3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Materials for cell culture and seeding

Material	Supplier
DMEM	Gibco Life Technologies, USA, No. 41966-029
PBS	Gibco Life Technologies, USA, No. 14190-169
Fetal bovine serum	Sigma-Aldrich, USA, No. F 2442
PSG solution	Gibco Life Technologies, USA, No. 10378-016
Trypsin-EDTA	Gibco Life Technologies, USA, No. 25200-056
L-ascrobic acid 2-phosphate	Sigma-Aldrich Chemie GmbH, Germany
Pasteur pipette	Merck Eurolab GmbH, Germany, No. 612 A 1702
10 ml polystyrene pipette	Wcp, Germany, No. 2421
75 cm² culture flask	Falcon, USA, No. 353111
10 cm² tissue culture dish	Sarstedt, Inc., USA, No. 83.1802.003
Polyethylene container	Merck Eurolab GmbH, Germany, No. 216A8155
1.5 ml Eppendorf tube	Eppendorf–Netheler–Hinz GmbH, Germany
50 ml polypropylene conical tube	Falcon, USA
Glass reservoir	Wheaton Inc,. USA, No. 355757
Silicon tubing	Cole Parmer, USA

3.1.2 Kits used in ECM quantitative analysis

Kit	Supplier
Collagen	Biocolor Ltd, NI, S1000
Elastin	Biocolor Ltd, NI, F2000

3.1.3 Antibody used in immunohistochemistry assay

Antibody	Supplier
CD31	DAKO corporation, USA, M823
CD34	DAKO corporation, USA, M824
Collagen I	Novocastra Lab.Ltd., UK, NCL-Coll-1p
Fibronectin	Novocastra Lab.Ltd., UK, NCL-Fib
a-Smooth muscle actin	DAKO corporation, USA, M851

3.1.4 Instrumentation

Instrumentation	Supplier
Incubator	Forma Scientific Inc., USA, Typ 3110
Olympus phase-contrast microscope	Olympus OPTLICAL CO., Japan, TYP CK 2
Respirator	Havard Apparatus Inc., USA, Model 613
Clean bench	Kendro-Heraeus, Germany, Typ HS12
Spectrophotometer UV mini 1240	Shimadzu Deutschland GmbH, Germany
Eppendorf centrifuge 5804 R	Eppendorf–Netheler–Hinz GmbH, Germany
Eppendorf centrifuge 5417 R	Eppendorf–Netheler–Hinz GmbH, Germany
Speed Vac	Savant Instuments Inc., USA
Philips XL-30 electron microscope	FEI CO., USA
HCP-2 critical point dryer	Hitachi Ltd., Japan
JEC-1100 ion sputter	Hitachi Ltd., Japan
Vibrofix VF1 shaker	Janke & Kunkel, Germany

3.1.5 Polymer scaffold

Polymer scaffolds used in this experiment were made of P4HB (Tepha Inc.,USA). P4HB is a rapidly absorbable biopolymer biologically derived from bacteria, that is strong and pliable. P4HB is a semicrystalline, thermoplastic elastomer with a melting point of about 60°C and a glass transition temperature of –50°C. Because of the thermoplastic properties of P4HB, it can be molded into almost any shape. In addition, the P4HB–based tissues showed supraphysiologic mechanical strength, an importantant finding for surgical applications. Complete biodegradation of P4HB occurs after 4 to 6 weeks. P4HB is a highly porous polymeric material (pore size 50-100 μm) (Figure 2) [64]. Because the surface of the polymer scaffold was flat (Figure 3), we rubbed the surface of the scaffold with a metal brush in order to enhance attachment of the seeded cells to the polymer. The polymer scaffold was cut in round form (diameter 50 mm, thickness 0.4-0.5 mm) and sterilized with ethylene oxide (at 37°C) because of the low melting point of P4HB.

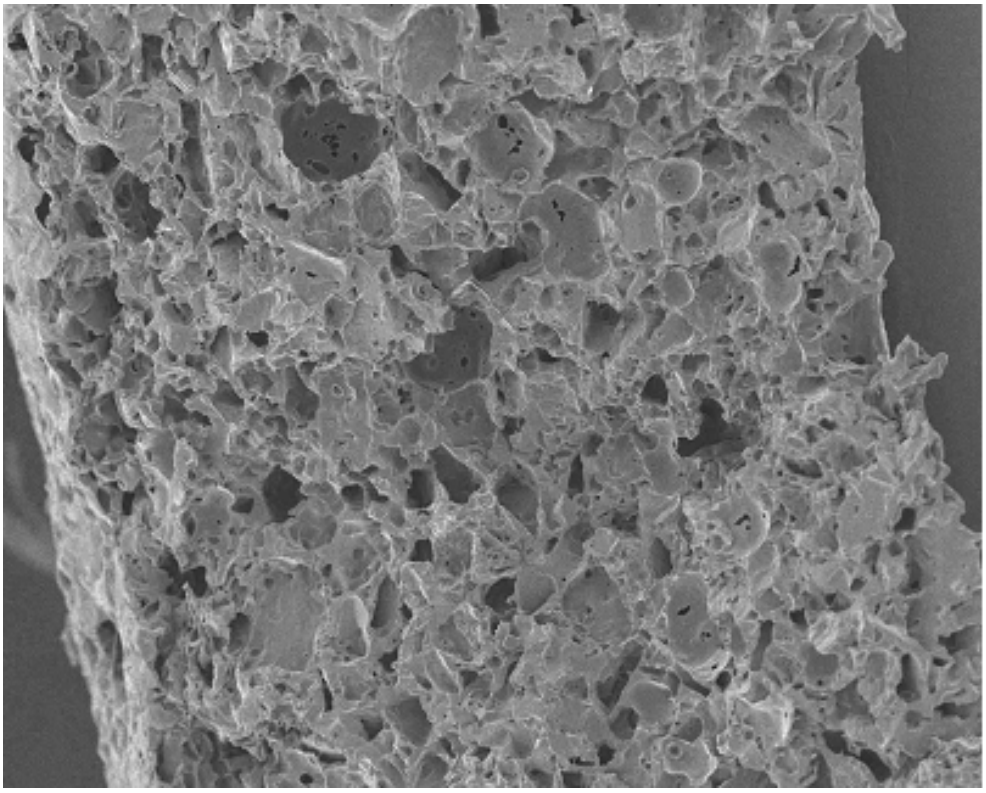

Figure 2 Ultrastructure of P4HB under electron microscopy. P4HB is a highly porous polymeric material (pore size 50-100 μm).

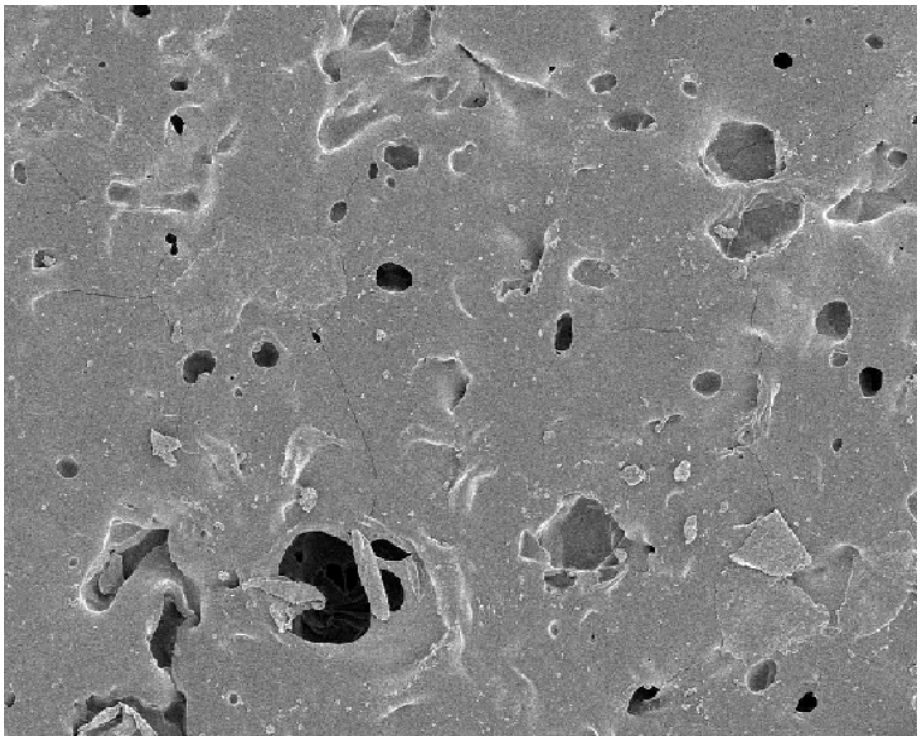

Figure 3 Surface of P4HB polymer under electron microscopy. The surface of the polymer scaffold is smooth and glazed and therefore has to be roughened before cell seeding.

3.1.6 Metal rings used in cell seeding

We designed two metal rings made of stainless steel to enhance the effect of cell seeding (Figure 4). The principle is illustrated in Figure 5.

Figure 4 Two metal rings used in cell seeding. The left ring with a 7mm wide gap was positioned under the patch with outer diameter of 44 mm and inner diameter of 40 mm. The second ring was placed upon the patch with outer diameter of 44 mm and inner diameter of 34 mm. The height of both rings was 5 mm.

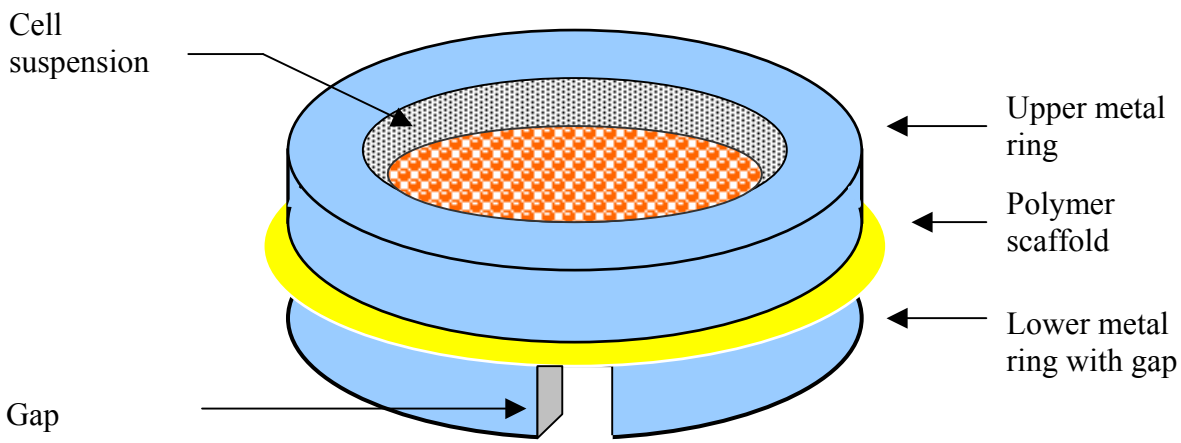

Figure 5 Schema illustrating the principle of metal rings used in cell seeding. The scaffold was positioned between two metal rings, which concentrated the cell suspension on the seeded area of patch. The lower metal ring had a gap, which was used for deairing of the space under the patch and allowed the cell medium subsequently to enter through it because two sides of the patch were seeded. The upper metal ring without gap fixed the polymer and circumscribed the cell suspension within the seeding area.

3.1.7 Bioreactor

3.1.7.1 Bioreactor design

The patch bioreactor system used in this experiment is self-developed and has been described in detail. It is made of acrylic glass (Berlin Heart AG, Berlin). The bioreactor, shown in Figure 6, consists of three different chambers: the air chamber (I) and two cell culture medium chambers (II and III). Chamber (I) is separated from chamber (II) by a silicon diaphragm (6) and connected to an air-driven respirator pump (7) with a single silicon tube. The patch construct (3) divides chamber (II) from chamber (III) and is surrounded by cell culture medium that is continuously recirculated through a closed loop, which is connected to a reservoir filled with cell culture medium. The patch constructs function as a diaphragm between chamber (II) and chamber (III), which can be tightly fixed by pressing an acrylic glass cylinder (2) on to the outer edge of the patch. The cell culture medium inlet (4) is connected to chamber (II) and is pointed directly at the patch. The medium outlet (5) is on the opposite side to the medium inlet and is connected to the cell culture medium reservoir by a silicone tube. The whole system can be sterilized with ethylene oxide and assembled with a standard screwdriver [76].

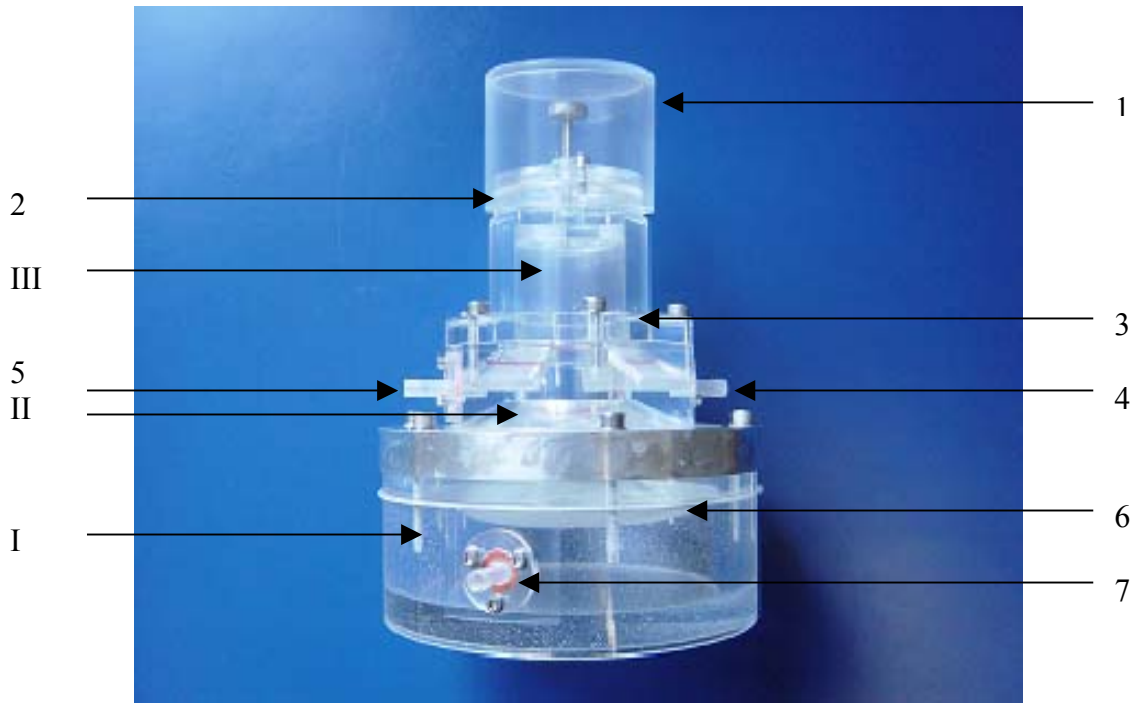

Figure 6 Pulsatile bioreactor for fabrication of tissue-engineered cardiovascular patches: I air chamber; II cell culture medium chamber; III cell culture medium chamber; 1 lid; 2 plexiglas cylinder; 3 patch construct; 4 cell culture medium inlet; 5 cell culture medium outlet; 6 silicon diaphragm; 7 air-driven respirator pump connector

3.1.7.2 Principles of bioreactor

The patch bioreactor is a closed loop perfused system [76]. The air chamber (I) is connected to an air-driven respirator pump while the cell culture medium chamber (II) is connected to a medium reservoir via a valved inlet and a valved outlet tube. In the first phase, by pumping air into air chamber (I), the silicon diaphragm is lifted up and presses the cell culture medium against the patch construct, which arches into chamber (III) as another diaphragm. At same time, the cell culture medium in chamber (II) is pumped into the reservoir. In the second phase, the air in chamber (I) is sucked up by the respirator and the silicon diaphragm arches into chamber (I). In parallel, the cell culture medium is aspirated from the reservoir and the patch construct arches into chamber (II). In this way, we establish the patch tissue conditioning, which combines continuous, pulsatile perfusion and mechanical stimulation by periodically stretching the tissue-engineered patch construct (Figure 7) .

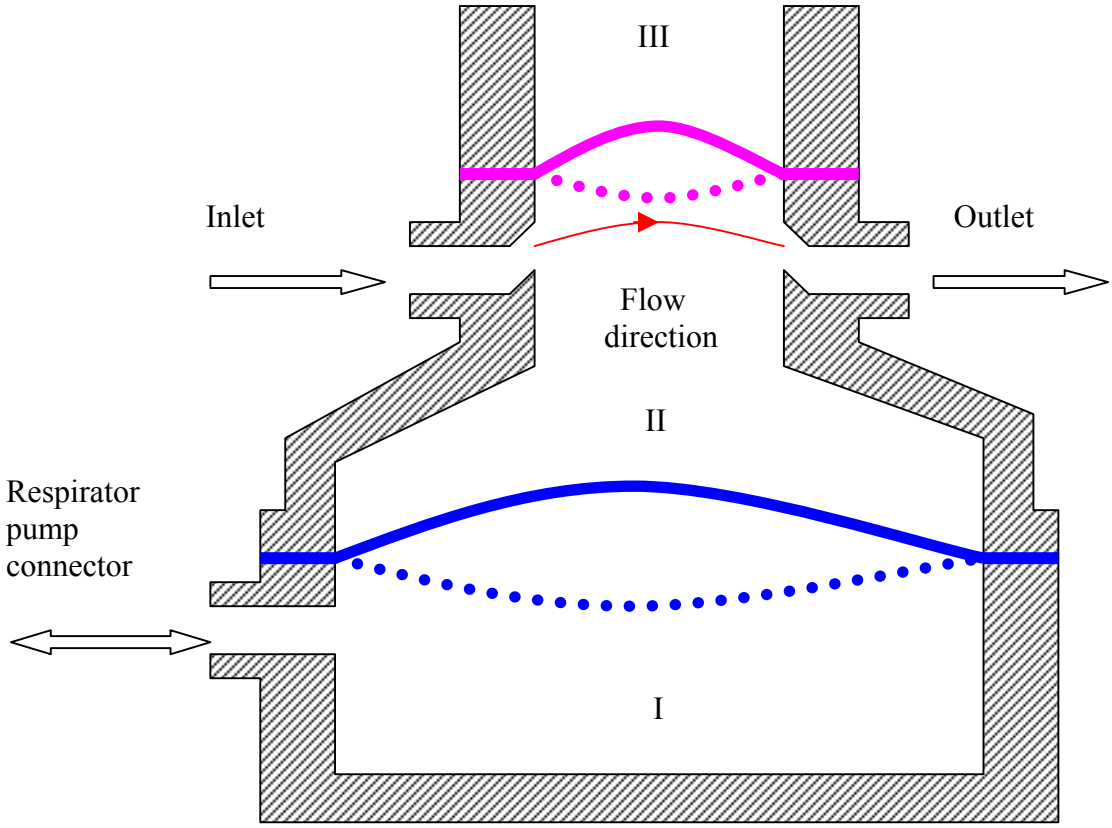

Figure 7 Periodical stretching and flow exposure of the patch

patch position in first phase of the system

patch position in second phase of the system

silicon diaphragm position in first phase of the system

silicon diaphragm position in second phase of the system

3.2 Methods

3.2.1 Cell culture

Cell harvesting was approved by the ethics committee of the Charité Medical School, Humboldt University, Berlin, Germany. Tissue of human ascending aorta was harvested from pediatric patients undergoing heart transplantation or corrective or reconstructive aortic surgery. Cell culture was performed on a clean bench. After harvesting, the arterial tissue was rinsed of blood with PBS and the tunica adventitia of the aorta was peeled off. Using a scalpel the tissue was then minced into 1 mm x 1 mm pieces, which were distributed over the bottom of 10 cm² Petri dishes for primary culture. The cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamin solution. The explants were placed in a humidified incubator at 37°C with 5% CO_2. After 7 -10 days the cells grew out from the explant (Figure 8). Then the cell culture medium was replaced. The spent medium was removed using a Pasteur pipette and 10 ml fresh medium was added using 10 ml sterile plastic pipettes and an automatic pipette aid. Thereafter the medium was changed every 4 days. The cultures were observed daily under an Olympus phase-contrast microscope.

After 4 weeks, when the cell population had grown into an 80-100% confluent monolayer (Figure 9), the primary culture was passaged. The spent culture medium was aspirated from petri dishes using Pasteur pipette connected to a vacuum line and the confluent cell monolayer was washed with 10 ml PBS. In every dish 400 µl 0.25% Trypsin/EDTA solution was added. Immediately the trypsinization was observed using a phase-contrast microscope. When the majority of cells were detached from the plastic (1-3 min), the dish was gently tapped against the palm of the hand a number of times to dislodge the remaining adherent cells. Following the trypsinization, 10 ml DMEM with 10% FBS was added to stop the reaction and to suspend the cells. Next the cell suspension was transferred into 75 cm² vented polystyrene cell culture flasks which were subsequently placed in the incubator. The cells were serially passaged and subcultured to obtain sufficient cell numbers for cell seeding of a P4HB patch. Sufficient cell numbers for cell seeding on bioabsorbable polymer scaffolds were obtained after 21-28 days (passage 3-4). The medium was changed every 4 days.

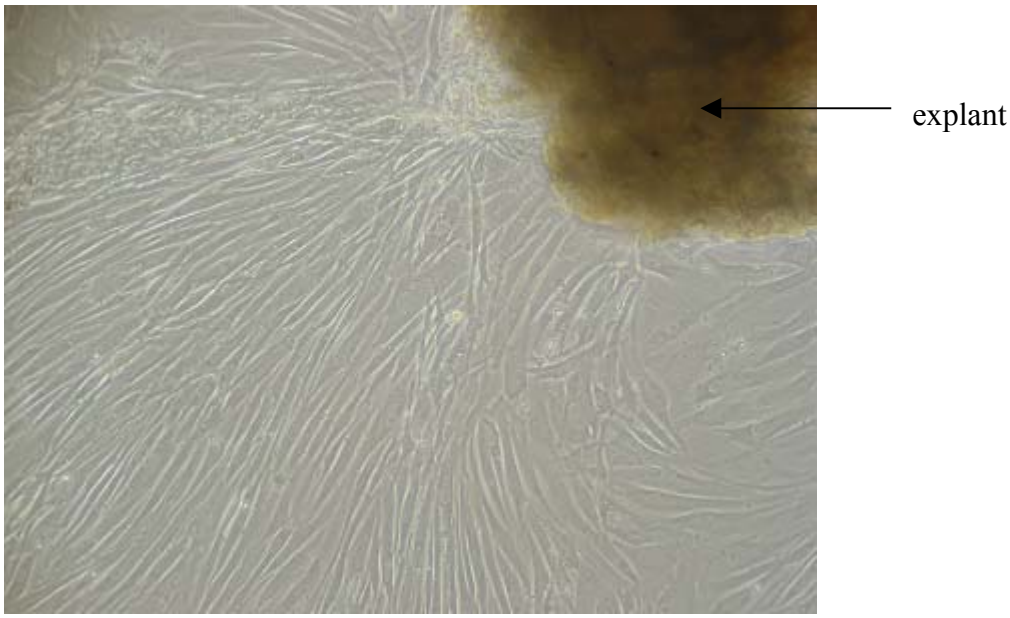

Figure 8 Primary cell culture. After 7–10 days the cells grew out from the explant.

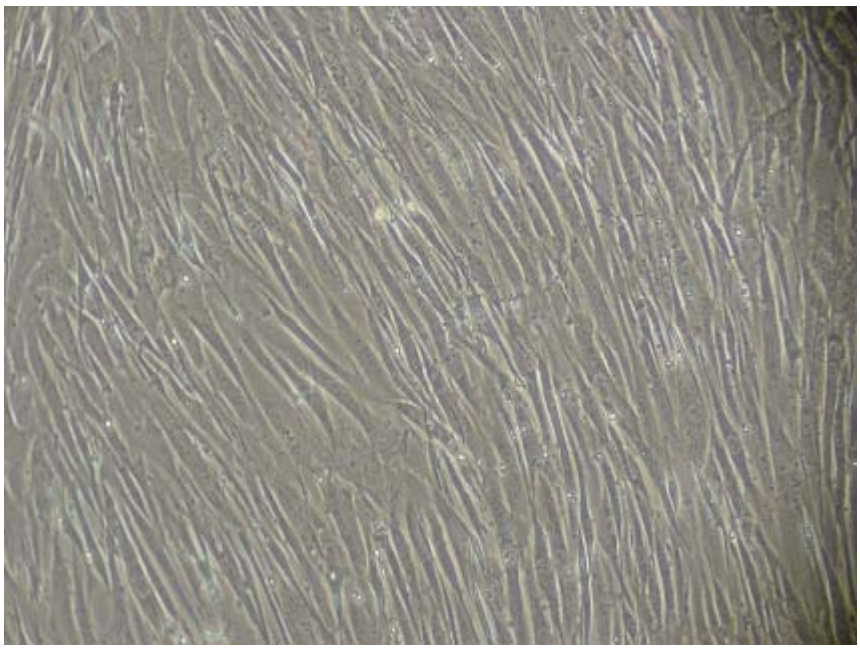

Figure 9 SMC confluent monolayer. After 4 weeks of cell culture, cell population grew into a 100% confluent monolayer. The SMCs are bipolar in shape and look like a fish shoal.

3.2.2 Cell seeding

All scaffolds were soaked in PBS solution at 37°C for 4 hours and subsequently in DMEM for 20 hours to prewet them. The cells were trypsinized from culture flasks. Following the trypsinization 10 ml DMEM with 10% FBS was added to flasks to suspend the cells and the cell number was counted with a hemocytometer. The cell suspension, which contained about 8-10 million cells, was transferred into 50 ml conical tubes and centrifuged at 8000 g for 8 minutes at 6°C using an Eppendorf centrifuge. The supernatant was removed and the cell pellet was resuspended with 1 ml culture medium. The cells were dripped with an Eppendorf pipette onto one side of the polymeric scaffold, which was positioned between two metal stainless rings (Figure 10). After the cell-polymer construct had been incubated for 1.5 h, 30 ml culture medium was added into the polyethylene container of the patch to cover the polymer completely. The polymers were seeded with 8 million SMCs each day on 3 consecutive days and were then incubated for 4 days. From the 8th to the 10th day the other sides of the scaffolds were seeded with cells in the same way. The cell-polymer constructs were further incubated in a bioreactor or in static nutrient media as controls.

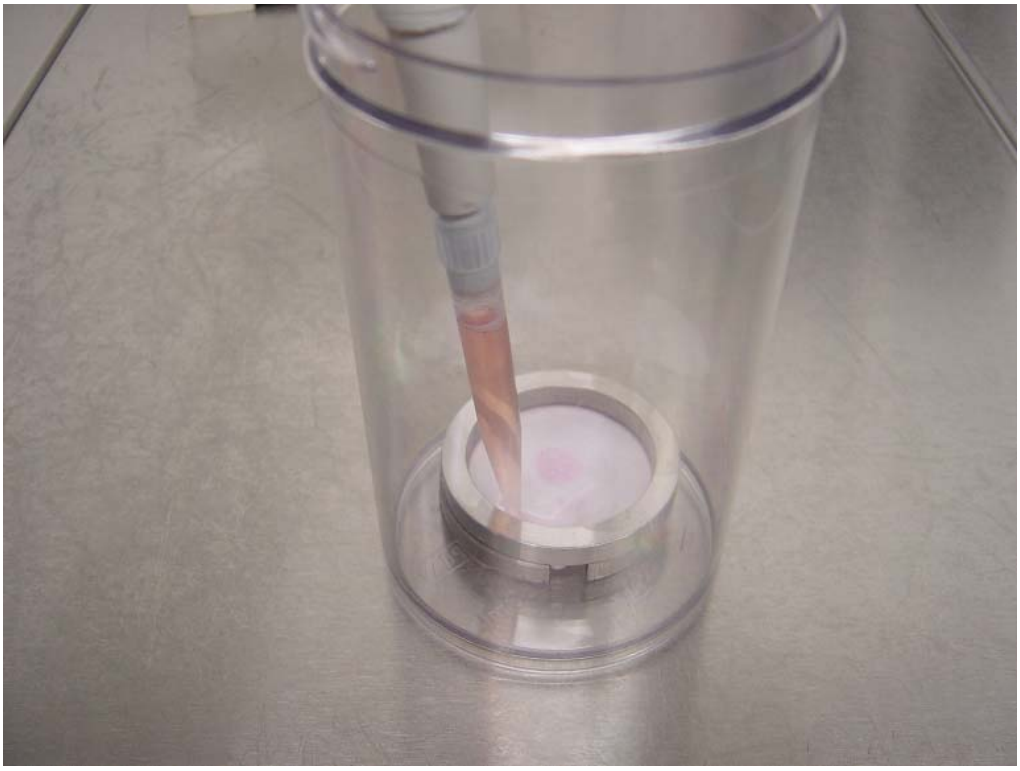

Figure 10 Cell seeding. The scaffold was positioned between two metal rings which were placed in a polyethylene container. Using an Eppendorf pipette the cell suspension was dripped evenly onto one side of the P4HB scaffold.

3.2.3 Cell seeded patch cultivation

3.2.3.1 Static cultivation

From the 15th day, four cell-polymer constructs were incubated in static nutrient medium in the humidified incubator at 37°C with 5% CO_2 for 14 days. The culture medium was DMEM supplemented with 10% FBS, 1% penicillin-streptomycin-glutamin solution and 26mg/100ml L-ascrbic acid 2-phosphate. The cell culture medium was changed every 4 days.

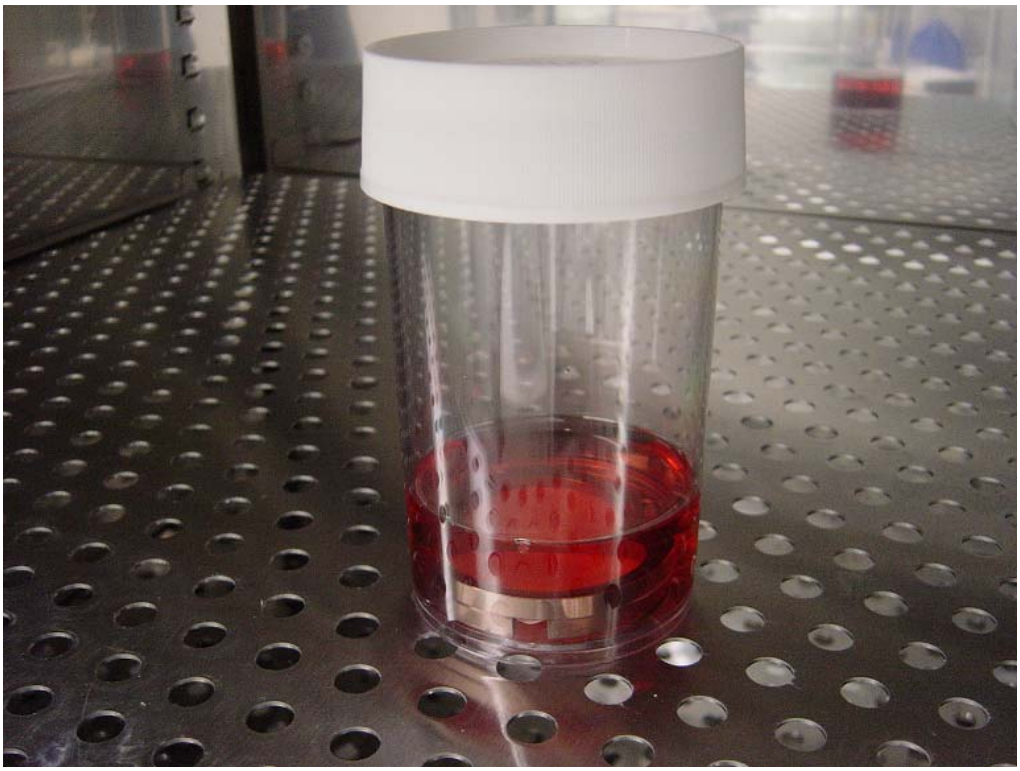

Figure 11 The polyethylene container containing the cell seeded patch construct was placed in a standard cell culture incubator for static tissue cultivation.

3.2.3.2 Dynamic cultivation

From the 15th day, four cell seeded polymer constructs were incubated in static nutrient medium in a humidified incubator at 37°C with 5% CO_2 for 7 days. On the 22th day, the polymer constructs were transferred into the bioreactor under sterile conditions and incubated for 7 days more. The culture medium was the same as in the static culture group and was changed every 4 days.

3.2.3.3 Experiment setting of bioreactor

The bioreactor with polymer construct was placed in a humidified incubator (Figure 12). The whole system was air-driven by a respirator, which was outside the incubator. By adjusting the stroke volume, the stroke rate and the inspiration/expiration ratio of the respirator, various pulsatile flows and biomechanical stresses were established. The flow rates ranged from 60 ml/min to 2.5 L/min and pressures from 5 mmHg to 250 mmHg. In this study, the frequency of the respirator was set to 5 cycle/minute. The inspiration/expiration rate was 3/7, and the stroke volume was 50 ccm/hub.

Figure 12 Experimental setting inside a humidified incubator: 1 Outlet tube; 2 Inlet tube; 3 Bioreactor; 4 Silicon tube connecting air chamber of the bioreactor and the respirator pump; 5 Cell culture medium reservoir

3.2.4 Analysis of the tissue-engineered patches

3.2.4.1 Histology

Sections of the tissue-engineered patches were fixed in 5% phosphate-buffered formalin and embedded in paraffin. Paraffin sections were cut at 5 μm thickness and stained with hematoxylin and eosin. Briefly, the slide with the section was placed in a ceramic staining rack and immersed in filtered Harris hematoxylin for 1 minute. Then the rack was removed to a beaker with tap water and tap water was exchanged until the water was clear. After sections were immersed in eosin stain for 1-2 minutes, the rack was removed to a beaker with tap water and the tap water exchanged until the water was clear. Subsequently the section was dehydrated in ascending alcohol solutions (50%, 70%, 80%, 95% x 2, 100% x 2) and cleared with xylene (3 - 4 x). The sections were mounted in glycerol gelatin under glass coverslips.

3.2.4.2 Immunohistochemistry

Individual cells contact and interact with other cells and with the extracellular matrix (ECM) which helps cells to bind together and regulates a number of cellular functions, such as adhesion, migration, proliferation and differentiation. ECM is formed by macromolecules locally secreted by resident cells. The two main classes of macromolecules are polysaccharide glycosaminoglycans and fibrous proteins of two functional types, structural (collagen, elastin) and adhesive (fibronectin, etc.) [77].

For immunostaining the alkaline phosphatase/anti-alkaline-phosphatase (APAAP) method was utilized. After deparaffinization and rehydration through xylene, graded alcohols, distilled water and PBS (pH 7.4), sections were incubated in primary monoclonal antibodies overnight in a humidified chamber at 4°C, washed three times with PBS and incubated with rabbit anti-mouse IgG (1:100 dilution) at room temperature for 1 hour. Sections were then washed three times in PBS and incubated for 1 hour in APAAP complex (1:100 dilution) at room temperature. After washing three times with PBS, immunoreactivity was tested by the addition of freshly prepared alkaline phosphatase substrate (0.2 mg/ml naphthol AS-MX phosphate containing 0.1 mg/ml fast red TR and levamisole in 0.1M TRIS-HCl (pH 8.2) for 10 minutes. The sections were counterstained in Harris's hematoxylin for 1 minute and mounted in glycerol gelatin under glass coverslips.

CD31,CD34

CD31 and CD34 are endothelial markers [78]. Platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) is a 130-kda integral membrane glycoprotein found at the intercellular junctions of endothelial cells. PECAM-1 functions in forming or stabilizing the vascular bed and as a cell-cell adhesion molecule [79]. CD34 is a heavily glycosylated type I transmembrane protein that is expressed on endothelial cells and has a potential adhesion function [80]. There is no expression of CD31 or CD34 on SMCs.

Collagen

Collagens and elastin are the main protein constituents of vessels. The biomechanical properties of vessels, particularly of the major arteries and veins, are largely dependent on the absolute and relative quantities of these two constituents [81]. Among the 19 different collagen types, collagen I and III are the major fibrillar collagens detectable in vessels, representing 60% and 30% of vascular collagens respectively [82-84]. Fibroblasts are the principal collagen-producing cells in the heart, producing the major fibrillar collagen types I and III. In vessels, the fibroblasts in the adventitia and medial smooth muscle cells produce these collagens [85]. They form fibrils and provide most of the connective material for the adhesion of cells and other structures [86] and a network of physical support that maintains cardiovascular structure and determines the cardiovascular stiffness. In addition to its structural role, collagen plays a critical role in the regulation of cell phenotype [85]. Collagen IV and laminin are the major constituents of basement membrane [87].

Fibronectin

Fibronectin is a high molecular weight glycoprotein that is present in normal arteries. It is produced by endothelial cells and SMCs and therefore is found both in the basement membrane and the interstitial matrix surrounding SMCs [88]. Fibronectin has a remarkably wide variety of functional activities besides binding to cell surfaces through integrins. It binds to a number of biologically important molecules that include heparin, collagen/gelatin, and fibrin. Fibronectin mediates a wide variety of cellular interactions with ECM and plays important roles in cell adhesion, migration, growth and differentiation [89].

alpha-Smooth muscle actin

alpha-Smooth muscle actin is one of the smooth muscle cytoskeletal markers produced by myofibroblastic cells [90]. The expression of alpha-Smooth muscle actin in fibroblastic cells is generally related to the development of structural features typical of myofibroblasts [91].

3.2.4.3 Ultrastructure

Additional samples of each patch were fixed in 2.5% glutaraldehyde and studied by scanning electron microscopy. Using PBS the probes were rinsed for 5 minutes three times. After that, the probes were dehydrated using a series of increasing concentrations of ethanol (from 50% to 90%) for 5 minutes respectively, followed by 100% ethanol for 5 minutes three times and 100% isoamyl acetate for 5 minutes three times. Following dehydration, pieces of the probes were critical point dried in CO_2 using a Hitachi HCP-2 critical point dryer. The dried samples were sputter-coated with a thin gold layer in a Jeol JEC-1100 ion sputter. The samples were examined with scanning electron microscopes (Philips XL-30 ESEM).

3.2.4.4 Quantitative tissue analysis

Collagen

Collagen was measured using biocolor collagen assay kits. Collagen standard aliquots (6.25, 12.5, 25, 50 µl volumes) and two reagent blanks (100 µl) were added to duplicated labelled 1.5 ml capacity conical microcentrifuge tubes. Using extraction buffer, the volume in all tubes was adjusted to 100 µl. Subsequently 1000 µl sircol dye reagent was added to each tube. Using a vortex mixer, the contents of each tube was mixed for 30 minutes to form collagen-dye complex. After 5 minutes, centrifuging, collagen-dye complex was packed at the bottom of the tubes. The unbound dye solution was removed and 1000 µl of alkali reagent were added to the collagen-dye complex to release the bound dye into solution. When the bound dye had been dissolved, the samples were ready for measurement. After setting the wavelength of spectrophotometer to 540 nm, the absorbance of reagent blanks and collagen standards were measured. Using graph paper, the standard curve was established.

A specimen was cut from the TE patch (5x5 mm) using a scalpel and lyophilized using Speed Vac; the dry probes were transfered to Eppendorf tubes. To each tube 100 µl 0.5 M acetic acid was added. After precipitation and formation of collagen-dye complex 1.0 ml 0.5 M NaOH was added to each tube to release the bound dye into solution. When the bound dye was dissolved, the samples were ready for measurement.

Elastin

Elastin is the most abundant protein of the large arteries, which are subjected to great pulsatile pressure generated by cardiac contraction. It also can be detectable in resistance arteries and veins. The function of elastin is elasticity that is essential in arteries [92, 93].

Elastin was quantified with biocolor elastin assay kits. Elastin standard aliquots (6.25, 12.5, 25, 50 µl volumes) and two reagent blanks (100 µl of distiled water) were added to duplicated labelled 1.5 ml capacity conical microcentrifuge tubes. Using distilled water, the volume in all tubes was adjusted to 100 µl. To each tube 1.0 ml elastin precipitating reagent was added. All tubes were placed in ice-water bath for 3 hours, while still cold, and centrifuged at 12000 g for 10 mins to pack the precipitated elastin. The supernatant was carefully decanted and discarded. Then 1.0 ml elastin-dye reagent and 100 µl 90% saturated ammonium sulphate was added to each tube to form elastin-dye complex. After 10 minutes, centrigfuging, elastin-dye complex was precipitated at the bottom of the tubes. The unbound dye solution was removed and 1.0 ml of elastin-destain reagent was added to the elastin-dye complex to release the bound dye into solution. When the bound dye had been dissolved, the samples were ready for measurment. After setting the wavelength of the spectrophotometer to 513 nm, the absorbance of reagent blanks and collagen standards were measured. Using the standards, a standard curve was prepared.

A specimen was cut from the TE patch (5x5 mm) using a scalpel and lyophilized. The dry probes were transfered to Eppendorf tubes. To each tube 100µl distilled water and 1.0 ml elastin precipitating reagent was added. After the precipitation and formation of elastin-dye complex 1.0 ml of elastin-destain reagent was added to each tube to release the bound dye into solution. The tubes were placed in a vortex and shaken for 60 minutes. When the bound dye was dissolved, usually within 20 minutes, the samples were ready for measurement.

3.2.5 Statistics

Data results were expressed as mean ± standard deviation. Comparisons between the conditioned TE patch group and the static control group were performed with paired t-test (Student's *t* test). Statistical significance was set at $p < 0.05$. Data and graphs were produced with MS Excel 2000 (Microsoft Corporation, USA).