Tissue engineering of aortic valves

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A literature review

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1. Introduction

The need for a long-lasting aortic valve substitute is high: annually in the USA, nearly 20 thousand people die as a result of valvular dysfunctions. The economic costs for the treatment and care of patients are estimated at several billion dollars\textsuperscript{1}. The current substitute heart valves have proven to be clinically successful over the short term, but show several poignant long-term disadvantages\textsuperscript{2}.

In this review, the relevant topics concerning the tissue engineering of an aortic heart valve are discussed. First the anatomy, functionality and mechanical characteristics of the aortic valve are dealt with.
Second the current treatments for aortic valve failure, and the shortcomings and complications of these treatments are elucidated.
Next, the emphasis is put on the concept of tissue engineering. This concept is subdivided in 3 main topics: the cells, the scaffold and the stimuli. Each of these aspects is evaluated separately. Finally conclusions are drawn towards the improvement of tissue engineered heart valves.
2.1. The aortic valve

The aortic valve is situated between the left ventricle and the ascending aorta (figure 1). The valve closes after each systole in order to prevent blood from flowing back into the ventricle. The opening and closing of the valve is a passive process induced by the pressure gradient over the valve. On average, the valve opens and closes 70 times per minute; this is approximately one hundred thousand times each day which adds up to thirty-seven million times a year.\(^3\)

The valve consists of three anatomical entities: three leaflets, three sinus cavities and an aortic ring\(^4\) (figure 2). The three leaflets, or cusps, are attached to the aortic wall like half-round pockets to a jacket. Behind the three leaflets three cavities can be found in the aortic wall, called the sinuses of Valsalva. The line of attachment of the leaflets to the aortic wall is referred to as the aortic ring. Since these attachment lines, also called commissures, are U-shaped, the aortic ring resembles a crown-like form, rather than a circular ring.
When the valve closes the three cusps bulge toward the central axis of the aorta, and they meet in the middle. In closed position, adjacent cusps contact with a substantial part of their surface area, up to 40%\(^2\). This phenomenon is called coaptation. Despite the large pressure gradient over a closed valve, prolapse is prevented through coaptation. The surface area near the free edge that coapts with 2 adjacent leaflets is called lunula, the remainder of the leaflet surface area is referred to as the load bearing leaflet portion (figure 3). In the middle of the free edge of each leaflet, a thickening is observed, called the nodulus of Arantius.

![Figure 3: Free edge (FE), lunula (R), line of coaptation (C), line of attachment (A), load-bearing surface (L), circumferential direction (CD), radial direction (RD).](image)

2.2. Leaflet structure

The aortic valve leaflet consists of three layers (from cranial to caudal direction): the lamina fibrosa, the lamina spongiosa and the lamina ventricularis (figure 4). Three distinct cell phenotypes have been identified in the aortic valve: fibroblasts, myofibroblasts and smooth muscle cells\(^5\). The surface of the leaflet is covered with a layer of endothelial cells.

![Figure 4: the three layers of the cusp](image)

Smooth muscle cells and myofibroblasts are essentially localized in the fibrosa, whereas different fibroblasts subtypes are expressed in the ventricularis and spongiosa. The cells are attached to the extra cellular matrix (ECM). Two important proteins that form the ECM are elastin and collagen (resp. 12% and 50% of its dry weight\(^6\)). The composition of the ECM differs in each layer, according to its function. The ventricularis is predominantly composed of elastin with radially aligned elastic fibers. During the closure phase of the cycle, the elastic fibers are stretched as the cuspal edges are drawn towards the other cusps, allowing coaptation.
The spongiosa contains some loosely arranged collagen fibers, but its main component is glycosaminoglycans. These macromolecules absorb water and cause the layer to swell. It thus forms a sponge-like tissue (which explains the name of the layer), capable of dissipating shocks during the closure of the valve.

The fibrosa is composed mainly of circumferentially aligned collagen fibers. Because of this configuration it can effectively transfer stress to the adjacent aortic wall. This enables the cusps to withstand the high pressures and bear the load.

Blood vessels are sparse in aortic valve cusps because the thin cusps are adequately perfused by the blood bathing them.

### 2.3 Mechanical characteristics

Extensive research on the mechanical characteristics of the aortic heart valve has been carried out. Both the characteristics of the complete heart valve, the separate layers and the contribution of the different matrix components have been determined.

#### 2.3.1. The complete valve

As mentioned before, heart valves have a layered, complex architecture and a highly specialized, functionally adapted extracellular matrix. The composition of the matrix is mainly responsible for the mechanical characteristics. Each of these components, collagen, elastin and proteoglycans, contributes to the performance of the total valve.

The design-features of the valve enable the cusps to be extremely soft and pliable when unloaded, but virtually inextensible when back pressure is applied. These features include: corrugations of the fibrosa, an array of collagen cords in the fibrosa which radiate primarily from the commissures and confer strength and coiling of the collagen fibers (figure 5). Elastin and collagen are the proteins that mainly determine the mechanical characteristics of aortic heart valves. Elastin structures occur in forms of sheets, tubes or fibers. Elastin is a highly extensible protein, having a stiffness ~2000 times less than that for collagen.

![Diagram of cusp geometry](attachment:image.png)

In the diastolic phase, the valve closes, and the forces on the valve increase. In the first step of this process the tissue offers negligible resistance to elongation and only elastic fibers provide force transmission. The collagen fibers start to uncoil. In the second step, the collagen fibers get uncoiled in increasing amounts and contribute...
increasingly to the force transmission. Next, all the collagen is uncoiled and contributes to the force transmission. In case the force would increase even more, the tissue eventually ruptures.

During this process the corrugations flatten and the collagen crimps expand in the radial direction. These phenomena are accomplished by fiber rearrangements and permit initial increase in dimension with minimal stress (figure 6). During diastolic loading, the collagen fibers realign and the cusps can extend beyond 50% strain. In a cardiac cycle, the leaflet length changes between the systolic to diastolic phase, but no changes occur during systole or diastole.

![Figure 6a characteristics of collagen and elastin](image1)

![Figure 6b typical characteristics of soft biological tissues](image2)

In figure 6a the stress/strain relation is shown for collagen and elastin separately. Figure 6b depicts the typical load-elongation curve for soft biological tissues in uniaxial tension. When the curves of collagen and elastin of figure 6a are combined they form a graph similar to figure 6b. The latter figure follows the loading steps described before. Three important features used in the characterization of tissues are indicated: the extensibility, the stiffness and the failure tension.

The structural orientation of the various compounds within the valve is nonrandom, leading to highly anisotropic properties. The mechanical properties of the valve can be subdivided into radial and circumferential orientation, as described in figure 3.

It needs to be stated that different mechanical testing methods have been reported. Thubrikar performed uniaxial loading tests. These tests however, do not duplicate the natural biaxial loading conditions. Lo and Vesely performed biaxial loading tests. Aortic cusps were placed in an experimental setup and marked with black dots. During the loading protocol, the three-dimensional position of the dots was recorded. The acquired data provided the stress-strain relationship in both the radial and circumferential direction. Comparing the results of the uniaxal to the biaxial testing results, the uniaxial test overestimated the extensibility of the leaflets by a factor 2, in both the radial as the circumferential direction.

The biaxial test revealed that the extensibility of the aortic valve is larger in the radial direction than in the circumferential direction, respectively 23% and 10% (table 1.1). The results also showed that the valve cusps were highly anisotropic in the central region, while the basal region was relatively isotropic. The cusp as a whole was asymmetrical in its distensibility. Interactions between the radial and
circumferential forces cause a larger stiffness during the diastolic phase. This contributes to the prevention of leaflet prolapse.

Carew et al. (2000) described the necessity to precondition the samples prior to the loading experiments. The isolated valves that are subjected to loading protocols will show changing characteristics in the initial phase of the experiment. After this ‘onset-phenomenon’ the characteristics will reach a constant value. These latter values are comparable to the values of valves in the natural biological environment, since they are constantly subjected to loads. So, in order to reflect the true characteristics of aortic valves, they have to be preconditioned before experimental data is acquired.

2.3.2. The different layers

To offer a better understanding of the valve mechanics, the contributions of each of the three layers must be evaluated. Due to the different matrix compositions of the three different layers, their mechanical characteristics differ. As mentioned before, the fibrosa is mainly composed of collagen, while elastin is prevailing in the ventricularis.

Because of these differences in configurations, the fibrosa and ventricularis are preloaded by virtue of their attachment to each other. The fibrosa is preloaded under compression and the ventricularis under tension. These internal stresses were verified by Vesely et al. The two layers of pig aortic valves were separated from each other. The separation induced dimensional changes in the cusps. The fibrosa expanded radially by 30%, whereas the ventricularis contracted by 12%. The ventricularis also contracted circumferentially by 13%.

Vesely and Noseworthy revealed the differences in mechanical characterizations of the separate layers. The ventricularis was stiffer circumferentially than radially (7.41 kPa vs 3.68 kPa), and was more extensible radially (62.7% vs 21.8% strain). The fibrosa was also stiffer circumferentially than radially (13.02 kPa vs 4.65 kPa) but had uniform extensibility. These data are represented in figure 7, and summarized in table 1.1.
2.3.3. The different components

The role of the different components of the matrix in the mechanics of the heart valve has been studied by different groups\textsuperscript{7-13}. This is done either by isolating one component, or by removing one component and observing the changes in mechanical behavior.

Using a hot-alkali digesting protocol, Scot and Vesely\textsuperscript{14} isolated the cusps elastin. SEM-images of the specimens revealed that the ventricularis contains a large continuous sheet of amorphous or compact mesh elastin that covers the entire layer. Elastin in the fibrosa is much more complex, consisting of large tubes that emerge from the aortic attachment and extend circumferentially across the cusp. The tubes, constructed of amorphous fenestrated sheet and loose mesh elastin, likely surround the large circumferential collagen bundles observed in the fibrosa.

<table>
<thead>
<tr>
<th></th>
<th>Extensibility (% strain)</th>
<th>Elastic modulus (kPa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radial</td>
<td>Circumferential</td>
<td>Radial</td>
</tr>
<tr>
<td>Whole valve</td>
<td>23% 10%</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>Ventricularis</td>
<td>62.7% 21.8%</td>
<td>3.68 7.41</td>
<td>Vesely, Noseworthy (1992)\textsuperscript{13}</td>
</tr>
<tr>
<td></td>
<td>63.7% 13.5%</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td></td>
<td>43% 12%</td>
<td>1.3 10.6</td>
<td>Lee, Medura (2001)\textsuperscript{7}</td>
</tr>
<tr>
<td>Fibrosa</td>
<td>27.8% 19.4%</td>
<td>4.65 13.02</td>
<td>Vesely, Noseworthy (1992)\textsuperscript{13}</td>
</tr>
<tr>
<td></td>
<td>29.5% 8.2%</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>Whole valve</td>
<td>18% 7%</td>
<td>2.6 11.9</td>
<td>Lee, Medura (2001)\textsuperscript{7}</td>
</tr>
<tr>
<td>without elastin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutaraldehyde treated</td>
<td>49.6% 15.2%</td>
<td>3.95 8.04</td>
<td>Vesely, Noseworthy (1992)\textsuperscript{13}</td>
</tr>
<tr>
<td>ventricularis</td>
<td></td>
<td></td>
<td>Vesely, Lozon (1993)\textsuperscript{12}</td>
</tr>
<tr>
<td></td>
<td>35.4% 14.7%</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>Glutaraldehyde treated</td>
<td>46.1% 12.1%</td>
<td>2.32 13.91</td>
<td>Vesely, Noseworthy (1992)\textsuperscript{13}</td>
</tr>
<tr>
<td>fibrosa</td>
<td></td>
<td></td>
<td>Vesely, Lozon (1993)\textsuperscript{12}</td>
</tr>
<tr>
<td></td>
<td>39.2% 12.8%</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
</tbody>
</table>

Table 1.1: mechanical characteristics of the aortic valve.

Since the amount of elastin in the aortic valve is far less than the amount of collagen, it is long believed that the influence of elastin is negligible on the valve mechanics. However, research has proven this assumption false.
Lee et al. treated cusps with elastase, which resulted in the elimination of the elastin contribution. Removing the mechanical contribution of elastin altered the mechanical behavior of the aortic valve cusp, primarily in the radial direction. The resting geometry of the cusps changed; the cusps elongated after sequential digestion. This again indicates the preloaded resting state of the cusps.

Elastin damage produced a decrease in radial and circumferential extensibility (from 43% to 18% strain radially and from 12% to 7% strain circumferentially), with a slight increase in stiffness (1.3 – 2.6 kN/m fro radial and 10.6 – 11.9 kN/m for circumferential direction). These data are summarized in table 1.1. Elastase treatment reduced the failure strength of the circumferentially orientated samples by 43%. No significant drop in failure strength was observed in the radial samples.

Vesely isolated intact aortic valvular elastin structures by digesting other components with NaOH. This procedure also revealed the cusps preloaded state, since the elastin structures shrank 10-30% during digestion. Isolated elastin was found to be extremely extensible with a very low stiffness: the circumferential stiffness of isolated elastin is roughly 1/1700 of that of the whole cusp, and its extensibility is well over 100%. The fracture tension of the fibrosal elastin was almost an order of magnitude greater than of the ventricularis, in both directions (table 1.2).

<table>
<thead>
<tr>
<th></th>
<th>Fracture tension (N m⁻¹)</th>
<th>Fracture strain(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Circumferential</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>N 15</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Mean 2.72</td>
<td>143</td>
</tr>
<tr>
<td></td>
<td>S.E.M. 0.29</td>
<td>10</td>
</tr>
<tr>
<td>Fibrosa</td>
<td>N 7</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Mean 0.33</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>S.E.M. 0.06</td>
<td>12</td>
</tr>
<tr>
<td>Ventricularis</td>
<td>N 8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Mean 2.94</td>
<td>152</td>
</tr>
<tr>
<td></td>
<td>S.E.M. 0.29</td>
<td>12</td>
</tr>
<tr>
<td><strong>Radial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole</td>
<td>N 8</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Mean 1.65</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>S.E.M. 0.36</td>
<td>17</td>
</tr>
<tr>
<td>Fibrosa</td>
<td>N 10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Mean 0.14</td>
<td>147</td>
</tr>
<tr>
<td></td>
<td>S.E.M. 0.01</td>
<td>13</td>
</tr>
<tr>
<td>Ventricularis</td>
<td>N 10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Mean 2.81</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>S.E.M. 0.30</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 1.2. Fracture parameters for elastin isolated from whole cusps and layers.

### 2.4 Summary

Concluding, the three cusps of the valve cooperate to fulfill their function. The geometry of the cusps is developed in a way that allows for coaptation. This prevents prolapse and contributes to the load carrying properties of the whole valve. The composition of the valve is complicated and non-isotropic. The highly organized configuration enables the valve to withstand large pressure gradients and shear stresses. The three different layers all contribute to the optimal functioning of the valve, each in a specific manner.
The fibrosa is considered the main load carrying structure because of its high collagen compound\(^9\). Collagen provides to cusps of sufficient strength to withstand the loads applied to it. The elastin structures were found to contribute significantly to the mechanical function of the cusps at low loads and low strains. Elastin, mainly localized in the ventricularis, is responsible for returning the collagen fiber structures back into their radially compressed state between successive loading cycles\(^{15}\). The main task of the spongiosa is to dissipate the shocks caused by the closure of the valve. All of these properties combined produce a reliable valve that is able to withstand large pressure gradients.
3.1 Aortic valve substitutes

In case of heart valve failure it is common to replace the diseased valve. Several different heart valve substitutes have been used. These valve substitutes can be categorized into two groups: mechanical prostheses and bioprostheses. The latter can be either xenografts or homografts. All of these replacements have their advantages and disadvantages. All of the bioprosthetic designs have shown to function for only a relatively short time span. Within 10-15 years, bioprosthesis-associated problems make a reoperation necessary or cause death in at least 50-60% of the patients. The overall rate of failure is similar for the different designs, but the major causes of failure differ. Mechanical valves show good dynamics and durability, but have several negative side-effects.

The advantages and disadvantages of the different aortic valve replacement designs are elucidated in the following chapter.

3.2 Mechanical prostheses

The mechanical heart valve is the most used aortic valve substitute so far. In the US in 1999, approximately 60% of the implanted valves were mechanical valves. Three types of mechanical heart valves have been used: the ball and cage valve since 1960, the tilting disc since 1969 and the hinged bileaflet introduced in 1970 (figure 2.1).

![Figure 2.1: the ball and cage valve, the tilting disc valve and the hinged bileaflet valve.](image)

The ball and cage valve is generally seen as ‘old fashioned’ but there are no data to prove that its performance is inferior to other mechanical valves. Some ball and cage designs have been implanted in the aorta descendens. In several cases the valve has shown to function for up to 30 years after implantation without significant wear. In the third world, ball and cage valves that can be positioned in the mitral position, continue to be used because of its reasonable costs.

The tilting disc valve has a wide opening. This feature is believed to make it less thrombogenic.

The bileaflet valve is the most popular. It has excellent haemodynamics, it opens easily, offers minimum resistance to onward flow and when closed has trivial regurgitation.

The main advantage of mechanical heart valves is their high structural reliability: they have a very low incidence of mechanical failure and a very high durability. The key drawback to mechanical valves is their susceptibility of causing thromboemboli and thrombolic occlusion due to their nonphysiological surfaces. Moreover, they show flow dynamics that are distinctly different from those of natural heart valves. The trombogenic properties of mechanical heart valves necessitate the life-time use of
anticoagulation therapy. This increases the risk of spontaneous bleeding and embolism. Furthermore, the risk of prosthetic valve endocarditis, a fatal infection due to immune responses against the foreign body material, is quite high with these kinds of valves. Overall, clinical studies showed that 10% of all mechanical heart valve recipients needed reoperation within 11 years due to the abovementioned side-effects.

### 3.3 Xenografts

Two types of xenografts are generally used as aortic valve substitutes; porcine aortic valves and bovine pericardial valves. Porcine valves are valves removed from porcine cadavers. Pericardial valves are composed of parietal pericardium attached to a frame. (figure 2.2). Both types of valves need to be pretreated before implantation.

Because the pericardial valve is an engineered valve, it lacks the anatomical constraints imposed by a porcine valve, and it can be fabricated in al desired sizes. As can be seen in figure 2.2, the geometry of the pericardial valve differs from that of the porcine valve. In contrast to the porcine valve, the free edge of the pericardial valve is nearly horizontal. This configuration implies that the tensile forces in the free edge are significantly greater than those in the porcine valve. Reducing the angle of the free edge away from the horizontal would decrease the tensile forces. However, this adjustment proves to be impossible with the relatively inextensible pericardium. Moreover, dropping the leaflet angle would reduce the coaptation area and therefore increase the risk of leakage.

The main advantages of xenografts are their sufficient availability, and their low incidence of thromboembolism. As opposed to mechanical valves, no anticoagulation therapy is needed. The main disadvantage is the less optimal durability and structural dysfunctions. According to Schoen and Levy, less than 1% of the implanted porcine aortic valves dysfunctions within 5 years after implantation. However, 20-30% fails within 10 years, and between 12 and 15 years after implantation over 50% of the implanted xenografts become dysfunctional. The risk of structural failure turns out to be highly age dependent. Children and adolescents under 35 years of age have the highest risk. Thus, bioprostheses are attractive in the elderly, because they have an especially high risk of anticoagulant-related hemorrhage.

Xenografts require pretreated prior to implantation. The aim of chemical pretreatment of the bioprosthesis is preservation of the tissue by enhancing the resistance of the material to enzymatic or chemical degradation, and reducing the immunogenicity of the material. Glutaraldehyde is the most widely used chemical preservative for
xenografts. Glutaraldehyde reacts with the ε-amino group of lysyl residues in proteins such as collagen, the main load bearing fiber in the leaflet. This induces the formation of interchain crosslinks, and stabilizes the tissue against chemical and enzymatic degradation. Besides enhancing material stability, this preservation technique inhibits autolysis, allows prolonged shelf-life and permits surgeons to have valves of various sizes readily available for implantation. Furthermore, chemical crosslinking also diminishes antigenicity while maintaining both thromboresistance and microbial sterility.

The use of glutaraldehyde as a crosslinking agent also proves to have a lot of drawbacks. The fabrication and pretreatment techniques cause several changes in the configuration of the cusps.

First, the endothelial layer is damaged or removed during preservation. The blood-cusp interface has become subendothelial connective tissue devoid of endothelial cells. As a consequence, various proteins cover the surface after implantation. Host endothelial cells do not typically grow onto bioprosthetic tissues when implanted into patients. Crosslinking chemicals have been shown to leak slowly from tissue derived bioprostheses fixed in glutaraldehyde, producing cytotoxic effects. This cytotoxicity presumably contributes to the lack of re-endothelialization.

Second, the configuration is locked at one phase of the cardiac cycle, preventing normal functional cyclical rearrangements. The natural collagen crimping is eliminated. The geometry in which the valve is fixed depends on the pressure over the valve during the fixation process. When a diastolic pressure gradient (~80 mmHg) is used during the fixation process, the valve is preserved in a closed position. When the systolic pressure (2-4 mmHg) is imposed on the valve, it will be fixed in an open configuration. Varying the conformation of the fixed valve leads to different mechanical properties. This way, the heart valves may be engineered according to their specific medical application.

Glutaraldehyde-treated tissue exhibits altered mechanical properties compared to untreated tissue. Glutaraldehyde-crosslinked porcine aortic valves tend to be stiffer than fresh tissue and have stress-relaxation rates 60% of those of fresh tissue. Treated tissues also show increased apparent tensile extensibility associated with shrinkage during flexion.

The influence of fixation on the mechanical properties of the different layers has been investigated. The ventricularis became less extensible after fixation (35.4% vs. 63.7% strain). The fibrosa became more extensible (39.2% vs. 29.5% strain radially and 12.8% vs. 8.2% strain circumferentially). The elastic modulus of the fibrosa changed in radial orientation after glutaraldehyde fixation from 4.65 kPa to 2.32 kPa. The elastic modulus of the ventricularis remained unchanged.

Another important difference with the native valve is that the interstitial cells are made non-viable, so no remodeling can take place. Chemical pretreatment produces crosslinked cellular debris which acts as foci for calcification. As elucidated below, calcification is one of the major causes for bioprosthetic failure.

### 3.4 Failure processes

Cuspal tissue deterioration is the major cause of bioprosthetic valve failure. Two distinct yet potentially synergistic processes are causal: calcification and noncalcific or mechanical degradation. Both processes have different initiators. Calcification is the formation of calcium-containing mineral deposits, which results in cusp stiffness, loss of pliability and blockage of the valve. The exact mechanism by
which cusp calcify remains unknown. A lot of effort is put into better understanding of the underlying pathways of calcification, and their initiators. Two types of initiation have been distinguished: intrinsic and extrinsic mineralization. Intrinsic mineralization occurs deep within the substance of the cusps, whereas extrinsic mineralization implies calcification of adherent thrombi or infective vegetations. These mineralization processes cause large calcific growths that can impede leaflet motion and cause stenosis, but often result in leaflet tearing and hence massive regurgitation.

Initiation, or nucleation, is most likely the reaction of calcium derived from plasma with phosphorus provided by plasma membranes and membrane bound organelles that are rich in phospholipids. Due to devitalization of cells subsequent to tissue fixation, cellular calcium regulatory mechanisms are disrupted and unimpeded calcium influx in the cells follows. Once inside the cell, the vast amount of calcium will react with phosphorus and nucleate calcific crystals.

After initiation of the calcification, the propagation phase follows. Propagation of crystal formation depends on the available concentrations of Ca$^{2+}$ and PO$_4^{3-}$ and the balance of calcification inhibitors and accelerators. It is likely that calcification is mediated not only by the remnants of dead porcine fibroblasts, but also by the death of the host fibrocytes and macrophages that come into contact with the toxic valve material.

Several attempts have been made to reduce the rate of calcification. Incorporating positive charges (e.g. Fe$^{3+}$ and Al$^{3+}$) into the tissue might prevent the migration of Ca$^{2+}$ ions into the valve tissue. Pretreatment of aortic valve cusps with ethanol prior to glutaraldehyde-crosslinking significantly inhibits calcification. Moreover, methods are being explored based on removing cellular components from the ECM, that are believed to promote calcification. It has been shown that more thorough removal of cellular debris attenuates calcification. To a certain extend, removal of loose proteoglycans reduces the degree of calcification. Although these various treatments seem to retard calcification of glutaraldehyde-treated tissues, in most cases the mineralization process is not completely inhibited. The glutaraldehyde-crosslinking reagent on its own, regardless of cells and soluble proteins in the tissue, likely contributes to the formation of calcium salts.

Mechanical damage also plays an important role in the deterioration of bioprosthetic heart valves. In the current generation of porcine bioprosthetic heart valves, calcific degeneration is a relative minor issue, with the majority of the damage occurring strictly for mechanical reasons. In addition, bovine pericardial valve failure is most frequently caused by noncalcific cuspal perforations and tears. Free edge tears occur often in the absence of calcification. Areas that experience highly localized mechanical forces are prone to mechanical failure. In pericardial valves, tears most frequently occur in the attachment points of the cusps. These are indications of a purely mechanical mode of failure, related to specific features of the valve design. Glutaraldehyde-fixation contributes to the feasibility of mechanical valve failure. The leaflets of pretreated xenografts are much stiffer than those of natural valves and therefore likely to experience abnormal flexure patterns during valve opening. This flexure-induced damage is probably a major contribution factor to noncalcific damage. Furthermore, fixation inhibits the naturally occurring structural
rearrangements in the cuspal tissue, inducing noncalcific damage. Another mechanism contributing to noncalcific damage might be the degradation of elastin. Because elastin cannot be crosslinked with glutaraldehyde, the antigenicity will not be completely diminished, and host scavenger cells can migrate into the heart valve tissue and damage the elastin. As discussed in chapter 1, damaging elastin causes significant changes in the mechanical properties of the cusps.

Calcification and noncalcific degradation can occur independently from each other or synergistically. Collagen disruption through mechanical damage could expose or create new nucleation sites and create internal spaces, which facilitate the growth of calcium crystals. On the other hand, mechanical damage can result from structural disruption, induced by calcification or stress concentrating in the cusp.

3.5 Homografts

Homografts are human aortic valve allografts, obtained from human cadavers. After removal from the body, they are cryopreserved by freezing, followed by storage at -196 °C in the vapor over liquid nitrogen. Aortic valve allografts have excellent haemodynamic profiles and a low incidence of thromboembolic complications, even without chronic anticoagulation. The quality of cryopreserved valvular tissue depends on details of freezing and thawing protocols, the interval from death to harvest, and additional warm and cold ischemic intervals. Progressive degeneration limits their long-term success. The most frequent modes of failure are cusp rupture and perforations. It has been indicated that cryopreserved allografts are unable to grow, remodel, or exhibit active metabolic functions. Another shortcoming of the homografts is the limited availability, and they are more difficult to insert. Despite all these draw-backs, they perform slightly better than conventional porcine bioprosthetic valves: approximately 50-90% valve survival at 10-15 years for allografts compared to 40-60% for porcine bioprostheses. In an echocardiographic follow-up study of 570 patients, 14.7% of the implanted allografts showed significant regurgitation, and aortic allograft stenosis was present in 3.2%. Overall, aortic valve replacement with allografts yields adequate mid-term results. Allografts still have a limited life span especially in young patients.

3.6 Autografts

In 1967, Ross described the replacement of the aortic valve using the patients own pulmonary valve, called pulmonary autograft. This intervention is known as the Ross procedure. The removed pulmonary valve is usually replaced by an allograft pulmonic valve. The pulmonary valve performs satisfactorily in the aortic position. It is less rigid and allows for sizing mismatch. Clinical analysis revealed that (between 1987 and 1998) the mortality for patients after the Ross-procedure is 2.5%. Reoperation for all valve-related complications is low, only 5.4%, with an autograft explant rate of 1.9%.

3.7 Summary

Many efforts have been taken to provide an adequate solution for aortic valve dysfunctions. Both mechanical and bioprosthetic valve substitutes have a satisfactory short-term performance, but show several major disadvantages on the long term.
Animal derived bioprostheses show good haemodynamical characteristics, and are relatively easy to obtain. However, they have shown to calcify resulting in stenosis or rupture of the cusps. In most cases, patients with an implanted bioprosthetic heart valve need reoperation within 10-15 years after surgery. Mechanical heart valves show satisfying haemodynamic properties, and have an excellent durability. However, due to immunogenic responses, life time anticoagulation therapy is a necessity for the recipient of a mechanical heart valve. It has become clear that another type of aortic valve substitute needs to be developed which will function for a longer period of time, and has the ability to grow, repair and remodel.
4.1. Tissue engineering of heart valves

In the previous chapters it is motivated why there is a need for a heart valve prosthesis that can grow, repair and remodel. The concept of tissue engineering offers good prospects into the development of such a device. The goal is to create an anatomical structure comparable to the native valve. Ideally, the tissue-engineered valve must equal the native valve in physiological function, biomechanical integrity, reparative ability and growth potential.

To create a functional living tissue construct, cells are seeded onto an appropriate carrier, the scaffold, in the shape of the desired tissue. The created tissue is viable, and thus able to repair and remodel. Furthermore, the risk of failure can be reduced, depending on the immune response of the body against the scaffold material and the strength and maturation of the tissue.

The most important issues concerning tissue engineering are the cell source, the scaffold material and the stimuli that have to be given to the tissue in order to make it strong enough for implantation. These three general issues, depicted in figure 3.1,

![Diagram](image)

figure 3.1: the building blocks for the creation of a living artificial heart valve alternative (LAHVA). Adapted from Flanagan 2003.

will be elucidated separately in the following paragraphs.

4.2 Cells

In an ideal tissue engineered construct, the cells are derived from the patient itself. By using autologous cells, the risk of immunologic complications after implantation is diminished. The cells used to seed the developing tissue should be nonimmunogenic, highly proliferative, easy to harvest and have the ability to differentiate into application-specific cell types with specialized functions.
Depending on the cell source used, cells have to be harvested from the tissue from the patient through digestion of the ECM of the tissue and separated from the digested tissue mixture. The desired cells can be isolated using a fluorescence-activated cell sorter (FACS). These cells have to be expanded using regular cell culture methods to obtain an amount large enough for cell seeding. A large variety of cell types has been used to create a tissue engineered heart valve (see paragraph 4.2.2).

In the next paragraph, the cellular composition of the natural heart valve is described.

4.2.1 Cells in the natural heart valve

The principal cell types in the heart valve are the valvular interstitial cells (VICs) and the valvular endocardial cells (VECs). VICs are believed to be responsible for the maintainance of valvular structure.

Three distinct VIC phenotypes have been identified in heart valves: fibroblasts, myofibroblasts and fetal-type smooth muscle cells (SMC). SMCs and myofibroblasts are essentially localized to the fibrosa, whereas fibroblasts are segregated in the ventricularis. The spongiosa is in general the poorest cell layer and displays a slightly different fibroblast pattern. The cellular configuration of the heart valve is depicted in figure 3.2.

![Figure 3.2: The cellular configuration of an aortic valve leaflet.](image)

The myofibroblasts are characterized by prominent stress fibers, associated with smooth muscle α-actin expression, and are thought to be involved in proliferation and migration. Fibroblasts are characterized by prominent synthetic and secretory organelles and are thus believed to be important in matrix regulation. They synthesize collagen, elastin, proteoglycans, fibronectin, growth factors, cytokines as well as matrix metalloproteinases (MMPs) and their tissue inhibitors. SMCs are able to contract in order to maintain a limited intrinsic valvular force and withstand haemodynamic pressures.

The VECs form a functional envelope around each of the heart valves leaflets. Presumably, VECs act to maintain a nonthrombogenic valve surface, similar to the vascular endothelium. VECs are also believed to regulate the underlying VICs.

4.2.2. Currently used cell types for tissue engineered heart valves

A great variety of cell types has been used to create a tissue engineered construct (table 3.1). In most studies, first (myo)fibroblasts are seeded on the scaffold to provide initial cell attachment and ECM production. Thereafter, endothelial cells are seeded on the construct to provide endothelial covering. Mostly, these cells are obtained from arteries.
Shinoka et al. (1996)\textsuperscript{32} seeded SMCs and fibroblasts obtained from an ovine femoral artery followed by ovine femoral artery endothelial cells. This resulted in a structure resembling the native valve architecture. Shinoka et al. (1997)\textsuperscript{33} also used mixed populations of dermal fibroblasts and endothelial cells. However, dermal derived cells proved to produce less organized structures in comparison to arterial derived cells. Human dermal fibroblasts have been used by Zeltinger et al. (2001)\textsuperscript{34}. In this study, recellularization of the matrix was 45\% compared to native valves, and evidence has been reported of ECM production. Ovine carotid artery myofibroblasts and endothelial cells were used by Steinhoff et al. (2000)\textsuperscript{35}. This resulted in repopulation of the valve scaffold matrix and complete endothelial lining. Human ascending aorta myofibroblasts were used by Jockenhoevel et al. (2001)\textsuperscript{36} which resulted in a structure with a gross appearance comparable to the native valve.

Even cells directly isolated from heart valves have been used\textsuperscript{37}, but this will not be an option for tissue engineering human heart valves, since it is to difficult and perilous to obtain them.

Considering the cell source, some studies describe the use of endothelial cells obtained from veins instead of arteries. Cebotari et al. (2002)\textsuperscript{38} and Bader et al. (1998)\textsuperscript{39}, describe the seeding of endothelial cells from the human saphenous vein on matrix scaffolds. This resulted in the formation of a viable confluent endothelial monolayer. Schnell et al. (2001)\textsuperscript{40} compared the use of myofibroblasts from peripheral veins to those of the aortic wall as possible cell sources for cardiovascular tissue engineered constructs. Constructs seeded with peripheral vein myofibroblasts were superior regarding collagen formation and mechanical stability compared to constructs seeded with aortic myofibroblasts. These results represent a promising easy-to-acces cell source for cardiovascular tissue engineering in the future.

There is no evidence that fibroblasts perform better than myofibroblasts in their use in cardiovascular constructs. It is, however, clear that endothelial cells are needed for the surface of the heart valve and the only way to obtain them is from a blood vessel. So probably, the use of myofibroblasts from the same blood vessel might be considered more efficient than using regular fibroblasts.

Hoestrup et al. (2002)\textsuperscript{41} and Kadner et al. (2002)\textsuperscript{66} used human umbilical cord cells to seed on a matrix. They demonstrated excellent growth properties and tissue formation with mechanical properties approaching the native tissue. This cell source avoids the invasive harvesting of intact vascular structures.

A relatively new cell source is human bone marrowstromal (BMSCs) cells. The use of BMSCs offers several advantages: They are relatively easy to collect by a simple bone marrow puncture. They show the potential to differentiate into multiple cell lineages, and they demonstrate good immunological characteristics\textsuperscript{42, 43}. The use of BMSCs in tissue engineered constructs showed morphological features and mechanical properties comparable to the native valve.

All the studies that are mentioned above are summarized in table 3.1.

4.3 The scaffold

Besides choosing the right cell source, the choice of an appropriate scaffold material is essential for the creation of functional tissue. The scaffold matrix determines the shape in which the growing tissue develops. It guides the orientation of the tissue and provides support against mechanical forces. Moreover, most cell types need anchorage points for the cells to attach to and will die if not provided an adhesion substrate.
There are several types of scaffold material. They can be subdivided into two categories: biological scaffolds and synthetic scaffolds. These two types of scaffold will be discussed separately.

**4.3.1. Biological scaffold materials**

Naturally derived biomaterials offer many mechanical, chemical and biological advantages. In contrast to synthetic matrices, biologically derived matrices possess important extracellular matrix proteins, which regulate cell adhesion and tissue regeneration\(^35\).

A promising concept concerning biological scaffolds is the use of decellularized allogeneic or xenogeneic heart valves. Many studies have been performed on the use of these matrices (table 3.1). By either chemical, enzymatic or mechanical treatment therapies, the cells are removed from the matrix. By depleting the heart valve from cells, the cellular antigens are removed, making the constructs less immunogenic\(^17\).

The remaining material, composed essentially of extracellular matrix components, can serve as an intrinsic template for cell attachment and growth\(^27\). Besides maintaining their mechanical properties, acellular matrices promote remodeling of the prosthesis. Bader et al. (1998)\(^39\) used a detergent and enzymatic strategy to produce decellularized porcine aortic valve matrices. After recellularization with human endothelial cells, a confluent monolayer of viable cells was observed. The same method of decellularization was used by Steinhoff et al. (2000)\(^35\). Carotid artery myofibroblasts and endothelial cells were seeded on the scaffold and implanted into ovine models. A dense population of myofibroblasts had infiltrated the construct, and endothelial lining was observed for up to 12 weeks.

The commercially available SynerGraft\(^{TM}\) was based on the same principle of decellularization. However, after the decellularization procedure, it is not reseeded with cells. It showed good in vitro results, as well as in vivo results in porcine models. However, early failure of the valve has been reported in human trials\(^44\).

Another biological material used as scaffold material is fibrin gel\(^45, 46\). Fibrin is a biodegradable polymer which can be produced from the patients own blood. Fibrin is formed by a polymerization of fibrinogen, mediated by thrombin and calcium chloride (CaCl\(_2\)). The degradation rate of fibrin gel can be controlled by the addition of the protein aprotinin\(^45\), or epsilon-aminocaproic acid (EACA)\(^47\). By addition of growth factors and proteins (TGF\(\beta\), insulin, plasmin) to the fibrin gel, the collagen synthesis of SMCs, incorporated in the fibrin gel, can be increased\(^48\). Jockenhoevel et al. (2001)\(^36\) have developed a moulding technique to produce trileaflet heart valve geometries, made out of fibrin gel.

Collagen has also been investigated as a suitable scaffold material\(^49, 50\). Either porcine or human SMCs were seeded in a synthetic collagen matrix. The structure was then seeded with endothelial cells, and cultured for 28 days. A tissue like morphology was observed in all samples. Evidence has been shown for the production of ECM components\(^49\). It is demonstrated that collagen sponge is a suitable biodegradable scaffold that can maintain viable interstitial cells and appears to enhance the capacity of the cell to express its original phenotype\(^50\).

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\(^{17}\) Bader et al. (1998)

\(^{35}\) Steinhoff et al. (2000)

\(^{39}\) Bader et al. (1998)

\(^{36}\) Jockenhoevel et al. (2001)

\(^{44}\) Bader et al. (1998)

\(^{45}\) Jockenhoevel et al. (2001)

\(^{46}\) Jockenhoevel et al. (2001)

\(^{48}\) Jockenhoevel et al. (2001)

\(^{50}\) Jockenhoevel et al. (2001)
<table>
<thead>
<tr>
<th>Scaffold material</th>
<th>Seeded cells</th>
<th>Seeding method</th>
<th>Culture method</th>
<th>Implantation</th>
<th>ECM production</th>
<th>Result</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Woven PCLA/narrow PGA</td>
<td>Ovine femoral artery smooth muscle cells, fibroblasts and endothelial cells</td>
<td>$10^6$ vascular cells seeded during 7 days, followed by $2 	imes 10^6$ endothelial cells once</td>
<td>static</td>
<td>For up to 11 weeks in the pulmonary position in lambs</td>
<td>Evidence for elastin and collagen production, increase in collagen content over time</td>
<td>Increasing tensile strength (up to 2.68 MPa), confluent lining with ECs. Collagen content approaching native valve</td>
<td>Shinoka 1996, Ref: 32</td>
</tr>
<tr>
<td>Woven PCLA/narrow PGA</td>
<td>Mixed population of dermal fibroblasts and endothelial cells</td>
<td>static</td>
<td></td>
<td>Implanted in the pulmonary position in lambs</td>
<td>Evidence for elastin and collagen production, Collagen content 36% of that of native valve</td>
<td>Less organized structure than leaflets of arterial cell origin, Mild regurgitation</td>
<td>Shinoka 1997, Ref: 33</td>
</tr>
<tr>
<td>Copolymer of 90% PGA and 10% PLA covered with pure PGA.</td>
<td>Human fibroblasts, bovine aortic endothelial cells</td>
<td>$10^6$ fibroblasts seeded each day for 12 days, then, $3 	imes 10^6$ EC's once</td>
<td>static</td>
<td>Not implanted</td>
<td>Not reported</td>
<td>Solid sheets of tissue with monolayer of endothelial cells. Soft and fragile tissue</td>
<td>Zund 1997, Ref: 37</td>
</tr>
<tr>
<td>Sheep heart valve interstitial and endothelial cells</td>
<td>$10^6$ interstitial cells seeded each day for 12 days, then, $3 	imes 10^6$ EC's once</td>
<td>static</td>
<td></td>
<td>Not implanted</td>
<td>Not reported</td>
<td>Histological resemblance with native tissue.</td>
<td></td>
</tr>
<tr>
<td>Decellularized porcine pulmonary valve.</td>
<td>Human saphenous vein endothelial cells</td>
<td>$10^5$ cells per cm$^2$ once</td>
<td>static</td>
<td>Not implanted</td>
<td>Not reported</td>
<td>Grossly loosened matrix structure. Confluent layer of endothelial cells. No ingrowth of cells observed</td>
<td>Bader 1998, Ref: 39</td>
</tr>
<tr>
<td>Triplekt porous PFO scaffold</td>
<td>Mixed cells from ovine aortic artery, endothelial cells from jugular vein</td>
<td>$10^6$ arterial cells each day for 4 days, after 10 days incubation $2 	imes 10^6$ endothelial cells once</td>
<td>static</td>
<td>Implanted in the pulmonary position in lambs</td>
<td>$11.6%$ of native valve collagen, $73%$ of native valve DNA content after 17 weeks</td>
<td>Smooth surfaces and neovalvulization stable. Stress strain relations resembled that of native tissue. Mild stenosis and regurgitation in all animals.</td>
<td>Sodian 2000, Ref: 53</td>
</tr>
<tr>
<td>Triplekt porous PFO scaffold</td>
<td>Ovine artery vascular cells</td>
<td>$2 	imes 10^6$ cells each day for 4 days</td>
<td>static</td>
<td>Not implanted</td>
<td>High DNA content and collagen production. Confluent cell layers, oriented in the direction of the flow. No elastin detected.</td>
<td></td>
<td>Sodian 2000, Ref: 50</td>
</tr>
</tbody>
</table>

Table 3.1: Overview of previously performed studies on tissue engineered heart valves
<table>
<thead>
<tr>
<th>Scaffold material</th>
<th>Seeded cells</th>
<th>Seeding method</th>
<th>Culture method</th>
<th>Implantation</th>
<th>ECM production</th>
<th>Result</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acellularized ovine valve conduit</td>
<td>Ovine carotid myofibroblasts and endothelial cells</td>
<td>6 days seeding of myofibroblasts, followed by 2 days seeding of endothelial cells</td>
<td>static</td>
<td>Implanted in the pulmonary position in lambs for up to 12 weeks</td>
<td>Indication of active matrix synthesis</td>
<td>Complete endothelial lining. Repopulation of valve matrix. Calcification and inflammation of conduit tissues</td>
<td>Steinhoff 2000 Ref: 33</td>
</tr>
<tr>
<td>non-woven PGA, coated with P4HE</td>
<td>Ovine carotid myofibroblasts and endothelial cells</td>
<td>Myofibroblast density of 5x10^6 cells per cm^2, followed by 1.5x10^6 endothelial cells</td>
<td>Static for 4 days, in pulse duplicator system for up to 28 days</td>
<td>Implanted in the pulmonary position in lambs for up to 28 weeks</td>
<td>ECM content higher than that of native valves after 20 weeks</td>
<td>Well functioning cusps, consisting of 3 layers. Mild to moderate regurgitation reported.</td>
<td>Heerstrup 2000 Ref: 55</td>
</tr>
<tr>
<td>Acellularized porcine valve conduit</td>
<td>Human skin fibroblasts</td>
<td>Circulation of cell suspension with 1x10^6 fibroblasts for 24 hours</td>
<td>Opening and closing of valve in bioreactor for 8 weeks</td>
<td>Not implanted</td>
<td>Synthesis of human ECM reported</td>
<td>45% recellularization compared to native valves.</td>
<td>Zeltinger 2001 Ref: 34</td>
</tr>
<tr>
<td>non-woven PGA, coated with P4HE</td>
<td>Human bone marrow stromal cells</td>
<td>5x10^6 human cells per cm^2</td>
<td>Static for 4 days, in pulse duplicator system for up to 14 days</td>
<td>Not implanted</td>
<td>25% collagen content and 35% GAG content of that of native valve. DNA content &gt; 300%.</td>
<td>Synchronous opening and closing of leaflets in bioreactor. Leaksless competent during valve closure.</td>
<td>Heerstrup 2002 Ref: 43</td>
</tr>
<tr>
<td>Acellularized human heart valve</td>
<td>Human endothelial cells harvested from saphenous vein</td>
<td>2x10^5 cells per cm^2</td>
<td>Exposure to flow in bioreactor for 7 to 10 days</td>
<td>Not implanted</td>
<td>Not reported</td>
<td>Formation of a viable confluent monolayer with high metabolic activity.</td>
<td>Cebotari 2002 Ref: 38</td>
</tr>
<tr>
<td>Patches of non-woven PGA, coated with P4HE</td>
<td>Mixed population of human umbilical cord artery and vein cells</td>
<td>-</td>
<td>In laminar flow system for 14 days</td>
<td>Not implanted</td>
<td>Collagen production observed, 34% GAG content of that of native pulmonary artery. DNA content &gt; 300%</td>
<td>Layered tissue structure with irregular tissue ingrowth</td>
<td>Koehler 2002 Ref: 66</td>
</tr>
</tbody>
</table>

Table 3.1 (continued): overview of previously performed studies on tissue engineered heart valves.
4.3.2. Synthetic scaffold materials

Instead of using biological matrices, synthetic biocompatible and biodegradable biomaterials can be used as a scaffold. The cells will attach to the polymer, multiply and develop into tissue. The polymer gradually degrades as the cells secrete their own ECM and develop into tissue. Synthetic polymers are advantageous in that their chemistry and material properties are controllable. These properties are highly important in the design of the scaffold. The main properties include:

- **Porosity**: Cells must have the ability to invade the scaffold in order to provide an adequate cell distribution. Thus, the scaffold needs an interconnected pore network, with a porosity of 90%\(^\text{51}\). This also promotes the flow of nutrients and metabolic waste.

- **Biocompatibility and bioresorbability**: The scaffold should not induce negative side effects on the cells or the surrounding tissue. Preferably, the scaffold material gradually degrades at a controllable rate, matching the tissue replacement. The degradation products must be non-toxic and must preferentially be eliminated or excreted by the body.

- **Mechanical properties**: The mechanical properties of the scaffold must match the mechanical properties of the tissue at the site of implantation. Over time, the load bearing function of the scaffold must be transferred to the developing tissue.

- **Manufacture**: The scaffold must be easy to manufacture in the desired shape and size.

In synthetic polymeric scaffolds, the porosity, pore size, mechanical stability and degradation rate can be well controlled\(^\text{28}\). Moreover, some types show little or no immune response, and they have the potential for defined integration and time-controlled release of bioactive compounds from the matrix\(^\text{52}\). The disadvantage however, of synthetic polymeric materials, is the difficulty of creating a complex shape. They can produce toxic degradation products and give rise to the possibility of inflammatory responses on the material.

Several synthetic polymers have been used as scaffold materials (table 3.1). The most widely used polymers in tissue engineering are poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and a copolymer of those; poly(lactic-co-glycolic acid) (PLGA)\(^\text{28}\). PGA and PLA belong to the class of biodegradable polymers called poly(hydroxy alkanoates) (PHAs). They are resorbable, flexible and induce only a minimal inflammatory response\(^\text{28}\). Also belonging to this class are poly(4-hydroxybutyrate) (P4HB) and poly(hydroxyoctanoate) (PHO). As can be seen in table 3.1, both of these polymers have been used as heart valve scaffold materials. PHO is a thermoplastic polymer and can be moulded into almost any shape\(^\text{53}\). PHO has proven to degrade more slowly than PGA/PLA\(^\text{54}\). Højerstrup \textit{et al.} (2000)\(^\text{55}\) and (2002)\(^\text{43}\) coated a PGA
matrix with P4HB. This resulted in a flexible scaffold with a more rapid degradation time than PHO. This material showed to be mouldable into a trileaflet heart valve geometry.

4.4 Stimuli

In a normal biological situation, heart valves are exposed to several stimuli. Both biological and mechanical stimuli play a role in the physiology of the heart valve. Both tensile and shear stresses, as well as bioactive agents, mediate the growth and maintenance of the valve. Without the proper stimuli, it is not possible to regenerate tissue. This chapter will describe the role of stimuli on the formation of tissue engineered heart valves.

4.4.1. Mechanical stimuli

Biological heart valves are continuously exposed to mechanical stresses. These mechanical stresses are mainly shear stress, caused by the blood flow, and cyclic stretching, during the opening and closing of the valve. These mechanical stimuli are important in the formation and remodeling of the valve. Endothelial cells orient and elongate in the direction parallel to the flow when directed to shear stress. Subsequent exposure to flow in a direction perpendicular to the cellular alignment causes progressive reorganization of the cytoskeleton, with a corresponding change in the direction of the alignment in the direction of the flow. In contrast, vascular smooth muscle cells (VSMCs) align in the direction perpendicular to the fluid flow, when fluid shear stress is applied without a pressure gradient. This VSMC alignment is dependant on the magnitude of and the exposure time to fluid shear stress. It was shown that shear stress induced VSMC alignment is a continuous process that is initiated within several hours of the onset of fluid flow. Porcine valve leaflets show no increase in protein, glycosaminoglycan (GAG), and DNA synthesis after exposure to flow. It was indicated that exposure to flow maintains leaflet synthetic activity near normal levels, but that the inclusion of another force, such as bending or backpressure, may be necessary to preserve the expression of contractile proteins such as smooth muscle α-actin (SMA). VSMCs that were embedded in a collagen gel showed increased cell proliferation and SMA expression after the exposure to 10% cyclic strain, as compared to the statically cultured cells. Hoerstrup et al. seeded human marrow stromal cells on a trileaflet synthetic polymer scaffold. Constructs cultured in a pulse duplicator system were compared to statically cultured constructs. In contrast to statically cultured constructs, which were fragile and lost structural integrity after 14 days, dynamically cultured constructs showed gross tissue formation. All leaflets were intact, mobile and pliable, and the valve constructs were competent during closure. The presence of GAGs, actin/myosin filaments and collagen fibrils was demonstrated. Sodian et al. demonstrated that exposure to a pulsatile flow stimulated cell proliferation and GAG formation in a tissue engineered heart valve construct. Valvular cells were mostly viable and formed connective tissue between the inside and the outside of the porous scaffold. It is obvious that mechanical stimulation, both shear stresses and compressive/tensile stresses, are a necessity in the engineering of a heart valve construct. By applying the appropriate forces, the cells in the construct are stimulated to synthesize their own
matrix, which is a crucial step in the development of tissues. This, however, can also be accomplished by using biochemical stimuli, as is elucidated in the next paragraph.

4.4.2. Biochemical stimuli

Bioactive agents play an important role in the development of a tissue. In the tissue engineering process, these bioactive agents can either be administered in the surrounding medium, or incorporated in the scaffold matrix. It has become clear that the ECM plays an mediating role for cellular activities\textsuperscript{61}. The cell surface contains receptors to respond to extracellular signals. As soon as the ligand-receptor interaction is established, the biochemical machinery involved in the control of gene expression starts. Cell adhesion is crucial for tissue formation. Several ECM-receptors are known that promote adhesion. Proteoglycans, among which CD36 and CD44 are the most widely studied, can form receptor proteins. These cell surface receptors bind to collagen, fibronectin and thrombospondin, thus stimulating cell adhesion. A large variety of receptor molecules belong to the group of integrins. Their structure consists of a non-covalent association of $\alpha$ and $\beta$ subunits. Many different integrins have been identified, given the fact that some $\alpha$ subunits can combine with several $\beta$ subunits. As soon as ECM molecules bind to their specific integrin receptors, a change in the cytoplasmic domain of the receptor occurs, which associates with the cytoskeleton\textsuperscript{61}. Besides ECM components, bioactive agents can bind to cell surface receptors such as integrins. Cytokines are a group of regulatory molecules that function as mediators of cell communication. Via interaction with specific receptors, they exert multiple biological functions. The family of cytokines includes interleukins, interferons, tumor necrosis factors and growth factors\textsuperscript{62}. The latter are interesting agents for the process of tissue formation. Scaffolds can be incorporated with cell adhesive ligands to promote or improve cell adhesion. These ligands comprise short peptide chains that can be recognized by several cell types. A widely used agent is the RGD peptide. But also other peptides, such as RGDS and REDV have shown to increase cell attachment\textsuperscript{63}. However these active peptides have shown to have a negative effect on the ECM synthesis by the cells, when they are used in high concentrations. This may pose limitations for the use of these agents in tissue engineering scaffolds\textsuperscript{63}. Growth factors may provide a solution to this problem. Several growth factors have proven to be useful in cardiovascular tissue engineering. Transforming growth factor $\beta$ (TGF$\beta$) can be used in scaffolds to dramatically increase matrix production in VSMCs\textsuperscript{64}. Platelet derived growth factor (PDGF) has proven to increase the proliferation of VSMCs in a collagen matrix. However, it decreased the SMA expression, which was stimulated by mechanical stimulation (as mentioned before). Thus, PDGF can inhibit the effects of mechanical stimulation. TGF$\beta$ strongly inhibits cell proliferation of VSMCs, but increases the expression of SMA, especially in the presence of mechanical strain\textsuperscript{59}. In a study performed by Fu et al.\textsuperscript{65}, TGF proved to increase the DNA content tissue engineered constructs seeded with human aortic cells. Basic fibroblast growth factor (bFGF) also increased the DNA contents of tissue constructs. Moreover, addition of bFGF led to markedly higher collagen synthesis. Constructs supplemented with bFGF
revealed a more dense, organized tissue development with pronounced matrix protein formation.

4.5. Summary

The three aspects of tissue engineering have been discussed. When designing a tissue engineered construct, a decision is to be made about what cell type(s) are to be seeded on the scaffold. Because of the abundance of (myo)fibroblasts in the natural heart valve, their capability of synthesizing ECM, and their relatively easy harvesting and culturing, many researchers have chosen (myo)fibroblasts to seed on their scaffold. This is often followed by seeding with endothelial cells, to mimic the natural situation. In the future, cells that still have the capability to differentiate might prevail, but the process of differentiation is still poorly understood.

The type of scaffold is also to be chosen. The advantage of synthetic scaffolds on biological scaffold is the fact that many characteristics of synthetic scaffolds can be controlled, e.g. their geometry, porosity and rate of degeneration. The major disadvantage of synthetic scaffold is the inflammatory response they, or their waste products, can induce.

The next important issue in tissue engineering is the stimuli that are to be applied to the construct. Cell phenotype can be regulated in cardiovascular tissue constructs by applying selected combinations of biochemical and mechanical stimuli. Yet, however, it is unclear which combinations provide the optimal environment for tissue development. Some stimuli, either mechanical or biochemical, seem to be synergistic, while other seem to counteract.
5. Conclusions

Tissue engineering is a field that supplies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain or improve tissue function. It is a challenge to develop a tissue engineered construct that shows a maximal resemblance with the native tissue, both anatomical, biochemical and mechanical. As discussed in this review, three main aspects have to be considered in the process of tissue engineering: the cells, the scaffold and the applied stimuli.

The ideal tissue engineered heart valve possesses the following characteristics:

- Endothelialized blood-contacting surface
- Cellular potential for extracellular matrix production, remodeling and repair
- Appropriate heterogeneity, anisotropy, and amount of extracellular matrix
- Stable geometry but potential growth with patient
- Immunological and inflammatory responses are not induced
- Resistance to calcification
- Stable mechanical properties, comparable to the characteristics of healthy natural valves
- Large effective orifice area
- Prompt and complete closure
- Easy and permanent implantation

A great deal of research still has to be done before a tissue engineering heart valve is developed that satisfies the demands listed above.

In the research, subsequent upon this review, a trileaflet polycaprolacton scaffold will be seeded with mice fibroblasts. After several days of static seeding, the construct will be subjected to pulsatile loads. The emphasis will be put on optimizing the extracellular matrix synthesis. Performing uniaxial loading tests provides information about the mechanical characteristics of the tissue engineered construct. Ideally, these characteristics coincide with the observed matrix production. Combining results from the mechanical characterization with the matrix production might provide insight in the optimal culture conditions.
6. References


