Collagen Orientation by Mechanical Stimuli in Tissue-engineered Heart Valves
-- A Literature Review
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Introduction

Each year about five million Americans are diagnosed with valvular disease. More than 18,500 patients die each year in the United States. In about 38,000 deaths valvular heart disease is a contributing factor. Most of the patients (63%) suffer from an aortic valve disorder. About 14% of the patients have a mitral valve disorder. Deaths due to pulmonic and tricuspid disorders are rare (3%). There are a number of different types of aortic valve disease, which include the following: aortic stenosis (narrowing, stiffening, thickening, fusion or blockage of the valve), aortic regurgitation (improper closing of the valve causes a backflow of blood) and aortic atresia (valve developed improperly and is completely closed upon birth). Many patients can be treated successfully with medications such as ACE inhibitors, antiarrhythmics, antibiotics, anticoagulants, diuretics and cardiotonics. If medications are unsuccessful surgery or other interventions may be required. The procedure may include heart valve repair or replacement. Percutaneous balloon valvuoplasty is a procedure to repair the diseased valve. It involves a nonsurgical, catheter-based treatment for valvular stenosis. Another method is valvulotomy, an open-heart surgery in which the surgeon cuts into a valve to repair valvular damage. A surgical repair of a defective valve through a small incision, which involves partial removal of the upper breastbone, is called minimally invasive heart valve surgery. Instead of a heart valve repair, a heart valve replacement could be performed. This is an open-heart surgery in which a biological or mechanical valve is used to replace a defective heart valve. For readers interested in aortic valve surgery and replacement reviews by [Braunwald 2000, David 1999 and Schoen & Levy 1997] are suggested.

Until now two basic types of valve replacement are most common. Biological valve replacement may be an autograft (i.e. from the patient’s own body), a xenograft (i.e. from a pig or cow) or a homograft (i.e. from a human donor). These biological valves last for about 10 years. In February 2001, the FDA approved the use of a new type artificial heart valve, made from a segment of a porcine aorta including its aortic valve. The second type is a mechanical valve substitute. This valve is built from man-made synthetic materials or metals. These valves can last for over 30 years. However, all these valve substitutes, except for the autograft, are seen as foreign objects by the body. They may cause blood clot formation. To prevent this, patients have to take anticoagulants for the rest of their lives. Furthermore, in case of a mechanical valve substitute, patients are asked to record the make, model and serial number of their artificial heart valve in case there are any problems with it in the future. If the autograft solution is chosen, usually the mitral valve is explanted and used to replace the aortic valve. The problem is thus shifted and a double operation is necessary. The mitral valve is then replaced. Also, the availability of homografts is limited. [www1]

Tissue engineering offers many new possibilities to create an aortic valve substitute. Cells can be grown in a biological and/or synthetic scaffold under mechanical and chemical stimuli into a suitable shape. Ideally, the properties of the prosthesis would match the mechanical, physiological and chemical responses of a native valve. Furthermore the replacement should be non-thrombogenic and should integrate into the surrounding tissues and exhibit natural wound healing, growth and regeneration. In a tissue engineered valve, based on products from the patient’s body, immune responses and medication are minimized. It is estimated that each year, almost 80,000 people will need a heart valve replacement. The search for the ideal aortic valve substitute continues.
2 The aortic valve

A tissue engineered heart valve can not be built from scratch. Knowledge of the natural anatomy and physiology is required. One of the biggest concerns with engineered tissues is their strength. Collagen is the main fiber if it comes to mechanical stiffness of the valve. In this study especially the mechanical triggers influencing collagen fiber development will be explored. It is not a goal of this research to mimic embryonic development. This involves too many unidentified chemical stimuli. Furthermore, the valve is actually growing in this stage. To grow a complete valve would consume too much time for clinical application.

2.1 The heart

The heart is composed of four heart chambers, as is depicted in figure 2.1.1. It contains two chambers that collect the incoming blood, the left atrium and the right atrium. From the atria the blood is transferred to the respective chambers, called ventricles. Upon electrical stimulation the right ventricle pumps the blood to the lungs for oxygenation. The oxygen-rich blood is then collected in the left atrium. Through the left ventricle it leaves the heart again, but this time the blood flows to all organs to supply them with oxygen and nutrients and to carry away their waste products. Four valves are present in the heart to maintain a blood flow in one direction.

![Normal Heart](image)

Figure 2.1.1: Drawing of the heart showing its chambers and valves. The arrows indicate the direction of blood flow. [www1]

The tricuspid valve prevents flow from the right ventricle to the right atrium. The pulmonary valve makes sure the blood flows from the right ventricle into the pulmonary artery. Blood flow from the left atrium to the left ventricle is assured by the mitral valve. The aortic valve prevents back-flow from the aorta into the left ventricle during diastole. The valve requires rather strong backflow for a few
milliseconds before it closes. Because of rapid closure and ejection, the edges of the semilunar valves (commissures) are subjected to great mechanical abrasion. [Guyton & Hall 1996]

The tricuspid and the mitral valve are also called the atrioventricular valves because they are located between an atrium and a ventricle. Likewise the pulmonary and the aortic valves are called arterioventricular valves. The latter valves are also called semilunar valves as their leaflets look like a half moon. A difference with the atrioventricular valves is that the semilunar valves have no papillary muscles or chordae tendinae to actively support them. In contrast to what was believed in the past, the aortic valve also has active parts.

2.2 The aortic valve

The aortic valve can be divided into two structural parts: the aortic root and the leaflets (figure 2.2.1). Anatomically, the aortic valve can be described as a three-layered leaflet. An endothelial layer surrounds the fibrosa layer and the spongiosa layer.

![Figure 2.2.1: Schematic of the aortic root and the leaflets. On the left a top view of the leaflets and the root is shown. On the right a side-view of one leaflet and the sinus is given. [Thubrikar 1990]](image)

2.2.1 The aortic root

The aortic root is the part of the aortic valve that provides anchorage of the leaflets to the heart. The root contains three aortic sinuses, outwardly expanded parts of the aortic wall, and the leaflet anchorage: the annulus fibrosis. In two of these sinuses a coronary artery originates to supply the heart with blood. Accordingly, the three sinuses of Valsalva together with their leaflets are named: right and left coronary sinuses and leaflets and the non-coronary or posterior sinus and leaflet. The sinus tissue roughly contains three layers like the arterial wall, but is thinner. The intimal layer is characterized by a line of endothelial cells and intermixed collagenous and elastic tissue. The medial layer is much thicker and contains alternating layers of smooth muscle and elastic tissue. The media is the main contributor to the elastic properties of the sinuses. The most important task for the adventitial layer is the supply of blood to the medial musculature. It is composed of various tissues, ranging
from loose, fatty tissue to extensions of cardiac muscle. Some intermixed collagenous fibers give the root mechanical strength. Most of the collagenous fibers seem to be more longitudinally oriented than the muscle and elastic fibers. The muscle fibers are long cells oriented circularly around the vessel wall.

In humans the annulus fibrosis consists of tightly packed collagenous fibers oriented circularly, interwoven with fibers continuing into the leaflets. The leaflet anchorage is located between the sinuses and the left ventricle. Around the leaflets the annulus has a u-shape and all three together give it a coronal appearance. At the crown tops the commissure points are located. It forms an incomplete ring, as the collagenous tissue is interrupted twice by cardiac muscle tissue. This allows deformation following the cardiac cycle.

### 2.2.2 The leaflets

The leaflets are tissue sheets that form cavities together with the sinuses during diastole. The leaflets are anchored to the wall at one end and the other end is referred to as the free edge. In the closed state the load bearing parts of the leaflets can be distinguished. A thickening of the free edge ensures extra closure at the center of the closed valve. These thickenings are the nodes of Arantius (figure 2.2.2.1). The leaflets of the aortic valve contain mainly connective tissue that is surrounded by endothelial cells. The connective tissue is built of two layers: the lamina fibrosa and the lamina spongiosa (figure 2.2.2.2).

![Leaflet layers](www2)

The lamina fibrosa is located at the aortic side of the leaflet. In this broad layer of dense connective tissue, thick parallel collagen fibers run in the circumferential (from commissure to commissure) direction (figure 2.2.2.3). They run from the commissures to the center of the leaflet. In the center they branch into smaller fibers and create a fine network. From the free edge towards the basis of the leaflet the collagen fibers are more cross-woven instead of individually arranged. The thick fibers result in a ribbed surface of the leaflet on the aortic side. At the basis of the leaflet radially oriented small diameter collagen fibers provide the anchorage of the leaflet to the sinus wall. The lamina fibrosa is the mechanically strongest layer in the leaflet. Therefore, it carries most of the aortic pressure during diastole.
The onset thickness of the lamina spongiosa at the basis of a leaflet converges towards the free end of a leaflet. This layer consists of loose connective tissue with proteoglycans as its main component. The large amount of proteoglycans attracts water (90% of a valve cusp is water). The few collagenous fibers as well as the cells in this layer are oriented radially. Some of these thin radially oriented fibers are attached to thick fibers from the lamina fibrosa. The swollen layer may dampen the vibrations caused by closure of the valve. Furthermore, the lamina spongiosa is mechanically weak because of the lack of strong fibers, like collagen fibers.

The fibrosa and the spongiosa are surrounded by extensions of the arterial intima and the ventricular endocardium. These layers are the arterialis and the ventricularis, named after the respective side of the leaflet that they are facing. The arterialis is not present continuously on the aortic side. It consists of very small collagenous and elastic fibers that are loosely arranged. The ventricularis consists mainly of sheet elastin that is radially oriented. It provides the tensile recoil necessary to retain the folded shape of the fibrosa. This relationship between the fibrosa and the ventricularis requires the fibrosa to remain preloaded in compression (to retain its corrugated state) and the ventricularis to remain in tension (to hold the fibrosa in compression). Elastin is believed to be primarily responsible for generating the preload in the ventricularis, and for maintaining the collagen fibre architecture in its neutral state. It possibly has a deeper layer of circumferentially oriented collagenous fibers as well. The superficial elastin orientation causes a smooth ventricular surface of the leaflet and thus a laminar blood flow passing the leaflets during systole [Bronneberg 2001, Thubrikar 1990]

**2.2.3 Cells and fibers in the leaflets**

Endothelial cells on the surface of the leaflets are oriented circumferentially (perpendicular to the direction of blood flow), on the ventricular side as well as the arterial side. The endothelial cells show a moderate synthetic activity. The basal lamina beneath the endothelium on both sides is variable in thickness and multilaminar.

In the fibrosa parent fibroblasts are sparse and a few elastic fibers occur, randomly spread among the collagenous fibrils. Lengthwise overlapping and cross-linking produce axial periodicity of fibrils. The collagenous fibrils are arranged parallel to each other and not rippled, suggesting a continuing tension to straighten the fibrils. The circumferential orientation of this collagenous tissue indicates that the dominant
tensions on the leaflet occur parallel to the free edge. Among the fibrils proteoglycans are present, some of which are attached to the fibrils. They would thus mechanically link the fibrils together and contribute to their strength. The hydration by the proteoglycans gives the tissue flexibility.

The lamina spongiosa is populated by more numerous fibroblasts than the fibrosa but relatively less collagenous and elastic tissue. The collagenous content of this layer is organized in small fibers and decreases towards the ventricular side. Elastic fibers are laced through the collagen fibers. The spongiosa collagenous and elastic fibers are loosely arranged, as are the proteoglycans and amounts of water. This suggests that the spongiosa is a highly flexible layer. The cells are extremely elongated and connected to each other and to collagenous fibers. The cell-fiber connections might transfer stresses to the cells when the valve opens or closes.

The ventricularis consists superficially of a few fibroblasts and fine elastic fibers, oriented in radial direction (two or three layers). Internal to these elastic fibers, a few layers of circumferentially arranged collagenous fibers can be found (figure 2.2.3.1). At the center of the free edge, where all leaflets meet each other, a dense tissue is present. This tissue is the leaflet nodule. Here, myofibroblasts populate intermixed collagenous and elastic fibers embedded in a dense hydrated proteoglycan network.

The synthetically active sites in the valve correlate well with regions of stress in the regularly functioning valve. Tissue turnover is one of the factors involved in the operational longevity of natural valves. [Thubrikar 1990]

Figure 2.2.3.1: Fiber orientations in the leaflet layers. On the left the collagen orientation in the lamina fibrosa is schematically depicted. The fiber orientation is mainly circumferential, except from the anchorage where the fibers are more radially aligned. In the picture on the right the fiber orientation in the lamina spongiosa and ventricularis is drawn. Here, fiber orientations are radially. The topside of both drawings represents the free edge.
3 ECM remodeling and cell alignment

Tissue engineering is based on the concept that a tissue can be developed using cells, a scaffold and external triggers (e.g. mechanical, chemical, electrical). In natural tissue the scaffold consists of the extracellular matrix. The extracellular matrix is the direct environment of the cells. Cells and matrix coexist in a continuous adapting process. A lot of matrix remodeling activity can be observed during embryonic development and wound healing responses. For more information on the embryological valve development the reader is referred to Mol [2001]. In this study the role of the matrix component collagen is emphasized. All kinds of influences on matrix modeling will be discussed. A short introduction on matrix and cytoskeleton fibers will be given. Furthermore, a literature search is performed to test the hypothesis that mechanical stimulation is a major factor in regulating collagen deposition and orientation.

3.1 The extracellular matrix

An extracellular matrix (ECM) surrounds cells in connective tissues (figure 3.1.1). The matrix binds the cells together and influences their growth, state of differentiation, development, polarity, metabolic responses and behavior. The matrix contains a ground substance, fibers and tissue fluid. The ground substance is highly viscous to inhibit spreading of microorganisms. The extracellular matrix macromolecules can be divided into four classes: proteoglycans, structural glycoproteins, collagens, and elastin.

![Figure 3.1.1: Image of the epithelial extracellular matrix in the cornea [Abrams 2000].](image)

Except for elastin all classes consist of a family. In the ground substance glycosaminoglycans (GAGs) are present. These GAGs are a heterogeneous group of negatively charged polysaccharide chains. These chains (except for hyaluronan) are covalently linked to proteins to form proteoglycans. The proteoglycans have acidic ends which can bind to the basic groups of the amino acids of the collagenous fibers.
The structural proteins are also part of the ground substance. The fibers in the matrix are collagenous and elastic (figure 3.1.2).

![Figure 3.1.2](image)

Figure 3.1.2: On the left collagen fibers on different scales can be seen. On the right an elastin fiber is depicted in coiled and uncoiled situation. [Alberts 1994]

Specific receptors on the cell surface link the cell to the ECM components. These receptor families are the integrins and the syndecans. Integrins are the principle receptors used by animal cells to bind to the extracellular matrix. The working of integrin receptors is illustrated in figure 3.1.3.

![Figure 3.1.3](image)

Figure 3.1.3: The coupling and uncoupling of integrins to matrix components. On the left the assembly of intracellular components is shown as integrins are coupled to fibronectin of the extracellular matrix. On the right the intracellular components are named. [Alberts 1994]

Integrins are heterodimers that mediate bidirectional interactions between the ECM and the actin cytoskeleton. They also function as signal transducers, activating various intracellular signalling pathways when activated by matrix binding. A cell can control the adhesive activity of its integrins by altering either their matrix-binding site or their attachment to actin filaments. The ECM production is regulated by growth factors and cytokines, vitamins and hormones and by cell-to-cell contact. [Alberts et al 1994 and Haralson & Hassell 1995] On the other hand, matrix metalloproteinases are known to
have the capability of degrading all the ECM components [Crean et al 1997, Majima 2000].

### 3.1.1 Collagen fibers

Collagen is the most abundant protein in the animal kingdom, representing approximately one-third of all protein in tissues. The collagen family includes at least thirty distinct gene products that are present in at least eighteen distinct types of collagen. The characteristic feature of a typical collagen molecule is its long, stiff, triple-stranded helical structure, in which three collagen polypeptide chains, called $\alpha$ chains, are wound around one another in a ropelike superhelix (figure 3.1.1.1).

![Collagen structure](image)

Figure 3.1.1.1: Schematic representation of the structure of type I procollagen [Haralson & Hassell 1995]

Collagens are extremely rich in proline and glycine and also contain much lysine. In table 3.1.1.1 an overview of the composition and functions of the collagen types that are present in aortic valves is given. All of these collagen types belong to the group of the fibrillar collagens.

<table>
<thead>
<tr>
<th>Collagen type</th>
<th>Chain type</th>
<th>Molecular species</th>
<th>Organization aggregates</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$\alpha_1(I)$ $\alpha_2(I)$</td>
<td>$[\alpha_1(I)]_2 \alpha_2(I)$</td>
<td>Large-diameter banded fibrils</td>
<td>Supporting fibers, recognized by integrin and syndecan surface receptors</td>
</tr>
<tr>
<td>III</td>
<td>$\alpha_1(III)$</td>
<td>$[\alpha_1(III)]_3$</td>
<td>Small-diameter banded fibrils</td>
<td>Small supporting fibers; forms copolymers with type I collagen</td>
</tr>
<tr>
<td>V</td>
<td>$\alpha_1(V)$ $\alpha_2(V)$ $\alpha_3(V)$</td>
<td>$[\alpha_1(V)]_3 [\alpha_1(V)]_2 \alpha_2(V) \alpha_1(V)\alpha_2(V)\alpha_3(V)$</td>
<td>Probably fine-diameter banded fibrils</td>
<td>Small supporting pericellular fibers; possible core for type I molecules</td>
</tr>
</tbody>
</table>

Table 3.1.1.1 Overview of collagen types found in aortic valves and their properties. [Haralson & Hassell 1995, Kielty et al 1993]

The collagen content of aortic valves has been determined as 74% collagen type I, 24% collagen III and some collagen type V [Mol 2001]. Electron microscopic observations showed that the collagenous fibrils in the leaflets have a diameter of 300-500 Å. This is an unusually small dimension for the fibers [Thubrikar 1990]. For more information on collagen structure, chemical composition and collagen assembly, the reader is referred to a chapter by [Kielty et al 1993].
3.1.2 Elastin fibers
A network of elastic fibers gives tissues resilience to recoil after transient stretch. It is a complex of its main component elastin with several non-elastic glycoproteins termed fibrillar components; the most notable of which are fibrillin and lysyl oxidase. Elastin is highly hydrophobic and is, like collagen, rich in proline and glycine but, unlike collagen, is not glycosated and contains little hydroxyproline and no hydroxylysine. After secretion from the cells elastin becomes highly cross-linked. Desmosine and isodesmosine form the intermolecular cross-links. [Alberts et al 1994, Haralson & Hassell 1995] More information concerning the role and of elastin in the aortic valve can be obtained from [Schoen 1997].

3.1.3 The cytoskeleton and stress fibers
The extracellular matrix contains the tissue’s backbone outside the cells that maintains tissue organization and architecture. Inside the cell the network is called the cytoskeleton. Three types of proteins spatially organize the cell cytoskeleton. The fiber proteins form a network of three types of filaments: microtubules, actin filaments and intermediate filaments. Microtubules are stiff proteins and have one end anchored in the centrosome and the other free in the cytoplasm. In many cells these microtubuli are highly dynamic and they alternateely grow and shrink by the addition and loss of tubulin subunits. Actin filaments are also dynamic structures, but they exist in bundles or networks rather than as single filaments. A layer called the cortex is formed just below the cell surface from actin filaments and a variety of actin-binding proteins. The cortex controls the shape and surface movements of cells. Intermediate filaments are relatively tough, ropelike structures that provide mechanical stability to cells and tissues.

Generally, cells are polarized when a transmembrane signal is induced on the plasma membrane. The actin cortex then reorganizes beneath the activated cell surface. The affected part of the cell membrane pulls probably on microtubuli to move the centrosomes towards the area. Now, the centrosomes organize the network in a polarized way. One way of regulating the actin polymerization is that extracellular signals bind to cell-surface receptors that act through heterotrimeric G proteins and the small GTPases Rac and Rho. [Alberts et al 1994]

Prominent components of the cytoskeleton of fibroblasts in culture are stress fibers. They are temporary contractile bundles of actin filaments and myosin-II. The ability to form microfilament bundles or stress fibers is required for matrix organization [Mar et al 2001]. Stress fibers form in response to tension generated across a cell and are disassembled at mitosis when the cell rounds up and loses its attachments to the substratum. Stress fibers in fibroblasts within tissues are thought to allow the cells to exert tension on the collagen matrix that surrounds them. They also disappear rapidly if tension is released by sudden detachment from their focal contact (i.e. special attachment site on plasma membrane), using a laser beam [Alberts et al 1994]. According to [Hayes et al 1999], in embryonic development, the stress fibers direct the initial elongation of cells and control the deposition of oriented extracellular matrix. At this stage oriented collagen fibers are deposited between cells. After birth, the stress fibers disappear again, supposedly because the cells get orientational cues from the matrix now.

Fibroblasts in attached collagen lattices developed stress fibers, surface-associated fibronectin fibrils, and a fibronexus-like transmembrane association interconnecting
the two structural components. Release of the attached collagen lattice from its points of attachment resulted in a rapid, symmetrical contraction of the collagen lattice. The rapid contraction resulted in a shortening of the elongate fibroblasts and compaction of the stress fibers with their subsequent disappearance from the cell [Tomasek et al 1992]. Interestingly, Takemasa et al [1998] found that stress fibers in endothelial cells under directional cyclic strain rearrange their stress fibers in an oblique direction relative to the stretch direction. In this way stress fiber length alterations were minimized during cyclic deformation. Furthermore, the oblique angle was only dependent on the strain amplitude.

3.2 Matrix remodeling

Many different factors influence fiber remodeling inside as well as outside a cell. From different disciplines people found different triggers for fiber remodeling. In this section an attempt has been made to give an overview of various factors that control fiber remodeling at different levels.

3.2.1 Influence of mechanical stimuli on matrix remodeling

Collagen reorganization involved only 5% of matrix degradation [Guidry et al 1985]. Preexisting collagen fibrils are rearranged rather than resynthesized. Several factors influence the orientation of the newly organized fibers. Timmenga et al [1991] found that stress induced stronger and more organized scars. Cells change their orientation and reorganize their surrounding matrix in response to changes in their mechanical situation. Stretch also affects the mechanical properties of the tissue. Fibrillar orientation, packing density and ultimate tensile strengths can be increased by stretch [Pins et al 1997].

Not only the extracellular matrix is remodeled by stress; the internal cytoskeleton is reorganized as well. The proliferation rate, gene transcription and the synthesis of matrix proteins and degradation enzymes are altered. Even the cellular phenotype can be changed by mechanical stimuli [Lambert et al 1992].

Kanda and Matsuda showed the different influences of static and dynamic stresses on arterial smooth muscle cells in 1994. They found that matrix reorganization under static as well as dynamic stress involved cell orientation parallel to the strain direction (after four weeks). Also, collagen was predominantly oriented in that same direction. Moreover, only periodic stretch on arterial smooth muscle cells induced a phenotypic change to the contractile cell type. Under static stress and in stress-free controls the phenotype of the cells remained synthetic. Dynamic mechanical stimulation of fibroblasts induces a much greater response than static stimulation [Prajapati et al 2000]. On the other hand dynamic loading can be more damaging to the tissue than its static equivalent because of an acceleration of proteolysis (that is if proteolytic enzymes are present) [Ellsmere et al 1999].

3.2.2 Other factors influencing matrix arrangement

Not only mechanical stimuli affect cell behavior. Some other factors are listed below.

- Guidry et al showed in 1985 that serum is necessary for collagen remodeling by fibroblasts in collagen gels. Fibroblast contraction was also promoted by the
presence of serum [Tomasek et al 1992]. Platelet-derived growth factor is believed to be the active component in serum [Clark et al 1989]

- In 1985 Doillon et al found that in dermal wound healing unorganized fibers were deposited and after that reorganization took place. It took the tissue 180 days after injury to get close to normal skin in strength and collagen fiber morphology. Collagen fibril and fiber diameters appeared smaller within wound and scar tissue than in normal skin. This might explain the low strength of the wound tissue. It is clear that one cannot wait 180 days for matrix modeling alone. It is suggested that any process that accelerates the remodeling process should increase collagen fiber diameters.

- Platelet-derived growth factor and transforming growth factor $\beta_1$ stimulate fibroblasts in wound healing to express integrin receptors [Singer & Clark 1999]. This may accelerate binding to the extracellular matrix components and speed up cellular alignment in wound healing.

- The different collagen subtypes are recognized by specific integrins. The expression pattern of these different collagen receptors is supposed to regulate the shift of the cell phenotype from a low expression of collagen combined with a high proliferation rate to increased collagen synthesis and matrix turnover rate [Heino 2000].

- Insulin-like growth factors regulate collagen production by heart fibroblasts as well [Kanekar et al 2000].

- Contact guidance is another tool that may be used to accelerate matrix remodeling. A comparison of remodeling responses has been made between cells that were aligned perpendicular to applied loads and cells that were aligned parallel. The alignments were generated by enforced contact guidance. The cells that were held in perpendicular alignment showed an increased remodeling response [Mudera et al 2000].

- Not only the speed of matrix remodeling is of interest in tissue engineering. Another important property is the mechanical strength of the newly assembled matrix. A powerful tool to achieve greater strength is cross-linking of collagen fibers. Decorin has been shown to increase ultimate tensile strength of uncross-linked fibers. Based on the observed results it was hypothesized that the proteoglycan, decorin, facilitates fibrillar slippage during deformation and thereby improves the tensile properties of collagen fibers [Pins et al 1997 and McCormick 1999].

- The influence of fiber modeling on the mechanical properties of the ECM is demonstrated by [Christiansen et al 2000]. Lateral fusion of fibers appears important in generating resistance to deformation at low strain, while linear fusion leading to longer fibrils appears important in the ultimate mechanical properties at high strain.

### 3.2.3 Cellular alignment

In most human tissues a well-defined structural organization can be observed. Especially in wound healing and bone formation the influences of mechanical stimulation is obvious. Contact guidance is believed to be an important aspect in cellular alignment and thus tissue architecture. Contact guidance is defined as the directed locomotory response of cells to an anisotropy of the environment [www3]. An example is the tendency of fibroblasts to align along ridges or parallel to the alignment of collagen fibers in a stretched gel. If contact guidance is taken into
account the collagen orientation could be considered equal to the cellular orientation. This implies that the cellular orientation can be taken as an indicator for the collagen orientation. This is especially convenient since cellular orientations after mechanical stimuli are reported in literature whereas collagen orientations are rarely reported. The general thought concerning cellular orientation after mechanical triggering was that cyclic stretching caused perpendicular cellular alignment. This theory was usually based on the principle of strain avoidance by the cells. Buck reported a cellular alignment perpendicular to the principal stretching direction in 1980. However, from several experiments it has become clear that oblique alignment could be triggered by application of cyclic mechanical strain as well [Takemasa et al 1998, Dartsch et al 1986 and Neidlinger-Wilke et al 2001]. This cell alignment occurred when strain exceeded a cell-specified threshold. For aortic smooth muscle cells this threshold was between 2% and 3.5% [Dartsch et al 1986, 1992], whereas for skin fibroblasts this was not further narrowed than between 1% and 4% [Neidlinger-Wilke et al 2001]. An upper limit for the fibroblasts’ strain tolerance (4.2%) existed as well. The cells did not align completely perpendicular to the direction of the applied strain. A tendency was observed with strain values above the threshold. The angle of orientation depended on the stretching amplitude: the larger the stimulus, the more pronounced the angle between the longitudinal cell axis and the direction of cyclic stretching [Neidlinger-Wilke 2001, Takemasa 1998, Dartsch et al 1986]. Dartsch et al [1986] showed also that the degree of cellular orientation increased with experimental time. The alignment of the fibroblasts was mainly caused by large strain that was applied in the axial cell direction. As cells are oriented by their cytoskeletons it was suggested that the actin cytoskeleton played a role in the mechanosensory mechanism as it is connected to integrin receptors that bind to the extracellular matrix. In contrast to these findings, Kim et al found in 1999 that cyclic mechanical strain induced fiber alignment predominantly parallel to the direction of applied strain. This is according to what Kanda et al concluded before in 1994. Alignment of the engineered aortic smooth muscle cells was subsequently generated by contact guidance. The period of application of dynamic strain influenced the alignment like it was the case in Dartsch’s experiments [1986]. After 20 weeks the fibers were almost oriented in the strain direction. It was concluded that extracellular matrix fibers tend to align parallel to the direction of strain application. Subsequently, the smooth muscle cells align along the fibers by contact guidance. To address this difference in alignment after strain application Wang and Grood [2000] hypothesized that the cellular orientation process is not dependent on the surface strain direction, and that contact guidance provided by microgrooves can maintain cell alignment in the presence of cyclic stretching in another direction. In their experiments they used engineered skin fibroblasts. Their study showed that the cellular orientation response is the cells’ avoidance to substrate deformation (i.e., strain-direction independent). It also suggested that the failure of fibroblasts to change orientations in vivo might result from the contact guidance provided by collagen fibers. In table 3.2.3.1 a summary is given of experimental input-settings and the orientational responses of the cells.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Frequency</th>
<th>Cell type</th>
<th>Period</th>
<th>Alignment</th>
<th>Scaffold</th>
<th>Reference</th>
</tr>
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<tbody>
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<td>-5 to 5 %</td>
<td>60 bpm</td>
<td>ASMC</td>
<td>4 wk</td>
<td>0º</td>
<td>3D collagen I gel</td>
<td>Kanda 1994</td>
</tr>
<tr>
<td>7 %</td>
<td>60 bpm</td>
<td>Eng. ASMC</td>
<td>10 wk</td>
<td>53º</td>
<td>Collagen I sponge</td>
<td>Kim 1999</td>
</tr>
<tr>
<td>7 %</td>
<td>60 bpm</td>
<td>Eng. ASMC</td>
<td>20 wk</td>
<td>20º</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 %</td>
<td>50 bpm</td>
<td>ASMC</td>
<td>14 d</td>
<td>Random</td>
<td>Collagen coated Silicone membrane</td>
<td>Dartsch 1986</td>
</tr>
<tr>
<td>5 %</td>
<td>50 bpm</td>
<td>ASMC</td>
<td>12 d</td>
<td>61º</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 %</td>
<td>50 bpm</td>
<td>ASMC</td>
<td>6 d</td>
<td>76º</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>ASMC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 %</td>
<td>60 bpm</td>
<td>FB</td>
<td>24 h</td>
<td>Random</td>
<td>Pronectin coated silicone dishes</td>
<td>Neidlinger 2001</td>
</tr>
<tr>
<td>4, 8, 12 %</td>
<td>60 bpm</td>
<td>FB</td>
<td>24 h</td>
<td>Stretch avoidance</td>
<td>Silicone coated silicone dishes</td>
<td>Wang 1995</td>
</tr>
<tr>
<td>12 %</td>
<td>60 bpm</td>
<td>Mel</td>
<td>24 h</td>
<td>60</td>
<td>Pronectin coated silicone dishes</td>
<td>Wang 2001</td>
</tr>
<tr>
<td>4, 8, 12 %</td>
<td>60 bpm</td>
<td>FB</td>
<td>24 h</td>
<td>Stretch avoidance</td>
<td>Silicone dishes</td>
<td>Wang 2000</td>
</tr>
<tr>
<td>0 %</td>
<td>60 bpm</td>
<td>FB</td>
<td>3 h</td>
<td>Random</td>
<td>Pronectin coated silicone membranes</td>
<td>Wang 2001</td>
</tr>
<tr>
<td></td>
<td>Equibiaxial</td>
<td>HAEC</td>
<td>3 h</td>
<td>Random</td>
<td>Pronectin coated silicone membranes</td>
<td>Wang 2001</td>
</tr>
<tr>
<td>Simple elongation 10%</td>
<td>3 h</td>
<td>70</td>
<td></td>
<td>Pronectin coated silicone membranes</td>
<td>Wang 2001</td>
<td></td>
</tr>
<tr>
<td>Uniaxial 10%</td>
<td>3 h</td>
<td>90</td>
<td></td>
<td>Pronectin coated silicone membranes</td>
<td>Wang 2001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 bpm</td>
<td>FB</td>
<td>18-24 h</td>
<td>90</td>
<td>Silicone rubber</td>
<td>Buck 1980</td>
</tr>
</tbody>
</table>

Table 3.2.3.1: Summary of orientational cellular responses upon dynamical strain application. ASMC: arterior smooth muscle cells, eng: engineered, FB: fibroblasts, (HA)EC: (human aortic) endothelial cells, Mel: melanocytes.

Below the angles and periods mentioned in the table are depicted in a graph.

The differences among the alignment results were not only due to differences in species, cell density, growth cycles, strain tolerance in sub-confluence and in confluence, cell type and source of cells, culture conditions and the coating protein. The extracellular matrix is one of the major factors influencing cellular alignment. Alignment parallel to the strain direction can occur due to contact guidance when
smooth muscle cells are embedded in a collagen gel [Kanda & Matsuda 1994]. The type of mechanical loading proved to be of importance, like the magnitude and rate of the strain [Wang et al 2001]. For the early cellular response, the stretching magnitude was predominantly determining the rate and extent of reorientation. For the later cell orientation response, stretching rate influenced cellular orientation. Apart from stretching loads, compression also caused stretch avoidance responses of fibroblasts.

### 3.2.4 Modeling fiber organization

To rearrange cells pull on fibers, but even in a nonmotile state cells exert traction on the matrix fibers and generate a tension [Roy et al 1999]. This tensional homeostasis has been measured as $40-60 \times 10^{-5}$ N per million fibroblasts [Eastwood et al 1998]. Cell traction can also cause compaction. Cell alignment results in anisotropic migration and traction. This has been modeled by a cell orientation tensor that is a function of a fiber orientation tensor, which is defined by the network deformation tensor [Barocas & Tranquillo 1997]. A literature discussion [Driessen 2001] is suggested for readers interested in modeling fiber amount and orientation in connective tissues. Dallon and Sherratt [1998, 1999 & 2000] performed a great deal of mathematical modeling of fiber reorientation. They consider the extracellular matrix as a continuum containing discrete cells. A basic assumption is orientation by contact guidance. Cells are guided by the fibers and their speed is also determined by the matrix. The cells deposit oriented matrix fibers as well as degrading them (remodeling). A nonlinear anisotropic finite element model for heart valves is presented by [Li et al 2001]. A fluid-structure interaction model of the aortic valve is presented by De Hart et al [2000, 2001].

### 3.2.5 Mechanotransduction and mechano-sensory

Cells are influenced by their mechanical environment. They align and they rearrange their surrounding matrix in response to mechanical changes. The precise remodeling process and the mechanisms are still unknown. Several receptors and proteins, which may play a role in mechano-sensory or mechanotransduction, have been identified (figure 3.2.5.1). Chiquet [1999] suggests that integrins are key players in mechanotransduction, via MAP kinase and NF-κB pathways. The response to a change in stretch is rapid and reversible, and is reflected on the mRNA level. Mechanical stress rapidly induced phosphorylation of PDGF receptor, integrin receptor, stretch-activated cation channels, and proteins, which might serve as mechanosensors [Li & Xu 2000]. Furthermore, mechanical stresses can directly stretch the cell membrane and alter receptor or G-protein conformation, thereby initiating signalling pathways, usually used by growth factors.
3.3 Fibroblasts, myofibroblasts and smooth muscle cells

Fibroblasts belong to the family of connective-tissue cells, together with cartilage cells, bone cells, fat cells and smooth muscle cells. Fibroblasts seem to be able to transform into any of the other family members and sometimes this is a reversible process. It is unclear if one single fibroblast type is capable of all the different transformations or that the differentiation potential is spread over a mixture of fibroblast types, each capable of a single interconversion. The transformations are dependent on the extracellular matrix composition, cell shape, hormones and growth factors [Alberts et al 1994].
Myofibroblasts exhibit a phenotype that is an intermediate between a fibroblast and a smooth muscle cell. They express α-smooth muscle actin and are contractile like a smooth muscle cell and unlike a fibroblast. Their emergence from fibroblasts can be induced by transforming growth factor β (TGF-β). [Roy et al 2001, Becker et al 2000] According to Mattey et al [1997] interleukin 4 (IL-4) evokes the same differentiation of the fibroblasts. Basic FGF (fibroblast growth factor) can inhibit the transformation. Balance between the cytokines TGF-β, IL-4 and FGF may be able to modulate fibroblast behavior.

Not only chemical factors affect cell phenotype. Intermittent stretch in vitro can cause myofibroblast differentiation towards vascular smooth muscle cells [Efendy et al 2000]. Fibroblasts cultured in stabilized collagen gels generate stress by pulling on the extracellular matrix (cell traction). When the same cells are cultured in freely floating gels no stress is generated [Halliday & Tomasek 1995] and no stress fibers were developed in the relaxed gel. [Kessler et al 2001] recently concluded that mechanical stimuli trigger a phenotypical shift of fibroblasts towards a synthetic state and simultaneously to an inhibition of matrix degradation.
4 Microscopy & characterization/quantification of collagen

In this chapter methods will be discussed for the determination of collagen content and collagen orientation in a sample. For the determination of the total collagen content, the sample can be sacrificed. A continuous monitoring of the collagen fiber modeling and orientation is desired. So, no fixation is possible. Staining of the sample may influence cellular behavior and remodeling and is thus undesirable.

4.1 Determination of collagen content

If a protein contains a particular amino or imino acid that other proteins do not, this amino acid can be used as a marker for that protein. Through determination of such an amino acid in a protein mixture the content of the desired protein can be measured. For collagen this marker amino acid is hydroxyproline. The only other protein that contains this marker is elastin. Collagen contains about 14% of hydroxyproline, whereas this is 1.6% in elastin. If sufficient collagen is present in a hydrolysate of a protein mixture, hydroxyproline determination can give a quantitative measurement of the collagen content. A protocol for hydroxyproline determination is described by the method of Neuman and Logan modified by Leach to improve reproducibility [Eastoe & Courts 1963]. Nowadays, a common method to measure the collagen content of a specimen is to use radioactive proline to isotopically label the different collagen types. A protocol for this procedure is presented in [Haralson & Hassell 1995]. The protocol can be extended for identification of the sample content of collagens I, III and V. The respective amounts of the different collagen types can be identified and quantified by ion-exchange chromatography of pepsin-derived collagen chains. Another method is based on identification of specific CNBr and V8 protease-derived peptides. If no radioactive proline can be used the three collagen types can be directly separated by HPLC. Another way to determine collagen content without the help of labeled proline is the Sirius Red assay. The dye Sirius Red F3BA precipitates collagen in acetic acid solution. The absorbance decrease of a Sirius Red/acetic acid solution is used to determine the collagen content by spectrophotometrical evaluation. The assay can be applied in a protein concentration range from 1 to 50 micrograms. The method is specific, sensitive, simple and rapid [Marotta & Martino 1985]. There are ways to determine collagen content without sacrificing the sample. These methods are based on analysis of microscope images. This may be less accurate and more time consuming than the techniques presented above. However, this analysis could be applied for qualitative determination of collagen content during the experiments.

4.2 Determination of fiber orientation

Microscope stems from the Ancient Greek μικρος (mikros) meaning small and σκοπειν (skopein), which means to look. Nowadays, the term microscope refers to a compound (more than one lens) microscope for visible light as the image-forming
agent [James & Tanke 1991]. The image in a compound microscope is magnified by the objective and a real inverted intermediary image is created between the objective and the eyepiece (figure 4.2.1).

![Figure 4.2.1: Image formation in a compound microscope. [James & Tanke 1991]](image)

In literature various methods to visualize collagen fiber orientation are presented. Most microscopic methods, like TEM (figure 4.2.2), SEM (figure 4.2.3) or AFM (figure 4.2.4), require fixation or staining of the sample.

![Figure 4.2.2: Transmission electron micrograph of mouse corneal stroma. Collagen fibrils are seen in longitudinal section (left). Fine filaments, probably Type VI collagen, showing a 100 nm periodicity (arrows), are seen to interweave along and between the fibrils. Scale bar=200 nm. Fibrils in cross-section are shown on the right. Scale bar=100 nm. [Meek & Fullwood 2000]](image)

![Figure 4.2.3.a: Scanning electron micrograph showing the arrangement of collagen lamellae in the mid-stroma. Scale bar=10 µm. [Meek & Fullwood 2000]](image)  ![Figure 4.2.3.b Scanning electron micrograph of collagen fibers. The natural waviness of collagen is evident. The highly organized nature of collagen is responsible for its birefringence. [www2]](image)
If imaging of living dynamic systems is needed, other techniques are necessary. The easiest way to visualize collagen would be by light microscopy. Although no resolution of individual collagen fibers is possible with this technique, collagen can be detected with the dye Sirius Red F3BA.

The first report on time-lapse confocal reflection images of collagen fiber assembly was by Brightman et al in 2000. When reflection is combined with confocal microscopy three-dimensional reconstruction is possible. The images were taken and sequenced into a movie to generate a four-dimensional structural analysis [www4]. Unlike a well-established spectrophotometric method, time lapse confocal reflection imaging allowed qualitative and quantitative evaluation of structural parameters and 3D-organization (fiber diameters and density). This method can be applied without staining or fixation of the specimen. Confocal reflection microscopy has also been used by [Voytik-Harbin et al 2001] to gain four-dimensional representations of collagen fibrillogenesis (note that Voytik-Harbin was a co-author in the Brightman article).

Small angle light scattering (SALS) is another method that has been applied for visualizing fiber orientation. SALS can be used for the analysis of quantitative differences in collagen fiber architecture [Bowes et al 1999]. In SALS an unpolarized wave laser beam is passed through the specimen and the light is scattered by the planar fiber structure (figure 4.2.5). The main fiber direction and an orientation index (OI), defined as the angle that contains 50% of the total number of fibers, can be obtained from the intensity. Oriented networks will have low OI-values, while more randomly oriented fiber networks will have larger OI-values [Sacks & Gloeckner 1999].
Another imaging option is polarized light microscopy. Polarized light consists of a bundle of light rays having a common propagation direction and common vibration directions. Polarized light can either vibrate in one plane, have a rotary vibration, or is vibrating elliptically. Naturally polarized light, like moonlight and skylight, is rather imperfectly polarized. Birefringence is the property to appear bright white in a dark field when examined with polarized light. It is based on the difference in refractive indices for a substance. When polarized light enters a birefringent material, it is split up into two rays that vibrate along the two principal directions of the anisotropic crystal lattice. One of these rays is called the fast ray, and the other the slow ray. By the time the rays emerge from the specimen, one of them has been retarded more than the other. This angle of retardation can be used to deduce the microstructure of the material. Such retardation measurements are typically applied to crystallography. Many biological materials that have a well-aligned molecular structure are also birefringent. Collagen fibers are an example of birefringent materials while elastin and ground substance are not.

Polarization microscopic studies are usually carried out on unstained tissues [Puchtler et al 1973]. As collagen has a natural birefringence, no extra staining is necessary. Extra staining, however, is possible with picrosirius. This is a birefringent enhancement stain specific for collagen. Optical retardations of the collagen bundles increase by a factor 5-6 after staining with picrosirius [Vidal et al 1982]. However, information on collagen orientation is limited in these images, as demonstrated in figures 4.2.6 and 4.2.7 on the next page.
Figure 4.2.6: Light microscopy of the cornea. (a) Cross-section of bovine sclera using conventional polarised light microscopy (no wave plate). The section thickness was 14 µm. (Magnification 100×) (b) Colour contrast polarised light microscopy of the same section as in (a) with a full wave plate at a small angle (about 7.5°) to the polariser. Interference colours are related to different optical path differences and hence to different collagen orientations. The blue regions show collagen at right angles to the collagen in the yellow regions. [Meek & Fullwood 2001]

Figure 4.2.7: Circularly polarized light image of an aortic valve cusp. Bright regions are collagen. [www2]

Collagen can also be excited by laser light (between 400 and 550 nm) to send its own frequency back. This phenomenon is called autofluorescence. In fluorescence radiation is absorbed to produce radiation of longer wavelengths, i.e. lower energy. Collagen autofluorescence is very weak and has a short lifetime. Therefore, (immuno)fluorescent labeling and other techniques are usually preferred.

4.2.1 Confocal microscopy

In confocal illumination the microscopic field is scanned by a small focused light spot. Computer-controlled mirrors guide this spot across the specimen. A correctly positioned diaphragm ensures an image only of the part of the specimen that is in focus. Light beams from above or below the focal plane can not contribute to image formation, as depicted in figure 4.2.1.1. Confocal imaging can be combined with fluorescence microscopy and reflection microscopy (figure 4.2.1.2).
An advantage of confocal imaging is that out-of-focus light rays are restricted from image formation by the diaphragm. This results in improved image contrast in lateral and axial direction. This property provides the possibility to create three-dimensional images of the specimen. A second advantage (over light microscopy) is a 1.4 times increased resolving power. With respect to fluorescence microscopy not only the improved contrast is good, but the autofluorescence of objects that are out-of-focus is reduced. On the other hand a high intensity excitation light is required to get a decent image. This is due to the ‘loss’ of light intensity in areas surrounding the desired imaging spot. If the exciting beam is too intense, photobleaching occurs; i.e. a focused laser beam depletes fluorescence in a local region in a living cell.

Meek and Fullwood [2001] discussed various microscopic imaging methods for collagen visualization. Light microscopy, confocal microscopy, scanning electron microscopy, transmission electron microscopy and atomic force microscopy are compared. Most of these methods required staining and/or fixation of the sample. The reader is referred to an article by Böhnke and Masters [1999] for an extensive review of confocal microscopy.
5 Mechanical test system

In 2000, Thomas Brown published a review on techniques for mechanical stimulation of cells in vitro. Several loading systems were described. Longitudinal stretch systems were discussed as devices that conduct uniaxial distention (figure 5.1) to deformable substrates [Vandenburgh et al 1989, Neidlinger-Wilke et al 1994]. Out-of-plane circular substrate distension involved culture systems in which strains are imposed by motions of deformable circular substrates. Hasegawa is thought to be the first one that reported such a device in 1985. The load in this system was transduced as in figure 5.2.a.

The platen displacement is transferred to the substrate as deformation. The principle in figure 5.2.b was applied by Vandenburgh [1988]. A vertically pulsating prong was used to form a membrane tent. The vacuum alternative (figure 5.2.c) was introduced by Banes et al in 1985. This device was commercialized in 1987 under the name Flexercell®. Characterization of the actual stimulus was performed by Gilbert et al [1990, 1994], including experimental strain measurements on the substrate culture surface and a linearized quasi-static finite element analysis of substrate strain anisotropy and heterogeneity. Pedersen et al [1992, 1993] elaborated the computational and experimental verification of the substratum behavior. Their findings led to a reviewed design in 1995, the BioFlex-II®, which more closely approximated ideal membrane behavior.
Finally, positive pressure has been applied to induce cellular mechanical stimulation like depicted in figure 5.2.d. In Thibault’s group [Winston et al. 1989] the deformation behavior of 100 μm circular polyurethane membranes was thoroughly analyzed. They also used fluorescently labeled cell surface markers to show that cell strain and substrate strain were not equal. Cellular elongation was ~60% of the substrate strain in their set-up. This constant seemed to depend on in vitro cell age and morphology.

5.1 Mechanical set-up

A mechanical loading pattern resembling the natural loading of aortic valve leaflets is required. The total mechanical load acting on an aortic valve is composed of three basic loads. A high axial pressure is built up as the valve is closed during diastole (120 mmHg). A second load acting on the valve is the radial pulsation that causes the commissures to stretch apart. The third mechanical component consists of shear exerted by the blood flow. In this study no shear stresses will be taken into account, as it is believed that the other two loads imposed on a natural valve are of greater influence on the valve deformation and cellular orientations in the lamina fibrosa. The cellular orientations in turn influence collagen organization.

The first set-up (figure 5.1.1) will not be too complicated and only impose the load felt by the valve after closing. So, the load that forces the commissures apart is not yet taken into account. A circular membrane is chosen to model the valve. A star of three lines supports the membrane upon loading. On top of the membrane (a polyurethane named desmopan 588) fibroblasts are cultured under DMEM. Under the membrane air channels and wires will be connected to a pump. The pump generates a cyclic loading (1 Hz, ~10% strain) of the membrane and thus the cells.

![Figure 5.1.1: Set-up for mechanical loading of fibroblasts resembling the closing behavior of a natural valve. On the left a view from the side to illustrate the application of mechanical loading through cyclic air displacement. On the right a three-dimensional impression of the set-up is shown with the three-legged stars.](image-url)
5.2 Preliminary studies - Cell attachment

To evaluate the cellular attachment to desmopan membranes and the toxicity of the membrane a cell experiment has been performed.

5.2.1 Materials and methods

The membrane was made of desmopan. This polyurethane has been tested for its toxicity and its mechanical properties are known. To evaluate the cell attachment an experiment has been performed. A sheet of desmopan 588 was cut into circles to fit into a nine-wells plate and sterilized for 24 hours in 70% ethanol. Three membrane circles were conditioned in medium (DMEM + 10% FBS + 1% penstrep) for another 24 hours and the other three stayed in alcohol. Subsequently, the membranes were washed in sterile PBS and placed into the wells. 100,000 CHO-K1 fibroblasts were added per well. Three wells without membrane served as blanco. On days 2, 4 and 8 the cells and membranes were checked under the light microscope.

5.2.2 Results

On the day 2 cells were attached to all membranes as well as to the bottom of the wells in the control wells. The membrane is striped due to its processing. The cells did not seem to align after these ridges. To make sure this was not influencing cell orientation, membranes without stripes will be used in future experiments. In the wells with membranes that were not conditioned in medium more pollution was present. However, this did not affect cell proliferation or attachment. On the fourth day the cells in the blanco wells were confluent (figure 5.2.2.1). This was almost the case in the wells with membranes due to the area that was available to cells to grow on. In the wells with membranes cells attached to the well bottom (under the membrane) as well as to the membrane. So, the area to grow on was twice as high. Still no differences in cell morphology were observed between the cells cultured with or without membranes. On the eighth day the cells were over-confluent in all wells. Unfortunately, no viability tests like trypan blue testing were performed. Note also in the image that focusing the cells on the membrane was not possible in the complete field of view as the membrane was floating.

Figure 5.2.1: A light microscopic image of the blanco (left) and desmopan with fibroblasts (right) on day four of the experiment. Objective magnification 40x.
5.2.3 Conclusions

Desmopan 588 can serve as cell carrier in experiments. It does not seem to affect cell proliferation or viability. Cells attach well to this polyurethane. Conditioning of the membrane after sterilization with medium does not affect cellular attachment but seems to help to get rid of some pollution that is in the ethanol (70%). The initial cell seeding density of 100,000 cells per well was too high.

5.3 Preliminary study - Finite element simulation of test set-up

To determine the principle strain directions in the experimental set-up a finite element analysis has been performed. Material parameters and material laws were implemented into an existing SEPRAN® program by van Oijen [2001/2]. Material parameters were obtained from a report on desmopan’s mechanical properties by van der Laan [2001]. Incompressible Mooney Rivlin material behavior was assumed. The mesh is built from 96 Taylor-Hood elements into a third of a circle (figure 5.3.1). The program is used to calculate the principle strain fields in the mesh and thus the membrane. In the experimental set-up strains of about 10% are desired. This amount of strain equals the naturally occurring strain in the heart valve.

![Figure 5.3.1: Top view of the mesh for numerical computation of principle strain fields. The mesh has two element layers in its thickness.](image)

Although the relation between substrate (membrane) deformation and cellular deformation is not known and differs for cell types and substrate types, this amount of strain should induce cellular deformation without causing damage to the cells. By applying a pressure difference of 0.25 bar the desired amount of strain was created in the membrane. The magnitude and direction of the three principle strains in this static problem are depicted in figure 5.3.2. A mechanically rather simple set-up was evaluated by Gilbert et al [1994]. A circular membrane is subjected to a pressure difference. The cells tend to align circumferentially. Simulation of this test in the SEPRAN® program mentioned before, leads to the conclusion that the principle strain acts radially rather than circumferentially. This implies that either the hypothesis of collagen alignment in the principle strain direction is wrong; or that collagen and cells do not align parallel in this stage of the remodeling process (figure 5.3.3).
Figure 5.3.2: Simulation of the proposed set-up. At the right the orientations of the principle strains in each node are depicted and on the left the magnitude of the corresponding strains is shown. The top two graphs represent the first principle strain. The middle graphs depict the second principle strains and the two bottom graphs show the third principle strain.

Figure 5.3.3: Simulation results on Gilbert [1994] set-up. At the right the orientations of the principle strains in each node are depicted and on the left the magnitude of the corresponding strains is shown. The top two graphs represent the first principle strain. The middle graphs depict the second principle strains and the two bottom graphs show the third principle strain.
Collagen is mechanically the most important fiber in soft tissues. Collagen fibers are degraded and synthesized continuously. Externally applied mechanical loads can influence fiber deposition. Quantitatively, mechanical loading increases the amount of collagen deposited by cells. Among mechanical loading patterns, the highest collagen deposition occurs when dynamic strain is applied. From a qualitative point of view, the cellular and fiber orientations are influenced by cellular deformation. The principle of contact guidance implies that the cellular and fiber orientations are equal with time. Cells move along the fibers after adhering to them. So it seems that the fibers determine cellular orientation. But what determines fiber orientation? The striking thing in literature is that all of a sudden oriented fibers seem to appear. Cells move towards these fibers and attach to them. Then cells migrate along these oriented fibers and deposit oriented fibers. It is trivial, however, that fibers are produced by cells and do not appear. What can be found in literature, is that in development (of the intervertebral disc) sheets of oriented fibroblasts deposit these ‘suddenly appearing’ oriented fibers. Stress fibers direct the initial cell elongation and control matrix deposition until birth. Still, there is no complete explanation for how or why tissue orientation originates. The knowledge of how and why these oriented fibers appear is useful in tissue engineering. It can provide tools to manipulate cells to orient in a specific direction and to produce a well-defined matrix structure. By controlling the matrix design, the desired mechanical properties of tissue engineered grafts could be regulated as well.

The question arises if this collagen orientation can be predicted. If it is assumed that cells respond to mechanical triggers by an avoidance reaction, they align away from the stretch direction. Thus, fibers would be deposited in a direction not equal to the loading direction. In true biology this is not the case. If collagen would be oriented away from the principal stretch direction, biological tissues would not have the strongest fiber organization in that direction. Bones would grow the wrong way, etc. From the various studies in literature it is clear that not only one factor (e.g. mechanical stimulation) determines cellular and fiber orientations. Furthermore, multiple orientations can coexist. The magnitude of strain and the period of time that the cyclic deformation is applied seem to be two important factors. Above a certain threshold, the cells respond to the strain until an upper limit is exceeded. The factor time includes several complex, biological signaling pathways and changed mechanical situations due to cell and fiber reorientation. The activation of pathways has long-term and short-term effects on the cell behavior.

In order to study the orientational response, mechanical loads have to be applied to cells. For applying loads to cells various methods are presented in literature. Depending on the problem definition a certain experimental set-up is chosen. A pressure difference over a membrane is required to simulate the closed-state loaded aortic leaflet. The hypothesis that collagen is deposited in the direction of the principal strain will be tested by comparing the experimental cellular reorientation to the strain patterns of the numerical simulations. It has never been proved that collagen orientation and cellular orientation actually are aligned. As collagen is the main load-bearing fiber in soft tissues, one would expect that the collagen fiber orientation is in the principal strain direction experienced by the tissue. From several studies in literature it becomes clear that cells seem to respond in a different way in experiments
which seem to provide comparable culturing and loading conditions. An explanation for the variation in experimental results on cellular orientations can not be given straight away. The cell type is likely to influence cellular behavior. The hormone content in the medium that the cells are in can also determine orientational processes. The surface properties of the substratum influence the attachment of the cells. A strong adherence to the substratum is more likely to cause a gradual reorientational response than a minimal degree of attachment. In this study, these effects will not be explored. Only the fiber orientation response after mechanical triggering will be considered and compared to the principle directions of strain.

If the impacts of all important variables were known, a proper prediction of collagen orientation would be possible. An assumption for this study is that the mechanical stimulation of the cells causes collagen and cellular reorientations. It would be interesting to investigate the relationship between fiber and cell alignment over time (during the course of the experiment). Furthermore, imaging of the in vitro collagen deposition by cells will be a challenge. A finite element program needs to be developed to evaluate the strain patterns in the experimental cell set-up. Special attention will be paid to the principle directions of strain. By comparing the numerical results to the experimental results hopefully conclusions can be drawn on cellular responses to fields of cyclic deformation. The great advantage of such a program would be that predictions could be done for the collagen orientation in tissue engineered devices. Mechanical strength in the desired areas and orientations of a tissue engineered aortic valve could be computed. This saves a lot of time as numerical simulations replace the need for experimental tests. In this way, a tool can be created for designing tissue engineered aortic valves with optimally oriented collagen fibers.
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