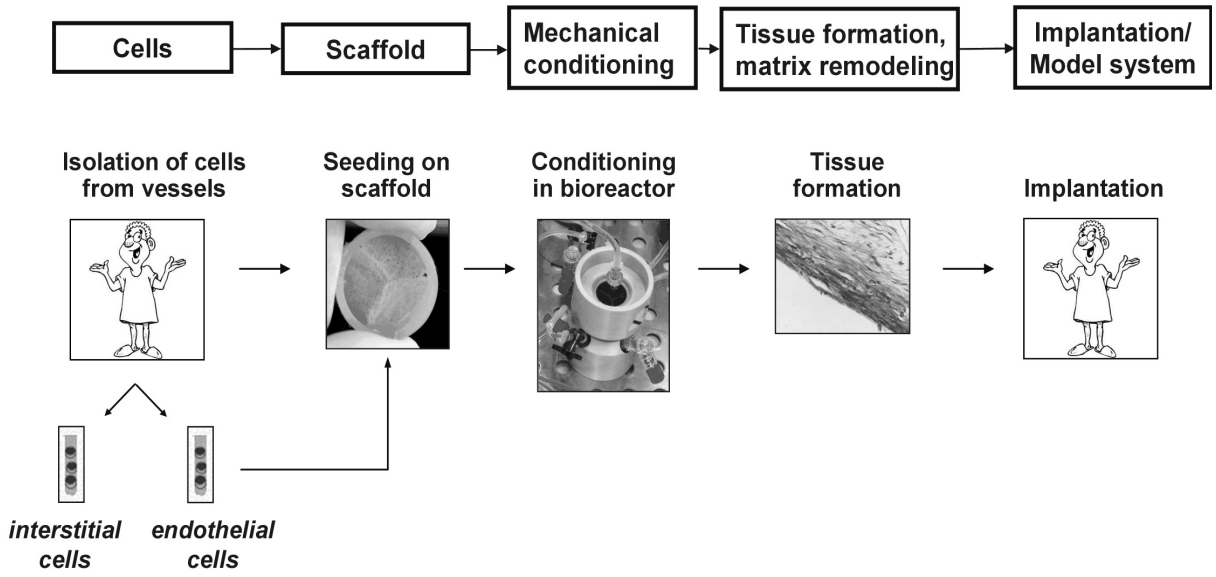


Fig. 1.4: *Paradigm of heart valve tissue engineering. Cells are harvested from the patient, seeded onto a scaffold, and positioned in a bioreactor to apply biological and mechanical stimuli. Tissue formation and remodeling is enhanced and after several weeks of culturing the autologous heart valve can be implanted into the patient.*

So far, tissue engineering of heart valves has been relatively successful. Heart valves were cultured and implanted in the pulmonary position into sheep, showing good functionality and native-like tissue composition after five months (Hoerstrup et al., 2000b). In addition, engineered human heart valves could withstand physiological systemic pressures for up to four hours in an in-vitro set-up (Mol et al., 2006). Although mechanical properties of these valves seem to be sufficient, the quality of the tissue still needs improvement to ensure proper durability and functionality of the replacements prior to implantation. When native (aortic) valves are considered as a benchmark, cultured valves are too stiff at infinitesimal strains and are less anisotropic. This mainly results in a high flexural rigidity of the valve leaflets and, thus, in less optimal valve opening. Therefore, the main challenge in heart valve tissue engineering is to optimize the mechanical characteristics of engineered valves towards native benchmarks.

Different approaches have been used to optimize the mechanical behavior of cultured heart valves. These approaches focus on the in-vitro culture and origin of

cells, the choice of the scaffold and the stimulation of tissue growth and development. First, the in-vitro culture methods and the origin of the cells are important factors which have a large effect on the outcome of a tissue engineering experiment. The technology of in-vitro cell culturing has made significant progress and allows control of tissue growth conditions. However, the ability of the cells to produce sufficient amounts of extracellular matrix proteins, in particular collagen and elastin, and to have the appropriate phenotype varies between patients and cell sources (Schmidt et al., 2007b). Myofibroblasts originating from the saphenous vein have been shown to be a suitable cell source (Schnell et al., 2001) and were used in many cardiovascular tissue engineering studies (Hoerstrup et al., 2000b; Jockenhoevel et al., 2002; Mol et al., 2005a; Stock et al., 2006; Boerboom et al., 2008; Stekelenburg et al., 2009).

Second, the choice of the scaffold material also has a significant effect on the final result of the tissue engineering process. The scaffold can be either based on biological or synthetic materials, but it must satisfy a number of requirements. These include biocompatibility, reproducibility, biodegradability, ability to be processed to complex shapes, ability to support cell growth and proliferation, and appropriate mechanical properties (Gunatillake & Adhikari, 2003). In addition, scaffolds need to be highly porous and need to have adequate pore sizes to ensure cellular ingrowth throughout the whole scaffold. Besides these fundamental requirements, the rate of scaffold degradation is a property that varies among different types of scaffold material. For example, non-woven polyglycolic acid (PGA) coated with poly-4-hydroxybutyrate (P4HB) has shown to lose its mechanical integrity within weeks in culture (Hoerstrup et al., 2002). The slowly degrading polyester poly-ε-caprolactone (PCL), however, retains its mechanical strength for months (Hoglund et al., 2007). With regard to the degradation rate of the scaffold material, two different tissue engineering strategies can be followed. The use of a stronger and slower degrading scaffold ensures sufficient mechanical strength of the engineered construct prior to implantation, but may induce a foreign body response due to the remaining scaffold. On the other hand, a faster degrading scaffold will be degraded at the time of implantation leading to a fully autologous implant. This approach requires that the in-vitro cultured tissue has sufficient strength at the time of implantation.

Third, mechanical conditioning of tissue-engineered heart valves largely influences tissue formation and quality. Research has demonstrated that dynamic mechanical stimulation of the developing tissue enhances growth and remodeling of functional tissue-engineered structures (Kim et al., 1999; Niklasson et al., 1999, 2001; Seliktar et al., 2001; Mitchell et al., 2001; Stegemann et al., 2003; Mol et al., 2003; Butcher et al., 2006; Ferdous et al., 2008; Boerboom et al., 2008; Stekelenburg et al., 2009; Rubbens et al., 2009). Such a dynamic mechanical environment is simulated in a bioreactor. In this study, a bioreactor is defined as a system that mimics a sterile environment for the creation, mechanical conditioning, and testing of cells, tissues, precursors, support structures, and organs in-vitro (Barron et al., 2003). In this thesis, myofibroblasts originating from the saphenous vein were chosen as a cell source and were seeded onto a fast degrading PGA-P4HB scaffold. Subsequently, a bioreactor system was employed to mechanically stimulate the developing heart valve tissue.

Table 1.1: *Overview of the bioreactor systems used in cardiovascular tissue engineering. An '?' indicates that it was not clear whether the applied load was measured or not. An '-' indicates the absence of a certain measurement or control, while the '+' is indicative for the presence and positive effect of the applied loads on the mechanical properties or tissue organization of the TE construct.*

Loading type	TE construct	Bioreactor	Applied load		Tissue response		Control	Effect on TE construct		References
			Type	Assessed	Type	Assessed		Mech. properties	Tissue organization	
Strain-based	strip	Flexercell or similar	force	-	strain	predefined & measured	-	+	+	Kim et al. (1999); Rubbens et al. (2008); Ferdous et al. (2008); Boerboom et al. (2008); Butcher et al. (2006)
Strain-based	strip	Uniaxial stretching device	force	-	strain	predefined	-	+	+	Mol et al. (2003)
Strain-based	square	Biaxial stretcher	force	measured	strain	predefined	-	-	+	Mitchell et al. (2001)
Strain-based	vascular graft	Pulsatile pressure bioreactor	pressure	measured	strain	predefined	-	+	+	Niklasson et al. (1999, 2001); Seliktar et al. (2001); Stegemann et al. (2003); Stekelenburg et al. (2008)
Strain-based	heart valve	Diastolic strain bioreactor	pressure	measured	strain	assessed afterwards	-	+	+	Mol et al. (2005, 2006)
Strain-based	heart valve	Diastolic strain bioreactor	pressure	-	strain	predefined & measured	-	+	+	Syedain et al. (2008); Syedain and Tranquillo (2009)
Flow-based	specimen, discs	Pulsatile flow chamber (Hoerstrup et al., 2000a)	flow	measured ?	strain, shear	estimated (shear)	-	-	-	Sodian et al. (2001); Yang et al. (2006)
Flow-based	vascular graft, heart valve	Pulsatile flow chamber (Hoerstrup et al., 2000a)	flow	measured ?	strain, shear	-	-	-	+	Sodian et al. (2002); Rabkin et al. (2002); Flanagan et al. (2007)
Flow-based	vascular graft	Laminar or perfusion flow bioreactor	flow	-	shear	-	-	-	+	Jockenhoevel et al. (2002); Aper et al. (2006); Cebotari et al. (2005)
Flow-based	vascular graft	Pulsatile flow bioreactor	flow	measured	strain, shear	-	-	-	+	Williams et al. (2004)
Flow-based	heart valve	Pulsatile flow bioreactor	flow	measured ?	strain, shear	-	-	-	-	Zeltinger et al. (2001)
Physiological	specimen	Flex-stretch-flow bioreactor	force, flow	-	strain, shear	predefined	-	+	+	Engelmayr et al. (2003; 2005; 2006; 2008)
Physiological	vascular graft	Pulsatile pressure - laminar flow bioreactor	pressure flow	measured (pressure)	strain, shear	-	pressure	-	-	Thompson et al. (2002)
Physiological	vascular graft	Pulsatile pressure - laminar flow bioreactor	pressure flow	measured (pressure)	strain, shear	measured (strain)	-	+	+	Hahn et al. (2007)
Physiological	vascular graft	Pulsatile flow - prestrain bioreactor	force, flow, pressure	measured (pressure)	strain, shear	measured (strain)	flow (manual)	-	-	Mironov et al. (2003)
Physiological	vascular graft	Pulsatile flow - prestrain bioreactor	force, flow, pressure	measured (force, pressure)	strain, shear	-	-	-	+	McCulloch et al. (2004)
Physiological	heart valve	Windkessel based bioreactor	pressure flow	measured (pressure, flow?)	strain, shear	-	pressure	+	+	Schenke-Layland et al. (2003)
Physiological	heart valve, vascular graft	Windkessel based bioreactor	pressure flow	measured	strain, shear	-	pressure, flow	-	+	Dumont et al. (2002); Hildebrand et al. (2004); Ruel et al. (2009); Narita et al. (2004)
Physiological	heart valve	Windkessel based bioreactor	pressure flow		strain, shear	measured (strain)	pressure, flow, strain	-	-	Vilendrer et al. (2003)

1.5 Bioreactors in cardiovascular tissue engineering

Since relatively less complex cardiovascular constructs such as tissue strips or vessels can be used as an in-vitro model system for heart valve tissue engineering, bioreactor systems in the complete field of cardiovascular tissue engineering were studied and discussed in this section. In this field, many bioreactors have been developed for the culturing and conditioning of tissue constructs. The design of a bioreactor is dictated by 1) the type of loading applied to the tissue-engineered construct and 2) the geometry of the tissue construct cultured in the bioreactor. The different types of loading are strain-based; predominantly inducing deformation in the cultured tissue, flow-based; primarily inducing shear in the tissue, or physiological; combining both strain- and flow-based loading in physiological ranges. Constructs vary in geometry from relatively simple configurations, such as strips, square pieces and disks, to more complex shapes, such as blood vessels or complete heart valves (table 1.1).

1.5.1 Strain-based loading

Strain-based bioreactor systems for relatively simple tissue construct geometries have shown a strong similarity to uni-, or biaxial tensile systems. Tissue-engineered pieces were positioned in a culture chamber and were exposed to strain-based static or cyclic loading. Kim et al. (1999), Boerboom et al. (2008), Rubbens et al. (2009) and Ferdous et al. (2008) used the Flexercell straining system to apply uniaxial straining to rectangular tissue-engineered strips. In this system, uniaxial loads were vacuum induced and predefined. Butcher et al. (2006) developed a similar system to apply mechanical stretching to tissue-engineered samples. In addition, Mol et al. (2003) constructed a device in which cultured samples were clamped in two steel grippers of which the movement of one clamp was operated and controlled. Mitchell et al. (2001) developed a bioreactor system that could apply biaxial loading. Tissue samples were attached to four loading rods and forces were applied and measured in orthogonal directions. In general, bioreactor systems designed for relatively simple tissue-engineered constructs can be considered accurate in the application of predefined strains. As a result of this strain application, tissue organization and mechanical properties of the strained constructs showed improvement.

Since vascular grafts have a more complex geometry than tissue strips, they were mechanically strained in more advanced bioreactor systems. Niklason et al. (1999; 2001) developed two types of pulsatile perfusion bioreactor systems for culturing and conditioning tissue-engineered vascular grafts. In the first system (Niklason et al., 1999) buffer-fluid was pumped through silicone tubing to apply predefined 5% radial strain to the tissue-engineered vessel. A similar approach was employed in the bioreactor developed by Stekelenburg et al. (2009) inducing 1% cyclic radial strain. The second set up by Niklason (2001) used air pressure to inflate the tubing and strain the tissue. The vascular constructs were not incorporated into the circulation loop, but were cultured over the silicone sleeves. Intraluminal pressures

were measured. Seliktar et al. (2000) built a similar bioreactor system to induce predefined cyclic strains, which was also used by Stegemann et al. (2003). In conclusion, in bioreactors in which cyclic straining was imposed to vascular grafts, applied strains were predefined. The studies showed that both tissue organization and mechanical properties of the vascular grafts were enhanced under influence of mechanical straining.

From a geometrical point of view heart valves are of an even higher level of complexity compared to tissue-engineered vascular grafts. Nevertheless, a few studies describe a bioreactor system in which a tissue-engineered heart valve was mechanically stimulated by cyclic straining in diastolic (closed) configuration (Mol et al., 2005a, 2006; Syedain et al., 2008; Syedain & Tranquillo, 2009). Here, dynamic pressure differences were applied across the engineered heart valve leaflets to induce local strains. In these studies, strains were not predefined and not measured. Since controlled strain application was considered of value, Mol et al. (2005a) used computational modeling to estimate the magnitude and distribution of dynamic strains in the heart valve leaflets. Syedain et al. (2008) did not assess local tissue strains either, but measured the circumferential stretching of the tissue-engineered valve root. In another study, Syedain & Tranquillo (2009) monitored the strain applied to the leaflets of bileaflet valvular equivalents in real-time during culture. Hence, in the developed strain-based bioreactors, tissue-engineered heart valves were mechanically strained in diastolic configuration. The applied strains were assessed after culture or measured in different heart valve locations, but not controlled. Again, these studies showed an increase in mechanical properties and an improvement of the tissue structure of the cultured heart valves due to the induced deformations.

1.5.2 Flow-based loading

In a flow-based bioreactor system, fluid flow is the main stimulus for tissue formation and development. In contrast to the strain-based bioreactor, it has been shown in literature that the complexity of the tissue-engineered construct has less influence on the design of the flow-based bioreactor. In general, a flow-based bioreactor set-up is composed of a mock circulatory loop, in which an actuation system, usually a peristaltic pump, and a culture chamber are included. During culturing, the pump drives an often pulsatile flow of culture medium along or through the tissue-engineered construct. Flow-based load application to tissue-engineered patches was performed by Sodian et al. (2001) who used the bioreactor concept of Hoerstrup et al. (2000). In the pulsatile flow chamber, dynamic flows were generated by a pneumatic pump pressurizing a silicone rubber diaphragm which propelled culture medium through the cardiovascular tissue. This set-up was also employed by Yang et al. (2006) who cultured circular constructs. However, in both studies, it was not mentioned if and how the application of flow was measured or controlled. Jockenhoevel et al. (2002) applied a variable laminar flow to tissue specimens, however, without direct control over absolute flow values. In vascular tissue engineering, Aper et al. (2006) cultured fibrin-based vessels by only applying a

predefined perfusion flow through the lumen of the vessels. Cebotari et al. (2005) also perfused their reseeded decellularized human allografts before implantation. The bioreactor of Williams et al. (2004) was capable of simulating a pulsatile flow through cultured vascular grafts. Flow was measured by two flow meters, but loading was non-physiological and uncontrolled. Vascular constructs and heart valves were cultured by Sodian et al. (2002; 2006) who again used the bioreactor concept of Hoerstrup et al. (2000). The bioreactor was also used in the tissue-engineered heart valve studies performed by Rabkin et al. (2002) and Flanagan et al. (2007). This bioreactor was able to apply pressures, ranging from 10 to 240 mmHg and flows, from 50 to 200 ml/min to the developing tissue-engineered heart valve. Nevertheless, it was not reported that load application was measured or controlled in real-time during culture. The set-up of Zeltinger et al. (2001) was able to generate static and dynamic flow conditions to heart valves but these again were not controlled. Although many flow-based bioreactor systems have been described in the literature, only a few were able to measure flow, but none of these were able to control the applied flows in real-time. The induced tissue response, e.g. deformation or shear, was neither monitored nor controlled during culture. While some of these studies indicated an improvement in tissue organization due to the flow-based loading, an increase or enhancement of the mechanical properties of these tissue-engineered constructs has not been reported.

1.5.3 Physiological loading

Bioreactor systems in which physiological loads are applied to tissue-engineered specimens, vascular grafts or heart valves, should be able to simulate all characteristics of in-vivo loading. Hence, in these systems mechanical conditioning is not limited to strain-based or flow-based load application alone.

Tissue-engineered samples were cyclically flexed by Engelmayr et al. (2003; 2005) in a dynamic flexural stimulation bioreactor. The system was further developed and was called the dynamic flex-stretch-flow bioreactor (Engelmayr et al., 2006; 2008). In this bioreactor system, it was possible to expose tissue-engineered constructs to predefined levels of cyclic flexure, stretch and shear. The different stimuli could be applied together or independently, allowing the simulation of physiological conditions. In these studies tissue organization and mechanical behavior of the tissue-engineered specimens showed improvement under influence of combined loading.

Vascular grafts were tissue-engineered under physiological loading conditions in studies of Thompson et al. (2002) and Hahn et al. (2007). In the bioreactor of Thompson et al. (2002), a pulsatile, laminar flow was directed through the vascular graft generating pressure waveforms similar to mammalian physiology. Pressure application was monitored and controlled by manually tuning. Hahn et al. (2007) developed a different system, composed of two pumps; a peristaltic pump to create the desired base flow and a pulsatile pump to produce pulsation profiles. The system produced physiological peak-to-trough pressure waveforms at both fetal and adult heart rates. These pressure waveforms were measured and the applied strains were calculated from the radial distension of the cultured vessels. Mironov et al. (2003) and McCulloch

et al. (2004) loaded vascular grafts in more advanced bioreactor systems. Next to pulsatile flow through the lumen of the vascular graft, an uniaxial straining device was employed to apply a fixed prestrain and/or cyclic strain, respectively, to the vascular construct. In this way both strain-based and flow-based load application were combined. In these approaches pressures were generally measured and sometimes controlled. However, flow was not monitored, but tissue deformation was. Moreover, only a few studies (McCulloch et al., 2004; Hahn et al., 2007) reported improvements in tissue organization of the engineered grafts as a result of physiological loading, while enhancement of the mechanical behavior was only reported once (Hahn et al., 2007).

Physiological flow and pressure profiles were applied to tissue-engineered heart valves in relatively large and complex systems mimicking the systemic circulation. The in-vivo systemic circulation was simulated by different components of the bioreactor set-up, based on the Windkessel model. The pulsatile pump represented the left ventricle and the systemic afterload was simulated by one or more Windkessel elements, representing the systemic compliance, peripheral resistance, aortic resistance and systemic inertia (Westerhof et al., 1971). Schenke-Layland et al. (2003) developed a hydrodynamic pulse duplicator system for culturing heart valves. The design and the arrangement of the system enabled the simulation of a pressure and flow profile identical to the native human aortic valve. Pressure values were measured after the valve but not controlled. It was unclear whether flow rates were monitored in this system. In addition, Dumont et al. (2002), Hildebrand et al. (2004), Ruel et al. (2009) and Narita et al. (2004) developed each a two-element Windkessel bioreactor to culture tissue-engineered heart valves in which pressure and flow values were measured and controlled. In the set-up of Narita et al. (2004), it was also possible to create and condition vascular grafts. The functioning of the other bioreactor systems to culture heart valves was described in detail but no publications were found in the literature in which these systems were used in-vitro. The same applies for the system of Enduratec (Vilendrer et al., 2003) which was developed for conditioning both heart valves as vascular grafts. The Enduratec system is highly advanced. Next to the generation and control of physiological pressures and flows, it was reported to be able to assess and control tissue deformation. However, in-vivo studies with cultured heart valves in the Enduratec system have never been published. Remarkably, only two physiological loading heart valve bioreactor systems were shown to be functional as an in-vitro set-up (Schenke-Layland et al., 2003; Narita et al., 2004). Both studies showed the beneficial effect of physiological loading on tissue formation, while improved mechanical behavior was solely indicated by Schenke-Layland et al. (2003).

1.5.4 Conclusion

The bioreactor studies in cardiovascular tissue engineering showed that an increase in the level of complexity of the tissue construct resulted in a decrease of control of the induced tissue response. For relatively simple constructs, such as strips, disks or squares, mechanical conditioning was applied in a well-defined way. The

resulting tissue response, i.e. deformation or shear, was predefined and kept constant during culture. In contrast, mechanical loading of tissue-engineered heart valves was less controlled, and the induced tissue response was generally unknown.

Furthermore, studies describing strain-based mechanical stimulation showed more correlation with enhanced tissue organization and mechanical properties when compared with reports on flow-based loading. Simulation of in-vivo loading conditions was reported in several bioreactor studies, however, the impact on tissue-engineered constructs was rarely investigated. The next step in heart valve tissue engineering would be to design a bioreactor system that is able to 1) accurately apply and control a strain-based loading protocol and 2) study the mechanical behavior of the tissue-engineered heart valve during culture in real-time and non-invasively.

1.6 Objective and Outline

In heart valve tissue engineering, the mechanical behavior of heart valves still needs improvement when native aortic valves are considered as a benchmark. Although it is known that cyclic straining enhances tissue formation, optimal loading protocols have not been defined yet. To obtain a better understanding of the effects of mechanical conditioning on tissue development, the induced tissue response, i.e. tissue deformation should be monitored and controlled in real-time. This lack of deformation monitoring and control during load application is the main shortcoming of current bioreactor systems in heart valve tissue engineering. Since the mechanical properties of a tissue-engineered heart valve change during culture, a preset loading amplitude does not necessarily induce a constant deformation. The effects of variation in applied deformation on tissue remodeling and mechanical properties are unknown. Therefore, it is desired to assess and control local tissue strains in real-time, non-invasively and non-destructively.

In general, the mechanical behavior of the engineered valves has been assessed by sacrificing the heart valves at the end-stage of tissue culture to perform destructive testing methods. To study the mechanical behavior of the heart valves during culture as a non-invasive quality check, it is also desired to assess mechanical properties in real-time, non-invasively and non-destructively. This leads to the objective of this work:

The development of a bioreactor system in which deformation of tissue-engineered heart valve leaflets is measured and controlled, and mechanical properties of tissue-engineered heart valve leaflets are assessed in real-time, non-invasively and non-destructively under diastolic loading conditions.

In chapter 2, an inverse experimental-numerical approach is proposed by which overall and local heart valve leaflet deformation is assessed during culture in a bioreactor system. Consecutively, in chapter 3, the experimental-numerical approach is further developed and applied to assess the mechanical properties of tissue-engineered valves, non-invasively and non-destructively. In chapter 4, the valve leaflet

deformation assessment method of chapter 2 is advanced by including a feedback control mechanism to control the applied deformation induced in the tissue-engineered valve constructs. In chapter 5, an overview of the macroscopic appearance and the additional analyses, i.e. biochemical assays and histology of all tissue-engineered heart valves described in chapters 3 and 4, is presented and discussed. Subsequently, chapter 6 describes the steps taken towards preclinical application of the developed bioreactor system. Its functionality is evaluated for implantable stented heart valves and heart valves created from animal-derived cells. In chapter 7, a general discussion is presented together with the conclusions based on the findings of the presented studies. Finally, appendix A reports a new bioreactor design in which all bioreactor characteristics discussed in chapters 2-5 are combined with the application of systolic flow conditions.

Chapter 2

Real-time, non-invasive assessment of leaflet deformation in heart valve tissue engineering

The contents of this chapter are based on J. Kortsmit, N.J.B. Driessen, M.C.M. Rutten, and F.P.T. Baaijens (2009), *Real-time, non-invasive assessment of leaflet deformation in heart valve tissue engineering*, Annals of Biomedical Engineering, Mar; 37(3):532-41.

2.1 Introduction

Contemporary tissue-engineered heart valves seem to have sufficient mechanical strength for implantation (Mol et al., 2006). However, mechanical properties, tissue structure and architecture still need to be improved. Research has demonstrated that dynamic load application to the developing tissue in a bioreactor system enhances the growth, remodeling and mechanical properties of tissue-engineered structures (Butler et al., 2000; Hoerstrup et al., 2000b; Isenberg et al., 2003; Engelmayr et al., 2005; Mol et al., 2006).

In heart valve tissue engineering, many bioreactors have been developed and almost all systems try to simulate physiological flow. These systems either apply mechanical loading in a wide physiological range, mimicking both systole and diastole (Dumont et al., 2002; Rutten et al., 2002; Hildebrand et al., 2004) or are characterized by simulation of the systolic or opening phase of the cardiac cycle (Hoerstrup et al., 2000a; Zeltinger et al., 2001; Rabkin et al., 2002; Schenke-Layland et al., 2003). However, the diastolic or closing phase represents the load bearing phase of the cardiac cycle, in which most strain is applied to the heart valve tissue. The positive effect of cyclic tissue straining on the developing construct has been demonstrated in recent studies. It enhances cell proliferation and functional tissue formation. Furthermore, an increase in ultimate tensile strength and tissue stiffness has been observed, compared to static control (Kim et al., 1999; Niklason et al., 1999, 2001; Seliktar et al., 2000; Seliktar et al., 2003; Mol et al., 2003).

In a recently developed bioreactor concept, the Diastolic Pulse Duplicator (DPD) (Mol et al., 2005a) dynamic strains were induced in the heart valve leaflets by applying a dynamic pressure difference over the closed valve. The strain-based conditioning approach was tested by culturing human heart valve leaflets in-vitro. The results showed more tissue formation and non-linear tissue-like mechanical properties in the strained valves when compared to unloaded valves. The strain-based conditioning approach of the DPD, in combination with its ease of use, offers new possibilities for bioreactor design and optimization of tissue properties in heart valve tissue engineering.

The main shortcoming of current bioreactor systems, including the DPD, is the lack of control during load application. Preset transvalvular pressures are applied to the developing heart valve while the induced deformations are unknown. Maximum strain values may vary during conditioning as a consequence of changing mechanical properties of the engineered construct. The effects of variation in applied deformation on tissue remodeling are yet unknown. Therefore, real-time, non-invasive measurement and control of local tissue strains is desired. Consequently, the objective of this study is to assess heart valve leaflet deformation, in real-time and non-invasively, during straining under diastolic loading conditions.

In this study, a method is presented to assess local tissue strains in heart valve leaflets. We hypothesize that local tissue strains can be determined from volume changes of the heart valve as a result of load application (Fig. 2.1). Volume changes are assessed non-invasively, using flow measurements, and represent the volumetric

deformation of the leaflets in a stented configuration. A computational model (Driessen et al., 2005a; Mol et al., 2005a) is employed to relate volumetric deformation to local tissue strains in the heart valve leaflet. The measurement method is validated and a tissue engineering experiment is performed to demonstrate the feasibility of the measurement method in-vitro.

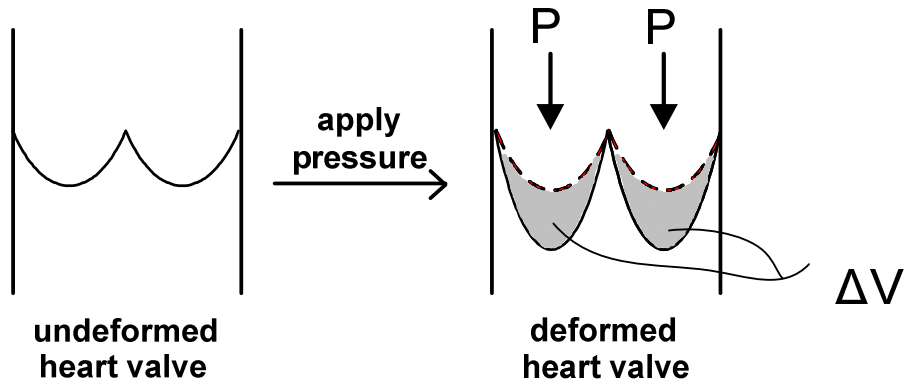


Fig. 2.1: *Volume change of the heart valve leaflets as a measure for heart valve deformation.*

2.2 Materials and Methods

2.2.1 Computational model

To test our hypothesis, a quasi-static computational model of the heart valve leaflets (Driessen et al., 2005a; Mol et al., 2005a) was applied to investigate the relation between volumetric deformation and local tissue strains in the leaflets. Furthermore, to study the influence of the model's input material parameters on this relation, a sensitivity analysis was performed.

Constitutive law

In the model, it is assumed that the leaflets are incompressible and therefore the total Cauchy stress ($\boldsymbol{\sigma}$) was split into a hydrostatic pressure (p) and an extra stress ($\boldsymbol{\tau}$):

$$\boldsymbol{\sigma} = -p\mathbf{I} + \boldsymbol{\tau} \qquad (2.1)$$

To model non-linear mechanical behavior, a non-linear Neo-Hookean model (Driessen et al., 2005a; Mol et al., 2005a) was used:

$$\boldsymbol{\tau} = G(\mathbf{B})(\mathbf{B}-\mathbf{I}), \qquad (2.2)$$

with the shear modulus G calculated from

$$G(\mathbf{B}) = G_0(\mathbf{I}_1(\mathbf{B})/3)^n, \qquad (2.3)$$

with G_0 and n material parameters. $\mathbf{I}_1(\mathbf{B})$= trace($\mathbf{B}$) represents the first invariant of the left Cauchy-Green deformation tensor, which is calculated from $\mathbf{B} = \mathbf{F}\cdot\mathbf{F}^T$, with $\mathbf{F}$ the deformation gradient tensor. The parameter n represents the degree of non-linearity of the constitutive equation: n>0 indicates stiffening of the material with increasing strains, whereas n<0 indicates softening. Note that the classical Neo-Hookean model is obtained for n = 0, with $G = G_0$.

Balance equations

After the material parameters were determined, finite element analyses (FEA) were performed to simulate the mechanical response of the engineered leaflets. The balance equations that were solved are conservation of momentum and mass for an incompressible solid:

$$\vec{\nabla}\cdot\sigma = \vec{0} \qquad (2.4)$$

$$J - 1 = 0 \qquad (2.5)$$

where J = det($\mathbf{F}$) represents the volume change between the undeformed, stress-free configuration and the deformed configuration. The finite element formulation (Bathe et al., 1996) was used to solve the set of balance equations. The computational framework and the constitutive equations were implemented in the software package SEPRAN (Segal et al., 1984).

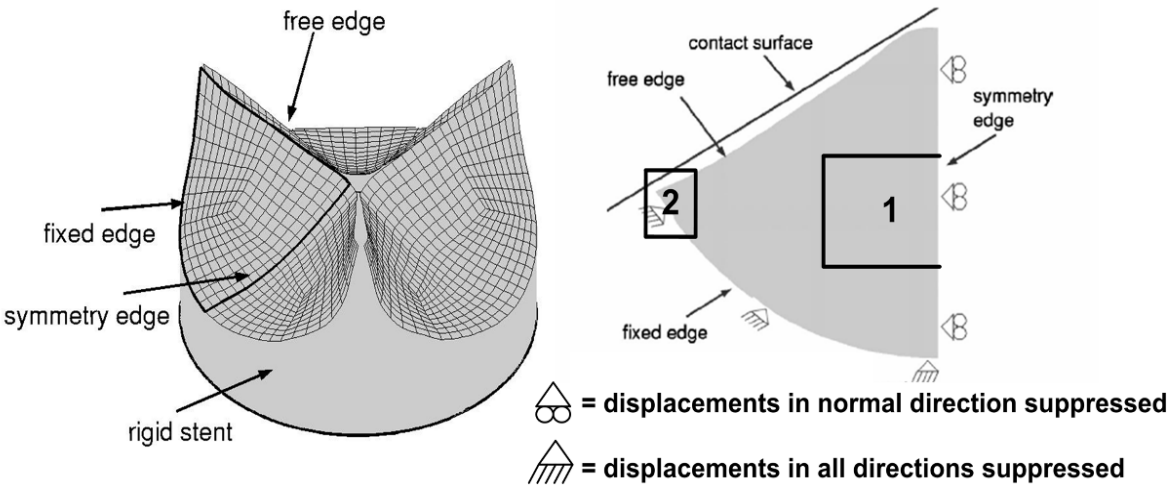


Fig. 2.2: *(a) Finite element mesh of the stented heart valve geometry in closed configuration. In the computations, only one half of a leaflet was used because of symmetry. (b) Schematic representation of one half of a heart valve leaflet, showing the boundary conditions. The gray area represents the top (aortic) side of the leaflet to which pressure is applied. A contact surface was defined to model coaptation of the leaflets (Driessen et al., 2005a; Mol et al., 2005a). Areas 1 and 2 represent the belly and commissural region of the heart valve leaflet, respectively.*

Geometry and boundary conditions

The finite element mesh of the leaflets in the closed configuration consisted of only one half of a leaflet, because of symmetry (Fig. 2.2a). At the symmetry edge, nodal displacements in the normal direction were suppressed (Fig. 2.2b). At the bottom (ventricular) side of the fixed edge, nodal displacements were suppressed in all directions. At the free edge, a contact surface was defined to model coaptation of adjacent leaflets. The radius of the leaflets was set to 12 mm. The belly and commissural regions of the heart valve are indicated by the selected areas 1 and 2, respectively. To model the diastolic transvalvular load, pressure was applied to the top surface of the leaflets. Subsequently, volumetric and local deformations were calculated.

Simulations

To relate volumetric to local deformations in the heart valve leaflets, simulations were performed. Increasing pressure differences, from 0 to 13 kPa were applied and both volumetric deformation and local tissue strains were computed. To assess regional variations, mean values of maximum principal strain were calculated for the entire valve leaflet, the belly region and the commissural region.

The magnitude of the input parameters; thickness (t), shear modulus (G_0) and degree of non-linearity (n) of the heart valve leaflets were chosen based on experimental data of tissue-engineered human valves after two to four weeks of culturing (table 2.1) (Driessen et al., 2005a, 2007). To investigate the influence of the model's input parameters on the relation between volumetric and local deformation, a parametric study was performed. The parameters were varied in a range to cover the experimental data (table 2.1). The thickness, shear modulus and degree of non-linearity were varied between 0.35 and 1 mm, 0.1 and 100 MPa, and between 1 and 15, respectively. The influence of each parameter was studied by varying it over the given range while the other two parameters were kept constant at 0.5 mm, 0.5 MPa and 10 for thickness, shear modulus and degree of non-linearity, respectively. The calculated volumetric-local deformation curves were averaged and error bars indicated the standard deviation due to the range in input parameters.

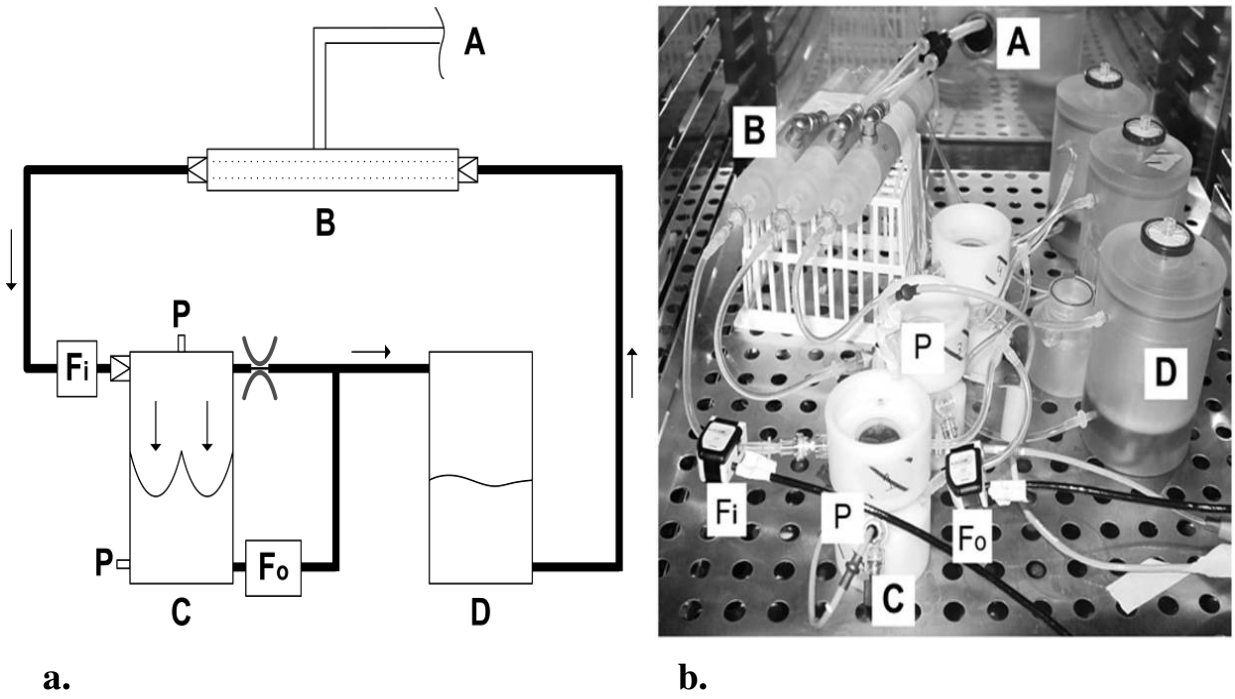
Table 2.1: *Experimental data and input parameters for the computational model; thickness (t), shear modulus (G_0) and degree of non-linear material behavior (n) of the heart valve leaflets.*

	t (mm)	**G_0 (MPa)**	**n (-)**
experimental data (Driessen et al., 2005a, 2007)	0.50	0.50	10
computational model	0.35 - 1.0	0.1 - 100	1 - 15

2.2.2 Experimental set-up

Bioreactor

The deformation measurement method was implemented in a bioreactor system similar to the set-up of the Diastolic Pulse Duplicator (Mol et al., 2005a). Briefly, the bioreactor system consisted of four main components: a proportional compressed-air valve (A), a pulsatile pump encompassing a flexible silicone rubber tube (B), a bioreactor, consisting of two chambers separated by the cultured valve (C) and a medium container (D) (Fig. 2.3a, b).



a. **b.**

Fig. 2.3: *(a) Schematic drawing of the bioreactor system and (b) a photograph of three systems in use simultaneously. One bioreactor system consists of a compressed air supply (A), a pulsatile pump (B), a bioreactor (C), including two pressure sensors (P) and two flow sensors (F_i , F_o), and a medium container (D).*

The whole system was filled with 150 ml of culture medium. The silicone rubber tube, which functioned as a pulsatile pump was compressed and decompressed by dynamic air pressure from the proportional valve. The amount, frequency and waveform of air release were controlled via a programmable multi-IO-card using LABVIEW software (National Instruments, USA). As a result, fluid was taken from the container and pumped into the upper chamber of the bioreactor, which caused a dynamic pressure difference over the heart valve. Consecutively, medium exited the bioreactor from both the lower and the upper chamber. Pressure sensors (P10EZ, BD, Franklin Lakes, NJ, USA) were positioned in both chambers, while two flow sensors (Transonic Systems Inc., USA) were attached to the tubing through which fluid entered (F_i) and exited (F_o) the bioreactor (Fig. 2.3a, b).

Deformation measurement method

Flow measurements were performed to assess volumetric deformation of the heart valve leaflets in a stented configuration. During load application, a pulsatile flow (F_i) was injected into the upper chamber of the bioreactor. In combination with the relatively large resistance of the upper chamber exit, this flow caused a dynamic pressure difference over the cultured heart valve. As a result, the heart valve leaflets deformed, and fluid in the lower chamber of the bioreactor was displaced and exited the bioreactor (Fig. 2.4a). After loading, the heart valve returned to its undeformed state and the displaced fluid reentered the lower chamber of the bioreactor (Fig. 2.4b). The volumetric deformation of the heart valve was defined as the amount of fluid exiting and subsequently reentering the bioreactor. This deformation was obtained after time-integration of the lower chamber exit flow (F_o). The net flow leaving the lower chamber was defined as the amount of fluid leaking through the valve. Furthermore, relative leakage was defined as the leak flow through the valve divided by the amount of fluid entering the bioreactor. By using this technique, volumetric deformation was distinguished from leakage (Fig. 2.4c).

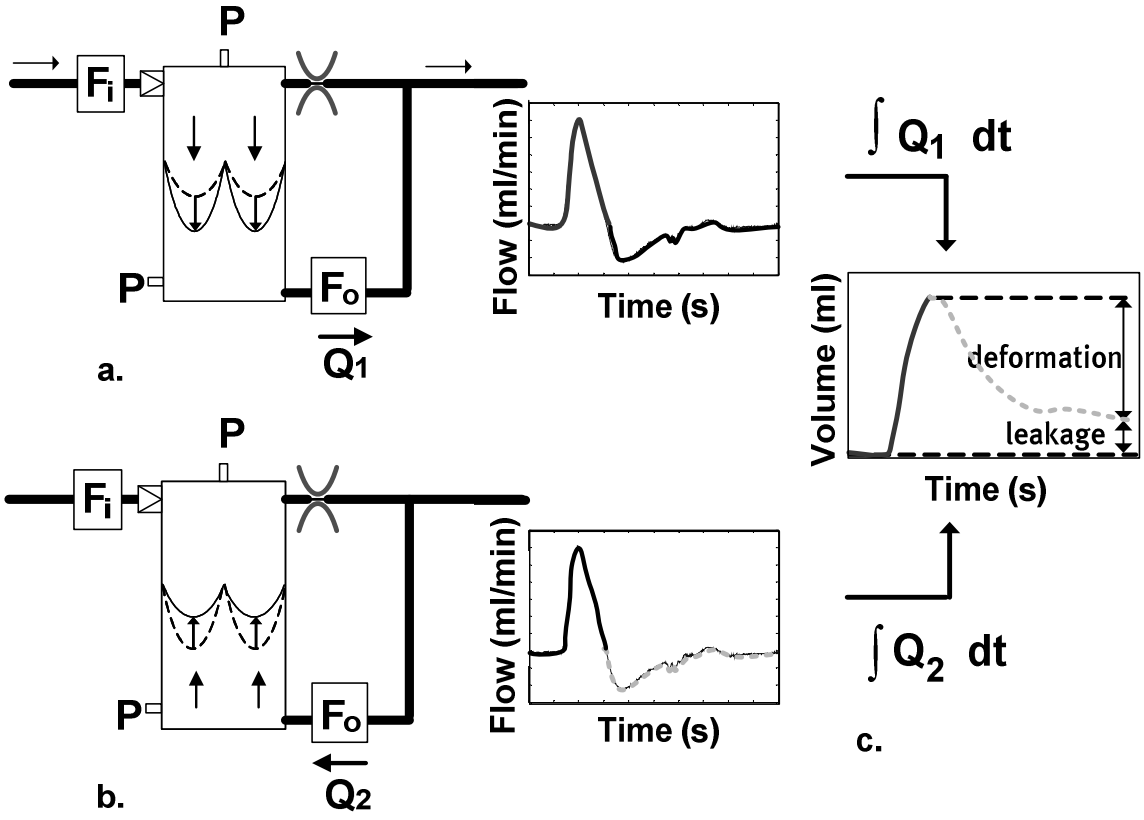


Fig. 2.4: *Flow-based deformation measurement principle. (a) Medium enters the bioreactor. A pressure difference over the heart valve is created which causes the valve leaflets to bulge down. As a result, a medium flow exits the lower part of the bioreactor (Q_1). (b) After deformation, the heart valve returns to its undeformed state and, consequently, medium reenters the bioreactor (Q_2). (c) By time-integration of the measured flow signal, volumetric deformation of the heart valve leaflets can be distinguished from the leakage through or along the valve.*

2.2.3 Validation

To verify the functioning of the deformation measurement method, a validation was performed in two steps. First, the experimentally measured deformation values of a non-leaking polyurethane rubber heart valve were compared to values calculated with the computational model. This was done to evaluate the applicability of the measurement technique to assess deformation of a heart valve geometry. Second, the influence of leakage on the measurement method was studied by simulation of different leak flows.

Polyurethane heart valve

To validate the deformation measurement method for a heart valve geometry, heart valve leaflets were made of polyurethane and bonded to a rigid polycarbonate stent (Fig. 2.5). Leakage of the heart valve was prevented by gluing the coaptation areas of the leaflets together.

Fig. 2.5: *Polyurethane heart valve leaflets fixed to a rigid polycarbonate stent.*

The valve was placed inside the bioreactor. Pressure differences increasing from 0 to 13 kPa were applied and the volumetric deformation was determined. The classical Neo-Hookean model (degree of non-linearity; n = 0) was applied to describe the mechanical behavior of the polyurethane valve. The stiffness of the rubber leaflets was chosen in the range of tissue-engineered human valve leaflets (Driessen et al., 2005a, 2007). Their thickness was measured (t = 0.08 ± 0.005 mm) and the shear modulus was determined by performing uniaxial tensile tests (G_0 = 7.2 ± 0.2 MPa). These parameters and the heart valve geometry were used as input for the computational model and a simulation was performed. The relation between pressure difference and volumetric deformation found in the experiment and the simulation was compared and correlated.

Leakage along a polyurethane disc

The influence of leakage on the flow-based deformation measurement technique was studied by loading a polyurethane membrane in the bioreactor system. The polyurethane membrane (diameter = 23 mm, thickness = 0.27 mm, Desmopan, Bayer AG, Germany) was clamped between two polycarbonate cylinders (height = 21 mm,

diameter = 25 mm) and inserted into the bioreactor. Increasing pressure differences were applied and deformation was determined by both flow measurement and using an optical technique based on laser Doppler. The laser Doppler velocimeter (Vibrometer OFV 3001, Polytec Optronics Inc., USA) was positioned above the bioreactor. The displacement of the centre was measured and the deformation of the membrane was assumed to be semispherical. Therefore, volumetric deformation was computed with:

$$V = \frac{1}{6} \cdot \pi \cdot h \cdot \left(3 \cdot r^2 + h^2\right) \qquad (2.6)$$

the volume (V) of a segment of a sphere, with r its radius and h its height.

Leakage was simulated by connecting the upper and lower chamber of the bioreactor with a bypass. The degree of leakage was controlled by clamping the bypass. Results obtained from both deformation measurement techniques were evaluated for various simulated leaks and applied pressure differences ranging from 0 to 13 kPa.

2.2.4 Tissue engineering experiment

To demonstrate the use of the deformation measurement technique in a tissue engineering experiment, stented heart valves with different initial mechanical properties were cultured. Anatomically shaped leaflets were cut out of non-woven polyglycolic acid (PGA) meshes (thickness: 1.0 mm). The dimensions were based on anatomical values measured in human specimens (Clark et al., 1974; Sauren et al., 1981; Thubrikar et al., 1990) and were similar to the computational domain applied in the computational model. The leaflets were coated with a thin layer of poly-4-hydroxybutyrate (P4HB) and molded in the shape of a trileaflet heart valve. Subsequently, they were attached to a rigid polycarbonate cylinder by sugar leaching of polycaprolactone (PCL).

Cells harvested from the human vena saphena magna were expanded using regular cell culture methods (Schnell et al., 2001) and seeded onto the scaffold using fibrin as a cell carrier (Mol et al., 2005b). The medium to culture these cells consisted of DMEM Advanced (Gibco, USA), supplemented with 10% Fetal Bovine Serum (FBS; PAN Biotech, Germany), 1% GlutaMax (Gibco, USA), and 0.1% gentamycin (PAN Biotech, Germany). The medium used for seeding and subsequent tissue culture contained 0.3% gentamycin and additional L-ascorbic acid 2-phosphate (0.25 mg/ml; Sigma, USA) to promote extracellular matrix production (Mol et al., 2005b). Before seeding, the scaffolds were disinfected in 70% ethanol and, subsequently, placed in culture medium for 24 or 72 hours. Scaffold degradation was promoted by exposure to medium and in this way two heart valves with different initial properties were created.

The constructs were placed in the bioreactor system and subjected to culture medium circulation at low speed (4 ml/min) for 5 days. Thereafter, a dynamic pressure difference with constant amplitude (at 1 Hz) was applied to the heart valve leaflets for 6 days. After 3 days of mechanical conditioning, the applied loading amplitude was

increased. The pressure difference over the valves, the volumetric deformation of the leaflets and the leak flow through the valves were measured daily. The measured volumetric deformation values were related to local tissue strains in the heart valve leaflets using the computational model.

2.3 Results

2.3.1 Computational model

The relations between volumetric deformation and mean local tissue strain show an increase in the local strain value as a result of an increase in volumetric deformation, in the entire leaflet, the belly region and commissural region (Fig. 2.6a, b, c). The strain values in the commissural region were considerably higher, up to a maximum strain of 22%, compared to the strain values in the entire leaflet (max 11%) and belly region (max 10%).

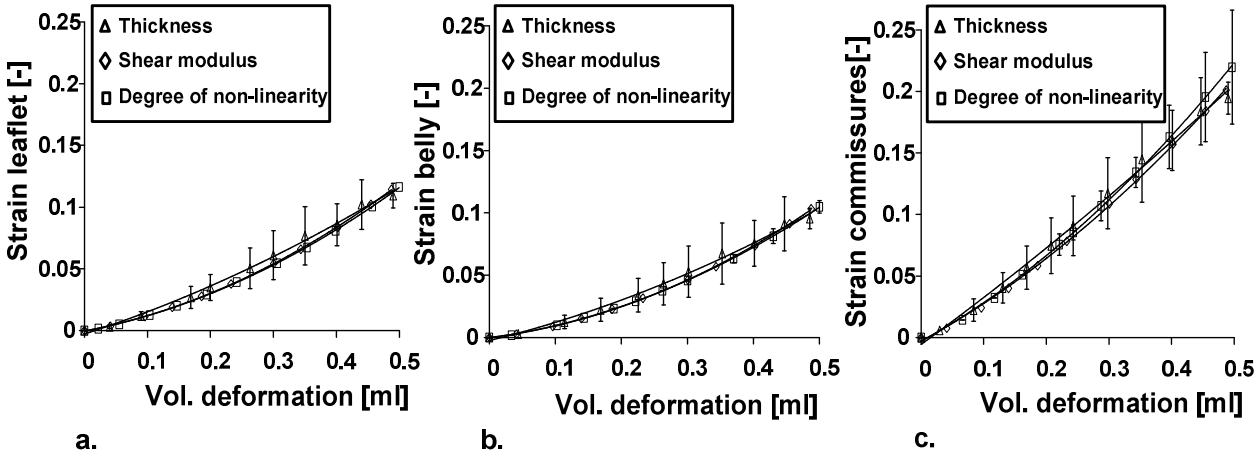


Fig. 2.6: *Mean maximum principal strain (-) depicted as a function of volumetric deformation (ml). Graphs were obtained by computation of strain and volumetric deformation values when one of the input parameters of the computational model was varied; thickness (t), shear modulus (G_0) or degree of non-linearity (n) in the range of t = 0.35 to 1 mm, G_0 = 0.1 to 100 MPa and n = 1 to 15, while the other two parameters were kept constant at t =0.5 mm, G_0 = 0.5 MPa and n = 10, respectively. These graphs are averaged and error bars are added to indicate the standard deviation of the curves due to a variation in thickness and non-linear material behavior (error bars). Results are shown for three regions of the valve; (a) entire leaflet, (b) belly region, and (c) commissural region.*

Variation in leaflet thickness led to a larger standard deviation in the calculated volumetric deformation - strain curve in all three locations, compared to variation of the other two input parameters. This standard deviation seemed to increase as volumetric deformation increased. In addition, a relatively large standard deviation was

also observed in the commissures when the degree of non-linearity was varied between 1 and 15. The standard deviation resulting from the variation in shear moduli was minimal and, therefore, not shown in figure 2.6.

2.3.2 Validation

Polyurethane heart valve

The experimentally and numerically obtained relations between volumetric deformation and applied pressure difference over the polyurethane heart valve show that an increase in applied pressure difference led to an increase in volumetric deformation (Fig. 2.7). A relatively good similarity was seen between the curves (R^2 = 0.98).

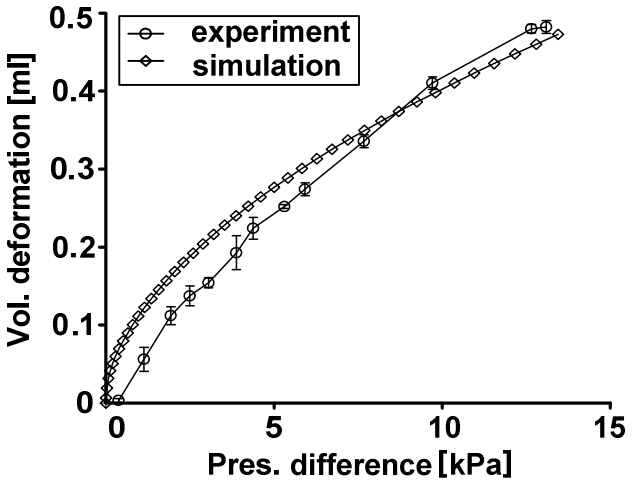
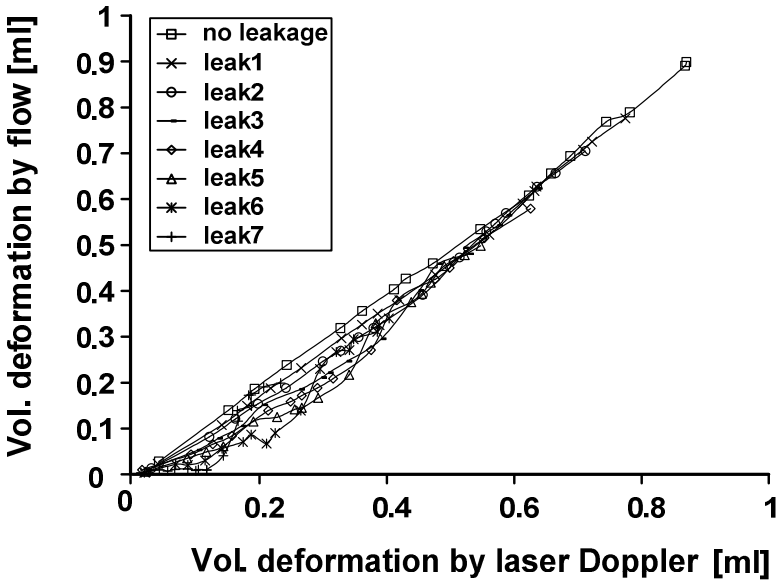


Fig. 2.7: *Experimentally and numerically obtained relations between volumetric deformation (ml) and applied pressure difference (kPa) over the heart valve. Experimental data (n=3) are depicted by open circles (O) and the standard deviation is represented by error bars. Numerical data are indicated by diamonds (◊).*

Leakage along a polyurethane disc

The volumetric deformation of a disc-shaped polyurethane membrane assessed by the flow measurement method was represented as a function of the volumetric deformation measured by laser Doppler velocimetry in figure 2.8. Results are shown for increasing simulated relative leak flows ranging from L_0 (no leakage) to L_7 (53 % leakage). A straight line through the origin with a slope equal to 1 would indicate a perfect resemblance between both measurement techniques and therefore an accurate validation of the flow-based deformation measurement. However, the degree of leakage simulated, seemed to affect the deformation measurements. As leakage increased, a slowly increasing offset in volumetric deformation was observed, which was indicated by a decrease of the coefficient of determination R^2 (Fig. 2.8). Furthermore, an increase in the leak flow resulted in a decrease of the imposed

maximum pressure difference over the membrane and, hence, in a downturn of the measured volumetric deformation values.



leak no.	leak-%	R^2
0	0	0.99
1	9	0.99
2	17	0.96
3	25	0.91
4	29	0.86
5	32	0.77
6	41	0.52
7	53	0.31

Fig. 2.8: *Volumetric deformation (ml) of a polyurethane, disc-shaped membrane assessed by the flow-based measurement method is represented as a function of volumetric deformation (ml) measured by laser Doppler. Deformation is determined at different simulated leak flows (leak 0 to 7) and the resemblance between both measurement techniques at these leak flows is indicated by R^2.*

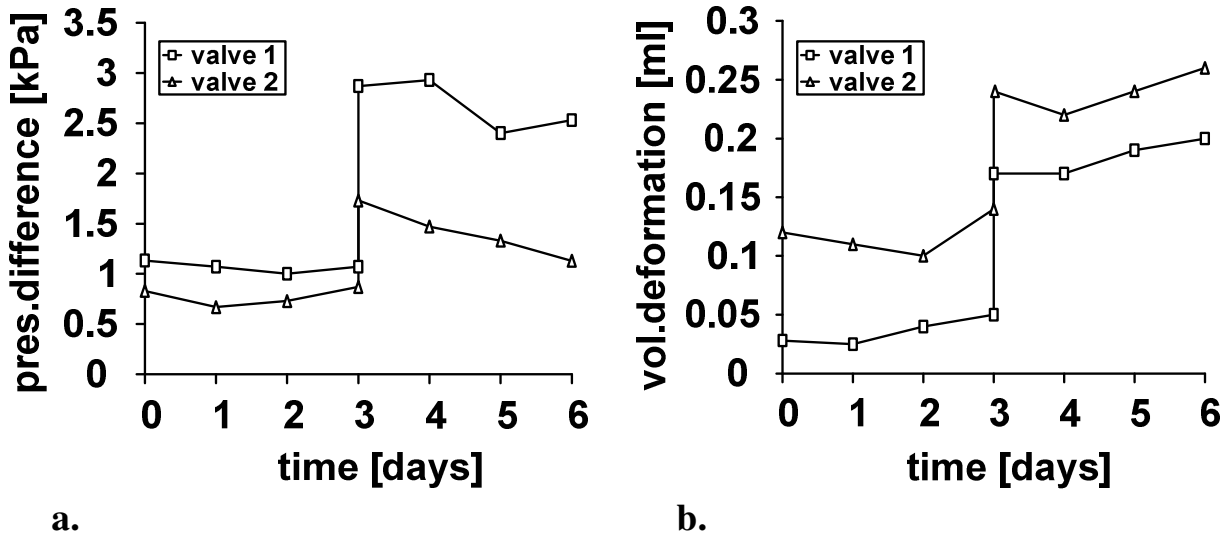


Fig. 2.9: *(a) Pressure differences (kPa) and (b) volumetric deformations (ml) represented as a function of dynamic culture time (days) for tissue-engineered valves 1 and 2.*

2.3.3 Tissue engineering experiment

The pressure differences over the cultured heart valves and the induced volumetric deformation values are represented as a function of culture time in figures 2.9a and b, respectively. The results show that for valve 1, a larger pressure difference led to a smaller deformation compared to valve 2. This indicated a larger stiffness of valve 1 in comparison with valve 2. Furthermore, the increase in load application on day 3 led to an increase in volumetric deformation values.

In figure 2.10, the calculated relative leakage through the valve is given as a function of culture time. The graph shows that the relative leakage did not exceed 15% and was most of the time even less than 10% for both valves.

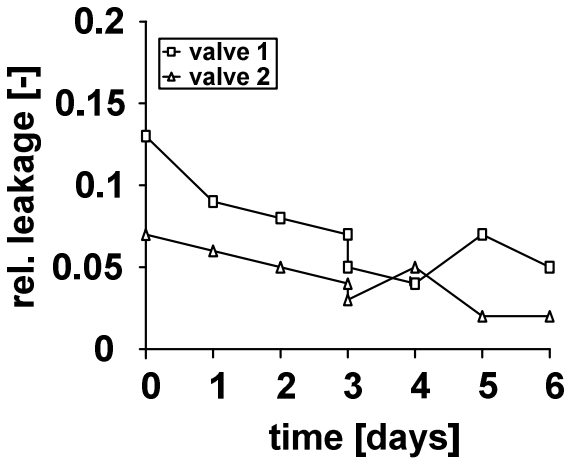


Fig. 2.10: *Relative leakage (-); leak flow (ml) of the tissue-engineered valve divided by the inflow (ml) in the bioreactor, depicted as a function of culture time (days) for valves 1 and 2.*

The mean local strain values in the entire leaflet, belly and commissures of valves 1 and 2, were determined by the computational model and are given as a function of culture time (Fig. 2.11). Comparing the three locations in the leaflet, strains in the commissural region appeared to be larger than in the belly and in the entire leaflet ($\varepsilon_{commissures} > \varepsilon_{leaflet} > \varepsilon_{belly}$). In addition, for all three locations in the leaflet, valve 2 experienced larger strains compared to valve 1, which also pointed to the larger stiffness of valve 1.

2.4 Discussion

In this study, a newly developed measurement method is presented by which leaflet deformation is assessed in heart valve tissue engineering. Deformation is measured using a flow-based measurement technique. This rather simple but new approach offers the possibility to measure deformation in real-time, non-invasively and non-destructively. No studies are known in which deformation of tissue-engineered heart valves was determined in such a way. The research in the literature that has been

performed includes studies in which relatively simple tissue-engineered constructs, 2D geometries (Engelmayr et al., 2003, 2005; Mol et al., 2003) or blood vessels (McCulloch et al., 2004; Mironov et al., 2004) instead of heart valves were subjected to preset deformational changes. Mechanical stimulation in these bioreactor systems shows strong similarity to the way load is applied by uni- or biaxial stretching devices. Tissue deformation was not measured but applied directly by adjusting mechanical loading. Deformation and motion of heart valve leaflets have been measured in several studies. Two cameras in combination with ink, graphite or projected markers on the valves surface were used to reconstruct 3D movement of the leaflet surface and/or to calculate 3D surface strains (Gao et al., 2000, 2001, 2002; Iyengar et al., 2001; Jensen et al., 2001; Sacks et al., 2002; Chen et al., 2004; Sun et al., 2005). Nevertheless, strain was only calculated when the markers were applied invasively, and the focus in these studies was on bioprosthetic valves instead of tissue-engineered heart valves. A study is known in which deformation of tissue-engineered valve leaflets was determined at several conditioning stages. In this study, however, cultured heart valves had to be sacrificed to estimate the applied strains afterwards (Mol et al., 2005a; Driessen et al., 2007).

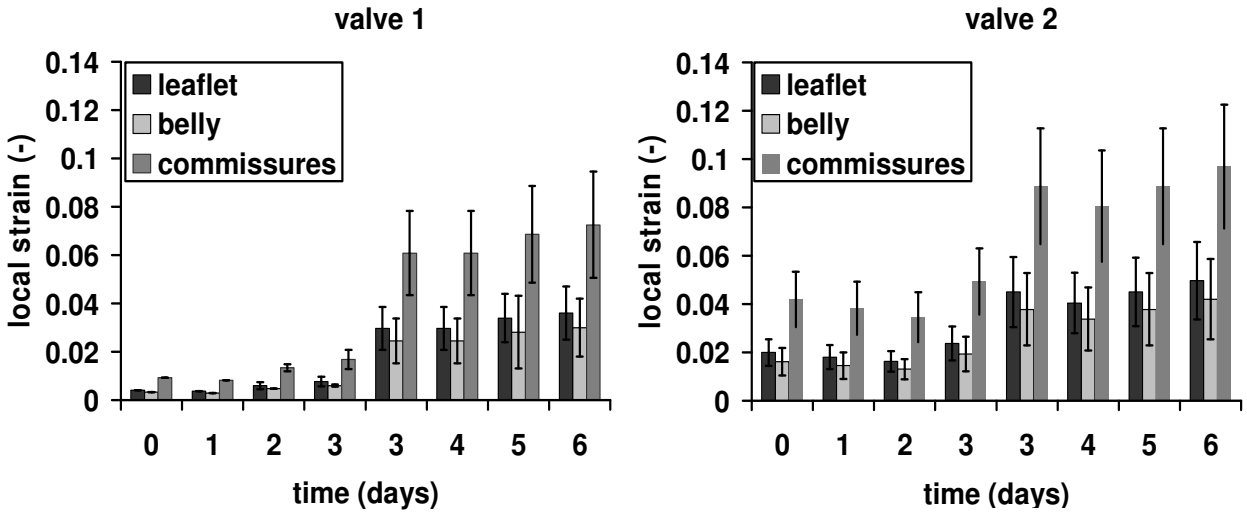


Fig. 2.11: *Mean local strain values (-) in three leaflet locations, given as a function of culture time (days) for (a) tissue-engineered valve 1 and (b) valve 2. Error bars indicate the range (maximum to minimum values) in local tissue strain values as discussed in the 'Computational model' section.*

Computational simulations indicated a relation between volumetric deformation and local tissue strains in the heart valve deformation range considered in this study. The relation was relatively insensitive to changes in material properties of the heart valve leaflets. Therefore, local tissue strains in the valve can be directly obtained from measured volumetric deformation values. The conventional numerical-experimental approach (Oomens et al., 1993) of determining material properties and local strain

values by using experimentally measured quantities is very time-consuming and can be avoided by using this direct relation. The relation between volumetric deformation and local tissue strain is most susceptible for variations in thickness; the maximum standard deviation in local strain values at a constant volumetric deformation was 25%. This is probably caused by the relatively large influence of thickness on the flexural behavior of the leaflets when the valve deforms. Furthermore, the largest standard deviation in the strain – volumetric deformation curves was found in the commissural region (maximum standard deviation was 20%). This part of the leaflet experiences highest strain values during loading and might therefore be more susceptible to changes in thickness and mechanical behavior.

The flow-based measurement method was validated by assessing deformation of a rubber heart valve. A good agreement was found between the curves in which volumetric deformation was plotted versus applied pressure differences for numerically and experimentally derived data (Fig. 2.7). In addition, the computational model was coupled to the experimental bioreactor set-up and thus local tissue strains were acquired from measured volumetric deformation values. Instead of experimental validation, a computational model was used to verify the measurement method. This model has proven its validity and the good match between the results of the experiment and the model is mutually valid.

The results of the second validation experiment, in which the influence of leakage was studied, showed a slightly growing offset in deformation values with increasing simulated leak flows (Fig. 2.8). This phenomenon was only seen in the volumetric deformation data obtained by the flow-based measurement technique. It was probably caused by the ratio between the amount of fluid exiting the lower bioreactor chamber as a result of leakage, and the amount of fluid reentering the bioreactor when the heart valve leaflets return to their unloaded configuration after load application. As leakage increases, this ratio increases and it becomes more difficult to distinguish the relatively small volumetric deformation from the large leak flow. Regarding figure 2.8, the flow-based deformation measurement method is less accurate for a relative leakage larger than 25%.

Volumetric deformation of engineered heart valve leaflets was related to local tissue strains by using a non-linear Neo-Hookean model to describe the mechanical behavior of the leaflets over time. In this model, the material properties were assumed to be homogeneous and isotropic. The relation between volumetric and local deformation was studied and was applied to tissue-engineered heart valves. Preceding research (Mol et al., 2005a, 2006) has indicated that the material behavior of these valves developed from being homogeneous and isotropic in the first weeks to inhomogeneous and anisotropic behavior after four weeks of culturing. Consequently, assuming homogeneous and isotropic material properties was not correct for the valve leaflets after four weeks of culturing. The use of a structurally-based anisotropic constitutive model could be a better option to describe material behavior in the final stages of culturing (Driessen et al., 2005b). When material properties in the circumferential and radial direction of the heart valve are significantly different, the anisotropic model can be applied to assess local deformations by using the

conventional numerical-experimental approach, mentioned earlier (Oomens et al., 1993).

The tissue engineering experiment demonstrated the possibility to measure volumetric deformation of the heart valve leaflets during mechanical conditioning, in real-time and non-invasively. Local strain values assessed by the computational model were maximal 6% (belly), 10% (commissural region) or 7% (entire leaflet). These values have been found to be realistic (Sun et al., 2005; Driessen et al., 2007). Furthermore, relative leakages of the valves were small, generally less than 10% (Fig. 2.10). So, according to figure 2.8, obtained deformation values can be considered accurate. Finally, as a result of various durations of scaffold degradation prior to seeding, two valves with different mechanical properties were cultured. This difference was observed from the pressure and deformation measurement data (Fig. 2.9 and Fig. 2.11). It indicates that the valve that was exposed to medium longest (valve 2), had the lowest stiffness.

2.5 Conclusion

The proposed flow-based deformation measurement method incorporated in the bioreactor system enables us to assess local tissue strains in the leaflets, in real-time, non-invasively and non-destructively, during culturing. By using this technique, the bioreactor system has great potential to systematically study the effects of mechanical loading on tissue development and, consequently, to design an optimal conditioning protocol for tissue engineering of aortic heart valve replacements.

2.6 Acknowledgements

This research was supported by the Dutch Program for Tissue Engineering (DPTE).

Chapter 3

Non-destructive and non-invasive assessment of mechanical properties in heart valve tissue engineering

The contents of this chapter are based on J. Kortsmit, N.J.B. Driessen, M.C.M. Rutten, and F.P.T. Baaijens (2009), *Non-destructive and non-invasive assessment of mechanical properties in heart valve tissue engineering*, Tissue Engineering Part A, Apr;15(4):797-806.

3.1 Introduction

The development of a tissue-engineered heart valve with appropriate structural and mechanical properties is a significant challenge. Tissue-engineered heart valves were cultured and successfully implanted in sheep at the low-pressure pulmonary side of the heart (Sodian et al., 2000; Hoerstrup et al., 2000b). Recently, human tissue-engineered heart valves were shown to have sufficient mechanical strength for implantation at the high-pressure aortic side (Mol et al., 2006). Nevertheless, the mechanical behavior of the engineered heart valves was less stiff at finite strains, and less anisotropic compared to native valves and, therefore, may still be improved.

Research has shown that mechanical stimulation has a beneficial effect on tissue properties (Kim et al., 1999; Seliktar et al., 2000; Isenberg et al., 2003; Mol et al., 2003; Engelmayr et al., 2005). Mechanical stimuli have been applied to the developing tissue in bioreactor systems, which are characterized by the type of conditioning. Bioreactors are specified as strain-based (Kim et al., 1999; Niklason et al., 1999, 2001; Seliktar et al., 2000, 2003; Mitchell et al., 2001) or flow-based (Conklin et al., 2000; Thompson et al., 2002; Jockenhoevel et al., 2002; Williams et al., 2004), or as systems in which the physiological environment of the tissue is mimicked (Hoerstrup et al., 2000a; Zeltinger et al., 2001; Rabkin et al., 2002; Rutten et al., 2002; Dumont et al., 2002; Schenke-Layland et al., 2003; Hildebrand et al., 2004; Narita et al., 2004; Flanagan et al., 2007). Most bioreactors that have been applied in heart valve tissue engineering are flow-based or physiological (Hoerstrup et al., 2000a; Zeltinger et al., 2001; Rabkin et al., 2002; Rutten et al., 2002; Dumont et al., 2002; Schenke-Layland et al., 2003; Hildebrand et al., 2004; Flanagan et al., 2007). However, studies have shown the enhancement of functional tissue formation as a result of cyclic tissue straining of the developing construct (Kim et al., 1999; Niklason et al., 1999, 2001; Seliktar et al., 2000, 2003; Mol et al., 2003). Hence, as a starting point of this study, the strain-based Diastolic Pulse Duplicator (DPD) (Mol et al., 2005a) is considered in which dynamic strains are induced in the heart valve leaflets in closed configuration. In human heart valve culture, this approach gave rise to enhanced tissue formation and non-linear tissue-like mechanical properties in the strained valves when compared to unloaded valves (Mol et al., 2005a).

The major drawback of the DPD and other bioreactor systems is the lack of control during load application. Pressure is applied to the developing heart valve while the mechanical properties of the valve are changing during culture. As a result, the induced tissue deformations are unknown, and vary during tissue culture.

An inverse experimental-numerical approach has been proposed by which leaflet deformation is assessed in real-time and non-invasively (Kortsmit et al., 2009a) during culture in a bioreactor system. This technique allows a systematic study of the effects of mechanical straining on tissue development. However, to assess the mechanical properties of the engineered valves, the constructs need to be sacrificed to perform tensile tests (Clark et al., 1973; May-Newman et al., 1995; Billiar & Sacks, 2000; Stella et al., 2007a, 2007b) or indentation tests (Cox et al., 2006). To study the mechanical behavior of the engineered constructs during culture and to test the

functionality of intact heart valves as a non-invasive quality check, it is desired to assess mechanical properties non-destructively. By further development of the experimental-numerical approach, a non-invasive and non-destructive assessment of mechanical properties of engineered heart valve leaflets is demonstrated here.

3.2 Materials and Methods

3.2.1 Computational model

To relate the combination of applied pressure and induced volumetric deformation of heart valve leaflets to the mechanical properties of the leaflets, a quasi-static computational model of the heart valve (Mol et al., 2005a; Driessen et al., 2005a) was employed.

Constitutive law

Assuming incompressibility of the leaflets, the Cauchy stress tensor ($\boldsymbol{\sigma}$) was split into a hydrostatic pressure (p) and an extra stress tensor ($\boldsymbol{\tau}$):

$$\boldsymbol{\sigma} = -p\mathbf{I} + \boldsymbol{\tau} \qquad (3.1)$$

where $\mathbf{I}$ represents the identity matrix. To model non-linear mechanical behavior, a non-linear Neo-Hookean model (Mol et al., 2005a; Driessen et al., 2005a) was used:

$$\boldsymbol{\tau} = G(\mathbf{B})(\mathbf{B}-\mathbf{I}), \qquad (3.2)$$

with $\mathbf{B}$ the left Cauchy-Green deformation tensor, and the shear modulus G defined by

$$G(\mathbf{B}) = G_0(\mathbf{I}_1(\mathbf{B})/3)^n \qquad (3.3)$$

with G_0 and n material parameters. $I_1(\mathbf{B}) = \text{trace}(\mathbf{B})$ represents the first invariant of the left Cauchy-Green deformation tensor, given by $\mathbf{B} = \mathbf{F}\cdot\mathbf{F}^T$. The deformation gradient tensor $\mathbf{F}$ is defined by

$$\mathbf{F}_{ij} = \partial\mathbf{x}_i / \partial\mathbf{X}_j \qquad (3.4)$$

with $\mathbf{x}_i$ the deformed position, and $\mathbf{X}_j$ the initial position.

The parameter n represents the degree of non-linearity of the constitutive equation: $n>0$ indicates stiffening of the material with increasing strains, whereas $n<0$ indicates softening. Note that the classical Neo-Hookean model is obtained for $n = 0$, with $G = G_0$.

Balance equations

The balance equations that were solved are conservation of momentum and mass for an incompressible solid:

$$\vec{\nabla} \cdot \sigma = \vec{0} \qquad (3.5)$$

$$J - 1 = 0 \qquad (3.6)$$

where J = det(**F**) represents the volume change between the undeformed, stress-free configuration and the deformed configuration.

Geometry and boundary conditions

The finite element mesh of the leaflets in the closed configuration is symmetric and, therefore, consisted of one half of a leaflet. At the symmetry edge, nodal displacements in the normal direction were suppressed. At the bottom (ventricular) side of the fixed edge, nodal displacements were suppressed in all directions. At the free edge, a contact surface was defined to model coaptation of adjacent leaflets. The radius of the leaflets was set to 12 mm. Pressure was applied to the top surface of the leaflets to model the applied diastolic transvalvular load. Subsequently, volumetric deformations were calculated.

Simulations - relation between applied load, deformation and mechanical properties

The computational model was employed to obtain the relationship between applied pressure, induced volumetric deformation and the mechanical properties of the loaded heart valve. The mechanical properties were defined as the product of thickness (t) and shear modulus (G_0) of the heart valve, and were used in different combinations as an input for the model. The range of these input parameters was chosen to cover experimental data from previous studies (Driessen et al., 2005a; Driessen et al., 2007) (table 3.1).

Table 3.1: *Range of input parameters for the computational model: shear modulus (G_0), thickness (t) and degree of non-linear material behavior (n) of the heart valve leaflets.*

	G_0 (MPa)	**t (mm)**	**n (-)**
input parameters	0.1 - 2.0	0.35 - 1.0	10

For every combination of thickness and shear modulus, physiological pressure differences, ranging from 0 to 15 kPa were applied to the valve and induced deformations were calculated. To acquire a relation in which every pressure-

deformation set will lead to one unique value for the mechanical properties, a polynomial function was fitted through the simulation results. The accuracy of the fit was investigated by plotting the fitted deformation values versus the numerically calculated deformation values.

Effect of inhomogeneity and anisotropy

The effects of inhomogeneity and anisotropy of the heart valve on the estimation of the mechanical properties were investigated. Inhomogeneity was simulated by choosing a gradient in shear moduli from the belly to the commissures of the valve. Three gradients were chosen in which the shear modulus ratio between the belly and commissures is equal to 2, 5 and 100. Modulus values increased from 1 to 2, 0.5 to 2.5 and 0.03 to 3 MPa. Consequently, for every gradient, the mean value of the shear modulus was kept constant at a physiological realistic value of 1.5 MPa. Thickness values were still assumed homogeneous and ranged between 0.35 and 1.0 mm.

To investigate the impact of anisotropy, a structurally-based fiber reinforced constitutive model (Driessen et al., 2005b) was used. The degree of anisotropy and other input parameters of the model corresponded to the parameters of tissue-engineered heart valves after two, three and four weeks of culturing in a previous study (Driessen et al., 2007).

3.2.2 Experimental set-up

To assess the mechanical properties of a cultured heart valve using the computational model, applied pressure and related deformation of the heart valve have to be measured. For this purpose, a previously developed bioreactor set-up was used (Kortsmit et al., 2009a).

Description of the bioreactor

The bioreactor system consisted of four main components: a proportional compressed-air valve, a pneumatic pump consisting of a cylinder in which a silicone rubber tube was placed, a bioreactor in which the valve was cultured, and a medium container (Fig. 3.1).

The whole system was filled with 150 ml of culture medium. The silicone rubber tube inside the polycarbonate cylinder, which functions as a pneumatic pump, was compressed and decompressed by pulsatile air pressure from the proportional valve. The amount, frequency and waveform of air release were controlled via a programmable multi-IO-card using LABVIEW software (National Instruments, USA). As a result, fluid from the container was injected into the upper chamber of the bioreactor, which caused a dynamic pressure difference over the heart valve. Consecutively, a fluid flow exited the bioreactor from both the lower and the upper chamber. Pressure sensors (P10EZ, BD, Franklin Lakes, NJ, USA) measured the pressure in both chambers. Two flow sensors (Transonic Systems Inc., USA) measured the flow in the tubing through which fluid entered the upper chamber (F_i) and left the lower chamber (F_o) of the bioreactor.

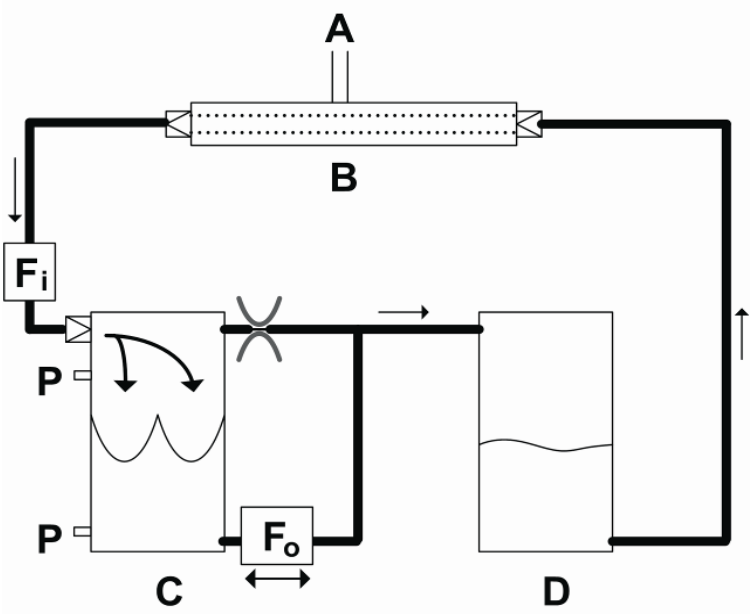


Fig. 3.1: *Schematic drawing of the bioreactor set-up. The bioreactor set-up consists of a compressed air supply (A), a pneumatic pump (B), a bioreactor (C), including two pressure sensors (P) and two flow sensors (F_i , F_o), and a medium container (D). Culture medium circulation is indicated by the arrows.*

Pressure and deformation measurement

During pulsatile load application, pressure and flow measurements were performed to assess the pressure difference over the heart valve and the resulting volumetric deformation of the heart valve leaflets. Pressure difference is defined as the pressure measured in the upper chamber of the bioreactor minus the pressure in the lower chamber. In addition, volumetric deformation is represented as the total amount of fluid displaced by the heart valve leaflets when load is applied, and can be distinguished from the leakage through the valve. Relative leakage is defined as the leak flow through the valve divided by the amount of fluid entering the bioreactor.

3.2.3 Tissue culture

To demonstrate and validate the assessment of mechanical properties of a heart valve in culture, six heart valves were tissue-engineered, using different conditioning protocols in two successive, but independent experiments. Two valves were cultured in the first experiment, while in the second experiment four valves were produced.

Heart valve scaffold

Stented heart valves were fabricated from anatomically shaped leaflets cut out of non-woven polyglycolic acid meshes (PGA; thickness 1.0 mm; specific density 70 mg/cm3; Concordia Manufacturing Inc, USA). The dimensions were based on

anatomical values measured in human specimens (Clark et al., 1974; Sauren et al., 1981; Thubrikar et al., 1990) and were applied in the computational model. The leaflets were coated with a thin layer of poly-4-hydroxybutyrate (P4HB; molecular weight: $1 \cdot 10^6$ Dalton; TEPHA Inc., Cambridge, MA) and molded in the shape of a trileaflet heart valve. Subsequently, they were attached to a rigid polycarbonate cylinder by sugar leaching of polycaprolactone (PCL; molecular weight: $8 \cdot 10^4$ Dalton; Sigma, USA).

Seeding procedure and mechanical conditioning

Cells harvested from the human vena saphena magna were expanded using regular cell culture methods (Schnell et al., 2001). After expansion, they were counted and seeded onto the scaffold. Cell densities ranged from 2.5 to 4.5 million (passage 7) per cm^3 scaffold. Higher cell densities did not improve the mechanical properties of the cultured tissue (unpublished results). Fibrin was used as a cell carrier (Mol et al., 2005b). The medium to culture these cells consisted of DMEM Advanced (Gibco, USA), supplemented with 10% Fetal Bovine Serum (FBS; PAN Biotech, Germany), 1% GlutaMax (Gibco, USA), and 0.1% gentamycin (PAN Biotech, Germany). The medium used for seeding and subsequent tissue culture contained 0.3% gentamycin and additional L-ascorbic acid 2-phosphate (0.25 mg/ml; Sigma, USA) to promote extracellular matrix production. Before seeding, the scaffolds were disinfected in 70% ethanol and, subsequently, immersed in culture medium for 24 hours to promote cell attachment. After seeding, the constructs were placed in the bioreactor system and subjected to culture medium circulation at low speed (4 ml/min) for 5-7 days to allow initial tissue development after seeding. Thereafter, dynamic pressure differences (at 1 Hz) were applied to the heart valve leaflets for 14-21 days (Fig. 3.2). Total culture time involved the time span in which both (low speed) medium circulation and dynamic loading were applied to the engineered valves.

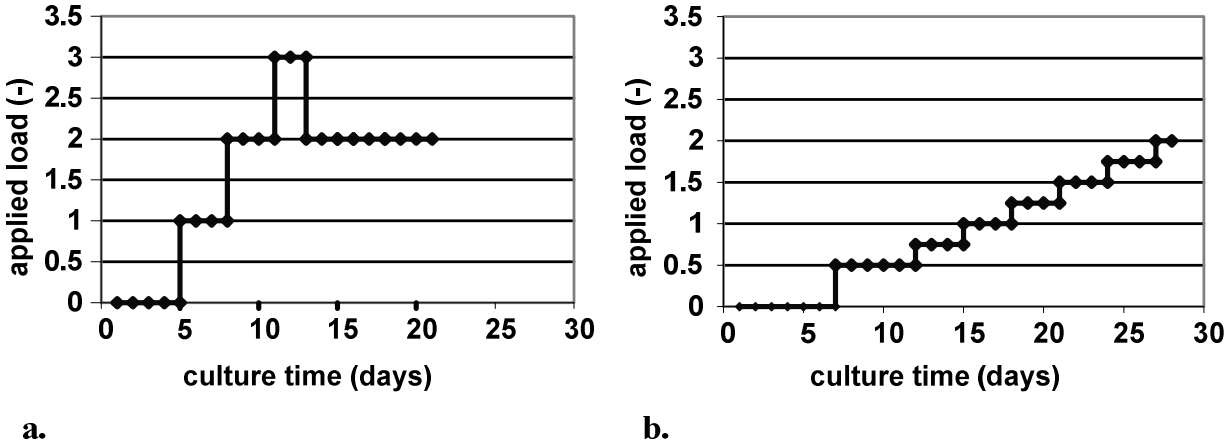


Fig. 3.2: *Conditioning protocol in which the applied load is given as a function of culture time for (a) tissue-engineered heart valves 1-1 and 1-2, and (b) valves 2-1 to 2-4.*

In the first experiment, dynamic load was applied stepwise for 9 days, increasing every 3 days. After 14 days, the applied load was decreased and kept constant at the level of day 8-11. Heart valves (valves 1-1, 1-2) were sacrificed after a total culture time of three weeks (Fig. 3.2a). In the second experiment, pressure was increased more gradually in relatively small three-day steps until the end of the experiment. Heart valve culture was finished after three weeks (valves 2-2, 2-4) and four weeks (valves 2-1, 2-3) (Fig. 3.2b).

Estimation of mechanical properties

After heart valve culture and just before sacrifice, tissue-engineered leaflets were subjected to increasing loads in the bioreactor set-up. Pressure differences and induced volumetric deformation values were measured to obtain a pressure-deformation (ΔP-ΔV) curve for each valve. This curve is representative for the mechanical properties of the leaflets. Using the method of least-squares, the curve was fitted to the relationship between applied load, deformation and mechanical properties, discussed in § 3.2.1, to obtain the product of shear modulus and thickness which corresponds to the best fit. Subsequently, the shear modulus of the heart valve was calculated by dividing this product by the average thickness of the heart valve.

Furthermore, correlation coefficients (R^2) were calculated that indicate the strength of the relation between the measured and predicted (fitted) pressure-deformation values. In the first experiment, the maximum pressure difference was equal to the applied pressure difference during the final stage of culturing (ΔP = 2.5 kPa). In the second experiment, a higher load was applied, trying to reach pressure values equal to physiological values (ΔP = 11 kPa).

Validation of mechanical properties assessment

To validate the assessment of the mechanical properties of the heart valve leaflets by the experimental-numerical method, cultured heart valves were sacrificed, cut into strips and uniaxial tensile tests were performed. For every valve, the strips were cut from the middle part of the leaflets (mainly belly region) in radial (n = 5) and circumferential (n = 5) direction, without including the leaflets' free or fixed edges.

First, thickness and width of the tissue samples were measured using a PLμ 2300 non-contact Confocal Imaging Profiler (Sensofar Tech S.L., Spain). After that, the tissue strips were tested until rupture at a constant strain rate (1/60 s^{-1}) using a tensile stage (Kammrath & Weiss GmbH, Germany) equipped with a 20N load cell. Force and elongation were measured during testing and were converted to Cauchy stress and strain, respectively. The slope of the resulting stress-strain curve in the first 0-15% strain range was defined as the tangent stiffness of the tissue specimen. For every valve, acquired stiffness values were analyzed for the circumferential and radial direction.

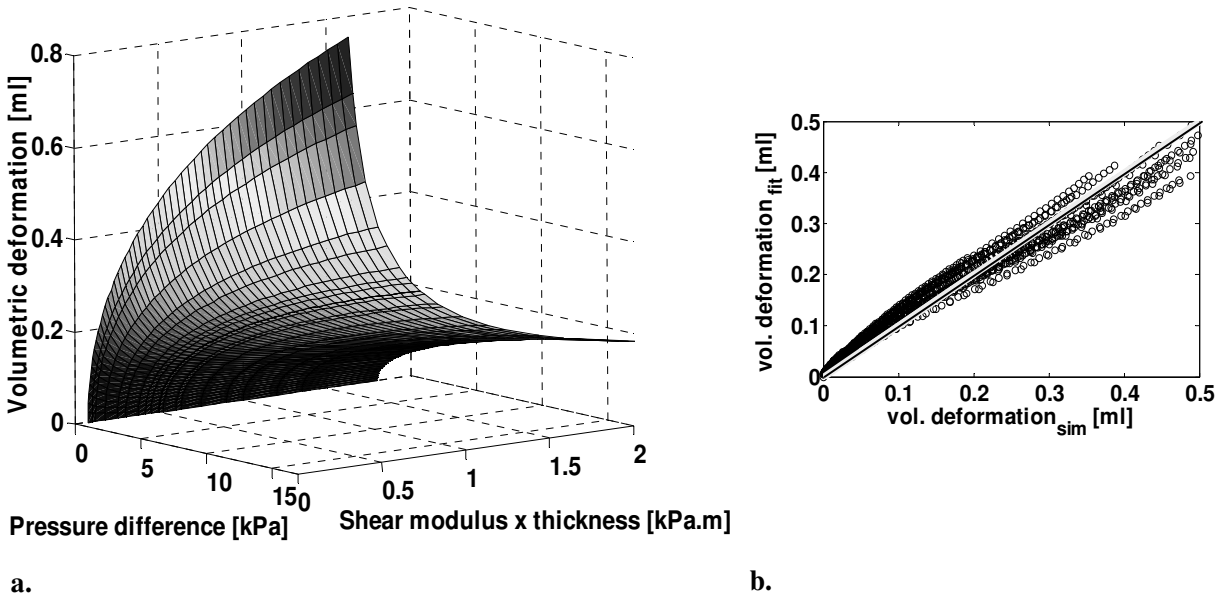
The uniaxial tensile test results, i.e. Young's moduli in radial and circumferential direction of the heart valve leaflets, were compared to the mechanical properties estimated by the non-invasive assessment method. Therefore, estimated shear modulus (G) values were rewritten to Young's modulus (E) values. For

incompressible materials and small strains, the Young's modulus (E) equals three times the shear modulus (G):

$$E = 3 \cdot G \qquad (3.7)$$

Statistics

The uniaxial tensile test data were not normally distributed and, therefore, tested non-parametrically and expressed as median ± 95% confidence interval. The Kruskal-Wallis test does not assume a normal population of the data and was applied for testing equality of population medians among groups. This test was performed to check whether the sample medians were significantly different at $p<0.05$. Data were analyzed using the Statgraphics Centurion XV software package.



a. **b.**

Fig. 3.3: *(a) Numerically obtained relation between the applied pressure difference [kPa] over the heart valve leaflets, induced deformation [ml] and mechanical properties [kPa·m], defined as shear modulus times thickness, of the valve. (b) Correspondence between deformation values calculated by the computational model and the deformation values obtained after fitting a surface through these simulation results. A perfect correspondence is indicated by the straight grey line through the origin with a slope equal to 1.*

3.3 Results

Simulations - relation between applied load, deformation and mechanical properties

The relation between the transvalvular pressure difference, the induced volumetric deformation and the mechanical properties of the valve leaflets, showed

that an increase in deformation at a constant transvalvular pressure difference resulted in a decrease in predicted stiffness of the valve, and vice versa (Fig. 3.3a). To obtain a smooth relation, the simulation data were described by a polynomial fit and the correspondence between the original and fitted data was shown in figure 3.3b.

In this figure, the straight line through the origin with a slope equal to 1 indicates a perfect resemblance between both data sets. It was shown that for small deformation values ($\Delta V<0.2$ ml) the fitted values were slightly overestimated while for larger deformations ($\Delta V>0.2$ ml) deformation values were underestimated.

Effect of inhomogeneity and anisotropy

The effect of inhomogeneity on the relation between applied load, induced deformation and mechanical properties of heart valve leaflets, was minimal for different degrees of inhomogeneity (Fig. 3.4).

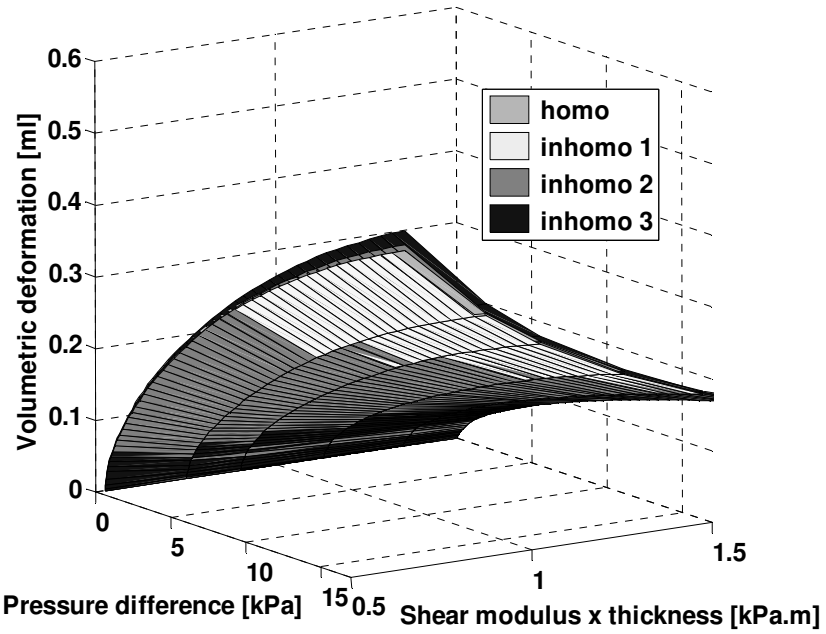


Fig. 3.4: *Numerically obtained relation between the applied pressure difference [kPa] over the heart valve leaflets, induced deformation [ml] and mechanical properties [kPa·m], defined as shear modulus times thickness, of the valve for four different degrees of inhomogeneity. Homogeneous material behavior is represented by 'homo', whereas 'inhomo1', 'inhomo2' and 'inhomo3' indicate the increasing degrees of inhomogeneity with shear moduli ranging from 1, 0.5 or 0.03 MPa in the belly to 2, 2.5 and 3 MPa in the commissures, respectively.*

The volumetric deformation was relatively insensitive to the degree of anisotropy for practical tissue engineering circumstances (Fig. 3.5). In particular, with anisotropies representative for two and three weeks of culture, the volumetric deformation was very close to the isotropic case. Anisotropy representative for four

weeks of culture did have a small effect. Especially for large pressure differences a small overestimation of the volumetric deformation was observed.

Estimation of mechanical properties

The measured pressure-deformation curves and their best fits were depicted in the same chart for every valve (Fig. 3.6 and Fig. 3.7). In the first experiment, it was shown that measured deformation values did not exceed 0.2 ml while the maximum applied pressure difference was equal to 2.5 kPa (Fig. 3.6). Furthermore, a good correlation between measured and fitted data was found for both valves (1-1 and 1-2), whereas the correlation coefficient for valve 1-2 was slightly higher than for valve 1-1. The relative leakage value was higher for valve 1-1 (10%) compared to valve 1-2 (7%) and the estimated mechanical properties indicated values of 0.28 and 0.22 kPa.m for valve 1-1 and 1-2, respectively (table 3.2).

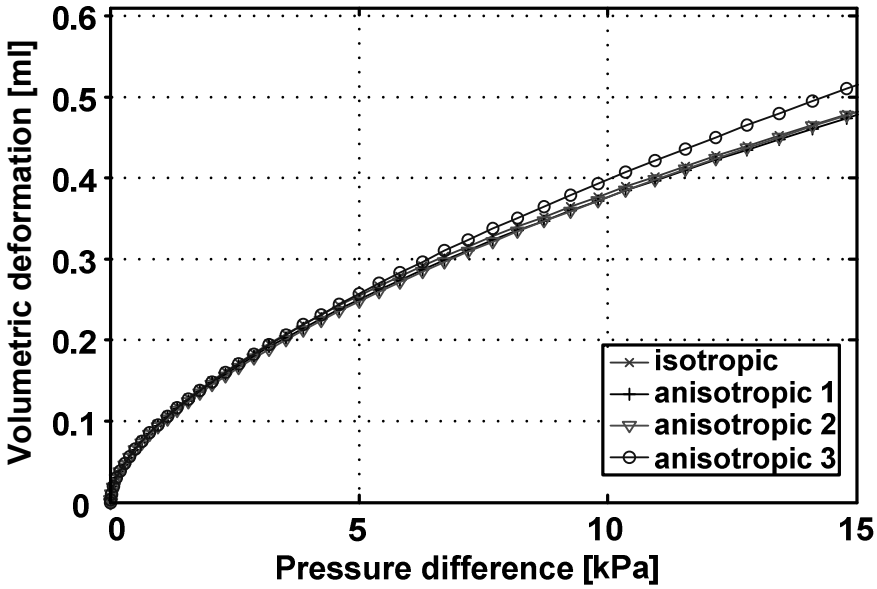


Fig. 3.5: *Relationship between applied pressure difference [kPa] and induced volumetric deformation [ml] of the heart valve leaflets, numerically obtained for four different degrees of anisotropy. Isotropic material behavior is represented by 'isotropic', whereas 'anisotropic1', 'anisotropic2' and 'anisotropic3' indicate the degrees of anisotropy estimated in a previous study in tissue-engineered heart valves after two, three and four weeks of culturing, respectively (Driessen et al., 2007).*

In the second experiment, physiological pressure differences (ΔP = 11 kPa), were imposed on three valves (2-1, 2-3 and 2-4), while for valve 2-2 physiological pressure buildup was not possible. Due to the high leak flow, the maximum applied load to valve 2-2 was only 5 kPa (Fig. 3.7).

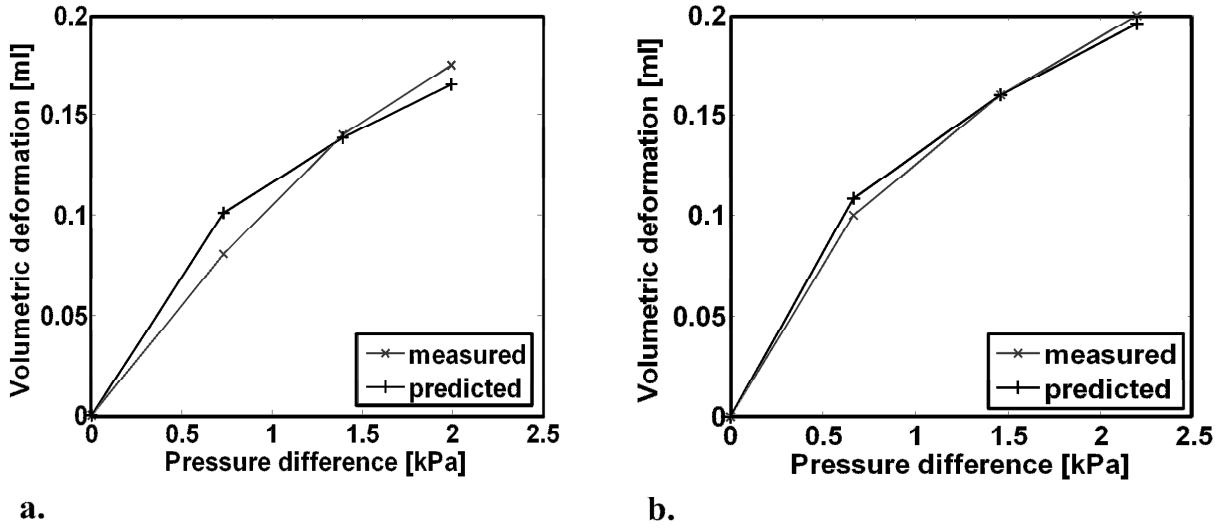


Fig. 3.6: *Measured and fitted (predicted) relationship, between applied pressure differences [kPa] and induced volumetric deformation [ml] of the cultured heart valve leaflets in the first tissue engineering experiment. Data were obtained after three weeks of culture and just before sacrifice for (a) valve 1-1 and (b) valve 1-2.*

Table 3.2: *Data obtained from the first (valves 1-1, 1-2) and second (valves 2-1 to 2-4) tissue engineering experiment. The relative leakage of the valves (% leakage), the estimated values of the product of shear modulus and thickness (G·t) and the estimation correlation coefficients (R^2) were assessed just before, and the mean thickness values after sacrifice of the heart valves. Thereafter, Young's modulus values were calculated from the estimated G·t and thickness values.*

valve	%-leakage	correlation coefficient R^2	estimated G.t (kPa·m)	mean thickness (mm)	Young's modulus (MPa)
1-1	10	0.97	0.28	0.87	0.97
1-2	7	0.99	0.22	0.86	0.77
2-1	9	0.98	0.67	0.90	2.2
2-2	32	0.88	-	0.80	-
2-3	0	0.98	0.46	0.70	2.0
2-4	1	0.99	0.36	0.83	1.3

In addition, the correlation between measured and predicted ΔP-ΔV curves was high for these three valves and relatively low for valve 2-2. Relative leakage values varied between 0% (valve 2-3) and 32% (valve 2-2). As seen in our previous study

(Kortsmit et al., 2009a), volumetric deformation measurements were not accurate when relative leakage values exceeded 25%. Consequently, a reliable estimation of the mechanical properties of valve 2-2 could not be made. In conclusion, the estimated product of shear modulus and thickness was equal to 0.67, 0.46 and 0.36 kPa·m for valves 2-1, 2-3 and 2-4, respectively (table 3.2).

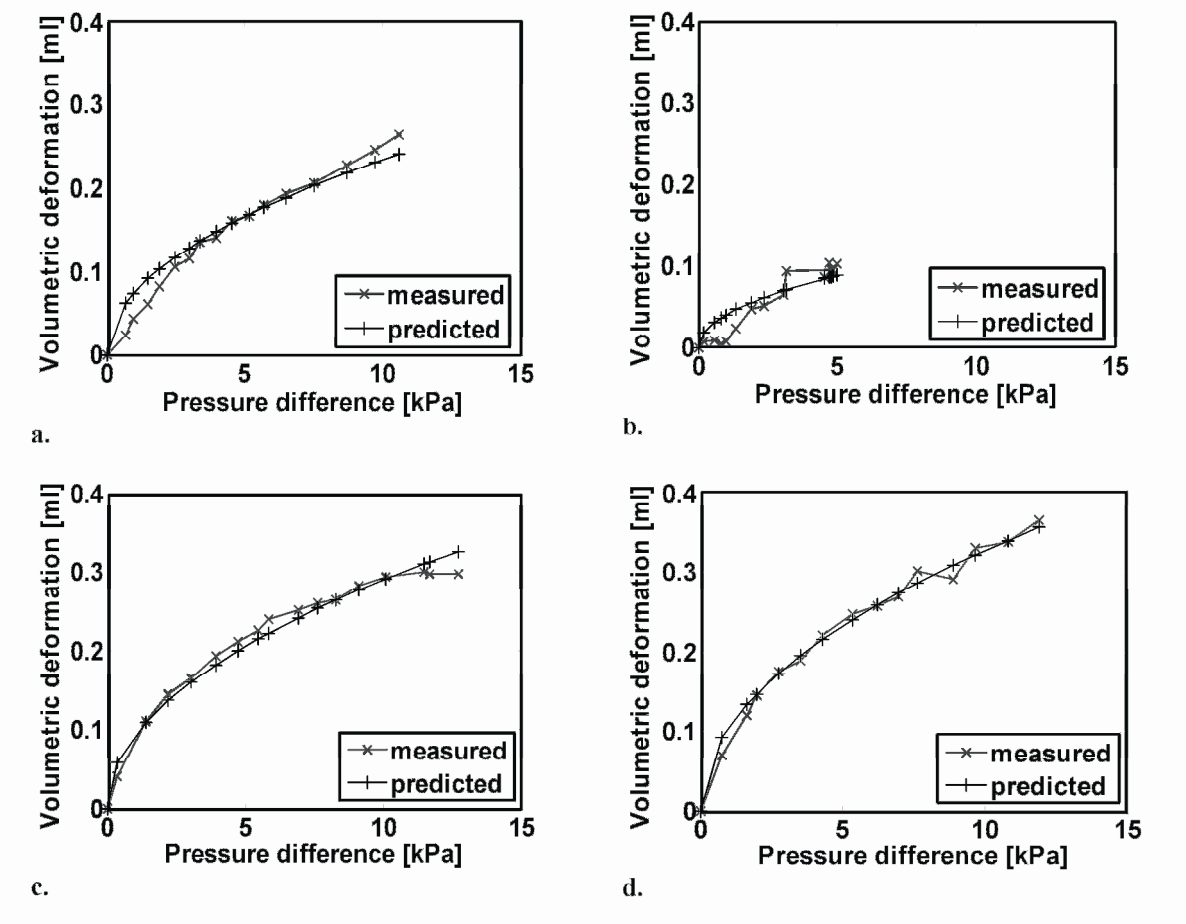


Fig. 3.7: *Measured and fitted (predicted) relationship between applied pressure differences [kPa] and induced volumetric deformation [ml] of the cultured heart valve leaflets in the second tissue engineering experiment. Data are obtained just before sacrifice after three weeks of culture for (b) valve 2-2 and (d) valve 2-4, and after four weeks of culture for (a) valve 2-l and (c) valve 2-3.*

Validation of mechanical properties assessment

Estimated mechanical properties of the cultured valves were compared with the data obtained from the uniaxial tensile tests. Young's moduli of valves 1-1 and 1-2, cultured in the first experiment, had estimated values equal to 0.97 and 0.77 MPa, respectively (table 3.2). In the second experiment, the cultured heart valves had higher estimated Young's moduli, ranging from 1.3 MPa (valve 2-4) to 2.2 MPa (valve 2-1) (table 3.2). For all valves except valve 2-2, the magnitude of the estimated stiffness was between the stiffness values obtained for the radial and circumferential direction

(Fig. 3.8). Significant differences ($p<0.05$) in Young's moduli were found between the radial and circumferential direction of tissue-engineered heart valves 1-2, 2-1, 2-3 and 2-4.

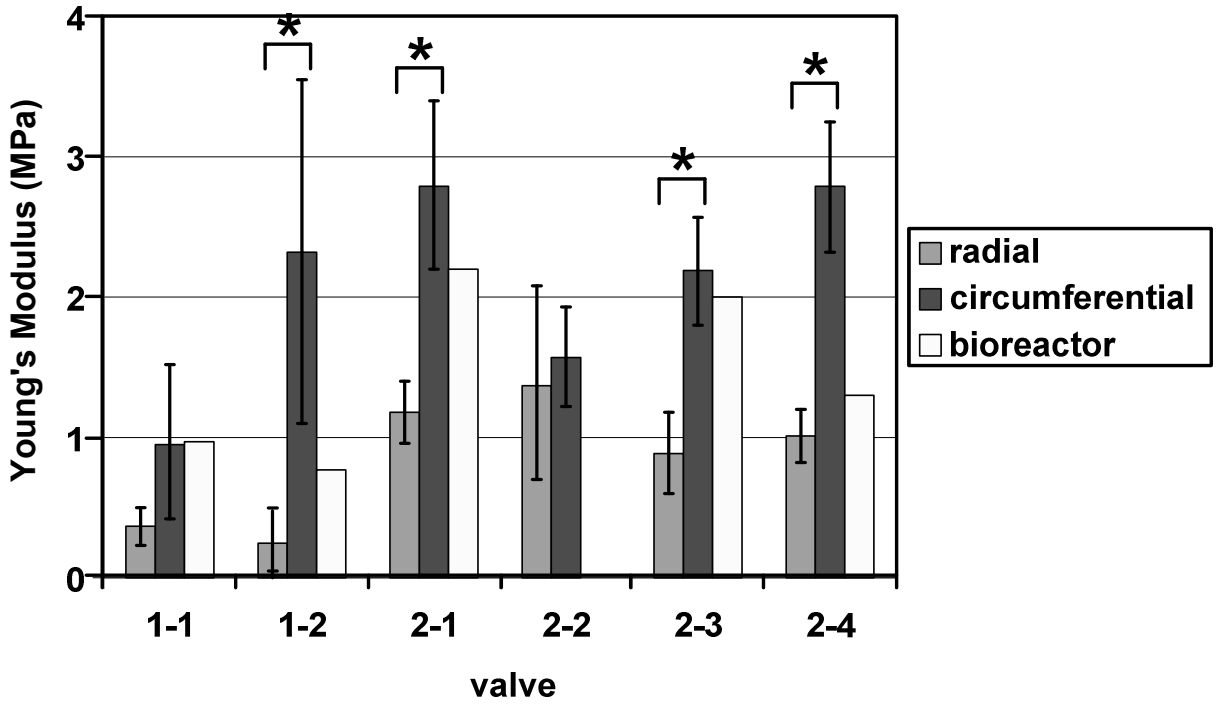


Fig. 3.8: *Young's modulus values [MPa] of valves 1-1, 1-2 and valves 2-1 to 2-4. The medians of the Young's moduli in radial and circumferential directions are represented. The error bars indicate the 95% confidence intervals of the data. Statistically significant differences ($p<0.05$) are marked with an asterisk. The bioreactor values represent the Young's moduli assessed by the inverse experimental-numerical estimation method.*

3.4 Discussion

In heart valve tissue engineering, the stiffness and strength of cultured heart valves still need additional improvement to reach mechanical characteristics and tissue composition of native aortic valves. One approach to enhance these parameters may be the application of well defined mechanical stimulation to the developing tissue. Tissue formation and development in cultured heart valves in response to mechanical loading have been evaluated in different ways. Functionality of the engineered constructs has been studied in bioreactor systems in which the physiological environment of the tissue is simulated (Schenke-Layland et al., 2003; Mol et al., 2006; Schmidt et al., 2007a, 2007b). Moreover, tissue quality has mainly been studied qualitatively or quantitatively by histology or biochemical assays, respectively (Sodian et al., 2000; Zeltinger et al., 2001; Rabkin et al., 2002; Schenke-Layland et al., 2003; Mol et al., 2006; Stock et al.,

2006; Flanagan et al., 2007; Schmidt et al., 2007a, 2007b). Additionally, in order to elucidate the effect of load application on the mechanical characteristics of the tissue-engineered construct, mechanical behavior of heart valve tissue has been evaluated by performing uniaxial or biaxial tensile tests or indentation tests (Hoerstrup et al., 2000b; Schenke-Layland et al., 2003; Mol et al., 2006; Schmidt et al., 2007a, 2007b). Unfavorably, these methods are destructive and can, therefore, only be performed at the end-stage of tissue culture. To study the development of mechanical properties of the tissue-engineered heart valve during culture, it is desired to monitor and control these properties in real-time and non-invasively. Hence, the objective of this study is to develop a bioreactor system in which engineered heart valve tissue is mechanically stimulated and in which, during and after culturing, the mechanical properties of the valve leaflets are assessed non-invasively.

The assessment of mechanical properties was performed by an inverse experimental-numerical approach. The relationship between the applied pressure differences, induced volumetric deformations and mechanical behavior was described by a polynomial function fit through the deformation values calculated by the model. Good similarity was found between the simulated and fitted volumetric deformation values. The minor deviations observed were probably due to the relatively large influence of thickness on the flexural behavior of the leaflets when the valve deforms. This phenomenon has been observed in particular for large deformations and has also been seen in our previous study (Kortsmit et al., 2009a). The effect of inhomogeneity on the mechanical properties estimation method was very small. The maximum gradient in shear moduli was varied by a factor 100 (0.03 MPa in the belly region versus 3 MPa in the commissures) and resulted in a negligible deviation with respect to the homogeneous shear modulus distribution. The minor effect of inhomogeneity was ascribed to the type of deformation that was considered. Overall deformation of the heart valve leaflets was used and, therefore, local stiffness differences in the leaflets were counterbalanced. This resulted in average mechanical properties that remained constant. The same phenomenon was observed for the effect of anisotropy. However, anisotropy had a larger influence on the estimation of mechanical properties. The highest degree of anisotropy, which was equal to the degree of anisotropy found in tissue-engineered valves after four weeks of culture (Mol et al., 2005a), resulted in a small overestimation of volumetric deformation values. The insensitiveness of the mechanical properties estimation method for changes in homogeneity or isotropy in the heart valve leaflets implies that the degree of inhomogeneity or anisotropy cannot be assessed using this estimation method.

In this study, six heart valves were tissue-engineered of which the mechanical properties were estimated by using the measured pressure-deformation (ΔP-ΔV) relation. In the first experiment, the ΔP-ΔV curves of both valves were acquired by applying pressure differences in a relatively small range (ΔP_{max} = 2.5 kPa), while in the second experiment the maximal applied pressure difference was physiological (ΔP_{max} = 12 kPa). The latter procedure is favorable in the estimation of mechanical properties, because more data points increase the accuracy of the fitting procedure. Furthermore,

load application up to physiological values provides a quality control to examine whether the cultured heart valve leaflets can sustain in-vivo aortic pressure differences.

The estimated mechanical properties of the engineered heart valve leaflets were validated using uniaxial tensile test data acquired in two orthogonal directions. However, uniaxial loading is non-physiological, the applied computational model is isotropic and, therefore, it was not necessary to perform biaxial tensile tests to validate the estimation method. In addition, tissue-engineered heart valves showed visco-elastic behavior after culture. However, the influence of preconditioning on the tensile test results was relatively small. The variation in stiffness values due to the presence or absence of preconditioning was smaller than the variation in stiffness values within a tissue-engineered heart valve. For this reason, preconditioning was not performed. Good agreement was found between both mechanical properties assessment methods when relative leakage values did not exceed 10%. This was also confirmed by the values of the correlation coefficients of the fitting procedure. The coefficients did not have values smaller than 0.97, except for valve 2-2 (R^2 = 0.88). An accurate Young's modulus estimation of this valve was not possible because its relative leakage value (32%) exceeded the leakage value (25%) above which volumetric deformation can not be measured correctly (Kortsmit et al., 2009a). Consequently, a relative leakage of 10% (valve 1-1) is still acceptable for the estimation of mechanical properties, but 32% (valve 2-2) leakage is not. The high leakage value of valve 2-2 was caused by the inappropriate fabrication of the scaffold of this valve. After culture, the valve leaflets themselves were intact but the attachment line between one of the leaflets and the polycarbonate wall was leaking. This leakage along the wall hindered pressure build-up, deformation measurement and, therefore, estimation of mechanical properties. Moreover, for all valves, except valve 2-2, estimated stiffness values were larger than the experimentally obtained radial tensile test values and smaller than circumferential values. This can be explained by the use of overall, volumetric deformation values for the estimation method by which local stiffness differences are evened out.

In this study, the first 0-15% strain range of the measured stress-strain curves was applied to assess Young's moduli of the radial and circumferential oriented heart valve tissue strips. This is in contrast to previous studies in which no strain range was defined but the steepest part of the stress-strain curve was chosen to determine the mechanical properties (Mol et al., 2003, 2005a, 2006; Driessen et al., 2007; Schmidt et al., 2007a, 2007b). However, maximum volumetric deformation of the cultured heart valve leaflets under physiological loading did not exceed 0.35 ml (valve 2-4), which corresponds to a mean strain value of approximately 7% in the leaflets (Kortsmit et al., 2009a). Therefore, the mechanical behavior of the cultured tissue is best characterized by a Young's modulus assessed in the lower 0-15% strain region of the stress-strain curve, which encompasses in-vivo-deformation.

Functionality of the cultured heart valve leaflets, except for valve 2-2 was shown by the small leakage values (<10%) and by the sustained physiological diastolic pressure differences (valves 2-1, 2-3 and 2-4). Furthermore, tissue-engineered heart valves examined by uniaxial tensile tests showed some development of anisotropy but did not exhibit the highly anisotropic and non-linear mechanical behavior seen in

native human aortic valves (Clark et al., 1973; Stradins et al., 2004; Balguid et al., 2007a). Young's moduli of the tissue-engineered valves were compared to native human aortic values (Balguid et al., 2007a) and appeared to be similar in radial direction (varying between 75% of native value for valve 1-1 to 112% for valve 2-4), but considerably smaller in circumferential direction (from 18% for valve 2-2 to 31% for valve 1-2). To be able to make this comparison, Young's moduli were not only determined from the lower 0-15% strain region of the obtained strain-stress curves, but also from the steepest part.

3.5 Conclusion

The proposed inverse experimental-numerical estimation method allows the assessment of overall mechanical properties of tissue-engineered heart valve leaflets in real-time, non-invasively and non-destructively, during and after culturing. Therefore, this method can serve as a real-time, non-invasive and non-destructive functionality and quality check and can be used to monitor and control tissue remodeling over time.

3.6 Acknowledgements

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Chapter 4

Deformation controlled load application in heart valve tissue engineering

The contents of this chapter are based on J. Kortsmit, M.C.M. Rutten, M.W. Wijlaars, and F.P.T. Baaijens (2009), *Deformation controlled load application in heart valve tissue engineering*, Tissue Engineering Part C Methods, Mar 10, Epub ahead of print.

4.1 Introduction

Heart valve tissue engineering is a promising, alternative technology to overcome the disadvantages associated with currently used heart valve replacements, such as lack of growth, repair, and remodeling capacities. At present, one of the main challenges is to culture a valve with sufficient mechanical integrity to replace a natural aortic valve. Cultured heart valves showed good functionality when implanted in the pulmonary position in sheep (Sodian et al., 2000; Hoerstrup et al., 2000b). In addition, human valves having sufficient mechanical strength for implantation in the aortic position have been cultured (Mol et al., 2006). However, compared to native aortic valves the tissue-engineered heart valves were stiffer at infinitesimal strain, less stiff at finite strains, and the degree of anisotropy was smaller. Therefore, enhancement of the mechanical properties is still desired.

Various studies showed that mechanical stimulation of tissue-engineered cardiovascular constructs improved tissue properties, although a complete understanding of the effect of loading on tissue development has not been established yet. To study the influence of specific conditioning protocols on tissue formation, the applied load should be well-defined and controlled. However, since the mechanical properties of a tissue-engineered construct change during culture, a preset loading amplitude does not necessarily induce a constant tissue deformation. Therefore, it is important and desired to monitor and control both the applied load and the tissue deformation during tissue culture.

In literature, the effect of mechanical conditioning on tissue formation and development was studied by using relatively simple, well defined (2D) configurations, such as strips, squares and discs (Kim et al., 1999; Mitchell et al., 2001; Mol et al., 2003; Isenberg et al., 2003; Butcher et al., 2006; Engelmayr et al., 2006, 2008; Ferdous et al., 2008; Boerboom et al., 2008; Rubbens et al., 2009). In these studies, load application was controlled and generally strain-based, being either uniaxial (Kim et al., 1999; Mol et al., 2003; Ferdous et al., 2008; Boerboom et al., 2008; Rubbens et al., 2009) or biaxial (Mitchell et al., 2001; Butcher et al., 2006). Next to cyclic strain, Engelmayr et al. (2006, 2008) applied cyclic flexure and laminar flow to the tissue-engineered constructs. The controlled application of cyclic strains to the relatively simple (2D) tissue engineering constructs induced predefined and constant tissue deformations.

Fewer studies focused on mechanical conditioning effects in a more complex (3D) geometry, like a heart valve (Hoerstrup et al., 2000b; Zeltinger et al., 2001; Rabkin et al., 2002; Schenke-Layland et al., 2003; Mol et al., 2005a; Sodian et al., 2006; Stock et al., 2006; Flanagan et al., 2007; Syedain et al., 2008; Syedain & Tranquillo, 2009). In most of these studies, heart valves were conditioned in bioreactor systems that mimic physiological flow (Hoerstrup et al., 2000a; Zeltinger et al., 2001; Rabkin et al., 2002; Schenke-Layland et al., 2003; Sodian et al., 2006; Stock et al., 2006; Flanagan et al., 2007). Mechanical loading was induced by simulation of the complete cardiac cycle, mimicking both systole as well as diastole, or by simulation of the systolic phase only. In these studies, the induced tissue deformation was unknown,

and neither monitored nor controlled. Others have engineered heart valves with a strain-based loading protocol in closed configuration, mimicking the diastolic phase of the cardiac cycle (Mol et al., 2005a, 2006; Syedain et al., 2008; Syedain & Tranquillo, 2009; Kortsmit et al., 2009a, 2009b). A pressure difference was applied across the engineered heart valve leaflets, inducing local strains. Mol et al. (2005a) estimated after culture the induced deformations by fitting the stress-strain curves obtained from uniaxial tensile tests of the cultured heart valve in a computational model. Syedain & Tranquillo (2009), however, measured the stretching of the tissue-engineered valve root in real-time during culture. In a previous study, we showed the monitoring of deformation of tissue-engineered heart valve leaflets during mechanical loading, in real-time, and non-invasively (Kortsmit et al., 2009a). A combined experimental-numerical approach was applied to assess volumetric and local leaflet deformation of the cultured heart valve in diastolic configuration. However, the applied load was predefined and tissue strains were only measured rather than controlled.

In this study, this experimental-numerical approach was developed further. A feedback control loop to regulate tissue deformation was incorporated into the bioreactor system. The functionality of this approach was demonstrated in a heart valve tissue engineering experiment.

4.2 Materials and Methods

4.2.1 Experimental set-up

Description of the bioreactor

To assess the volumetric deformation of a tissue-engineered heart valve during culture, a previously developed bioreactor set-up was used (Kortsmit et al., 2009a, 2009b). The complete bioreactor set-up (Fig. 4.1) consisted of four independent bioreactor systems, positioned in a humidified incubator (37°C, 5% CO_2) (A). A roller pump (Masterflex®, Cole-Parmer, Vernon Hills, IL, USA) was employed for slow medium circulation (not shown). Proportional pneumatic valves (Festo, Esslingen Berkheim, Germany) (B) were used for dynamic load application, and the pressure, frequency and time-dependency of air release were controlled via a programmable multi-IO-card using LABVIEW software (National Instruments, Austin, TX, USA) (C). In addition, measurements were performed with flow (Transonic Systems Inc., Ithaca, NY, USA) and pressure sensors (P10EZ, BD, Franklin Lakes, NJ, USA), and corresponding amplifiers (D, E).

Each system (Fig. 4.2) enclosed a single cultured heart valve and was composed of the following components; a proportional pneumatic valve (A), a pulsatile pump encompassing a flexible silicone rubber tube (B), a bioreactor in which the valve was cultured (C) and a culture medium container (D) (Kortsmit et al., 2009a, 2009b). In the medium container, an open connection to the surroundings was created in which a sterile filter (0.2 μm, Schleicher & Schuell Bioscience, Dassel, Germany) was placed to oxygenate the circulating culture medium. The whole system was filled with 150 ml of

culture medium, the composition of which will be discussed in § 4.2.2. By cyclic inflation and deflation of the pump by the proportional valve, fluid from the culture medium container was injected into the upper chamber (c1) of the bioreactor, causing a dynamic pressure difference over the heart valve. Consequently, fluid flow exited the bioreactor from both the lower (c2) and the upper chamber (c1). Pressure sensors (P) measured the pressure in both chambers. A flow sensor (F) measured the lower chamber exit flow rate (Fig. 4.2b).

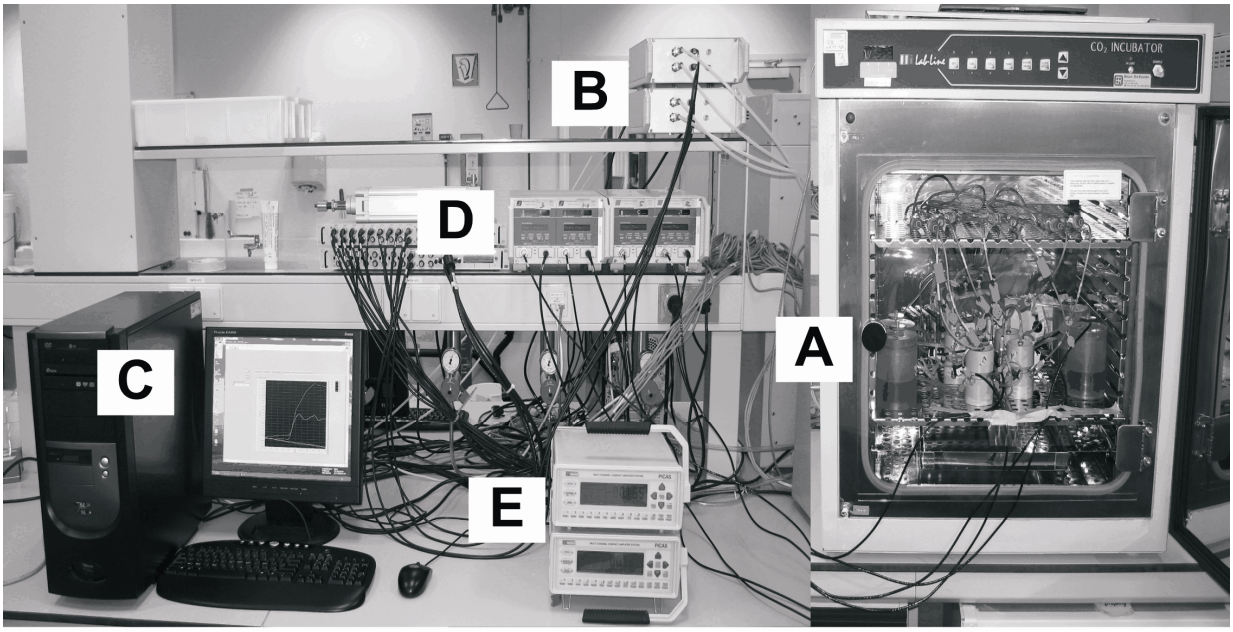
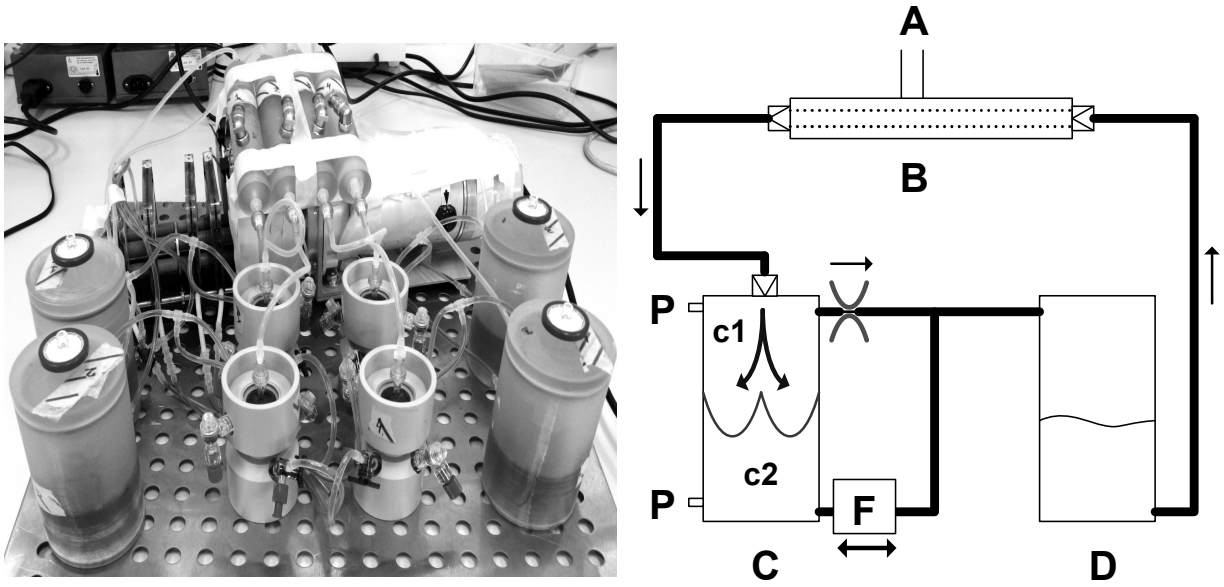


Fig. 4.1: *Complete bioreactor set-up, including four bioreactor systems in the incubator (A), proportional pneumatic valves (B), pc hard- and software (C), flow (D) and pressure (E) amplifier modules.*

Deformation and leakage measurement

During pulsatile load application, flow measurements were performed to assess the volumetric deformation and leakage of the heart valve leaflets (Kortsmit et al., 2009a). Volumetric deformation was defined as the amount of fluid exiting and subsequently reentering the lower chamber of the bioreactor in a single loading cycle. The net flow leaving the lower chamber was defined as the amount of fluid leaking through the valve. Volumetric deformation and leakage of the cultured valve were obtained after time-integration of the lower chamber exit flow (Fig. 4.3).Leak flow values were used as an indication of valve functioning and were assessed to check the accuracy of the deformation measurement method, which is known to decrease as leak flow increases (Kortsmit et al., 2009a).



a. **b.**

Fig. 4.2: *(a) Picture of four bioreactor systems. (b) Schematic drawing of a single bioreactor system, consisting of a pneumatic valve (A), a pulsatile pump (B), a bioreactor (C), consisting of an upper (c1) and lower (c2) chamber, and a medium container (D). In addition, two pressure sensors (P) and a flow sensor (F) were connected to the bioreactor (C).*

Deformation control

To apply controlled diastolic deformation to the cultured heart valves, the magnitudes of the applied loads were adjusted in such a way that prescribed deformation was induced in the tissue-engineered heart valve leaflets. Desired strains were obtained in the cultured valves by implementation of a deformation control loop in the bioreactor software, which controlled the pulsatile pump. The feedback mechanism consisted of a proportional–integral–derivative (PID) controller (Liptak, 1995). The PID controller corrects the difference or error (e(t)) between the measured and the prescribed volumetric deformation value at the end of each loading cycle by calculating and subsequently generating a corrective action (controller output) that adjusts the process. The PID controller is described by:

$$y(t) = \left(K_p \cdot e(t)\right) + K_i \int_0^t e(\tau) d\tau + K_d \frac{de}{dt} \qquad (4.1)$$

in which y(t) is the controller output, e(t) is the error in the volumetric deformation value, and K_p, K_i and K_d are the proportional, integral and derivative feedback control gains, respectively. These PID controller parameters were tuned manually to obtain the optimum values for the desired control response.

The controller output influenced the output of the process, i.e. volumetric deformation of the heart valve leaflets, until the difference between the measured and reference deformation value, i.e. the error value (e(t)), was negligible. The relative error values were determined during culture and averaged for every cultured heart valve. This value is a measure for the accuracy of the deformation control. After each loading period, the volumetric deformation of the heart valve was assessed by integration of the measured flow signal. Since the corrective action of the feedback mechanism was determined by the difference between the obtained and the reference deformation value, load application was controlled once every loading period. Therefore, the shape of the induced deformation signal within a loading period was not controlled, only its maximum value.

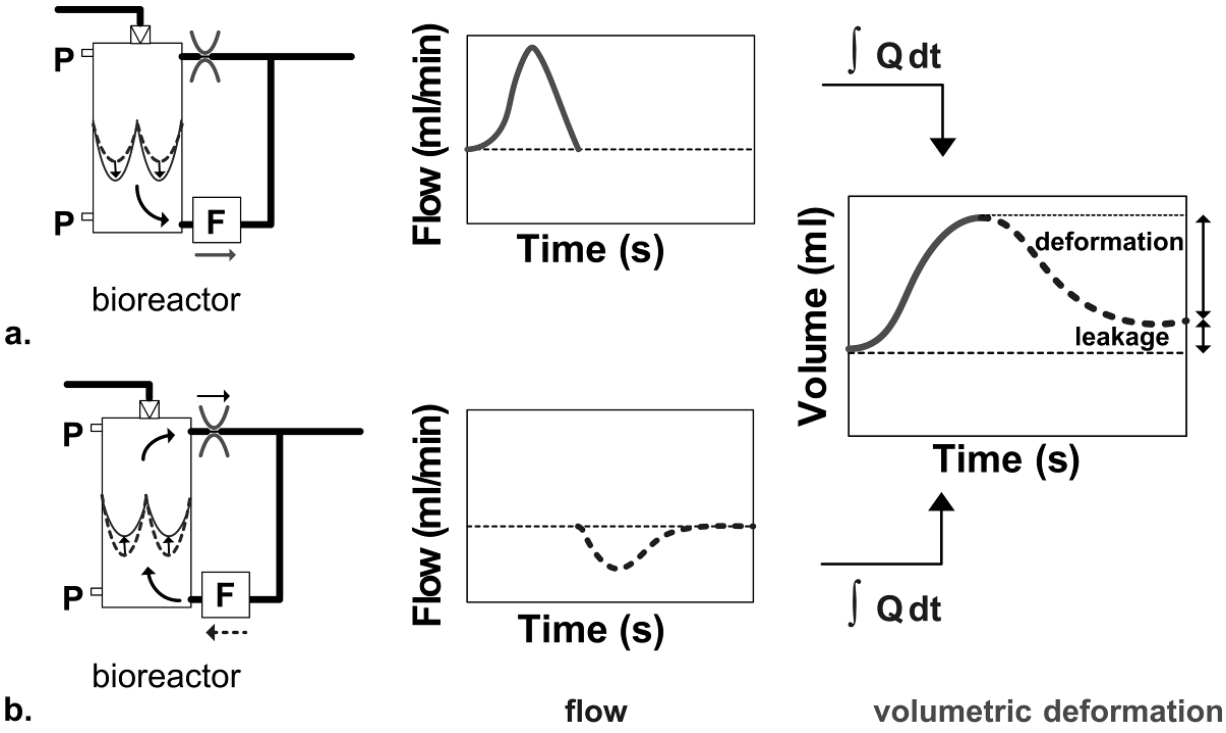


Fig. 4.3: *Flow-based deformation measurement principle. (a) A pressure difference over the heart valve causes the leaflets to bulge down; fluid exits the bioreactor (solid line). (b) After deformation, the leaflets return to their undeformed state; fluid reenters the bioreactor (dashed line). By time-integration of the flow signals, volumetric deformation is distinguished from leakage.*

4.2.2 Heart valve culture

A total of eight heart valves was cultured in two independent experiments (I and II). In each experiment four heart valves (A, B, C and D) were cultured by applying two different mechanical conditioning protocols. So, in each experiment two valves were made according to each protocol.

Heart valve scaffold

Heart valves were made of anatomically shaped leaflets and heart valve wall, cut out of non-woven polyglycolic acid meshes (PGA; thickness 1.0 mm; specific density 70 mg/cm^3; Concordia Manufacturing Inc, Coventry, RI, USA). The dimensions were based on anatomical values measured in human specimens (Clark et al., 1974; Sauren, 1981; Thubrikar, 1990). The leaflets and wall were coated with a thin layer of poly-4-hydroxybutyrate (P4HB; molecular weight: 1x10^6 Dalton; TEPHA Inc., Cambridge, MA, USA*)* and molded in the shape of a trileaflet heart valve. In experiment II, the focus of this molding process was to minimize the open areas between the leaflets at the commissural regions, while no extra attention was paid to that in experiment I. Subsequently, the heart valve wall was coated with porous polycaprolactone (PCL; molecular weight: 8x10^4 Dalton; Sigma, St. Louis, MO, USA) by sugar leaching, and was bonded with tetrahydrofuran (THF; Merck, Whitehouse Station, NJ, USA) to the inside of a rigid polycarbonate cylinder.

Seeding procedure

Cells harvested from the human vena saphena magna were expanded using regular cell culture methods (Schnell et al., 2001). Characterization of the cells was performed previously (Mol et al., 2006). Cells showed expression of vimentin, but showed no expression of desmin. A subpopulation showed expression of α-smooth muscle actin, which characterized the cells as myofibroblasts (Rabkin-Aikawa et al., 2004). After expansion, they were counted and seeded onto the scaffold in cell densities ranging from 4.0 to 5.5 million cells (passage 7) per cm^2 scaffold. Fibrin was used as a cell carrier (Mol et al., 2005b). The medium to grow these cells consisted of DMEM Advanced (Life Technologies, Carlsbad, CA, USA), supplemented with 10% Fetal Bovine Serum (FBS; PAN Biotech, Aidenbach, Germany), 1% GlutaMax (Life Technologies, Carlsbad, CA, USA), and 1% PEN-STREP (Lonza, Basel, Switzerland). The medium used for seeding and subsequent tissue culture contained additional L-ascorbic acid 2-phosphate (0.25 mg/ml; Sigma, St. Louis, MO, USA) to promote extra cellular matrix production (Hoerstrup et al., 1999). During tissue culture, medium was replaced twice a week. Before seeding, the scaffolds were disinfected in 70% ethanol and, subsequently, immersed in tissue culture medium for 24 hours to promote cell attachment.

Mechanical conditioning protocol

After seeding, the heart valve constructs were placed in the bioreactor system and subjected to culture medium circulation at low speed (4 ml/min) for 12 days to allow initial tissue development. Thereafter, dynamic pressure differences (at 1 Hz) were applied to the heart valve leaflets for 16 days. Total culture time included the application of (low speed) medium circulation as well as the application of dynamic loads to the engineered valves. Two different mechanical conditioning protocols were applied to the tissue-engineered valves in both experiments (Fig. 4.4).

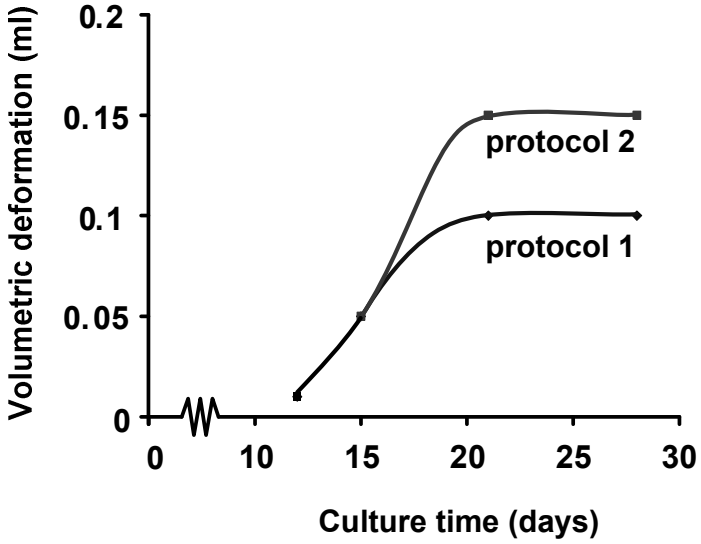


Fig. 4.4: *The two mechanical conditioning protocols applied to the tissue-engineered heart valves. The lower deformation profile represents protocol 1 and the upper profile represents protocol 2.*

Load application was deformation controlled, as described in § 4.2.1 (Deformation control). The magnitude of the dynamic loads, so the output of the proportional pneumatic valves was regulated in such a way that increasing leaflet deformation values were induced from day 13 to 21 of tissue culture. At day 21, volumetric deformation was kept constant at values of 0.1 ml (protocol 1) and 0.15 ml (protocol 2) until the end of culture. In both experiments, protocol 1 was applied to heart valves A and B and protocol 2 to valves C and D.

To determine the accuracy of the deformation control mechanism, relative error values were calculated by:

$$rel.error = \left| \frac{\Delta V_{measured} - \Delta V_{prescribed}}{\Delta V_{prescribed}} \right| \times 100\,\% \qquad (4.2)$$

in which $\Delta V_{measured}$ is the measured volumetric deformation, and $\Delta V_{prescribed}$ the prescribed volumetric deformation. The relative error values were obtained during controlled load application and were averaged for every valve.

The volumetric deformation values of 0.1 and 0.15 ml corresponded to local strain values in the commissural region of the valve equal to 3% and 5%, respectively (Kortsmit et al., 2009a). Since a beneficial effect of 4% straining on tissue development was observed in a previous study and application of higher strains possibly introduced damage to the cultured tissues (Boerboom et al., 2008), prescribed volumetric deformation values were based on local strains in the commissural region. In this region of the heart valve, local strain values are highest during diastolic loading. Finally, heart valves were sacrificed after a total culture time of four weeks.

Mechanical characterization

To assess the functionality and overall mechanical properties of the tissue-engineered heart valve leaflets at the end of culture, leaflets were subjected to increasing loads in the bioreactor set-up, up to physiological values (ΔP = 13 kPa). Functionality was defined as the ability of the cultured valves to sustain these aortic diastolic pressure differences without failure and with acceptable leakage (<5 ml/min). To estimate the mechanical properties, pressure differences and induced volumetric deformation values were measured to obtain a pressure-deformation (ΔP-ΔV) curve for each valve. The ΔP-ΔV curve was representative for the overall mechanical properties of the leaflets, estimated by an inverse experimental-numerical method (Kortsmit et al., 2009b). Since local leaflet deformations could not be measured, and were even counterbalanced by measurement of the volumetric deformation, the estimation method is not suitable to assess either local mechanical properties, or anisotropic characteristics of the cultured heart valve leaflets. Additionally, the accuracy of the inverse experimental-numerical estimation method showed to be relatively insensitive for changes in homogeneity or isotropy in the tissue-engineered leaflets (Kortsmit et al., 2009b).

To elucidate anisotropic properties, heart valve leaflets were cut into strips (length = 10 mm, width = 1.7 - 2 mm; area = 17 - 20 mm^2) after sacrifice and mechanical properties were determined in both radial and circumferential directions by uniaxial tensile tests (Fig. 4.5). The strips were cut from the middle part of the leaflets (mainly belly region) in radial (n = 6) and circumferential (n = 6) direction, without including the free or fixed edges of the leaflets.

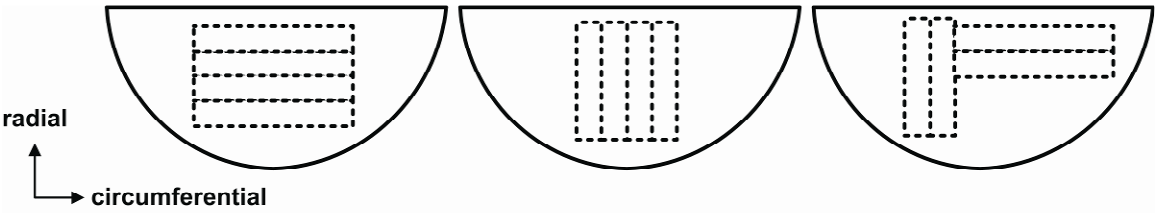


Fig. 4.5: *Uniaxial tensile tests were performed on tissue strips (length = 10 mm, width = 1.7 - 2 mm; area = 17 - 20 mm^2) from the three leaflets of each tissue-engineered heart valve in circumferential and radial direction.*

First, thickness and width of the tissue samples were measured using a PLμ 2300 non-contact Confocal Imaging Profiler (Sensofar Tech S.L., Terrassa, Spain). After that, the tissue strips were tested until rupture at a constant strain rate (1/60 s^{-1}) using a tensile testing device (Bose ElectroForce LM1) with a 2.5N load cell (Honeywell International Inc., Morristown, NJ, USA). Force and elongation were measured during testing and were converted to Cauchy stress and true strain, respectively. The slope of the resulting stress-strain curve in the 0-15% strain range was defined here as the tangential stiffness of the tissue specimen. Since the variation in stiffness values obtained with or without preconditioning was relatively small compared to the variation

in stiffness values within a tissue-engineered heart valve, preconditioning was not performed (Kortsmit et al., 2009b).

Statistics

The uniaxial tensile test data were expressed as mean ± standard deviation. Data were analyzed using an analysis of variance (ANOVA), followed by a Bonferroni post-hoc test to check whether the mean values of the experimental groups were significantly different when $p<0.01$ or $p<0.05$. The statistical analyses were performed using the Statgraphics Centurion XV software package.

4.3 Results

4.3.1 Deformation control

The prescribed conditioning protocols were applied to the tissue-engineered heart valves by using the PID controller. For both experiments the induced volumetric deformation values were measured during culture and are represented in figure 4.6. In experiment I (Fig. 4.6a) deformation was only controlled after 18 days of culture for valves A and B and after 14 days for valves C and D. Deformation control was postponed due to the large leak flows through these valves and was started when the leak flows decreased to an acceptable level. During this phase, constant dynamic loads were applied to the cultured heart valves. The start of the deformation controlled load application is observed in figure 4.6a as a sharp increase in measured volumetric deformation values. In experiment II (Fig. 4.6b), however, controlled loading was seen directly after day 12, at the start of the dynamic load application.

The measured volumetric deformation values had good resemblance with the prescribed values of the conditioning protocols (Fig. 4.4). The mean relative tracking error (table 4.1) ranged between 1.7% (valve D, experiment II) and 2.9% (valve B, experiment II), except for valve D of experiment I. For this valve, the mean relative error was relatively large and equal to 5.3%.

In both experiments, vertically arranged data points were observed at regular intervals during culture, generally showing deformation values smaller but sometimes also larger than prescribed. These deformation measurements were obtained when heart valve culture was interrupted by growth medium replacement or other maintenance activities. In those cases, controlled load application was shut down and restarted, after which it took some time for the system to reach steady-state induced deformations at the prescribed values.

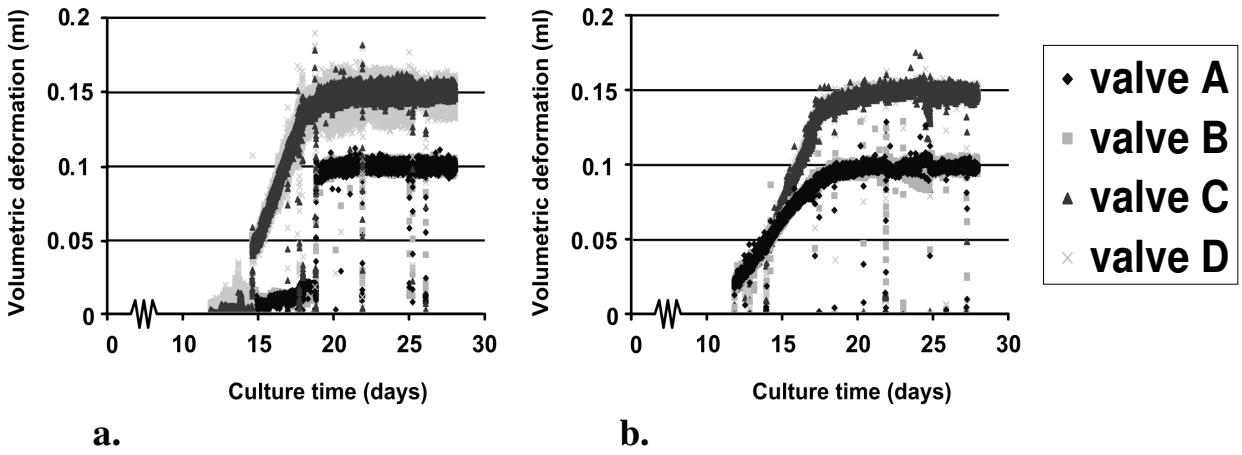


Fig. 4.6: *All measured volumetric deformation values (ml) of tissue-engineered heart valves A, B (lower profiles) and C, D (upper profiles), given as a function of the culture time (days) for (a) experiment I and (b) experiment II.*

Table 4.1: *The mean relative tracking error (%) of all valves in both experiments.*

Experiment	Valve	Relative error (%)
I	A	2.2
I	B	2.2
I	C	2.6
I	D	5.3
II	A	2.3
II	B	2.9
II	C	2.8
II	D	1.7

4.3.2 Leak flow

Absolute leak flow values were measured during culture and are represented in figure 4.7. In experiment I, valve leakages were maximal after 15 days of culture. For valves A, B, C and D these values were approximately 25, 15, 5 and 10 ml/min, respectively (Fig. 4.7a). The large leak flows at the start of dynamic load application were caused by the relatively high stiffness of the tissue-engineered valves in combination with the insufficient coaptation of the valve leaflets. As culture time increased, the leaflets became less stiff and more flexible which resulted in an increase in coaptation and a decrease in leakage. In experiment I, leak flows were reduced to 5-6 ml/min at the end of culture (Fig. 4.7a). In experiment II, large valve leakages were precluded by enhancement of the scaffold fabrication. Improvement was focused on maximum reduction of the open area between the leaflets, especially at the commissures to prevent non sufficient coaptation. This resulted in relatively small leak

flows as compared to experiment I. The largest value - for valve D - was approximately 5 ml/min after 14 days of culture. At sacrifice, all leakage values were smaller than 3 ml/min (Fig. 4.7b).

4.3.3 Mechanical characterization

Functionality of the cultured heart valves was demonstrated by their capability to withstand physiological load application (ΔP = 13 kPa) with minimal leak flows. Measured leak flows (table 4.2) were less than 1 ml/min for all valves except for valves A and D of experiment II and valve D of experiment I; leak flows through these valves were 3, 3 and 5 ml/min, respectively. The estimated Young's moduli, assessed by the inverse experimental-numerical estimation method yielded values ranging from 0.77 to 1.66 MPa (table 4.2). These moduli were also depicted next to the tensile test results (Fig. 4.8), and were averaged for each protocol (Fig. 4.9).

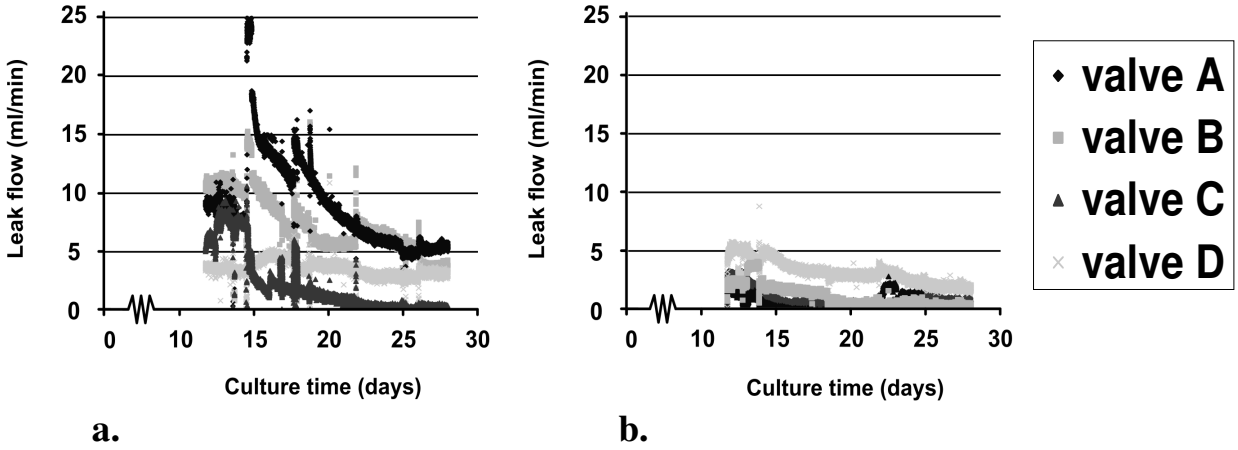


Fig. 4.7: *Measured leak flows (ml/min) of tissue-engineered heart valves A, B, C and D, given as a function of the culture time (days) for (a) experiment I and (b) experiment II.*

The Young's moduli determined by the uniaxial tensile tests were represented per cultured heart valve and averaged per direction (radial and circumferential) within the valve. In addition, these values were subdivided according to the two conditioning protocols (Fig. 4.8). No significant differences were found between the mechanical properties of the cultured valves independently. However, almost all valves showed a significant difference ($p<0.01$) in Young's moduli between the radial and circumferential direction. For these valves, the modulus in circumferential direction was larger than in radial direction. For valves A of the first and second experiment, however, the mechanical properties in both directions were not significantly different. For all valves except valve A of experiment II, the estimated stiffness was between the measured stiffness obtained for the radial and circumferential direction.

Table 4.2: *Leakages and Young's moduli of the tissue-engineered heart valves, arranged according to the applied loading protocols. The leakage of the valves (ml/min) was assessed at physiological load application and the Young's moduli (MPa) were estimated using the inverse experimental-numerical estimation method.*

Protocol	Valve	Leakage (ml/min)	Young's modulus (MPa)
1	I-A	0	0.82
1	I-B	0	1.14
1	II-A	3	1.66
1	II-B	0	1.07
2	I-C	0	1.22
2	I-D	5	1.36
2	II-C	0	0.77
2	II-D	3	1.26

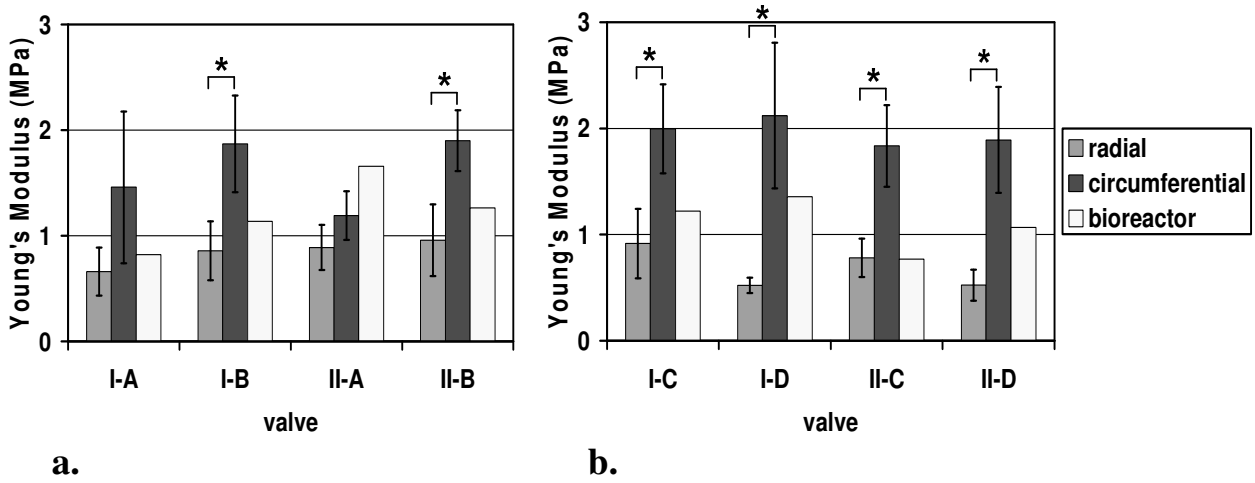


Fig. 4.8: *Young's moduli (MPa) of the tissue-engineered heart valves cultured according to (a) protocol 1; valves I-A, I-B, II-A and II-B, and (b) protocol 2; valves I-C, I-D, II-C and II-D. The average values of the Young's moduli in radial and circumferential directions are represented. The error bars depict the standard deviation of the mean. Statistically significant differences between radial and circumferential directions ($p<0.01$) are marked with an asterisk (*). The bioreactor values represent the Young's moduli assessed by the inverse experimental-numerical estimation method.*

The Young's moduli of the valves in both directions were averaged over all valves for each protocol, independent of the experiment (Fig. 4.9). Statistical analyses indicated no significant difference between conditioning protocols 1 and 2. In contrast,

the Young's moduli in circumferential direction were significantly larger ($p<0.01$) than in radial direction for both loading protocols. Additionally, the Young's moduli estimated during culture were in between the radial and circumferential values, as measured after sacrifice of the valves.

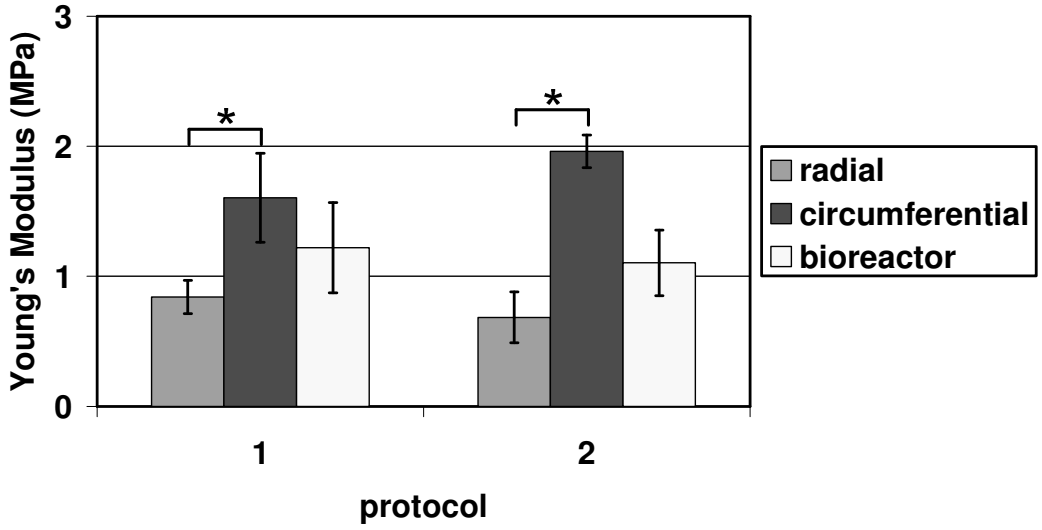


Fig. 4.9: *The average Young's moduli (MPa) of the tissue-engineered heart valves cultured according to protocol 1; valves I-A, I-B, II-A and II-B, and protocol 2; valves I-C, I-D, II-C and II-D. The average moduli, obtained by tensile tests in radial and circumferential directions are shown. The bioreactor values represent the average Young's moduli assessed by the inverse experimental-numerical estimation method. The error bars depict the standard deviation of the mean. Statistically significant differences between radial and circumferential directions ($p<0.01$) are marked with an asterisk (*).*

4.4 Discussion

One of the main challenges in heart valve tissue engineering is to improve the mechanical behavior of cultured heart valves towards native aortic valves. Mechanical stimulation of the developing tissue is known to enhance tissue formation and quality, and is widely used in cardiovascular tissue engineering. Strains in the cultured tissue as a result of load application are an important factor in tissue development. Because of this, and to obtain a better understanding of the effects of mechanical loading on tissue maturation, tissue strain monitoring and control are desirable. Many studies were performed in which relatively simple tissue-engineered configurations were cultured, such as strips, squared patches or discs (Kim et al., 1999; Mitchell et al., 2001; Mol et al., 2003; Isenberg et al., 2003; Butcher et al., 2006; Engelmayr et al., 2006, 2008; Ferdous et al., 2008; Boerboom et al., 2008; Rubbens et al., 2009). In these studies loads were applied to induce predefined deformations. However, during culture of heart valves the deformation in the leaflets as a result of the applied load were unknown, since real-time deformation monitoring and control were absent (Hoerstrup et al.,

2000b; Zeltinger et al., 2001; Rabkin et al., 2002; Schenke-Layland et al., 2003; Mol et al., 2005a; Sodian et al., 2006; Stock et al., 2006; Flanagan et al., 2007; Syedain et al., 2008). In a previous study (Kortsmit et al., 2009a) a combined experimental-numerical method was applied to assess deformation, in real-time and non-invasively. In this study, this method was further developed and the inclusion of a feedback control mechanism enabled both the assessment and the regulation of induced deformation in the cultured heart valve leaflets.

A PID feedback control loop was designed and tuned, and incorporated into the software of the recently developed bioreactor system. Eight heart valves were tissue-engineered in this bioreactor system and two different conditioning protocols were applied. The protocols represented prescribed deformation profiles and mechanical load application was controlled such that induced deformations followed the imposed protocol. The measured deformation values showed good correspondence with the prescribed deformation values in both experiments. The mean relative tracking error of all valves did not exceed 5%. Nevertheless, the error value of valve D in experiment I was relatively large compared to the error values of the other valves. This was probably caused by suboptimal tuning of the proportional pneumatic valve. In experiment II, this was improved, which resulted in a more accurate control. In experiment I, a delay in controlled load application was observed for all valves, which was caused by the large leakage through the valves, especially valves A and B, at the start of pulsatile load application. When leakages are very large, deformation measurements are less accurate (Kortsmit et al., 2009a) and deformation control is not reliable. For this reason, deformation controlled load application was postponed for 2 to 6 days in experiment I, until leak flows decreased to an acceptable level. In contrast, valve leakages were relatively small in experiment II. This resulted in a continuous and accurately controlled application of dynamic loads to the cultured heart valves.

The feedback regulation mechanism developed in this study was applied to control the induced deformation in the cultured heart valve leaflets. Since dynamic load application was performed at a frequency of 1 Hz, and (maximum) deformation was assessed by integration of the measured flow signal after the loading period, the PID control loop was only updated every second. Tissue development during culture is a slow and steady proceeding process and therefore, a control frequency of 1 Hz seemed to be sufficient to apply controlled loads to tissue-engineered heart valves. Due to the assessment of volumetric deformation of the heart valve after each loading period, the control feedback could not intervene directly in the loading process. Its control actions were based on the end result of each loading period. Nevertheless, as a result of the steady output of the proportional pneumatic valves in combination with the gradual progress of heart valve tissue development, control instabilities did not occur.

The cultured heart valves had mechanical properties in the range of previous tissue engineering studies (Mol et al., 2005a; Mol et al., 2006; Boerboom et al., 2008; Kortsmit et al., 2009b). Obtained Young's moduli were compared to native human aortic values (Balguid et al., 2007a). To be able to make this comparison, Young's moduli were also determined from the steepest part of the obtained strain-stress curves, and corresponded reasonably well in radial direction (varying between 88% of native

value for valve A, experiment I, to 155% for valve B, experiment II), but were considerably smaller in circumferential direction (from 23% for valve A to 34% for valve C, both experiment II). Anisotropy of the mechanical properties was indicated by the significant difference ($p<0.01$) between the radial and circumferential tensile test results for all valves, except valves A of both experiments. These valves were cultured according to conditioning protocol 1. No significant differences were found between both loading protocols, but the degree of anisotropy seemed to be smaller in the valves cultured with protocol 1. The absence of (significant) anisotropy in valves A confirmed this supposition. In addition, functionality of the tissue-engineered heart valve leaflets was shown by the sustained physiological diastolic pressure differences in combination with the small leakage values (<6 ml/min) at the end of the culture period. The estimated Young's moduli during tissue culture were in between the radial and circumferential moduli obtained via tensile testing (Kortsmit et al., 2009b).

Two loading protocols were applied to the tissue-engineered heart valves, but no significant differences in mechanical properties were found between the valves cultured by the dissimilar protocols. The applied deformation profiles were probably not distinctive enough. However, the focus of this study was not to investigate the effect of different conditioning protocols on tissue development, but to demonstrate the functionality of the developed deformation controlled feedback mechanism. Moreover, this study presents the possibilities to culture functional heart valves showing mechanical behavior similar to heart valves in previous tissue engineering studies, using the feedback mechanism.

The next step would be to use this deformation feedback mechanism to apply specific loading protocols to cultured heart valves to systematically study the effects of mechanical loading on tissue development. In this context, the application of intermittent loading protocols (Boerboom et al., 2008; Rubbens et al., 2009) may have a more beneficial influence on tissue formation than the protocols applied in this study.

In conclusion, the designed bioreactor system including the deformation feedback control is a well defined platform to study optimal conditioning protocols for tissue engineering of aortic heart valves.

4.5 Acknowledgements

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Chapter 5

Further evaluation of the heart valves cultured in the bioreactor system

5.1 Introduction

In the previous chapters, a bioreactor system was presented in which an inverse experimental-numerical approach was incorporated that enabled assessment and control of leaflet deformation and assessment of mechanical properties of engineered heart valves, non-invasively and non-destructively. The functionality of the developed bioreactor was validated and the in-vitro performance was shown by the culture of 14 human heart valves in four independent experiments (chapters 3 and 4). In addition to the elaborate mechanical and functional evaluations described in the previous chapters, analyzing tissue composition of the engineered valves on a microscopic and macroscopic level may provide further insight in the relation between mechanical conditioning and tissue development.

In this chapter, the qualitative and quantitative tissue analyses performed on the cultured valves from chapters 3 and 4 are described. First, the macroscopic appearance of all engineered heart valves after culture is shown. Additionally, the presence and distribution of the main structural matrix components of human aortic valves; collagen type I, collagen type III and elastin in the tissue-engineered heart valves are investigated by immunohistology. Conclusively, heart valve tissue formation is analyzed quantitatively by biochemical assays for the amount of DNA, as an indicator for cell number, glycosaminoglycans (GAGs) and hydroxyproline (HYP), as an indicator for collagen.

5.2 Materials and Methods

5.2.1 Heart valve culture

In chapters 3 and 4, a total of 14 heart valves was tissue-engineered in four independent experiments using four different dynamic conditioning protocols (table 5.1). The complete heart valve culture process and the used bioreactor system have been extensively described in chapters 2, 3 and 4. In chapter 3, six heart valves were cultured in two experiments. In the first experiment, two valves were cultured according to loading protocol 1 (Fig. 5.1a). In the second experiment, four valves were tissue-engineered according to loading protocol 2 (Fig. 5.1b); two valves for three weeks (protocol 2a) and two valves for four weeks (protocol 2b). Both loading protocols were predefined and not deformation controlled. In chapter 4, eight heart valves were tissue-engineered in two experiments. In both experiments, two different deformation controlled conditioning protocols (protocols 3 and 4) were applied to the tissue-engineered valves (Fig. 5.2).

5.2.2 Qualitative tissue evaluation

After culture, tissue-engineered heart valves were photographed for macroscopic appearance. Thereupon, representative samples of valves 5, 6 (chapter 3), 8 and 10 (chapter 4) were used for histology. Tissues were fixated in 10% phosphate-buffered formalin (Fluka, USA) and embedded in paraffin. Sections were cut at 10 μm thickness. To analyze the formation of collagen type I and III, and elastin in the tissue-engineered heart valve samples, immunofluorescent stainings were applied using the following primary antibodies: monoclonal mouse IgG1 anti-collagen type I (c2456, Sigma), monoclonal mouse IgG1 anti-collagen type III (c7805, Sigma) and monoclonal mouse IgG1 anti-elastin (ab9519, Abcam, UK). Goat anti-mouse IgG2 Alexa 488 was used as secondary antibody (Invitrogen). Sections were counterstained with DAPI (cell nuclei) (Invitrogen). Antigen retrieval was performed by pepsin incubation (0.04% pepsin and 0.5% milk powder in PBS, pH2, 8 minutes, RT) for elastin or incubated with hot TRIS/EDTA buffer (10mM Tris Base; 1mM EDTA solution; 0.05% Tween20; PH9.0, 20 minutes, RT) for collagen types I and III. Sections were blocked for 30 minutes in 1% bovine serum albumine (Roche) in PBS. Primary antibodies were incubated overnight at 4ºC, secondary antibodies for 30 min at RT. Sections incubated with only the secondary antibody served as negative control, and sections from porcine aortic valve leaflets were used as positive control (data not shown). Images were taken using a fluorescent microscope (Axiovert 200, Zeiss), mounted with a monochrome Axiocam, using appropriate filters and post-hoc color definition.

Table 5.1: *Overview of the tissue-engineered heart valves of chapters 3 and 4.*

TE heart valves	Chapter	Original valve names	Experiment	Protocol	
				#	Culture time (weeks)
1	3	1-1	1	1	3
2	3	1-2	1	1	3
3	3	2-2	2	2a	3
4	3	2-4	2	2a	3
5	3	2-1	2	2b	4
6	3	2-3	2	2b	4
7	4	I-A	3	3	4
8	4	I-B	3	3	4
9	4	I-C	3	4	4
10	4	I-D	3	4	4
11	4	II-A	4	3	4
12	4	II-B	4	3	4
13	4	II-C	4	4	4
14	4	II-D	4	4	4

5.2.3 Quantitative tissue evaluation - biochemical assays

Heart valve tissue formation was analyzed quantitatively by biochemical assays for DNA, as an indicator for cell number, glycosaminoglycans (GAGs) and hydroxyproline (HYP), as an indicator for collagen. Lyophilized samples were digested in papain solution (100mM phosphate buffer, 5mM L-cystein, 5mM ethylenediaminetetraacetic acid, and 125–140 mg papain per mL) at 60℃ for 16 hours. The amount of DNA was determined using the Hoechst dye method (Cesarone et al., 1979) with a reference curve of calf thymus DNA (Sigma). Subsequently, digested tissue samples were hydrolyzed in 6M hydrochloric acid (Merck, Whitehouse Station, NJ, USA) and used for amino acid analyses. The hydroxyproline (HYP) quantity was assessed using an assay modified from Huszar et al. (1980) and a trans-4-hydroxyproline (Sigma) reference. The hydroxyproline to collagen ratio was assumed to be 0.13. The GAGs content was determined using a modification of the assay described by Farndale et al. (1986), and a shark cartilage chondroitin sulfate reference (Sigma). The amounts of DNA, GAGs and HYP were expressed as μg per mg dry tissue weight. To obtain insight into the amount of GAGs and HYP produced per μg DNA, as an indicator for extracellular matrix synthesis of the cells, the GAGs and HYP content per mg dry weight were divided by the amount of DNA per mg dry weight.

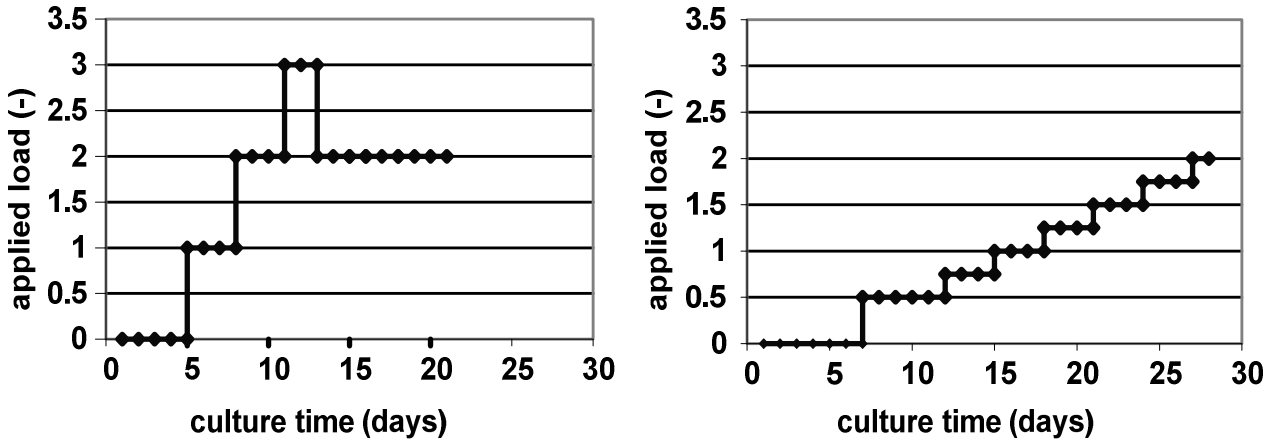


Fig. 5.1: *Conditioning protocols applied to the tissue-engineered heart valves in chapter 3; (a) protocol 1, (b) protocol 2a and b. The applied load is given as a function of culture time.*

5.2.4 Statistics

The data obtained from the biochemical assays were averaged per tissue-engineered heart valve (6-9 samples per valve) and expressed as mean ± standard deviation. In addition, heart valve data were also grouped according to their experiment, loading protocol and culture time. Analyses were performed using an analysis of variance (ANOVA), followed by a Bonferroni post-hoc test to investigate

differences between the experimental groups ($p<0.05$). The statistical analyses were performed using the Statgraphics Centurion XV software package.

5.3 Results

5.3.1 Qualitative tissue evaluation - macroscopic appearance

All tissue-engineered heart valves showed dense tissue formation and had a homogeneous, shiny, tissue-like appearance after culture (Fig. 5.3 and Fig. 5.4). Although scaffold leaflets were not attached to each other at the start of the experiment, they were in all cases completely grown together along the coaptation line, from the commissures towards the center of the valve. In general, the heart valve leaflets kept their initial bulged configuration, even though some valve leaflets showed flattening after culture, e.g. valve 6 (Fig. 5.3f). In addition, tissue-engineered valve 3 (Fig. 5.3c) was discussed in chapter 3 (valve 2-2) and showed high leakage when diastolic loads were applied at the end of culture. However, since leakage was caused by inappropriate attachment of one of the leaflets to the wall, valve failure is not detectable at the coaptation area.

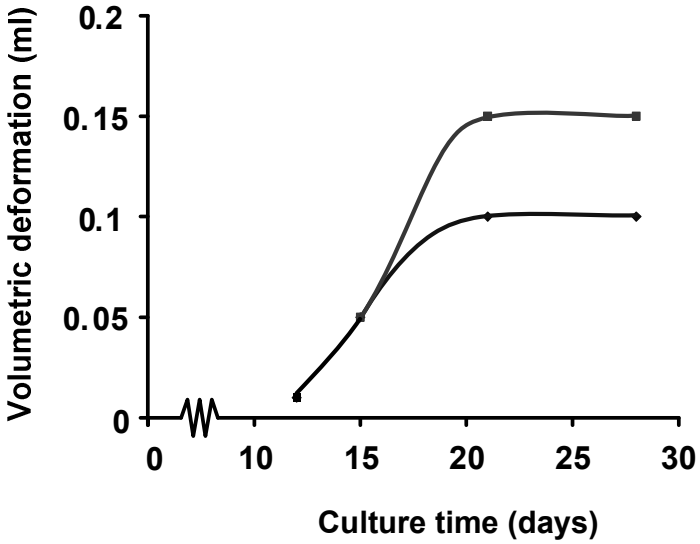
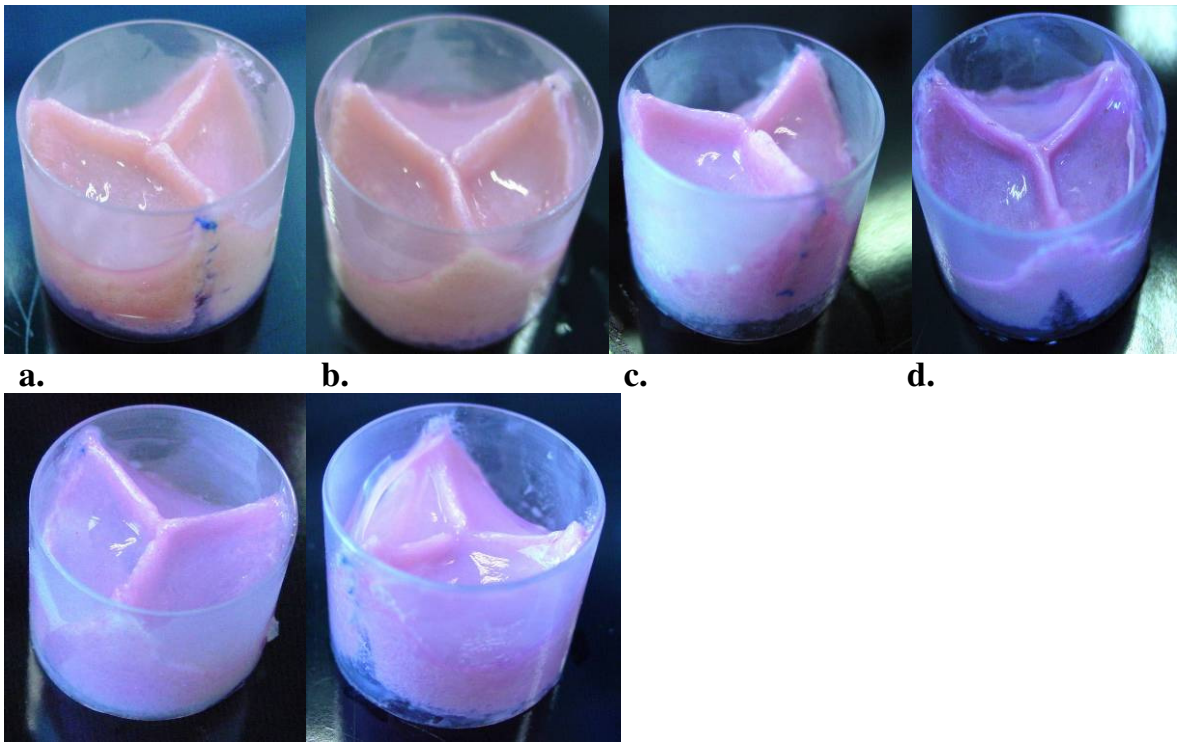


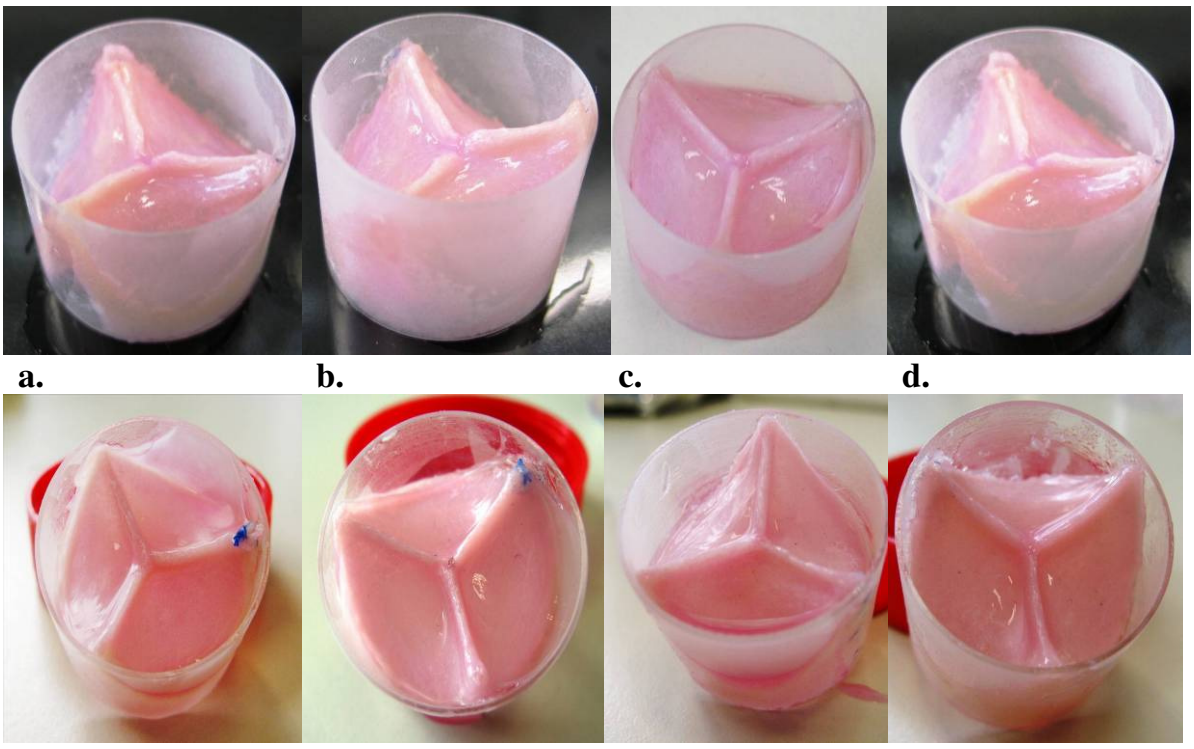
Fig. 5.2: *Conditioning protocols 3 (lower profile) and 4 (upper profile) applied to the tissue-engineered heart valves in chapter 4. The induced volumetric deformation is given as a function of culture time.*



a. **b.** **c.** **d.**

e. **f.**

Fig. 5.3: *Tissue-engineered heart valves 1 - 6 (a - f, respectively) [chapter 3].*



a. **b.** **c.** **d.**

e. **f.** **g.** **h.**

Fig. 5.4: *Tissue-engineered heart valves 7 - 14 (a - h, respectively) [chapter 4].*

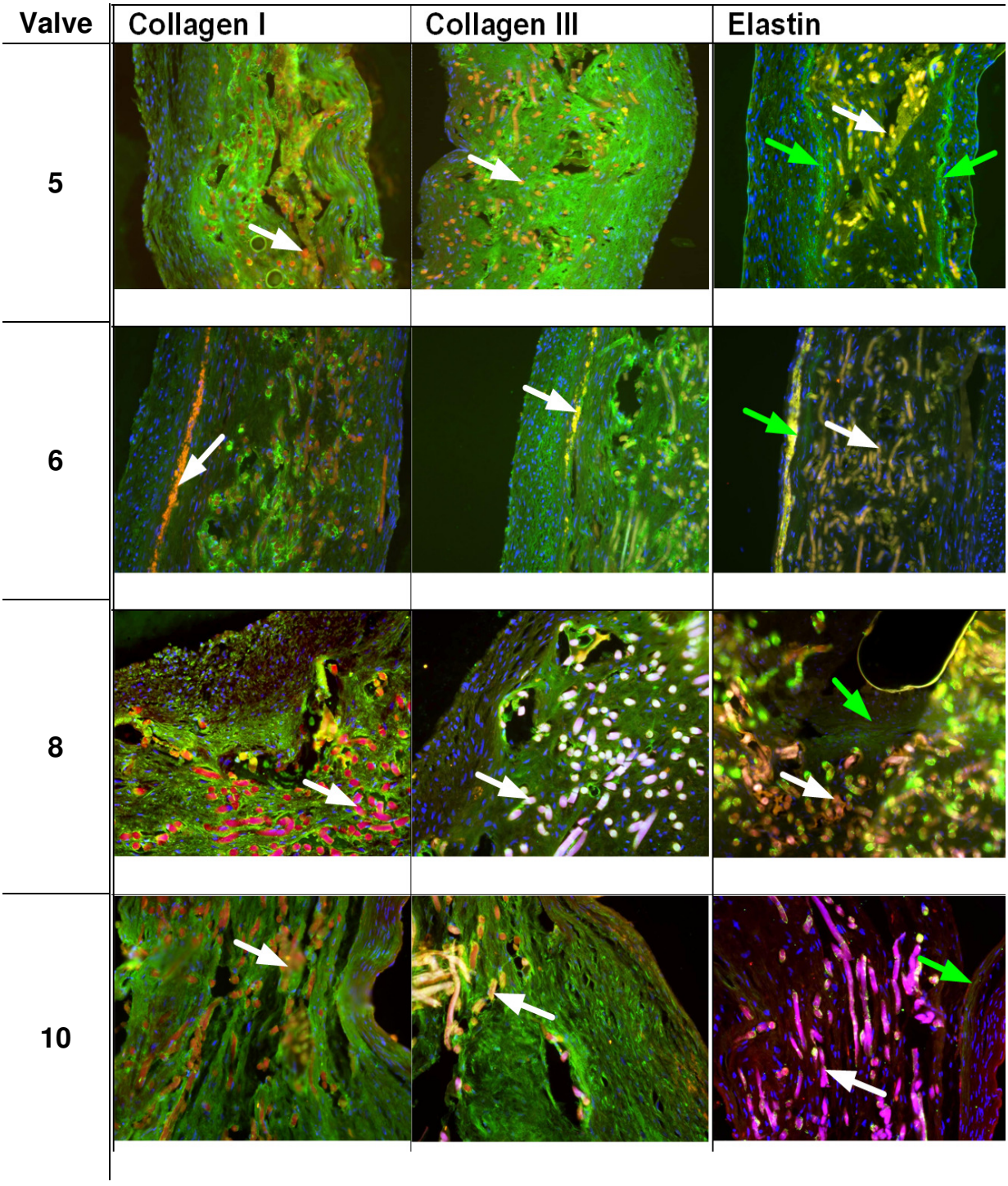


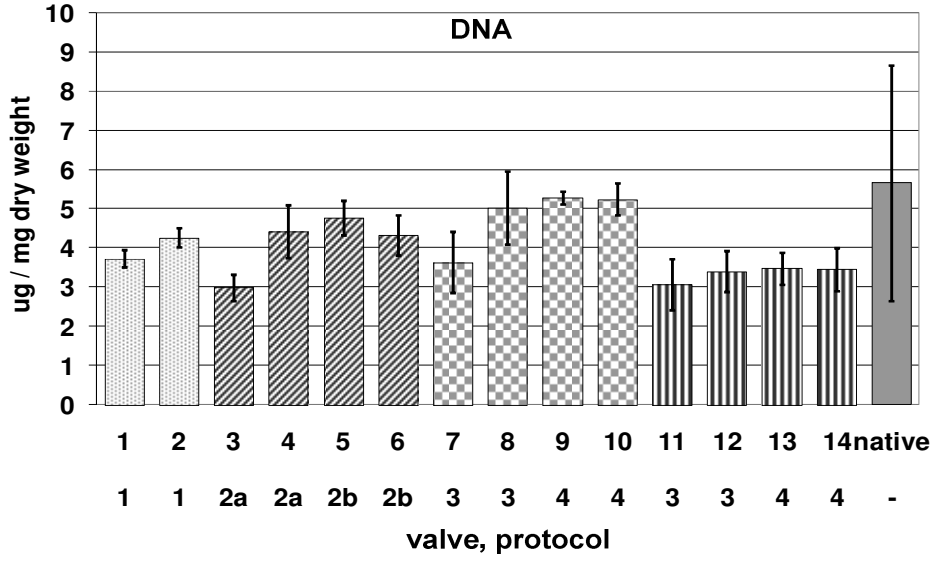
Fig. 5.5: *Immunofluorescent staining of tissue-engineered heart valves 5, 6, 8 and 10 (magnification: 20x). Collagen type I, III, and elastin are shown in green; elastin is also indicated by green arrows. Cell nuclei are depicted in blue. The staining of the heart valve tissue in other colors than green and blue represents scaffold remnants; also indicated by white arrows.*

5.3.2 Qualitative tissue evaluation - immunohistology

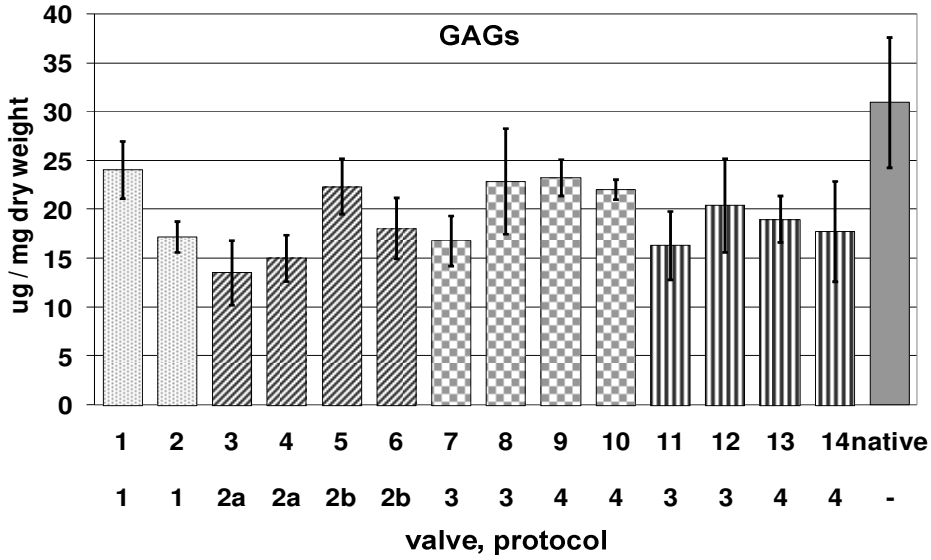
Representative immunofluorescent stainings of heart valves 5, 6, 8 and 10 are presented in figure 5.5. Tissue morphology appeared homogeneous and dense throughout the thickness of the heart valve leaflets. Cells were distributed through the valve tissue, but seemed more concentrated at the surface of the valvular constructs. In contrast, scaffold remnants were mostly located in the middle part of the tissue. The images show the formation of collagen type I and III as thick wavy bundles throughout the whole cross-section of the tissue-engineered heart valve leaflets. In addition, staining of elastin was observed in several leaflets. Elastin staining appeared as striated structures indicative of small elastin fragments, or as (thin) elastin bands. Two of those small elastin bands are clearly observed in valve 5 (Fig. 5.5).

5.3.3 Quantitative tissue evaluation - biochemical assays

The amounts of DNA, glycosaminoglycans (GAGs), and hydroxyproline (HYP) in microgram per milligram dry weight are represented in figures 5.6a, b and c, respectively, for all tissue-engineered heart valves of chapters 3 and 4, and human native aortic heart valves (Balguid et al., 2007b). All tissue-engineered data are compared with native values and differences resulting from a variation in experiment, protocol and culture time were studied.



a.



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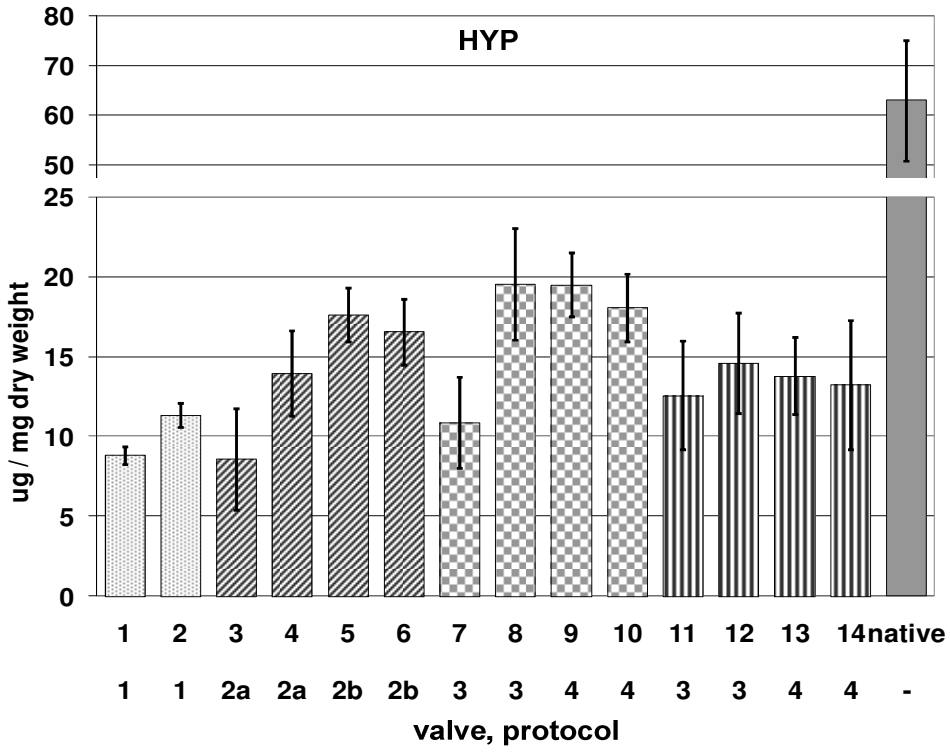


c.

Fig. 5.6: *The average amount of (a) DNA, (b) glycosaminoglycans (GAGs) and (c) hydroxyproline (HYP) in microgram per milligram dry weight in the tissue-engineered heart valves of chapters 3 and 4, and in native human aortic heart valves. The applied protocols are indicated below the heart valve numbering. The different patterns in the histogram represent the performed experiments; 1, 2, 3 and 4 (from left to right). The error bars indicate the standard deviation of the mean. The significance levels are not shown, see text for details.*

DNA

The overview of the amount of DNA in the heart valves (Fig. 5.6a) shows that cultured tissue had an average amount of 3.0 ± 0.34 (valve 3) to 5.3 ± 0.17 (valve 9) µg DNA per mg dry weight. Native heart valve tissue contained 5.7 ± 3.0 µg DNA per mg dry weight, which was significantly more DNA than the heart valves cultured in experiment 4 (valves 11 to 14) and valves 1, 3 and 7. In addition, the tissue of the heart valves in experiment 4 had significantly less DNA than the valvular tissue in the other experiments. Differences in the DNA amount resulting from mechanical conditioning by dissimilar protocols were not indicated.

GAGs

The average amount of GAGs (Fig. 5.6b) in the tissue-engineered constructs was between 13.6 ± 3.3 (valve 3) and 24.1 ± 3.0 (valve 1) µg per mg dry weight. The mean native GAGs value was 31 ± 6.7 µg per mg dry weight and was significantly larger for valves 1, 3, 4, 6 and 7, and all valves of experiment 4 (valves 11 to 14).

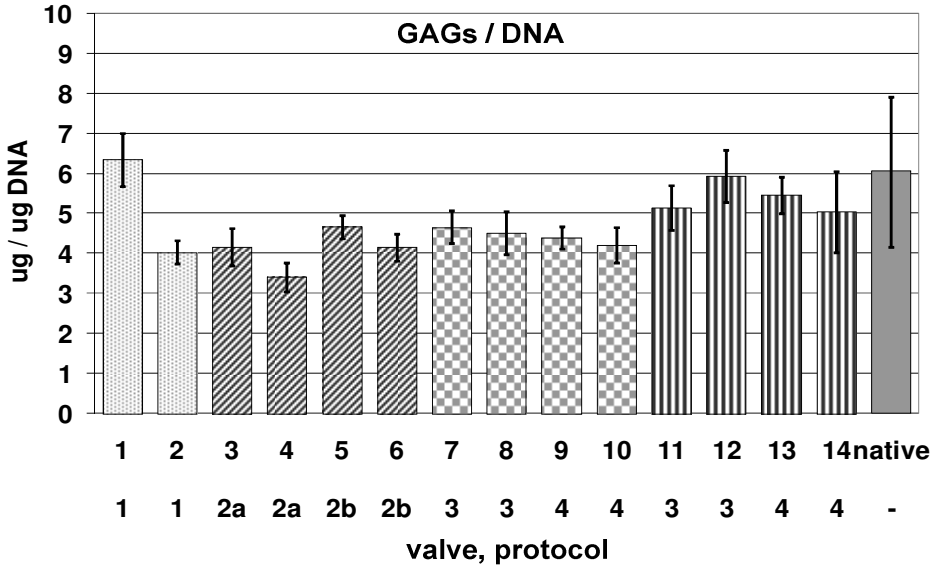
HYP

For the hydroxyproline (HYP) amount found in the cultured valvular tissue (Fig. 5.6c), average values ranged from 8.6 ± 3.2 (valve 3) to 19.6 ± 3.5 (valve 8) µg per mg dry weight. Most notable is the difference between the tissue-engineered HYP values and the native value, which was equal to 63 ± 12 µg per mg dry weight. Human native aortic valves contained significantly more HYP than all the in-vitro cultured heart valves. Statistical analyses also showed a significant increase in HYP content when heart valves were conditioned for four weeks instead of three weeks. Furthermore, the valves cultured in experiment 3 were composed of a larger amount of HYP than the valves in experiment 4.

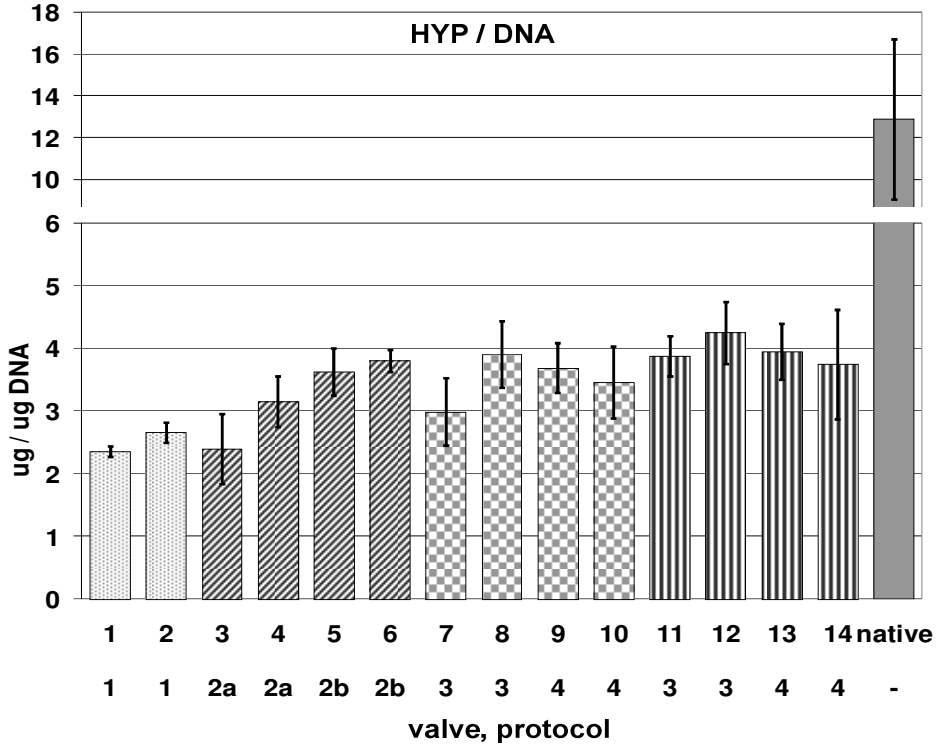
To detect any differences in extracellular matrix synthesis activity of the cells in the heart valve tissue, the amounts of GAGs and HYP in microgram produced per microgram DNA were presented in figures 5.7a and b, respectively, for all the tissue-engineered valves of chapters 3 and 4. These values were compared with each other and the benchmark values of human native aortic valves (Balguid et al., 2007b).

GAGs / DNA

The average tissue-engineered value of the amount of GAGs per DNA varied between 3.4 ± 0.37 (valve 4) and 6.4 ± 0.7 (valve 1) µg per µg DNA (Fig. 5.7a). These values did not significantly differ from the native GAGs per DNA value, which was 6.1 ± 1.9 µg per µg DNA, except for valves 2 and 4. Furthermore, the cells of the valves cultured in experiment 4 excreted significantly more GAGs per DNA than the valvular cells in the third experiment.



a.



b.

Fig. 5.7: *The average amount of (a) glycosaminoglycans (GAGs) and (b) hydroxyproline (HYP) in microgram per microgram DNA, in the tissue-engineered heart valves of chapters 3 and 4, and in native human aortic heart valves. The applied protocols are indicated below the heart valve numbering. The different patterns in the histogram represent the performed experiments; 1, 2, 3 and 4 (from left to right). The error bars indicate the standard deviation of the mean. The significance levels are not shown, see text for details.*

HYP / DNA

The synthesis activity for the production of hydroxyproline (HYP) by the cultured valve tissue ranged from 2.4 ± 0.07 (valve 1) to 4.3 ± 0.5 (valve 12) µg per µg DNA (Fig. 5.7b). The native HYP production per DNA was 12.9 ± 3.8 µg per µg DNA and was significantly larger ($p<0.01$) compared to all in-vitro cultured tissues. Again, the valves cultured for three weeks contained significantly less HYP per DNA compared to the four weeks cultured valves.

5.4 Discussion

In the preceding chapters of this thesis, a bioreactor system was developed of which the assessment and control features were demonstrated and validated in practice. Tissue-engineered human heart valves were cultured in various independent experiments. These heart valves were studied and evaluated based on their mechanical behavior and functionality under physiological diastolic conditions. Although the morphological appearance and composition of the developed heart valve tissue are important tissue quality criteria, these were not addressed in the previous chapters. Hence, in this study, the macroscopic configuration and the additionally performed analyses, i.e. immunohistology and biochemical assays, of the tissue-engineered heart valves were analyzed, quantitatively and qualitatively, and discussed.

The macroscopic outcome showed that the tissue-engineered heart valve leaflets had a similar appearance. Variation in the performed experiment, applied conditioning protocol or culture time did not have an apparent visible effect. The heart valve leaflets kept their shape during culture but some showed flattening, which was probably due to tissue compaction. This was previously observed in tissue-engineered heart valve leaflets (Mol et al., 2006) and was defined as shrinkage of the tissue due to contractile forces developed within the tissue. During culture, the valve leaflets grew together while they were initially separated in the scaffold design. Due to the applied diastolic straining protocol, heart valves kept their closed configuration during the complete culture period. The systolic phase was not included in the loading protocol and, therefore, periodic opening of the heart valve - which could have prevented tissue growth over the coaptation lines - did not take place. As a result of leaflet fusion, a prestress in the tissue could be developed, which stimulated tissue formation. When the valve leaflets were separated after culture, the release of prestress resulted in compaction of the leaflet tissue and, thus, reduced heart valve functionality. It was also thought that separation of the leaflets shortly before implantation in a sheep model was one of the causes of the local irregularities and thickening of the valvular tissue observed after explantation (Dijkman et al., 2008b). In future studies, the tissue growth between valve leaflets could be prevented by e.g. the inclusion of the systolic phase in the conditioning protocol or the use of a leaflet separation template.

The qualitative heart valve tissue analyses were focused on the presence and distribution of the main structural matrix components of native heart valves; collagen types I, III and elastin. Immunofluorescence showed the presence of collagen types I

and III throughout the entire tissue in thick wavy fibers. The structure of these main types of collagen in the heart valve was similar to native aortic heart valves. However, in native valves the collagen architecture was located in the ventricularis and fibrosa layers and not distributed throughout the entire leaflet (Aikawa et al., 2006). In addition, 74% of the native tissue is composed of collagen type I, while 24% is collagen type III (Mulholland et al., 1996). This ratio between both collagen types was not detectable by immunofluorescence. Elastin was indicated as fragments of striated structures in some tissue-engineered valve leaflets. No specific location was observed. In-vivo, striated elastin structures were mainly found in the ventricularis and sometimes in the upper fibrosa as interweaving fibers intermingled with collagen fibers (Latif et al., 2005). Although only fragments of elastin were shown in the histology of the cultured heart valves, few cardiovascular tissue engineering studies are known in which elastin synthesis was induced by mechanical stimulation (Isenberg & Tranquillo, 2003; Opitz et al., 2004; Patel et al., 2006). Besides histology, also quantitative evidence is needed to indicate the presence of elastin in the tissue-engineered heart valve tissue. Furthermore, the tissue-engineered heart valves did not have a layered structure as seen in native valves. The absence of such a structure and a similar extracellular matrix composition as shown in human fetal heart valves suggested a correspondence between the in-vitro cultured tissue and immature native valvular tissue (Aikawa et al., 2006).

Tissue formation of all tissue-engineered heart valves was analyzed quantitatively by biochemical assays for DNA, an indicator for cell number, glycosaminoglycans (GAGs) and hydroxyproline (HYP), as an indicator for collagen content. In general, the variation in biochemical assay results was minimal when all heart valves were compared. Some differences between heart valves were observed but due to diversities in experiment and protocol, and a relatively small sample size sound conclusions cannot be drawn. However, in experiment 4, the cultured heart valves (valves 11 to 14) contained significantly less DNA, GAGs, HYP and GAGs per DNA compared to the tissue-engineered heart valves in experiment 3 (valves 7 to 11). Since the applied protocols were identical in both experiments, it was thought that the observed inter-experimental differences were caused by the seeded cells at the start of the experiments. Cell seeding densities were similar, but possibly a difference in cellular phenotype initiated more tissue formation in experiment 3. The obtained results were correlated with values obtained from human aortic heart valves (Balguid et al., 2007b). The amounts of measured DNA ranging from 53% (valve 3) to 93% (valve 9) of native values, and GAGs, varying between 44% (valve 3) and 85% (valve 1), showed values close to values found for native aortic valves. In the engineered valves, however, HYP content was only between 14% (valve 3) and 31% (valve 8). The relative amounts of GAGs per DNA and HYP per DNA showed values ranging from 56% (valve 4) to 115% (valve 1), and from 19% (valve 3) to 33% (valve 12), respectively. The secretion of GAGs per DNA seemed sufficient as native values were set as benchmark. In contrast, the in-vitro HYP production per DNA is low and needs to be increased to reach native standards. The tissue analyses showed that such an increase might be achieved when culture time is prolonged, since both the absolute and relative amounts of HYP were increased by extension of the culture time from three to

four weeks. Next to the amount of HYP (collagen), more attention should be given to the organization of collagen in the cultured heart valves. A previous study even revealed a directive role of collagen crosslinks, rather than collagen content, in the biomechanical development of tissue-engineered constructs towards native tissue (Balguid et al., 2007b). Hence, in-vitro tissue formation in the valve leaflets can still be improved to mimic the tissue composition of native aortic valves, but was similar to heart valves (Mol et al., 2006) and other cardiovascular constructs (Boerboom et al., 2008; Stekelenburg et al., 2009) cultured in previous studies.

5.5 Conclusion

The 14 human tissue-engineered heart valves cultured in four independent experiments (chapters 3 and 4) all showed dense tissue formation and had a tissue-like appearance. The presence of collagen type I and III, throughout the tissue, and elastin, in striated structure fragments was indicated and showed similarities with immature (fetal) native valvular tissue. Lastly, quantitative and qualitative tissue composition was similar to previously performed cardiovascular tissue engineering studies, but needs improvement to reach native adult standards.

5.6 Acknowledgements

Mieke van Marion and Kang Yuen Rosaria-Chak were acknowledged for their contribution to this work. This research was supported by the Dutch Program for Tissue Engineering (DPTE).

Chapter 6

Bioreactor adjustments towards preclinical studies

6.1 Introduction

In this thesis, tissue-engineered heart valves were cultured to validate the functionality of the developed bioreactor system (chapters 2-4). The engineered heart valves were mainly used as a model system and were not intended for implantation. Since the valve wall consisted of a rigid polycarbonate cylinder, the engineered valves were not implantable. To make the step towards preclinical application, it should be possible to culture implantable heart valves in the developed bioreactor system. The tissue-engineered heart valves currently used for minimally invasive implantation purposes in ovine studies consist of a self-expandable vascular stent in which the valve wall and leaflets are integrated (Dijkman et al., 2008a, 2008b, 2009). To culture these heart valves in the developed bioreactor system, the bioreactor and heart valve design should be adapted without loss of the bioreactor functionalities.

In addition, for clinical application, implantations in animal models are needed to study the feasibility of tissue-engineered heart valves in-vivo. The functional mechanical behavior, in-vivo tissue response and remodeling capacity are relevant properties that require investigation during such studies. In order to implant autologous heart valves in animals, tissue-engineered heart valves need to be cultured from cells of autologous animal origin. Although tissue engineering protocols have been developed to culture human heart valves, the translation to the animal model appears not trivial. This can be attributed to differences in cell proliferation rate, gene expression and extracellular matrix synthesis (Driessen-Mol et al., 2009). To culture heart valves for animal models, the protocols needed to be revised and, therefore, the functionality of the bioreactor system is tested and re-evaluated.

Hence, to apply the developed bioreactor system described in this thesis in the next step towards preclinical studies, it should be possible to culture implantable stented valves and heart valves composed of cells of animal origin in the bioreactor, while its measurement and control features are maintained. This chapter describes the applicability of the developed bioreactor system for tissue engineering of heart valves based on the implantable valve design. In addition, the functionality of the system is evaluated for engineered heart valves composed of cells of animal origin.

6.2 Bioreactor application - stented, implantable heart valves

To employ the bioreactor system for the tissue engineering of stented implantable valvular constructs for future clinical application, the functionality of the system should be validated and tested for this ‘new’ configuration. Heart valve leakage was shown to be the most disturbing factor in the assessment of volumetric deformation and in the estimation of mechanical properties of the tissue-engineered heart valves (chapters 2-4). Therefore, the main challenge to culture the stented implantable heart valves in the bioreactor system was to prevent leakage through or alongside the heart valve.

For the implantable stented design, the polycarbonate cylinder, which functioned as the heart valve wall (chapters 2-4), was replaced by a self-expandable stent (EVO stent, 25X27 mm, Pfm - Produkte für die Medizin AG, Germany). The heart valve leaflets and the valve wall were composed of PGA and were sutured inside the stent. The resulting PGA scaffold was coated with 1% P4HB, dissolved in tetrahydrofuran (THF, Merck, Whitehouse Station, NJ, USA) and molded in the shape of a heart valve. After coating, the stented heart valve was inserted in a polycarbonate cylinder to prevent medium flow alongside the valve wall during culture. This cylinder was identical to the polycarbonate cylinders employed in previous experiments (chapters 2-4). The tissue-engineered heart valves were clamped and sealed in the bioreactor system using o-rings. The stented heart valve was not glued or attached to the polycarbonate cylinder but slightly crimped before insertion and, therefore, exerting an outward force to the inside of the cylinder (Fig. 6.1a).

a. **b.**

Fig. 6.1: *The stented implantable heart valve design used for heart valve culture in the developed bioreactor system; (a) heart valve scaffold before cell seeding, (b) one of the tissue-engineered heart valves after culture.*

In two tissue engineering experiments, a total of six heart valves was cultured using this heart valve design. Heart valves were cultured for four weeks. Scaffolds, inserted in the polycarbonate coverings were sterilized and seeded with fibrin and human vena saphena magna cells originating from another patient as in the previous studies (chapters 3 and 4). The seeded heart valve scaffolds were positioned in the bioreactor system, sealed with o-rings. First, a circulating culture medium flow was applied after which pulsatile loads were imposed to the tissue-engineered valves. From the start of dynamic conditioning, leak flow values were very small (<5 ml/min) and leaflet deformation was induced and controlled. However, relatively small pressure differences applied over the leaflets already caused significant volumetric deformations. Pressure values from both experiments were measured, and indicated a low stiffness of the valvular constructs. As a result, heart valves were easily deformable which, eventually, resulted in rupture of four out of six tissue-engineered valves within two to three weeks of culture. The other two cultured heart valves did not fail.

Deformation controlled loading was imposed on these two valves, but the desired (large deformation) protocols could not be applied due to the high risk of heart valve tissue rupture. Tissue morphology showed a less shiny, tissue-like heart valve appearance compared to the heart valves cultured in chapters 2-4 (chapter 5). The clear visibility of scaffold remnants and stent material through the tissue also indicated reduced tissue formation (Fig. 6.1b). Quantitative tissue evaluation confirmed this observation. The average amounts of DNA, GAGs and HYP measured in the valvular tissue were smaller than all of the previously cultured heart valves (table 6.1 and chapter 5). The mechanical properties of all heart valves in this experiment were obtained by uniaxial tensile tests after culture (table 6.1). Additionally, the mechanical behavior of the two heart valves that remained intact until the end of culture was also quantified by the mechanical properties estimation method (chapter 4). Results showed that the Young's moduli assessed in the low strain region (0-15%), were a factor two to three smaller than the moduli of the heart valves created earlier (chapters 3 and 4). Since stiffness values were not significantly different in radial and circumferential direction, tissue organization, i.e. anisotropy was limited.

A likely explanation for the inferior tissue formation is the use of a different human cell source in these experiments. Human vena saphena magna cells used in the preceding experiments which showed relatively good tissue formation were not available anymore and, therefore, cells were obtained from an other donor. These cells were successfully used before in vascular tissue engineering (Pullens et al., 2009a, 2009b). To create a standard control group for the heart valve tissue engineering experiments, rectangular tissue strips were cultured under static conditions (no mechanical conditioning) in parallel with the valves and seeded with the same cell population.

Quantitative tissue analyses of all tissue-engineered heart valves showed that DNA, GAGs and HYP values were in the same range as the values found for the cultured heart valves in the last two experiments (table 6.1). Similar conformity was found in the assessed mechanical properties of both tissue constructs. Consequently, the mechanical loading protocols applied in the bioreactor system, either controlled or not, did not have an (extra) positive or negative effect on tissue formation.

In summary, the formation and organization of heart valve tissue was much lower in comparison with previous experiments (chapters 3 and 4). The change in cell source was a probable reason for this experimental outcome. The relatively low mechanical properties of the valvular constructs resulted in rupture of four out of six heart valves when deformation controlled loading was applied. As a consequence, large deformation controlled loading protocols could not be applied to the tissue-engineered heart valves. Nevertheless, the heart valves based on the stented implantable design were mechanically conditioned in the developed bioreactor system, while deformation was induced, measured and controlled during culture. In addition, the mechanical properties of two out of six heart valves were estimated non-invasively in the bioreactor system before sacrifice.

Table 6.1: *Overview of the data obtained from the performed tissue engineering experiments in which the developed bioreactor system was employed for culturing stented implantable heart valves of human and ovine origin. In addition, the data are shown of the cultured strips that served as a control, all tissue-engineered heart valves of chapters 3 and 4, and an ovine study performed by Dijkman et al. (2009). The '✓' shows that the bioreactor functionality was present and applied. 'N/A' indicates that the bioreactor functionality or mechanical properties evaluation analysis was not applicable.*

TE experiment	TE construct	Valve design	Cell source	Functionality bioreactor		Quantitative tissue analyses (µg per mg dry weight)			Thickness (mm)	Mechanical properties; Young's modulus (MPa)		
				leak flow (ml/min)	deformation meas / control	DNA	GAGs	HYP		estimated	tensile tests	
											rad	circum
1 (§ 6.2)	4 valves	implantable	human (2)	< 5	✓	1.9± 0.2	12.5± 1.7	3.8± 1.3	0.42± 0.04	0.42± 0.06 (2 valves)	0.27± 0.03	0.32± 0.1
2 (§ 6.2)	2 valves	implantable	human (2)	< 5	✓	3.1± 0.2	13.5± 1.2	9.3± 0.8	0.51± 0.05	-	0.52± 0.13	0.63± 0.18
control 2 (§ 6.2)	4 strips	-	human (2)	N/A	N/A	3.2± 0.3	15.9± 1.8	6.2± 0.6	0.68± 0.03	N/A	0.38± 0.17	
all (ch 3+4)	14 valves	implantable	human (1)	6 valves: N/A 8 valves: <5	6 valves: N/A 8 valves: ✓	4.1± 0.79	19.4± 3.6	14.2± 3.6	0.76± 0.09	1.33± 0.31	0.90± 0.36	2.19± 0.90
1 (§ 6.3)	3 valves	implantable	ovine	2 valves: ~8 1 valve: 0	✓	2.4± 0.7	23.0± 4.0	18.6± 6.6	1.3± 0.25	- 3.6	1.2± 0.39	0.99± 0.33
control 1 (§ 6.3)	4 strips	-	ovine	N/A	N/A	1.3± 0.2	13.1± 1.2	13.6± 2.3	0.8± 0.12	N/A	0.88± 0.24	
ovine study*	16 valves	implantable	ovine	N/A	N/A	3.4± 0.1	12.3± 0.3	9.7± 0.3	>1.0	N/A	only high strain region	

* *Dijkman et al., 2009*

6.3 Bioreactor application - heart valves of animal origin

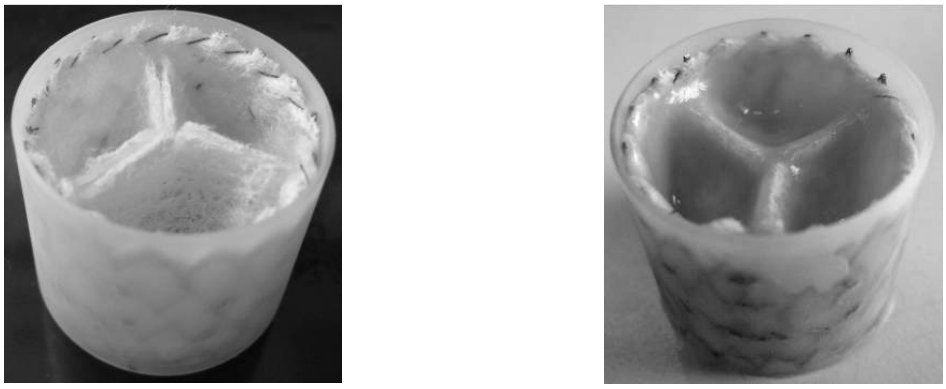
Since animal studies are essential towards clinical application of tissue-engineered heart valves, the functionality of the bioreactor system also needed to be evaluated for tissue-engineered heart valves cultured from cells of animal origin. As sheep are generally used as an animal model for evaluation of cardiovascular implants in preclinical studies (Jones et al., 1989; Hilbert et al., 1990; Ali et al., 1996; Ouyang et al., 1998), tissue-engineered valves were cultured using ovine cells originating from the jugular vein. These cells are characterized as myofibroblasts, like the cells obtained from the human vena saphena magna in previous studies (chapters 3 and 4). The cell proliferation rate and extracellular matrix synthesis of ovine cells are significantly higher than human cells. In addition, ovine cells react more intensely to mechanical conditioning (Driessen-Mol et al., 2009). The translation of in-vitro human heart valve culture protocols to sheep studies required quite some investigation, but eventually resulted in adaptation of the culture protocol. To prevent excessive tissue formation resulting in thick (>1 mm) heart valve leaflets and, thus, impaired leaflet flexibility, the culture medium contained considerably less growth serum (0.5%) in comparison with previous ovine (2.5%) (Dijkman et al., 2008a, 2008b, 2009) and human heart valve cultures (10%) (chapters 3 and 4). Seeded cell densities and culture time were similar to the human heart valves protocol.

The heart valve scaffold design was identical to the stented implantable design discussed before (§ 6.2), (Fig. 6.2a). Nonetheless, the poly-4-hydroxybutyrate (P4HB) concentration in tetrahydrofuran (THF) by which the polyglycolic acid (PGA) scaffold was coated, was increased. In ovine studies, the used P4HB concentration was 1.75% (Dijkman et al., 2008a, 2008b, 2009), while in human tissue engineering studies, a concentration of 1% P4HB in THF was used (chapters 2-4). Since P4HB is a biologically derived biopolymer by which adjacent PGA fibers are coated and physically bonded (Hoerstrup et al., 2000b), the amount or concentration of P4HB with which PGA fibers are coated, determines the stiffness of the PGA-P4HB scaffold. A higher concentration of P4HB increases the bonding between PGA fibers and, thus, the stiffness of the scaffold. Due to the higher proliferation rate and syntheses of extracellular matrix proteins by sheep-derived cells, tissue formation was more pronounced. As a result, the contractile forces exerted on the scaffold by the ovine cells, were higher. To counteract these forces an increase in P4HB concentration was desired to increase the initial stiffness of the scaffold in tissue-engineered ovine valves.

After scaffold fabrication and sterilization, cells were seeded onto three scaffolds using fibrin. Thereafter, the three seeded heart valves were positioned in the bioreactor system using the polycarbonate inserts as described before (§ 6.2). From the start of the experiment, the constructs were exposed to a circulating culture medium flow, after which dynamic loads were imposed to the tissue-engineered valves. The pulsatile load application directly led to heart valve leakage (~ 8 ml/min) measured in two out of three tissue-engineered heart valves. The third valve, however, did not show any leakage. During culture, the leak flows through the two valves were constant and deformation was measured and controlled. However, it was only possible to induce and

control a small deformation in the engineered valve leaflets. The application of an increasing load did not result in more tissue deformation but only in an increase of valve leakage. A strong tissue development overgrowing all three leaflets was observed in all sheep valves, so leakage through the valve was not occurring. The leak flows were probably forced through the space between the stent and the polycarbonate cylinder. Consequently, large deformation controlled protocols could not be applied to these valves. In contrast, the non-leaking third heart valve was subjected to controlled deformations which led to pressure differences equal to in-vivo diastolic conditions (~ 70-80 mmHg). The induced volumetric deformation in this heart valve was relatively small; 0.15 ml, which corresponded to an average strain of 2.5% in the leaflets (chapter 2). After four weeks, the mechanical properties of this cultured heart valve were assessed by the mechanical properties estimation method incorporated in the bioreactor system (chapter 4) and demonstrated a relatively high Young's modulus for the valvular tissue (table 6.1). The cultured valves were relatively thick, and valve morphology showed very dense tissue formation and a homogeneous, shiny, tissue-like appearance (Fig. 6.2b). Biochemical assays supported these findings and showed that the amounts of DNA, GAGs and HYP in the valvular tissue were larger compared to a previous ovine tissue engineering experiment (Dijkman et al., 2009). However, uniaxial tensile test data did not support these results; lower Young's moduli were found for all cultured heart valves compared to the estimated value. Moreover, tissue strips had a smaller thickness than the tissue-engineered heart valves. The strips contained less DNA, GAGs and HYP, but mechanical properties were similar as in the cultured heart valves. The difference between the estimated and measured Young's moduli could be explained by the relatively high thickness of the heart valves. In previous studies (Kortsmit et al., 2009a, 2009b), the large influence of thickness on the flexural behavior and, thus, on the deformation of the valve leaflets was discussed. So far, the thickness of tissue-engineered heart valves has not exceeded one millimetre and this resulted in accurate mechanical properties estimation (chapter 3). The effect of a larger valve thickness on the mechanical properties estimation method has not been studied, but appears to overestimate the stiffness of the valve leaflets.

When the tissue analyses of the cultured heart valves were compared with the tissue strips, larger protein amounts and thicknesses were observed for the heart valves while mechanical properties were similar. This suggested more tissue formation but less tissue organization in the cultured valves which was confirmed by the lack of anisotropy in the valvular tissue indicated by the tensile test results. Since ovine cells were known to react more intensely to mechanical conditioning than human cells (Driessen-Mol et al., 2009), it appeared that the effect of tissue growth outweighed the impact of mechanical conditioning. This thought was endorsed by the inability to apply large deformation protocols to the tissue-engineered heart valves.



a. **b.**

Fig. 6.2: *Ovine tissue engineering in the developed bioreactor system; (a) heart valve scaffold before cell seeding, (b) one of the tissue-engineered heart valves after culture.*

In summary, the tissue-engineered heart valves made from cells of ovine origin were conditioned in the developed bioreactor system through induction, measurement and control of deformation during culture. For one heart valve, mechanical properties were estimated in the bioreactor system after culture. However, for two out of three heart valves the induced deformations were minimal and valve leakage prevented the generation of larger deformations and the estimation of mechanical properties. Furthermore, despite the reduced serum concentration in the culture medium, tissue formation in the heart valves was still abundant. This resulted in relatively large valvular thickness values compared to human tissue-engineered heart valves, but these were similar as to previously engineered tissues (Dijkman et al., 2009). From a bioreactor functional point of view, the sheep valve experiment was successful. Controlled volumetric deformations were applied to the heart valves, up to limited pressure application. However, leak flows should be prevented when abundant tissue development is present.

6.4 Discussion

In the previous studies (chapters 2-4), the heart valves cultured in the developed bioreactor were not intended for implantation. To use the bioreactor system for clinical studies in the future, the functionality of the system should also be validated for implantable heart valves and heart valves created of animal-derived cells. The tissue-engineered valves used for minimally invasive implantation studies in sheep, were made of a self-expandable vascular stent in which the valve wall and leaflets were integrated (Dijkman et al., 2008a, 2008b, 2009). In this study, human heart valves based on this design were cultured in the developed bioreactor system, in which the bioreactor's measurement and control possibilities were successfully applied. Additionally, heart valves composed of cells of ovine origin were tissue-engineered in

the bioreactor system. Again, a proper functionality of the bioreactor with regard to deformation measurement and control was shown, provided that leakage was limited.

In the human tissue engineering experiments, inferior tissue development was observed. This was likely related to the alternative cell source utilized in the experiments. Despite good results with these cells in previous tissue engineering experiments (Pullens et al., 2009a, 2009b), the extracellular matrix synthesis and cell proliferation rate of the cells from this cell source was strikingly low. A similar degree of tissue formation was found in the tissue strips that were cultured parallel to the heart valves and acted as a control. The conformity between the outcomes of the strip and heart valve tissue engineering experiments suggested that the mechanical conditioning protocols applied to the heart valves had no additional influence on the cellular production of extracellular proteins. This inter-experimental variability may be caused by e.g. differences in cell batch, cell passage or storage time in liquid nitrogen. All of these factors may have had an influence on the cellular phenotype and may have affected cell proliferation rate, protein synthesis and, thus, tissue formation. Consequently, it is suggested to investigate the phenotypic differences between different cell sources and their corresponding tissue development capacity.

In the sheep experiments, two out of three tissue-engineered heart valves experienced continuous leakages during culture. Leak flows through the valves were prevented by the abundant and overall tissue formation, but leakage along the valve wall was likely occurring. This indicated two problems. The first problem involves insufficient sealing of the connection between the vascular stent (heart valve wall) and the polycarbonate insert. The second problem may be that excessive tissue growth outweighs mechanical straining by forcing fluid flow through the valve wall, where apparently less resistance is present. This phenomenon is especially relevant for the applicability of the bioreactor for the sheep model. These problems should be addressed by, first, closing the space between the stent and the polycarbonate cylinder and, second, further reducing tissue growth. A possible solution for the valve design problem could be to soften the interior wall of the polycarbonate cylinder by some kind of coating. It would improve the sealing of the connection between the valve wall and the bioreactor insert and, thus, would enable deformation of the ovine heart valve leaflets. Further reduction of tissue growth should also be established to increase accuracy of the mechanical properties estimation. An additional decrease or even a complete omission of the serum concentration in the culture medium would be a promising option to prevent abundant tissue growth in ovine tissue engineering.

6.5 Conclusion

In this chapter, heart valves based on a currently used stented implantable geometry were cultured in the developed bioreactor while the bioreactor's functionality was maintained. This was demonstrated in both tissue-engineered human and ovine heart valves. There are still some practical issues that need to be overcome. However,

these preliminary results are a step forward towards the use of the developed bioreactor system to culture heart valves suitable for future clinical implementation.

6.6 Acknowledgements

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Chapter 7

General discussion

7.1 Thesis recapitulation and main findings

Tissue-engineered heart valves are a promising alternative for currently used heart valve replacements. Despite their success, current valvular implants are made of non-living material and therefore do not have the ability to grow, adapt or remodel in response to a change in the valves' environment. In heart valve tissue engineering, the main goal is to create functional, autologous and living heart valves that overcome these limitations. However, the in-vitro formation of aortic heart valves has proven to be a significant engineering challenge. In a sheep model, tissue-engineered heart valves implanted in the pulmonary position showed sustained functionality for five months (Hoerstrup et al., 2000b). Heart valve functionality under systemic (human) conditions was also proven for cultured heart valve leaflets for at least four hours in an in-vitro set-up (Mol et al., 2006). Although the mechanical behavior of these valves appeared to be sufficient, they may need improvement. When native aortic valves are considered as a benchmark, cultured valves are too stiff at infinitesimal strains and are less anisotropic. Mechanical stimulation of the developing tissue in a bioreactor system is known to enhance tissue formation and quality, and is widely used in cardiovascular tissue engineering. More particular, inducing strains in the cultured tissue by load application appeared to be an important enhancer of tissue development. The effects of different mechanical straining protocols on tissue maturation have been studied in relatively simple tissue-engineered construct configurations (Kim et al., 1999; Mitchell et al., 2001; Engelmayr et al., 2003; 2005; 2006; 2008; Mol et al., 2003; Butcher et al., 2006; Ferdous et al., 2008; Boerboom et al., 2008; Rubbens et al., 2009). However, optimal conditioning protocols in heart valve tissue engineering have not been identified, yet. Current cardiovascular bioreactor systems are unable to induce predefined or controlled deformation to relatively complex constructs such as heart valves. A better understanding of the effects of mechanical loading on heart valve tissue development is necessary to design an optimal culturing protocol. In addition, the mechanical properties of the engineered valves have been generally assessed by sacrificing the heart valves at the end-stage of tissue culture to perform destructive testing methods (Hoerstrup et al., 2000b; Schenke-Layland et al., 2003; Mol et al., 2006; Schmidt et al., 2007a, 2007b; Cox et al., 2009). To investigate the mechanical behavior of the heart valves during culture and to test the functionality of intact valves as a non-invasive quality check, it is also desired to assess mechanical properties in real-time, non-destructively and non-invasively.

Therefore, advancement of the currently available bioreactor systems is needed to be able to measure and control induced deformations and resulting mechanical properties of the cultured heart valves. The Diastolic Pulse Duplicator (DPD) developed by Mol et al. (2005a) is one of the few bioreactor systems in which the cyclic tissue straining approach was employed, yet not deformation controlled, to human heart valve leaflets in closed (diastolic) configuration. This system was used as basis for the now presented bioreactor and was further improved in this study.

As a first step towards a feedback controlled bioreactor system, an inverse experimental-numerical approach was developed to measure volumetric and local heart

valve leaflet deformations during culture. This method was implemented in a modified version of the Diastolic Pulse Duplicator (chapter 2). Volumetric deformation was defined as the amount of fluid displaced by the deformed heart valve leaflets in a stented configuration. This was measured non-invasively using a flow sensor. A computational model was employed to relate volumetric deformation to local tissue strains in various regions of the leaflets (e.g. belly and commissures). The flow-based deformation measurement method was validated and the accuracy of the measurements proved to be good, provided that leakage through the valve was not excessively high. The functionality of the measurement technique was demonstrated in an in-vitro setting. Tri-leaflet, stented tissue-engineered heart valves were strained in-vitro, showing physiologically realistic values for volumetric and local deformation during mechanical loading.

Consecutively, the inverse experimental-numerical approach was further developed and applied to assess the mechanical properties of the tissue-engineered heart valves, non-invasively and non-destructively (chapter 3). A range of increasing pressure differences was applied and the corresponding induced volumetric deformations of the engineered heart valve leaflets were measured during culture. The correlation between these two data sets (pressure difference and deformation) served as input for the estimation of mechanical properties using the computational model. To validate the method, six heart valves were cultured, and the mechanical properties obtained from the inverse experimental-numerical approach were in good agreement with uniaxial tensile test data. Furthermore, the diastolic functionality of the heart valve leaflets was assessed in the developed bioreactor by studying the deformation and leakage of the cultured heart valves under physiological aortic diastolic pressure differences.

As a second step towards a controlled bioreactor system, the above described deformation assessment method was advanced by addition of a feedback control mechanism (chapter 4). The resulting technique enabled both measurement and control of the heart valve deformation in real-time and non-invasively. Functionality of this approach was demonstrated in two tissue engineering experiments in which a total of eight heart valves were cultured by application of two different deformation protocols. Results indicated a good correlation between the measured and the prescribed deformation values in both experiments. In addition, the cultured heart valves showed mechanical properties in the range of that was previously reported. However, no significant differences in mechanical properties were found between the valves cultured by the dissimilar protocols.

In chapters 3 and 4, 14 tissue-engineered heart valves were cultured to validate the developed deformation and mechanical properties assessment and control methods, and to show their feasibility in practice. In these chapters, the mechanical properties and diastolic functionality of all tissue-engineered heart valves were examined. To extend the scope of the qualification criteria, the cultured heart valve tissue was also analyzed qualitatively (macroscopic appearance and immunohistology) and quantitatively (biochemical assays), as discussed in chapter 5. The 14 heart valves cultured in four independent experiments all showed a dense, homogeneous tissue with

a smooth surface. Tissue composition was comparable to previously performed cardiovascular tissue engineering studies (Mol et al., 2006; Boerboom et al., 2008; Stekelenburg et al., 2009). Collagen type I and III were demonstrated throughout the tissue, as well as striated structure fragments of elastin, both of which are the main structural matrix components of natural heart valves.

The next step towards preclinical application of the controlled bioreactor system was to optimize the system to culture tissue-engineered heart valves suitable for (minimal invasive) implantation (chapter 6). First, implantable stented human heart valves were successfully cultured in the bioreactor system. Thereafter, the bioreactor system was employed to create ovine tissue-engineered heart valves suitable for animal studies. Bioreactor functionality was demonstrated for one of the three cultured ovine heart valves. The presence of leakage along the heart valves was still an issue in the other valves and needs further optimization.

7.2 Implications of the developed bioreactor system

In this thesis a feedback controlled bioreactor system has been developed, capable of inducing controlled deformations and assessing mechanical properties of tissue-engineered heart valves in real-time and non-invasively. In the literature, some studies appeared in which deformation and motion of heart valve leaflets were monitored, however this was performed invasively or not in real-time during culture (Lo & Vesely, 1995; Gao et al., 2000, 2001, 2002; Iyengar et al., 2001; Jensen et al., 2001; Sacks et al., 2002; Chen et al., 2004; Sun et al., 2005; Mol et al., 2005a; Syedain & Tranquillo, 2009). The assessment of the mechanical behavior of heart valve tissue has mostly been performed with uniaxial or biaxial tensile tests or indentation tests (Hoerstrup et al., 2000b; Schenke-Layland et al., 2003; Mol et al., 2006; Schmidt et al., 2007a, 2007b; Cox et al., 2009). These methods are destructive and can therefore only be performed at the end-stage of tissue culture and never on valves that will be implanted.

The possibilities to monitor and control heart valve deformation and to assess the valve's mechanical behavior are a large step forward to design an optimal protocol for tissue engineering of heart valves. These bioreactor functionalities are performed non-invasively and in real-time during culture without compromising sterility in the bioreactor, and are also an important step towards the increase in reproducibility of the quality of the tissue-engineered heart valves. In this context, heart valve quality also encompasses the ability of the tissue-engineered heart valve to withstand pulmonary and systemic diastolic pressure differences without rupture of the tissue or a significant increase in valve leakage. In the bioreactor system leak flow measurements are performed to check the valve's integrity under loading. The bioreactor system offers the possibility to monitor the tissue engineering process and to intervene, i.e. by adjusting the protocol when heart valve tissue development deviates from the planned trajectory. Unsatisfactory tissue development is detected early in an experiment and as

a result, tissue culture can be adjusted or terminated and restarted. This way, loss of time which may be crucial for the patient, and loss of money is prevented.

7.3 Limitations of the developed bioreactor system

The presented bioreactor system offers new opportunities in heart valve tissue engineering. Nevertheless, there are also some limitations. Leakage between the valve leaflets or alongside the valve stent is the most disturbing factor in volumetric deformation measurements of the tissue-engineered heart valves during tissue culture. It was shown that a rise in leakage, notably above 30%, results in an increase in the inaccuracy of the volumetric deformation measurement (chapter 2) and, therefore, the mechanical properties assessment (chapter 3). The amount of leakage through the cultured heart valves was indicated as a fraction of the fluid flow that entered the bioreactor to induce leaflet deformation (chapters 2 and 3), and as an absolute value (chapter 4). Although leak flow values could be measured precisely, tissue engineering experiments showed that at the end of tissue culture, heart valve leakage was either relatively large, hindering measurement of any deformation, or close to zero. Intermediate values were seldom found. When heart valve leakage was large from the start, leak flow could decrease during culture (chapter 4). This would typically occur within one week after the start of dynamic loading. However, a continuous large or abrupt leak flow through the heart valve during culture indicated permanent heart valve failure. Since the leak flow values have such a large influence on heart valve deformation measurement, it is very important that the heart valve perfectly fits inside the bioreactor. Even a small leak flow along the heart valve wall will lead to a less accurate deformation measurement and an overestimation of the leak flow through the valve. In case of relatively large leakage, volumetric deformation may not be accurately measurable and controllable, but may still be applied to the heart valve leaflets.

Another limiting factor in the accurate measurement of leaflet deformation is movement of the valve wall. Since volumetric deformation of the heart valve is defined as the amount of fluid displaced by the deforming valve leaflets, extra wall deformation would increase this amount and, thus, makes the leaflet deformation measurement less precise. For an accurate leaflet deformation measurement, it is therefore important for the heart valve wall to be rigid. When heart valve cultures are expanded towards stentless heart valves to include aortic sinuses for example, the deformation of the heart valve wall needs to be included in the volumetric deformation measurement. In that case, re-evaluation of the measurement technique is inevitable. In summary, both heart valve leakage and valve wall deformation have a negative influence on the accuracy of the volumetric deformation measurement. The abundant presence of either one of these factors during heart valve culture interferes strongly with measurement and control options of the developed bioreactor since the deformation measurement method is indirect. A direct measurement technique such as ultrasound, as used in vascular tissue engineering to measure vascular wall displacement after culture (Pullens et al., 2009b) would not have this drawback. However, this technique cannot assess 3D valve

deformation. Moreover, it cannot be used for continuous measurements during culture and is too large to fit in a standard incubator.

Several assumptions were made in the computational model to correlate volumetric deformation to local tissue strains (chapter 2) and to assess the mechanical properties of tissue-engineered heart valves (chapter 3). A non-linear Neo-Hookean model was used to describe the mechanical behavior of the leaflets. Material properties were assumed to be homogeneous and isotropic, and the thickness was assumed to be uniform throughout the valve leaflet. For native aortic heart valves these assumptions are not correct since the material properties of this tissue are inhomogeneous, anisotropic and the thickness is non-uniform (Stradins et al., 2004; Balguid et al., 2007a). However, for tissue-engineered heart valves the model's assumptions were correct in the first two weeks of culture. In this period, the mechanical behavior of the valve was predominantly determined by the scaffold material which was isotropic and homogeneous in thickness and material properties, and tissue development was still moderate. After three to four weeks of culture, anisotropy was increased but inhomogeneity or non-uniformity in the thickness was not directly observed (Mol et al., 2005a, 2006). In this thesis, the effect of inhomogeneity and anisotropy in the material properties, on the estimated mechanical properties was studied and found to be small in the computational model. The minor effect of inhomogeneity was ascribed to the type of deformation that was considered. The global deformation of the heart valve leaflets was used as input for the model. Since it was not possible to extract local leaflet stiffness values from this, only average stiffness values in the leaflets were assessed (chapter 3). Relatively large degrees of inhomogeneity were examined and anisotropy was varied in a range relevant for tissue engineering. However, the combined effects of inhomogeneity and anisotropy were not investigated. Since the estimated mechanical properties showed good correspondence with uniaxial tensile test data, the estimation by the model seemed to be accurate for the degrees of inhomogeneity and anisotropy found in the tissue-engineered heart valves after culture.

In chapters 2, 3 and 6, the influence of valve leaflet thickness on the flexural behavior and, thus, on the volumetric deformation of the leaflets was discussed. When leaflet thickness increases, the geometrical change of the valve results in a gradual decrease of the flexural behavior of the heart valve. This effect was particularly present when thickness exceeded one millimeter and/or large deformations were considered. Consequently, the direct relationship between volumetric and local deformations was most sensitive for a variation in thickness (chapter 2). Moreover, additional pressure was required to deform the heart valve when leaflet flexure was impaired by thickness. The mechanical properties estimation method (chapter 3) resulted in an overestimation of the stiffness of the valve leaflets, which was seen for the ovine heart valve in chapter 5. As long as the thickness of the tissue-engineered heart valves did not exceed one millimeter, an accurate estimation of the mechanical properties could be performed (chapter 3). However, the effect of a larger valve thickness on the mechanical properties estimation method was not studied and needs further investigation.

The mechanical properties of the tissue-engineered heart valves were estimated by using the combination of applied pressure differences and corresponding induced

volumetric deformations of the heart valve as input for the computational model. At physiological load, the maximum value of the induced volumetric deformation did not exceed 0.35 ml (chapter 3). This value corresponded to a mean strain value of 7.0 ± 1.8% in the leaflets (Kortsmit et al., 2009a). The estimated mechanical properties represented the mechanical behavior of the cultured tissue in the lower 0-15% strain region. Hence, the estimated value was validated by the Young's modulus assessed in the same lower strain region of the stress-strain curve obtained by uniaxial tensile testing. This is in contrast to previous studies in which the mechanical properties were assessed in the steepest part of the stress-strain curve at much higher strain values (Clark et al., 1973; Sodian et al., 2000; Mol et al., 2003, 2005, 2006; Stradins et al., 2004; Driessen et al., 2007; Schmidt et al., 2007a, 2007b). However, the low strain region encompasses physiological deformation of the tissue-engineered heart valve. Nonetheless, in-vivo deformation of native aortic valves under diastolic loading was larger, and studied by Driessen et al. (2005) using computational modeling. In this study, the biaxial mechanical data of Billiar & Sacks (2000) were used as input for the model. The simulations showed in-vivo strains of approximately 30% and 60% in circumferential and radial direction, respectively. However, fresh porcine heart valves loaded under diastolic conditions, experienced strains that were considerably smaller; 10% in circumferential and 23% in radial direction (Lo & Vesely, 1995). The induced strains in glutaraldehyde treated porcine valves were similar to the assessed strains in tissue-engineered heart valves. Diastolic pressurization led to strains of 2-4% and 3-10% in circumferential and radial direction, respectively (Adamczyk & Vesely, 2002), and an average strain value of 4-10% in the heart valve leaflets (Sun et al., 2005). Chemically fixed anisotropic tissue was described to become more isotropic (Zioupos et al., 1994) and less compliant than fresh tissue (Broom et al., 1982; Schoen et al., 1997; Billiar & Sacks, 2000) due to chemical crosslinking. These findings may explain why engineered and fixed native tissues have lower strains at physiological (diastolic) loading, compared with fresh native tissue. Furthermore, when the mechanical properties of the tissue-engineered heart valve were estimated at the end of culture, leaflets were grown together and a prestress was present in the tissue. This was observed by flattening of the bulged valve leaflet shape as a result of tissue compaction (chapter 5, Mol et al., 2006). Due to this compaction, the compliance of the tissue-engineered heart valve decreased and induced strains were smaller, and similar to the strains found in glutaraldehyde-treated valvular tissue. In addition, the presence of prestress in the tissue-engineered heart valve leaflets resulted in some overestimation of the mechanical properties. However, the successful validation of the in situ estimation method of the mechanical properties by uniaxial tensile tests indicated that this overestimation was small.

7.4 Future perspectives and recommendations

7.4.1 Control and standardization of the tissue engineering process

The presented bioreactor system can be considered a significant improvement compared to previous bioreactors. It provides more control in the heart valve tissue engineering process to increase the reproducibility of the cultured heart valves. Due to the controlled application of induced deformations, mechanical conditioning protocols can be employed in a well-defined way. However, apart from the mechanical conditioning protocol, other factors that have a large influence on tissue formation and tissue quality should also be controlled or standardized. These factors are related to 1) the cells; e.g. cell source (patient), cell-seeding density, cellular phenotype, cell passage number, 2) the culture medium; e.g. amount of different additives, batch-variability of medium and additives, or 3) the culture procedures; e.g. fibrin preparation, seeding technique, frequency of medium change. The factors associated with the culture procedures are relatively easy to standardize, however, to obtain more control over the cell related and culture medium related elements will be a great challenge in (heart valve) tissue engineering.

7.4.2 Optimization of conditioning protocols

In chapters 2-4 of this thesis, the heart valve leaflet deformation measurement and control techniques incorporated in the developed bioreactor system were presented and validated. Human heart valves were cultured to support this validation and, consequently, different deformation controlled conditioning protocols were applied. So far, these protocols have proven not to be sufficiently distinctive as they have not resulted in a significant improvement of the mechanical properties (chapters 2 and 3) or tissue composition (chapter 5) of the tissue-engineered heart valves when compared with previous studies. Since native aortic valves were set as benchmark for heart valve tissue engineering and this standard has not been reached yet, improvement of tissue formation and organization may be needed. This may be achieved by further optimization of the mechanical conditioning protocol. The developed bioreactor system is a well defined platform to systematically study the effects of mechanical straining on tissue development and, thus, to design an optimal conditioning protocol for heart valve tissue engineering.

In chapter 4, it was found that the mechanical properties were relatively insensitive to the applied deformation, at least within the range tested (0.10-0.15 ml). Inter-experiment variability had a larger effect. In the future, differences in maximal applied deformation between protocols should be increased to study the relation between the induced deformation and mechanical behavior of the tissue-engineered heart valves over a wider range (Cox et al., 2009). In addition, intermittent loading protocols appeared to be more beneficial for tissue development and organization, i.e. increased collagen production and crosslink formation, in tissue-engineered strips than continuous loading protocols (Boerboom et al., 2008; Rubbens et al., 2009). Based on

these findings, a conditioning protocol was proposed in which continuous dynamic loading is applied for short periods of time each day (e.g. two hours) followed by a resting period. The initially applied strain magnitude should not be too high (Boerboom, 2007).

Finally, the combination of strain-based and flow-based load application to cardiovascular tissue-engineered constructs has shown to have a synergistical effect on the mechanical properties of the tissue (McCulloch et al., 2004; Engelmayr et al., 2006, 2008; Hahn et al., 2007). Animal studies in which tissue-engineered heart valves were implanted showed an increase in leaflet flexibility which may be due to in-vivo tissue remodeling (Hoerstrup et al., 2000b, 2002). The additional application of physiological flow is thought to improve the valve's mechanical behavior in general, and leaflet flexibility in particular. Consequently, appendix A addresses a further advanced bioreactor design, which extends the revised bioreactor with systolic flow capabilities. The newly developed bioreactor system would allow the independent application of both controlled strain-based and physiological flow-based loads to tissue-engineered heart valves.

7.4.3 Application of the developed bioreactor system

To employ the developed bioreactor system to culture heart valves for preclinical animal studies and for future human clinical trials, the bioreactor system itself and the design of the heart valves cultured in it, need to be adjusted. In chapter 6, the first steps were made towards this ambition.

The non-invasive measurement and control possibilities of the bioreactor system were successfully applied to stented heart valves suitable for minimal invasive implantation studies. The valve design itself, in which valve leaflets were sutured to a radially self-expandable vascular stent was identical to the heart valve design used in ovine tissue engineering (Dijkman et al., 2008a, 2008b, 2009). However, adapting the design to fit the original valve in a polycarbonate cylinder was necessary for proper functioning of the bioreactor. After investigating and implementing the design, one experiment was conducted to culture ovine tissue-engineered heart valves in the bioreactor system. In this experiment just one out of three cultured valves did not leak and allowed deformation measurement and control. The presence of leakage was an issue in the other cultured valves. Since it was assumed that leakage was in between the stent and the polycarbonate insert, this connection was insufficiently sealed. Softening of the interior wall of the bioreactor insert could improve sealing and may stop leakage (chapter 6). Hence, from a design point of view the bioreactor was validated to culture stented implantable heart valves, however, the leakage alongside the valve wall in ovine tissue engineering needs to be resolved and further investigated.

7.5 Conclusion

In this thesis, a controlled feedback bioreactor system was developed to measure and control deformations, and to estimate the mechanical properties of tissue-engineered heart valve leaflets in closed (diastolic) configuration, non-invasively and in real-time. The functionality of the bioreactor system was successfully validated and demonstrated in-vitro. In the future, the set-up will be enhanced by addition of (systolic) flow generation. The bioreactor system concept offers many possibilities in heart valve tissue engineering with respect to the optimization of the mechanical conditioning protocols, and the performance of several heart valve functionality and quality testing procedures.

Appendix A

New bioreactor design for controlled diastolic straining and systolic flow application in heart valve tissue engineering

A.1 Introduction

Heart valve tissue engineering is a promising technology to overcome the drawbacks associated with currently used heart valve replacements. Despite recent progress, the mechanical behavior of tissue-engineered heart valves still needs improvement when native heart valves are considered as benchmark. The application of mechanical stimulation to tissue constructs in culture has appeared to be a successful approach to improve mechanical properties and tissue development. As a result, many different bioreactor systems have been developed in which heart valves were mechanically loaded. In most of these bioreactor systems, physiological flow was mimicked. Mechanical loading was either applied in a wide physiological range, mimicking both systole as well as diastole (Rutten et al., 2002; Dumont et al., 2002; Hildebrand et al., 2004; Ruel et al., 2009) or was characterized by simulation of the systolic or opening phase of the cardiac cycle (Hoerstrup et al., 2000a; Zeltinger et al., 2001; Rabkin et al., 2002; Schenke-Layland et al., 2003). The induced tissue response, i.e. tissue strains or shear stresses, was unknown and neither assessed nor controlled. Since the positive effect of cyclic tissue straining on tissue-engineered constructs has been demonstrated in literature (Kim et al., 1999; Niklason et al., 1999, 2001; Seliktar et al., 2003; Mol et al., 2003, 2005; Boerboom et al., 2008; Ferdous et al., 2008; Rubbens et al., 2009), a few studies developed bioreactor systems in which diastolic straining was simulated. In these studies, dynamic pressure differences were applied across the cultured heart valves in closed configuration inducing tissue deformation. However, tissue strains were applied but neither measured nor controlled. Mol et al. (2005a) estimated, by using finite element analysis, the amount and distribution of the dynamic local strains in the heart valve leaflets after culture. Syedain & Tranquillio (2009) did neither assess local tissue strains but measured the stretching of the tissue-engineered valve root in real-time during culture.

Hence, the main shortcoming of all current heart valve bioreactor systems is the lack of control during load application. Preset transvalvular pressures are applied to the developing heart valve while the induced deformations are unknown. Maximum strain values may vary during conditioning as a consequence of changing mechanical properties of the engineered construct. The effects of variation in applied deformation on tissue remodeling are yet unknown. Therefore, a bioreactor system was developed in our previous studies in which heart valves were cultured in diastolic configuration, and in which it is possible to measure and control heart valve leaflet deformation in real-time and non-invasively (Kortsmit et al., 2009a, 2009b). In addition, a combined experimental-numerical approach was applied to assess the mechanical properties of the tissue-engineered valves during culture, non-invasively and non-destructively (Kortsmit et al., 2009c).

Despite the new possibilities of this developed bioreactor system only strain-based loads can be applied by simulation of the diastolic phase of the cardiac cycle. However, it is desired to incorporate flow-based load application into the bioreactor concept to be able to mimic complete physiological loading as a pre-implantation functionality check of the tissue-engineered heart valve. In addition, to enhance the

mechanical behavior, i.e. flexibility of the tissue-engineered heart valves towards native benchmarks, the mechanical conditioning protocol needs to be optimized and flow-based loading seems to be essential. Therefore, in this study, the previously developed bioreactor concept of controlled diastolic straining of tissue-engineered heart valves was enhanced by addition of a systolic flow generator. The concept and design of this improved flow bioreactor system are presented here.

A.2 Bioreactor requirements

In an ideal bioreactor, a tissue-engineered valvular structure that is able to grow, repair and remodel and has tissue properties sufficient to withstand in-vivo conditions is obtained after a couple of weeks of culturing. To develop such a bioreactor, the system should meet the following requirements. The set-up itself should be simple, be small and easily sterilizable. In addition, easy refreshment of the culture medium should be possible as well as access to, and visualization of the tissue-engineered valve. The use of a pump should not disturb the climate in the incubator and the culture medium must be exposed to the controlled atmosphere in the incubator. Handling of the tissue-engineered construct should be without difficulty and completely sterile. With respect to the mechanical stimulation of the heart valve, the possibility to apply a wide range of pressures and flows is desirable. These values should be measured and controlled to produce stable and repeatable waveforms. Physiological loading should be feasible but also simulation of pathological, hypo- and hyper physiological conditions are required. Furthermore, it should be possible to apply both strain-, and flow-based loading, alternatingly and independently, for mechanical conditioning of the cells in the tissue-engineered construct. Loading profiles and their effects on the cultured tissue should be studied by measuring pressure, flow, deformation, and mechanical properties during culture in real-time, non-invasively and non-destructively. To be able to regulate the induced tissue response, it is essential to implement a control mechanism for tissue deformation and/or the mechanical properties of the cultured tissue. In addition, the bioreactor must allow the testing of tissue-engineered heart valves, non-invasively without disrupting sterility, as a functionality and quality check. Preferably, it should be able to perform testing procedures according to industry standards imposing design specifications and minimum performance specifications for heart valve substitutes. Lastly, the bioreactor system should proof its functionality in-vitro by performing successful tissue engineering experiments in the set-up (Dumont et al., 2002; Barron et al., 2003; Hildebrand et al., 2004; Ruel et al., 2009).

A.3 Newly developed flow bioreactor

A.3.1 Bioreactor design

The newly developed flow bioreactor is based on the previous bioreactor system for mimicking the diastolic phase of the cardiac cycle by applying strain-based loading (Kortsmit et al., 2009a). However, simulation of the systolic phase has been included by modification of the existing concept. The bioreactor system (Fig. A.1) consists of the following main components; a pulsatile pump (A) encompassing a flexible collapsible rubber tube, a culture chamber (B) in which the heart valve is tissue-engineered, a ball valve (C) positioned between the pump and the culture chamber, a height adjustable upper bioreactor part including a glass window (D) for heart valve visualization, outer stainless steel and silicone housing (E) and a small culture medium reservoir (F) including a control needle (G). All these bioreactor parts are composed of stainless steel; grade 316L, suitable for medical applications, except the culture chamber and outer housing. The culture chamber is made of polycarbonate, and silicone rubber is used for the outer mantle. Additionally, silicone rubber rings and tubing (t) have been used to seal and connect the bioreactor components, respectively, and the ball valve is lined with Teflon. The height of the bioreactor is 43 cm and its outer diameter is 12 cm, excluding the control needle. The total amount of culture medium in the bioreactor is 250 ml.

The pulsatile pump (length: 18 cm, inner diameter: 30 mm) in the bioreactor is driven by a proportional pneumatic-air valve (Festo, Esslingen Berkheim, Germany) (not shown) of which the amount, frequency, and waveform of air release is controlled. The proportional pneumatic-air valve periodically releases air and alternatingly compresses and decompresses the silicone rubber tube (thickness: 0.5 mm) of the pulsatile pump. As a result, fluid from the medium reservoir is pumped through the ball valve and injected into the culture chamber. The culture chamber (length: 70 mm, inner diameter: 25 mm) encloses the tissue-engineered heart valve, with its aortic side facing upwards. Dependent on the type of mechanical loading that is applied, the injected culture medium can enter the culture chamber above or below the tissue-engineered heart valve. This is determined by the configuration of the full port ball valve (length: 60 mm, inner diameter: 25 mm). A full port ball valve is a valve that opens by turning a handle attached to a ball inside the valve. The ball has a port through the middle so that when the port is in line with both ends of the valve, flow can occur. When the valve is closed, the hole is perpendicular to the valve, and flow is blocked. The tissue-engineered heart valve is loaded strain-based when the ball valve is closed and flow-based or physiologically when the ball valve is opened. Opening and closure of the ball valve is pneumatically controlled. Culture medium exits the culture chamber through silicone rubber tubing (t) or through the outflow opening between the culture chamber and the upper part of the bioreactor. The flow resistance of this outflow channel is manually adjustable and the opening and closure of the channel is pneumatically controlled. From the culture chamber, medium flows into the outer mantle of the bioreactor.

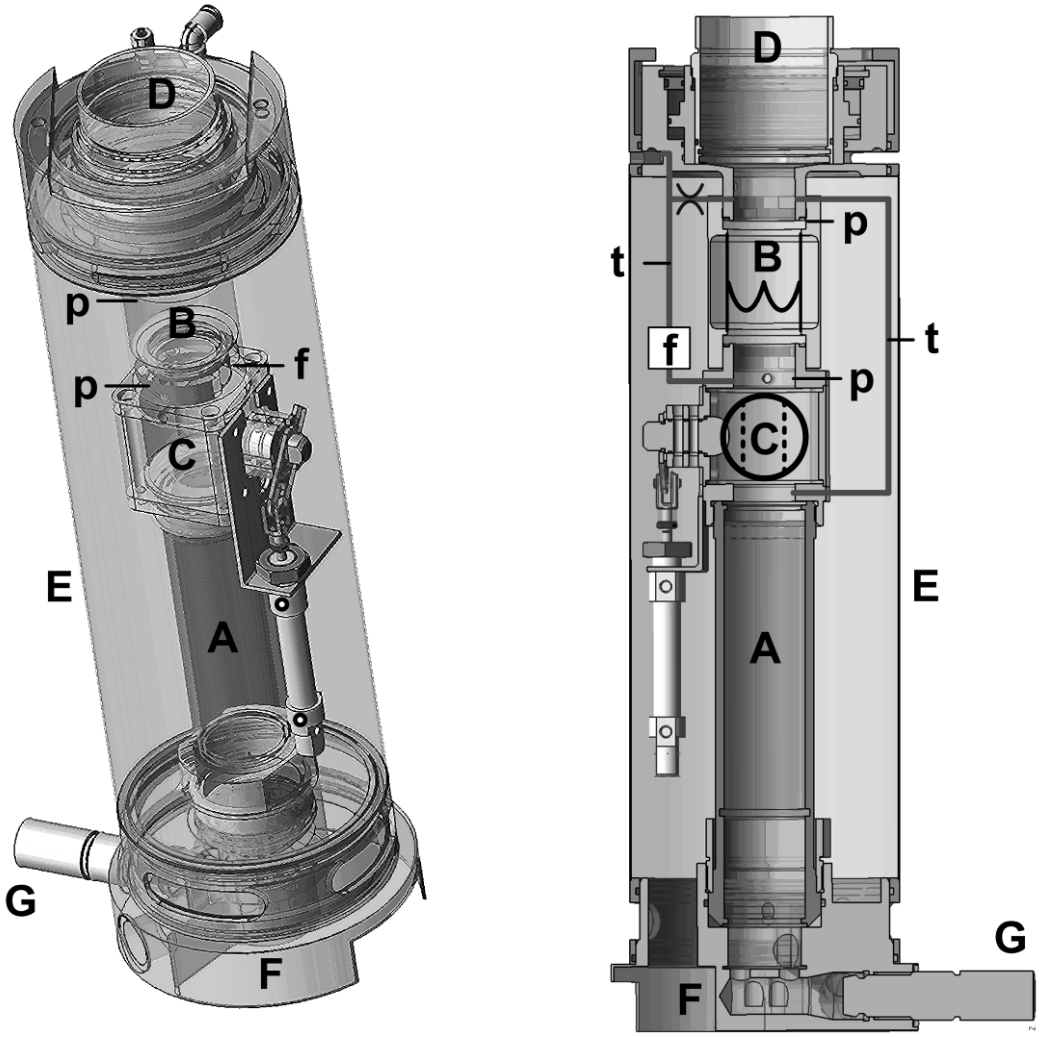

Fig. A.1: *Schematic drawings of the flow bioreactor design; (a) side view of the bioreactor, and (b) longitudinal cross-section, showing the pulsatile pump (A), culture chamber (B), open ball valve (C), upper part including a glass window (D), outer mantle (E), medium collector (F), and control needle (G). Also indicated are the positions of the pressure sensors (p) at both sides of the culture chamber, the flow sensor (f), and silicone rubber tubing (t) inside the outer mantle.*

This mantle is composed of a stainless steel column slid over the bioreactor's interior and, a silicone rubber sheet covering this column. So, medium is accumulated between the metal column and the rubber housing. This housing functions as a semi-permeable membrane to oxygenate the circulating medium when the bioreactor is positioned in a humidified incubator (37℃, 5% CO_2). Thereafter, the medium is collected in the small medium reservoir at the base of the bioreactor system and conducted to the pulsatile pump by compression and decompression of the silicone rubber tube of the pump.

A.3.2 Strain-based load application

For diastolic strain-based load application, the previously developed bioreactor concept is used (Kortsmit et al., 2009a). In the new flow bioreactor system this concept has been incorporated and effectuated by closure of the ball valve (Fig. A.2). Hence, the collapsible tube of the pulsatile pump (A) is compressed by air and the generated medium flow is not directed through the ball valve (C) but through silicone rubber tubing (t1) into the culture chamber (B). The medium is injected in the volume enclosed by the upper part of the bioreactor (D) and the aortic side of the tissue-engineered heart valve; the upper culture chamber. During diastolic straining, the upper bioreactor part is maximally lowered by pneumatic control. Consequently, the upper culture chamber is closed and only allows medium flow to exit through silicone rubber tubing (t2) into the outer mantle of the bioreactor. When the generated pulsatile flow enters the upper culture chamber, a dynamic pressure difference is created over the valve which causes the heart valve leaflets to deform. After deformation, the heart valve returns to its unloaded configuration and fluid flow exits the upper culture chamber through silicone rubber tubing (t2) and enters the housing to be oxygenated. Pressure sensors (p) measure the pressures at both sides of the heart valve leaflets, and a flow sensor (f) monitors the exit flow rate of the culture medium from the lower culture chamber; the volume confined by the closed ball valve and the ventricular side of the heart valve.

Deformation measurement and control

Flow measurements during strain-based load application are performed to assess the volumetric deformation and leakage of the heart valve leaflets as described in this thesis (Kortsmit et al., 2009a, 2009b, 2009c). Volumetric deformation is defined as the amount of fluid exiting and subsequently reentering the lower culture chamber of the bioreactor in a single loading cycle. The net flow leaving this lower chamber is defined as the amount of fluid leaking through the valve. Volumetric deformation and leakage of the cultured valve are obtained after time integration of the exit flow rate. Leak flow values are used as an indication of valve functioning and are assessed to check the accuracy of the deformation measurement method, which is known to decrease as leak flow increases (Kortsmit et al., 2009a).

To apply controlled diastolic deformation to the cultured heart valves, the magnitudes of the applied loads are adjusted in such a way that prescribed deformation is induced in the tissue-engineered heart valve leaflets. Desired strains are obtained in the cultured valves by implementation of a deformation control loop in the bioreactor soft- and hardware, which controls the pulsatile pump. After each loading period, the volumetric deformation of the heart valve is assessed by integration of the measured flow signal. Because the corrective action of the feedback mechanism is determined by the difference between the measured and the reference deformation value, load application is controlled once every loading period. Therefore, the shape of the induced deformation signal within a loading period is not controlled, only its maximum value.

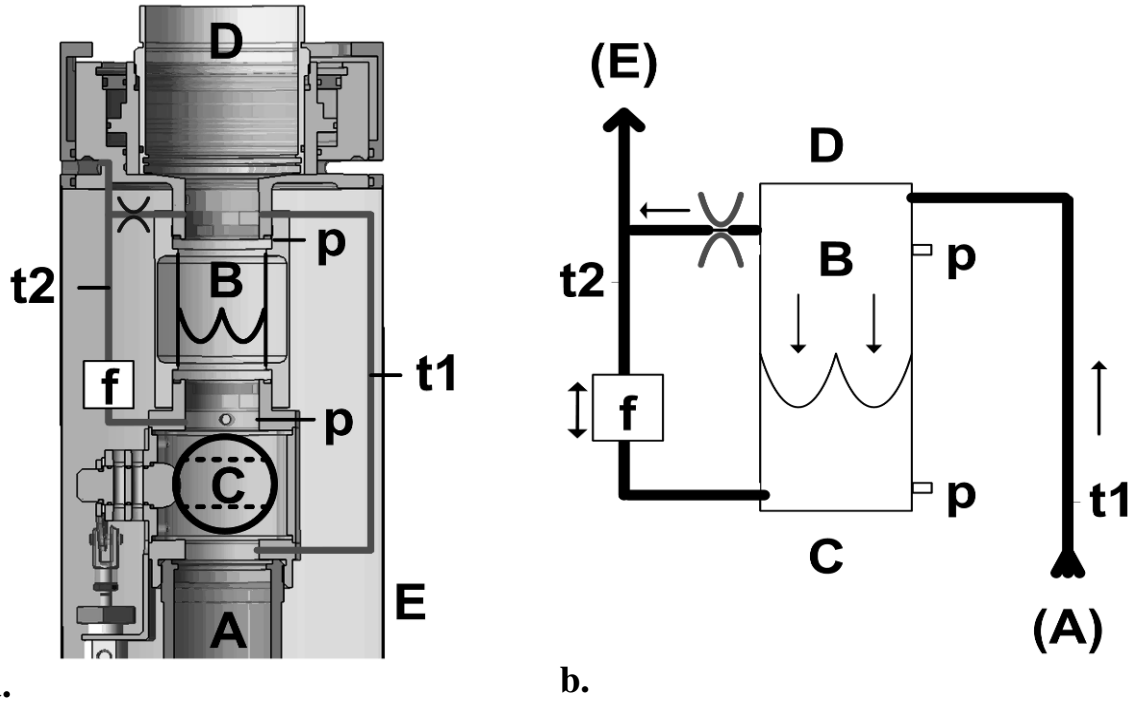

Fig. A.2: *Schematic drawings of the bioreactor section which is involved in diastolic strain-based load application; (a) longitudinal cross-section, and (b) simplified representation, showing the pulsatile pump (A), culture chamber (B), closed ball valve (C), upper bioreactor part including a glass window (D), and the outer mantle (E). Also indicated are the silicone rubber tubing (t1 & t2), the locations of the pressure sensors (p) at both sides of the heart valve and the flow sensor (f).*

A.3.3 Flow-based or physiological load application

The application of flow-based or physiological load application is simulated by complete opening of the ball valve resulting in an unrestricted flow of culture medium from the pulsatile pump into the culture chamber. The heart valve leaflets open and the fluid flow exits the culture chamber from the top. Since the systolic flow is relatively large, silicone rubber tubing is not sufficient to guide this flow outside the culture chamber. Consequently, the upper part of the bioreactor is pneumatically moved upwards to open the outflow channel. Thereafter, culture medium is collected in the outer mantle and conducted to the medium collector at the base of the bioreactor. The control needle regulates the flow between the medium collector and the pulsatile pump.
To produce physiological flows relevant for the aortic heart valve, the in-vivo systemic circulation is simulated by the different components of the bioreactor set-up. The pulsatile pump represents the left ventricle and pumps culture medium through the tissue-engineered heart valve. The systemic afterload is simulated by a four element Windkessel model, representing the aortic resistance, systemic inertance, systemic compliance and peripheral resistance. The height adjustable upper part of the bioreactor that regulates the outflow resistance from the culture chamber into the outer mantle

embodies the aortic resistance. The systemic inertance is mimicked by the inertia of the fluid column above the tissue-engineered heart valve. The flexibility of the silicone rubber covering the outer metal housing acts as a systemic compliance. Finally, the peripheral resistance is resembled by the resistance of the medium flow-through from the silicone housing to the medium collector at the bioreactor base and is directed by the control needle.

Physiological load application is simulated by regulation of the pressure signals measured at the aortic and ventricular side of the tissue-engineered heart valve. To obtain physiological pressure levels and pulse shapes, the afterload of the system needs to be tuned. In the bioreactor, only two of the four elements Windkessel model can be tuned manually; the aortic resistance and the peripheral resistance. The systemic inertance is constant since there is no possibility to vary the amount of fluid at the aortic side of the heart valve. In addition, the geometry and stiffness of the silicone housing is predefined and, therefore, the systemic compliance is not adjustable either.

A.3.4 Biocompatibility and sterilization

The bioreactor has been designed to be biocompatible. The construction materials that are in contact with the tissue-engineered heart valve and culture medium are stainless steel grade 316L, polycarbonate, silicone rubber and Teflon. These materials were tested and appeared to be not toxic (data not shown). Prior to operating the bioreactor system, the bioreactor components are sterilized. First, the complete set-up is taken apart in two main sections. The lower section comprising the pulsatile pump (A), the culture chamber (B), the ball valve (C), the small culture medium reservoir (F) including the control needle (G), and silicone rubber tubing (t1 & t2) (Fig. A.1). The top section holds the upper bioreactor part enclosing the glass window (D) and the outer metal and silicone rubber housing (E) (Fig. A.1). Thereafter, both sections are sterilized in an autoclave by subjecting them to high pressure steam at 121 °C for 30 minutes. After sterilization, the two bioreactor sections are transferred to a LAF cabinet in which, the silicon tubing, pressure and flow sensors are connected. Additionally, the top and lower bioreactor parts are reunited by screwing them together. The sterilization procedure is completed in one day. After sterilization and assembly, the bioreactor system is filled from the base with 250 ml culture medium.

A.4 Discussion

In heart valve tissue engineering, the main shortcoming of the current bioreactor systems is the lack of control during load application. Strain-based, flow-based or physiological loads were applied to the cultured heart valves without considering the tissue response, i.e. deformation of the valve leaflets. Therefore, a bioreactor system have been developed in our previous studies in which diastolic straining was imposed on the heart valves, and in which it is possible to measure and control heart valve leaflet deformation in real-time and non-invasively (Kortsmit et al., 2009a, 2009c). In addition, a combined experimental-numerical approach was applied to assess the mechanical properties of the tissue-engineered valves during culture, non-destructively and non-invasively (Kortsmit et al., 2009b). The possibilities of this bioreactor system were an improvement compared to preceding set-ups. However, flow-based load application was not incorporated. To be able to apply physiological conditions to the cultured heart valves as a heart valve quality and functionality test, and to enlarge mechanical conditioning options, it should be possible to generate flow in the bioreactor. Hence, in this study, the previously developed bioreactor concept of controlled diastolic straining of tissue-engineered heart valves was enhanced by addition of systolic flow-based load application.

The design of the newly developed flow bioreactor allows the combined and independent application of strain-, and flow-based loads. Both types of loads are imposed by a pulsatile pump to the tissue-engineered heart valve positioned in the culture chamber. The pump is pneumatically actuated and is an enlarged version of the one used in the previous bioreactor concept (Kortsmit et al., 2009a). The configuration of the ball valve, situated in between the pump and the culture chamber, and the outflow channel, at the top side of the culture chamber, determine the applied loading type. An open ball valve and an open outflow channel permit unrestricted flow from the pulsatile pump through the cultured heart valve and lead to systolic flow conditions. In contrast, by closure of the ball valve and the outflow channel, fluid flow generated by the pump is bypassed through silicone tubing. The tissue-engineered heart valve is diastolically strained by fluid injection into the culture chamber at the aortic side of the heart valve. Afterwards, fluid flow is diverted from the culture chamber through silicone tubing to the outer mantle. The pressure difference over the heart valve is measured and the volumetric deformation of the heart valve leaflets is assessed and controlled during culture.

The opportunities of the combined and independent application of strain-, and flow-based loads to tissue-engineered heart valves in this new flow bioreactor set-up are numerous and promising. Further optimization of the mechanical conditioning protocol to enhance the mechanical characteristics of the engineered valves towards native benchmarks, is one of these. Recent strain-based cultured valvular constructs were too stiff at infinitesimal strains and were less anisotropic compared to native heart valves (Mol et al., 2006; Kortsmit et al., 2009b). Previous studies (McCulloch et al., 2004; Engelmayr et al., 2006, 2008; Hahn et al., 2007) showed that the combination of strain-based and flow-based load application to cardiovascular tissue-engineered

constructs had a synergistical effect on the mechanical properties of the tissue. Furthermore, animal studies in which tissue-engineered heart valves were implanted showed an increase in leaflet flexibility due to in-vivo tissue remodeling (Hoerstrup et al., 2000b, 2002). Therefore, the additional application of flow, which results in frequent opening of the tissue-engineered heart valve leaflets, is thought to improve the valve's mechanical behavior in general, and leaflet flexibility in particular. In addition, periodic opening of the heart valve leaflets will prevent leaflet fusion during culture. This way, leaflets do not need to be separated before implantation, preventing possible in-vivo complications as a result of exposure of the disrupted leaflet surface layer to the blood (Dijkman et al., 2008a, 2008b). In-vitro flow-based conditioning also has an important function in the retention, formation and alignment of endothelial cells (Isenberg et al., 2006; Pullens et al., 2009a, 2009b) on the surface of cardiovascular constructs. The importance of endothelium with respect to the antithrombogenic response and modulation of underlying cells are well-known (Cebotair et al., 2002; Vesely, 2005) and might be a significant factor for the long-term in-vivo function of tissue-engineered constructs (Lichtenberg et al., 2006).

Besides the mechanical conditioning opportunities of the bioreactor system, the combination of both flow-based and strain-based loading offers possibilities to evaluate or test the tissue-engineered heart valve after or during culture. In the new flow bioreactor, the valve's mechanical properties and its diastolic functionality can be assessed in the same way as described before (Kortsmit et al., 2009c). However, the cultured heart valve can additionally be subjected to full in-vivo conditions in a wide range, human or otherwise mammal, to test the valve's overall mechanical behavior, functionality and integrity. Preferably, these testing procedures are performed according to official industry standards (ISO 5840, 2005).

The requirements of an ideal bioreactor system for heart valve tissue engineering, as discussed above (§ A.2) are summarized and represented in table A.1. In this table, the properties of the developed bioreactor system discussed in chapters 2-4, and the new flow bioreactor presented in this chapter are compared with the ideal bioreactor. The comparison shows that the majority of the requirements of an ideal bioreactor system are met by the bioreactor system presented in chapters 2-4. However, in this set-up the applied loads are solely strain-based and as a result the requirements, in which flow application was mentioned, are not fulfilled. In contrast, the new flow bioreactor nearly represents the ideal bioreactor system. Due to the wide range of loading options incorporated, however, the bioreactor set-up becomes relatively complex. Despite this, disassembly of the system in two parts is not difficult and facilitates the sterilization process. Moreover, the in-vitro functionality of the new bioreactor system has not been validated yet. Although the functionality of the previously developed bioreactor was extensively tested (Kortsmit et al., 2009b, 2009c) and the application of physiological flows was adopted from earlier studies (Rutten et al., 2002, 2007; Geven et al., 2004), the effect of additional flow application still needs evaluation.

Table A.1: *Checklist to indicate which requirements of the 'ideal' bioreactor (§ A.2) for heart valve tissue engineering are met by the newly developed flow bioreactor system. The requirements met by the flow bioreactor are pointed out by the symbol '✓', while the non-fulfilled requirements are expressed by the symbol '* ***X****'.*

Requirements of ideal bioreactor		Bioreactor (chapters 2-4)	Flow bioreactor (appendix A)
1.	Set-up is not complex	✓	**X**
2.	Set-up is small (it fits in an incubator)	✓	✓
3.	Set-up is easily sterilizable	✓	✓
4.	Easy refreshment of culture medium	✓	✓
5.	Easy access to tissue-engineered heart valve	✓	✓
6.	Easy and sterile handling of tissue-engineered heart valve	✓	✓
7.	Visualization of tissue-engineered heart valve	✓	✓
8.	No disturbance of incubator climate by pulsatile pump	✓	✓
9.	Culture medium exposed to climate in incubator	✓	✓
10.	Application of wide range of pressures and flows	**X**	✓
11.	Application of physiological loads	**X**	✓
12.	Application of strain and flow-based loads	**X**	✓
13.	Measurement of pressures and flows, non-invasively	✓	✓
14.	Measurement of deformation and mechanical properties, non-invasively	✓	✓
15.	Control of pressures and flows	**X**	✓
16.	Control of deformation and/or mechanical properties	✓	✓
17.	Testing/ evaluation of tissue-engineered heart valves, non-invasively	✓	✓
18.	Proven functionality (in heart valve tissue engineering)	✓	**X**

A.5 Conclusion

The newly developed flow bioreactor system allows the application of controlled strain-based and physiological flow-based loads, independently and alternatingly, to tissue-engineered heart valves. This concept offers many possibilities in heart valve tissue engineering with respect to the optimization of the mechanical conditioning protocol, and the performance of several heart valve functionality and quality testing procedures.

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Samenvatting

In 2006 werden er wereldwijd 300.000 patiënten waarvan 6.000 kinderen geopereerd om hun hartkleppen te vervangen door hartklepprothesen. Een belangrijke tekortkoming van de huidige hartklepprothesen is dat deze niet kunnen meegroeien met de patiënt of zich kunnen aanpassen aan een verandering in de belasting, zoals natuurlijk weefsel dat wel kan. Als een gevolg hiervan zijn voor jonge patiënten meerdere operaties nodig om de eerder geïmplanteerde hartklep te vervangen door een andere, grotere klep.

Tissue engineering is een veelbelovende techniek waarmee deze tekortkoming mogelijk teniet wordt gedaan. In hartklep tissue engineering wordt een patiënteigen, levende hartklep in het laboratorium gekweekt en daarna geïmplanteerd waardoor de klep wél in staat is mee te groeien met de patiënt en ook kan reageren op veranderingen in het lichaam. Eveneens bestaat deze hartklepprothese uit lichaamseigen weefsel en zal daarom geen afstotingsreactie veroorzaken.

Kort samengevat worden bij tissue engineering van hartkleppen cellen afkomstig van de patiënt op een dragermateriaal (Engels: scaffold) gezaaid dat de vorm heeft van een hartklep. Het geheel wordt in een bioreactor geplaatst waarin onder steriele omstandigheden de cellen worden blootgesteld aan allerlei biologische factoren en krachten die ook in het menselijk lichaam aanwezig zijn. Deze omstandigheden stimuleren de aanmaak en ontwikkeling van hartklepweefsel. Na een aantal weken kweken, is het dragermateriaal geheel vervangen door hartklepweefsel. Het eindresultaat is een levende hartklep gemaakt van cellen van de patiënt die klaar is voor implantatie.

De belangrijkste uitdaging in hartklep tissue engineering is het kweken van een hartklep die sterk genoeg is om de hoge bloeddrukken in het menselijk lichaam te kunnen weerstaan. Tevens dient de klep een goed opening- en sluitingsgedrag te vertonen na implantatie in het menselijk lichaam.

Uit eerder onderzoek is gebleken dat bevordering van weefselsterkte en andere weefseleigenschappen van de hartklep vooral wordt bewerkstelligd door het cyclisch vervormen van het hartklepweefsel tijdens de kweek in een bioreactor. Het probleem is echter dat dit in de huidige bioreactoren wordt uitgevoerd zonder dat men kan controleren of de hartklep wel vervormd zoals gewenst. Hartklepdeformatie wordt niet gemeten, laat staan gereguleerd. Hierdoor is het onmogelijk uit te zoeken wat nu het meest optimale belastingsprotocol is voor het kweken van hartkleppen. Daarnaast is het in de huidige bioreactoren niet mogelijk te weten hoe de weefseleigenschappen van de hartklep tijdens het kweekproces ontwikkelen. Nú is het zo dat de kwaliteit van de hartklep pas wordt onderzocht wanneer de hartklepkweek ten einde is, meestal na vier weken. De gekweekte hartkleppen moeten dan opgeofferd worden, waarna met destructieve methoden de weefselsterkte en andere hartklepeigenschappen worden bepaald. Hierna is de hartklep natuurlijk niet meer geschikt voor implantatie. Het algemene doel van het onderzoek dat beschreven is in dit proefschrift was dan ook om een bioreactor te ontwikkelen waarin weefseldeformatie en mechanische eigenschappen

van een hartklep kunnen worden gemeten en worden gecontroleerd tijdens de kweek. De klep blijft hierbij intact, en na het kweekproces kan gewaarborgd worden of de klep aan alle eisen voldoet om geïmplanteerd te worden.

Het eerste deel van het promotieonderzoek was gericht op het ontwikkelen van een bioreactor waarin het mogelijk is de opgelegde vervorming te meten en daarnaast te controleren tijdens de kweek. Om het kweekproces niet te verstoren moeten deze meting en controle steriel uitgevoerd worden zonder de hartklep te beroeren. Daartoe is een bioreactor ontwikkeld waarin de deformatiemeting wordt uitgevoerd door gebruik te maken van een stromingssensor. Met behulp van deze sensor kan de verplaatste hoeveelheid vloeistof als gevolg van hartklepvervorming gemeten worden. De meetmethode werd eerst uitgebreid gevalideerd met een plastic hartklep waarna de bioreactor succesvol werd toegepast in enkele humane hartklepkweken.

In het tweede deel van het promotieonderzoek lag de focus van het onderzoek op het ontwikkelen van een kwaliteitscontrole voor de gekweekte hartkleppen. Om deze doelstelling te bereiken zijn de mogelijkheden van de, in het eerste deel, ontwikkelde bioreactor verder uitgebreid. De deformatiemeting werd gekoppeld aan een numeriek computermodel teneinde de weefseleigenschappen van de hartklep tijdens de kweek te kunnen afschatten. Een cyclische vervorming werd aan de hartklep opgelegd in de vorm van een vloeistofdruk waarna zowel de opgelegde druk als de geïnduceerde hartklepvervorming werden gemeten. Deze meetwaarden werden vervolgens ingevoerd in het computermodel. Het model gebruikte de meetwaarden om de mechanische eigenschappen van de hartkleppen af te schatten. Ook deze bepaling kon steriel uitgevoerd worden en uitgebreide validatie met behulp van trekproeven aan gekweekt hartklepweefsel bewees dat de afschatting van de hartklepeigenschappen nauwkeurig is.

Samenvattend is er in dit promotieonderzoek een bioreactor ontwikkeld voor het kweken van hartkleppen, waarin enerzijds vervorming gecontroleerd kan worden opgelegd op de hartkleppen en anderzijds de eigenschappen van de hartkleppen gemeten kunnen worden. Beide methoden kunnen steriel en zonder het kweekproces te verstoren worden uitgevoerd in de bioreactor. Met deze kenmerken creëert de ontwikkelde bioreactor veelbelovende mogelijkheden voor hartklep tissue engineering in de toekomst, met name voor het ontwikkelen van een optimaal kweekprotocol en het vormen van een kwaliteitswaarborg van de te implanteren hartkleppen.

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Jeroen Kortsmit
Eindhoven, augustus 2009

Curriculum Vitae

Jeroen Kortsmit is geboren op 14 oktober 1978 in Hulst. In 1997 behaalde hij zijn VWO (Gymnasium) diploma aan het Reynaertcollege te Hulst. Aansluitend is hij Biomedische Technologie gaan studeren aan de Technische Universiteit Eindhoven. Als onderdeel van deze studie liep hij stage in de onderzoeksgroep van prof. Mark Ratcliffe en dr. Julius M. Guccione aan de University of California in San Francisco (UCSF), waar hij numeriek onderzoek deed naar hart remodellering. Zijn afstudeerwerk vond plaats bij Philips Research, Consumer Healthcare en richtte zich op het effect van wrijving op de huid. Dit onderzoek werd begeleid door dr. ir. Dirk Brokken (Philips) en dr. ir. Cees Oomens (TU/e). Na een half jaar te hebben rondgereisd in Indonesië en Australië, is hij in 2004 begonnen met zijn promotieonderzoek aan de faculteit Biomedische Technologie van de Technische Universiteit Eindhoven, resulterend in dit proefschrift. Het project maakte deel uit van het hartklep tissue engineering onderzoek dat uitgevoerd wordt binnen de groep Soft Tissue Biomechanics & Engineering.