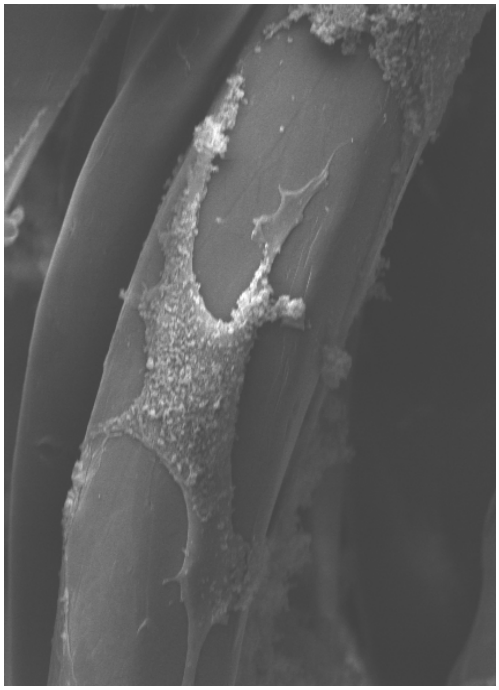
PROLIFERATION STUDIES AND EXTRACELLULAR MATRIX DEPOSITION IN DYNAMIC, 3-DIMENSIONAL FIBROBLAST CULTURE



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Proliferation studies and extracellular matrix deposition in dynamic, 3-dimensional fibroblast culture

Prolifieringstudier och deponering av extracellulärmatris i dynamisk, 3-dimensionell fibroblastkultur

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Abstract

Tissue engineering is a growing multidisciplinary field that works towards restoring, maintaining, and/or enhancing tissue and organ functions. One of the challenges is developing biodegradable and biocompatible scaffolds (matrices) that promote cell maturation and organization *in vivo*. There is a number of studies that demonstrate an increased matrix synthesis *in vitro* from cell cultures grown under mechanical stimulation.

This project investigated the effect of dynamic stimulation on murinae fibroblasts, cultured on a non-biodegradable, 3-dimensional scaffold, polyethyleneterephtalate (PET). Cell proliferation was evaluated with an MTT assay (Thiazolyl Blue Tetrasolium Bromide), and cell-extracellular matrix (ECM) distribution was studied with scanning electron microscopy (SEM), Scanning Laser Confocal microscopy, and histology.

A bioreactor and silicon tube with PET knitting implemented a dynamic cell culture. A physiological pressure on the cells was simulated by expansion and relaxation of the silicon tube with the help of a medium and a peristaltic pump.

Cell cultures in dynamic conditions showed a considerably higher proliferation and growth rate than cells grown in static conditions. Dynamically cultured fibroblasts increased their proliferation nearly 10 times within 1 week compared with static cultures. The static cultures reached their highest cell count at three weeks, which was half the dynamic proliferation's highest value.

The results showed that cells were tightly bound to the PET scaffold, and the ECM proteins had initiated a matrix bridging over the fibre distances in the PET scaffold. Immunostaining confirmed the production of ECM by verifying the presence of collagen type I.

Keywords: tissue engineering, fibroblasts, bioreactor, PET scaffold, dynamic stimulation, proliferation, extracellular matrix

Sammanfattning

Vävnadsteknik är ett tvärvetenskapligt område som strävar efter att återskapa, upprätthålla och/eller förbättra funktionen hos vävnader och organ. Bland utmaningarna finns framställning av matriser (stödstrukturer) som är vävnadsvänliga och resorberbara, samtidigt som de gynnar celltillväxt *in vivo*. Flera *in vitro*-studier visar på en ökning av matrissyntesen hos cellkulturer som har vuxit under mekanisk stimulering.

I detta projekt analyserades effekten av dynamisk stimulering på murina fibroblaster, odlade på ett icke resorberbart, 3-dimensionell matris, polyethyleneterephtalate (PET). Cellprolifieringen utvärderades med MTT-analys (Thiazolyl Blue Tetrasolium Bromide), och distributionen av cell-extracellulärmatris studerades med svepelektronmikroskopi (SEM), konfokalmikroskopi och histologi.

Den dynamiska cellkulturen utgjordes av en bioreaktor med silikonrör och en PET-matris. En växelvis utvidgning av silikonröret åstadkoms med hjälp av ett medium och en peristaltisk pump vilket simulerade ett fysiologiskt tryck på cellerna.

Cellkulturerna under dynamiska förhållanden visade betydligt högre prolifiering och tillväxthastighet än celler i statisk kultur. Prolifieringen hos dynamiskt odlade celler var efter en vecka 10 gånger större än hos statiskt odlade celler. Den statiska kulturen uppnådde högsta cellantalet efter tre veckor, vilket motsvarade halva värdet av den dynamiskt odlade kulturens högsta prolifiering.
Analyserna visade hur tätt bundna cellerna var till PET-stickningen, och hur ECM-proteiner överbryggde avståndet mellan PET-fibrerna. Immunoinfärgning styrkte ECM-produktion genom verifiering av kollagen typ I.

Nyckelord: vävnadsteknik, fibroblaster, bioreaktor, PET-matris, dynamisk stimulering, prolifiering, extracellulärmatris

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Introduction

1.1. Tissue engineering

The progress in molecular biology has created new opportunities to tailor *in vitro*-produced biomaterials. The merging of biology with materials science permits the design of biomaterials that contain specific biological activities, which can then be used in new medical products (Peppas & Langer, 1994; Walboomers & Jansen, 2001; Chen *et al.*, 2004; Vunjak-Novakovic *et al.*, 2004)

The purpose of tissue engineering is to regenerate or grow functional tissues in the laboratory, or to enhance tissue repair in a patient. On this basis, tissue engineering can be defined as a speciality where principles of engineering and life sciences are applied for the regeneration of biological substitutes meant to create or reinstate loss of organ function (Vacanti & Mikos, 1995). In addition to having a therapeutic application, tissue engineering can have diagnostic applications, where the tissue is made *in vitro* and used for testing toxicity, pathogenity, and drug metabolism and uptake (Peppas & Langer, 1994; Castner & Ratner, 2001).

Living tissues have a wide range of functions, and the intended biomaterial properties depend on the type of tissue it meant to replace. The biomaterial can be processed from natural or synthetic materials, or a combination of these. These biomaterials are generally known as matrices or scaffolds. It is essential that the scaffolds for tissue reconstruction and replacement match the topical tissue according to its structural, functional and mechanical properties (Badylak, 2002; Cukierman *et al.*, 2002; Vunjak-Novakovic *et al.*, 2004). The challenge is to develop a scaffold that mimics the natural architecture, and also to have a good interaction between the living cells and the scaffold. The scaffold surface should encourage cells to attach, proliferate and differentiate as they normally do *in vivo* (Castner *et al.*, 2001; Grinnell, 2003).

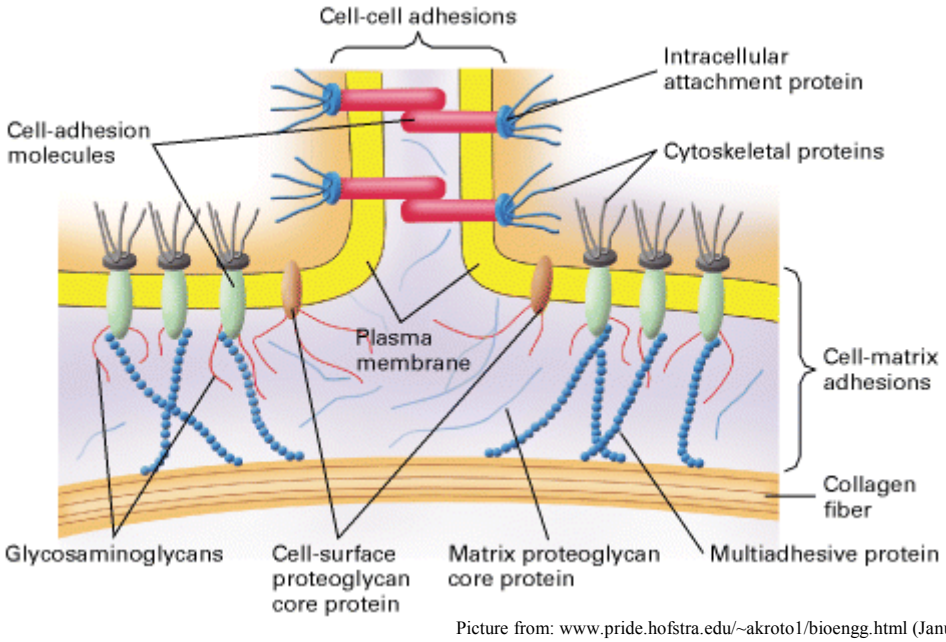
1.2. Extracellular matrix

Biopolymers or natural polymers are macromolecules created by fusion of many small, identical or similar molecules whose units form a repetitive sequence. Carbohydrates, amino acids, nucleic acids and proteins are all examples of biopolymers.

An extracellular matrix (ECM) consists of biopolymers which form a compex, 3-dimensional network within the extracellular space. Variations between these macromolecules give rise to matrices of different types (Grinnell, 2003; Stenmen & Vaheri, 1978). The two main classes of extracellular macromolecules are glycosaminoglycans and fibrous proteins. The arrangement of biopolymers within the extracellular space is shown in figure 1. The glycosaminoglycans (GAGs) are unbranched polysaccharide chains, which are linked to protein to form proteoglycan molecules. These links form a viscous gel substance in the extracellular space (Nowack *et al.*, 1976; Bosman & Stamenkovic, 2003). The proteoglycans resist pressures on the matrix while permitting diffusion of nourishment metabolites and hormones between blood and tissue cells. In this polysaccharide gel substance fibrous proteins are

embedded, such as fibronectin, laminin, elastine, and collagen (Cukierman *et al.*, 2002). These ECM components provide sites for cell adhesion, migration, and proliferation (Poole *et al.*, 2005). The ECM also acts as a reservoir for growth factors and cytokines (Bosman & Stamenkovic 2003).

With regard to ECM production, a number of studies have shown increased matrix synthesis *in vitro* from stretched cells. Studies have demonstrated that mechanical forces from outside the cell through cell-matrix and cell-cell contacts play an important role for cell growth, maturation, and organization (Walboomers *et al.*, 1998; Kessler *et al.*, 2001; Chen *et al.*, 2004). Mechanical strain induces various biological responses, such as the release of soluble factors which act as autocrine stimuli for ECM production (O'Callaghan & Williams, 2000; Grinnell & Ho, 2002). Further research into the mechanical stimulation of cells describes effects in DNA and mRNA expression (Kessler *et al.*, 2001) and protein synthesis such as α-smooth muscle actin (Wang *et al.*, 2004; Grinnell, 2000).



Picture from: www.pride.hofstra.edu/~akroto1/bioengg.html (January 2006)

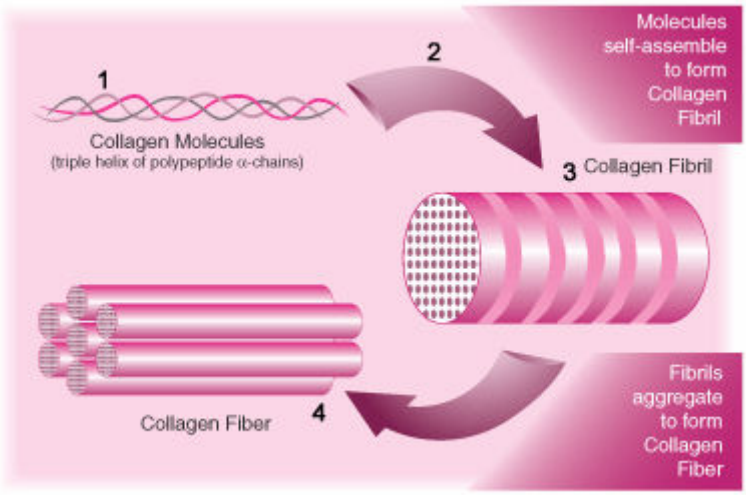
Figure 1. The matrix plays a fundamental role through its supporting, structural, and mechanical functions. The picture shows how cell receptors interact with the extracellular matrix. These cell-cell and cell-matrix interactions are important for cell adhesion, proliferation, and cell migration.

1.3. Fibroblasts and collagen

Fibroblasts are spread throughout the connective tissue and their content of endoplasmatic reticulum (ER) makes them specialized in secreting a collagenous extracellular matrix. When a tissue injury occurs, nearby fibroblast cells start to proliferate and migrate into the wound and start to produce large amounts of non-rigid matrix. This mobilization makes it possible to isolate the injury and repair the damage (Badylak, 2004).

In vertebrates collagen represents approximately 25% of the total cellular protein in the body. At present, more than twenty genetically distinct types of collagen are recognized (Bosman & Stamenkovic, 2003). Individual collagen polypeptide chains are synthesized

on membrane-bound ribosomes and are introduced into the lumen of the ER. After being secreted into the extracellular space, the collagen molecules assemble into higher order polymers called collagen fibrils. The assembly of collagen is shown in figure 2. Collagen type I is the most common form of fibrillar protein, which is widely distributed throughout the body.



Picture from: Orange Frazer Press (Reinhardt & Carey, 2000)

Figure 2. Collagen is formed from smaller precursors, pro-collagen. The pro-collagen is synthesized in the endoplasmatic reticulum (1) before secretion into the plasma membrane (2). In the plasma membrane, collagen fibrils form when pro-collagens self-assemble (3), and later on aggregate into collagen fibers (4).

Ascorbic acid is important for the normal production of pro-collagen (Nowack *et al.*, 1976). If there is insufficient ascorbic acid, the pro-collagen cannot maintain a stable helix configuration and no cross-linking occurs. The consequence is that collagen is poorly secreted from the cells, and the molecules that are secreted are more sensitive for enzymatic degradation and are deficient in strength. Insufficiency of ascorbic acid can give serious consequences in humans with gradual loss of normal collagen in the connective tissue. If this is allowed to progress, joints, muscles, and subcutaneous tissues may become the sites of hemorrhage.

1.4. Extracellular matrices as therapeutic scaffolds

Today's need of implants and organs is much larger than what can be provided from human donations. Scaffolds derived from xenogenic ECM have proved to be effective in the repair and reconstruction of several body tissues including the lower urinary tract, esophagus and, blood vessels (Reinhardt & Carey, 2000; Badylak, 2002; Badylak, 2004; Chen *et al.*, 2004). Still there are problems using xenogenic ECM for tissue reconstruction. There are ethical questions of using animal organs for human transplantation, and the risk of transmitting pathological diseases is controversial (Badylak, 2002). One of the major barriers to human donation is the presence of natural antibodies (Badylak, 2004). Concerning human donors, the compatibility between the receiver and donor must be taken into consideration. A significant problem with transplantation is the need of lifelong immunosuppressive medication for preventing immunological rejection (Vunjak-Novakovic *et al.*, 2004).

By utilizing the ability of cells to produce extracellular matrices and combining it with a biodegradable and biocompatible scaffold, a new type of biomaterial can be produced for tissue regeneration. Recent studies suggest that cells undergo different tissue development processes when they are grown in 3-dimensional (3D) scaffolds, compared with conventional 2-dimensional cell-culture systems (Reinhardt & Carey, 2000; Geiger, 2001; Chen *et al.*, 2004; Grayson *et al.*, 2004). The 3D arrangement of ECMs has made fibrous scaffolds the most commonly used type in tissue engineering applications (Ma *et al.*,1999; Cukierman *et al.*, 2002; Badylak, 2004), because of its high specific surface area, good mechanical properties, and high void volume (Ma *et al.*, 1999). Current research suggests that 3D cultures affect cellular processes like cell proliferation, signalling, differentiation, and morphology (Walboomers *et al.*, 1999; Ali *et al.*, 2005). The amount of expressed ECM proteins is also up-regulated in 3D cultures (Cukierman, 2002). The purpose of synthetic scaffolds is to function as artificial tissue with mechanical strain and cell compatibility. As the cells produce their own ECM and the tissue regenerates, the synthetic scaffold should gradually be resorbed (Walboomers & Jansen, 2001).

1.5. Aim of the study

The aim of this study was to examine how cells seeded on a 3D scaffold are distributed, and how they proliferate and deposit ECM proteins under dynamic stimulation. A static culture was used as a reference to the dynamic culture study.

The 3D scaffold that was used was a weft-knitted poly(ethyleneterephtalate) (PET) fabric. This is a well-known and non-biodegradable polyester with good compliance (Atthoff *et al.*, 2006). The purpose was to seed cells and let them grow *in vitro* in this PET scaffold that carried the mechanical load. The scaffold provided sufficient surface area for cell adhesion, and also space for cells to grow and develop in an ECM microenvironment. Uniform spatial cell distribution in a scaffold is advantageous for normal cell function and proliferation. The differentiation and migration of cells plays a fundamental role in determining of the phenotype of the final tissue construct (Li *et al.*, 2001).

The cell line that was used for this project was 3T3 mouse fibroblasts from ATCC. 3T3 is a well-known and well characterised cell line, commonly used for *in vitro* systems because of the cells' high sensitivity for contact inhibition. Fibroblasts secrete large amount of extracellular matrix protein as collagen, which is advantageous since matrix distribution in PET scaffolds is investigated in this study.

2. Materials and methods

2.1. Cell culture

Frozen stock cultures of 3T3 mouse fibroblasts were quickly thawed in a 37ºC water bath and transferred to a complete medium that contained Dulbecco's Modified Eagle Medium/F12 (DMEM-F12) (Gibco, Invitrogen Corporation), supplemented with 10% fetal bovine serum (FBS) (Hyclone, Labdesign AB) and 50 µg/ml gentamicin (Gibco,

Invitrogen Corporation). After a short centrifugation, $1x10^6$ cells were transferred to a T175 culture flask (Nunclon) with 50 ml of medium and cultured in a humidified atmosphere incubator, with 95% air and, 5% CO_2 at 37ºC to an approximate confluence of 80%. The medium was changed twice a week, and cells the cells were examined daily under a light microscope (Nikon Eclipse Te 2000-U).

When the cell confluence had reached approximately 80%, the cells were washed with phosphate-buffered saline (PBS) (Gibo, Invitrogen Corporation), detached by incubation in 0.25% trypsin and 0.38g/l ethylenediaminetetra acetic **(**EDTA) (Gibco, Invitrogen) and, collected in tubes before being centrifuged and re-suspended in fresh medium. Unless otherwise stated, the cells were split in new culture flasks, in proper ratios according to need. Cell counting was performed by Trypan Blue exclusion 0.4% (Gibco, Invitrogen Corporation) in a Bürker chamber. All experiments were performed with two to five generations of cultured cells.

2.2. Surface modification of PET

To improve cell attachment to the knitted PET, the surface was hydrolyzed. The surface hydrolysis was performed according to a previous description from Atthoff & Hilborn (2006). The fabrics were hydrolyzed in 2.5M sodium hydroxide for 90 minutes at 50ºC, rinsed with deionised water, and washed in 1M 10% acetic acid. Each fabric was 1 cm long and was turned inside out before surface modification.

2.3. Attachment study

To establish a time when a majority of the seeded cells were attached to the fabric surface, an attachment study was performed. The fabric was threaded on a small plastic stick and placed in a special plate with a hollow on each side of the wells, to avoid that the knitting touched the bottom. The kit was then autoclaved. In a volume of 80 µl medium, $1x10^6$ cells were seeded on seven knitted fabrics. During attachment, the knittings were incubated in 95% air and, 5% CO_2 at 37ºC. Every hour from the seeding moment, one fabric was rinsed with 5 ml medium. The procedure was repeated for six hours. The seventh knitting was left overnight and was rinsed with medium 12 hours after seeding. The evaluation of cell attachment was made through an MTT assay as describes below.

2.4. Growth study

2.4.1. MTT assay

An MTT assay (Thiazolyl Blue Tetrasolium Bromide) (SIGMA) is an easy method to evaluate cell proliferation and cell viability. The method is based on mitochondrial enzyme conversion of a salt to a crystal in viable cells. By dissolving the crystals, the colored product can be measured spectrophotometrically. The measured absorbance correlates to the number of viable cells (Sladowski *et al.*, 1993; Rollino *et al.*, 1995).

A stock solution of 5 mg/ml MTT was prepared by dissolving MTT in PBS, which was then filtered through a sterile filter (0.2 µm). The MTT solution was added directly to the assay plates in an amount corresponding to 10% of the culture volume. The cells were then incubated for 4 hours in 37ºC. After incubation, the medium was removed and the produced crystals were dissolved in dimethyl sulphoxide (DMSO) (SIGMA). The plates were preincubated in 37ºC for 5 minutes, after which the concentration of the colored product was measured with a Perkin Elmer Lamda 35 UV/VIS spectrometer at 570 nm (measurement) and 690 nm (reference). The measured absorbance correlates to the number of viable cells.

2.4.2. Standard curve

The cells were prepared by trypsination and counted in a Bürker chamber. Each cell density was made in triplicate, and the cells were seeded in suitable dishes as can be seen in table 1. The cell cultures were left overnight, and then checked by light microscopy the day after to inspect cell morphology before an MTT assay was performed.

Table 1. Cell cultures in different plates and volumes. Each set was performed in triplicate.

Number of cells	Plates	Medium	MTT	DMSO
0	24 wells	2 ml	200 µl	2 ml
50 000	24 wells	2 ml	200 µl	2 ml
100 000	24 wells + 6 wells	2 ml + 5 ml	200 µl + 500 µl	2 ml + 5 ml
500 000	6 wells	5 ml	500 µl	5 ml
1 000 000	10 cm diameter	10 ml	1 ml	10 ml
3 000 000	10 cm diameter	10 ml	1 ml	10 ml
5 000 000	10 cm diameter	10 ml	1 ml	10 ml
15 000 000	10 cm diameter	10 ml	1 ml	10 ml

2.4.3. Static study

Circular discs (16 mm in diameter) were punched out from knitted PET fabric and hydrolysed before being placed with the rough surface up in holders, as shown in figure 3. All experiments were performed with the rough side up, since previous tests show that this results in better cell attachment to the fabric. Holders with knittings were autoclaved for 20 minutes and dried before $1x10^5$ cells were seeded in a volume of 80 µl complete medium. The holders were kept in wells and incubated for four hours before complete medium was added to the wells. During the experiment, the plates with holders were placed in an incubator with a humidified atmosphere of 5% CO_2 37ºC. The medium was changed every second day during the first two weeks, and thereafter every day.

Cell viability was measured at time 0, and subsequently after 1 day, 3 days, and 1, 2, 3, 4 and, 5 weeks. The holders with the fabrics were washed once with PBS, moved to new wells, and evaluated with an MTT assay. Each set included all materials in triplicate.

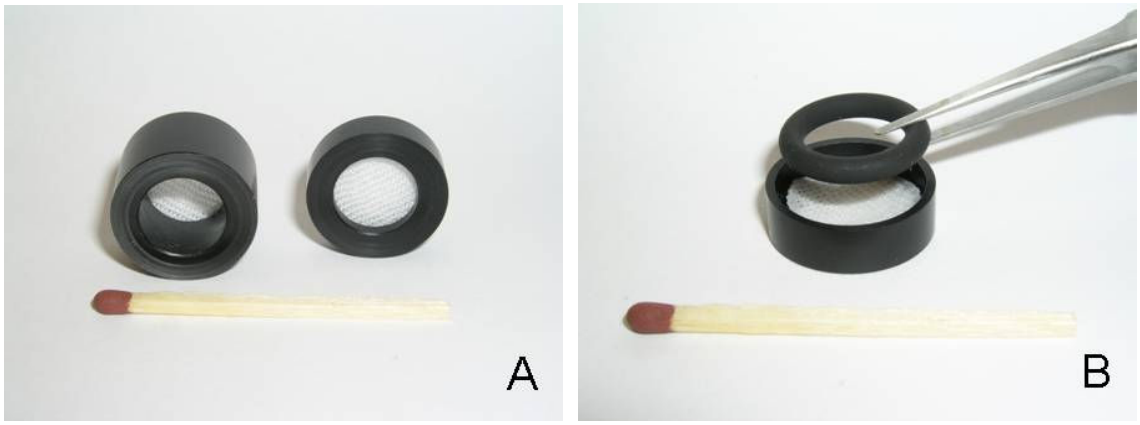


Figure 3. (A) Holders. **(B)** The knitting was held in place with a rubber O-ring tightly fitted into the holder. Because of the manufacturing procedure, the PET fabric has one smooth side and one rough side.

2.5. Bioreactor

The bioreactor used for the dynamic culture study is shown in figure 4. A silicon tube connects the two mandrels. To simulate a physiological pressure of about 120/70 mm Hg, the silicon tube is repetitively expanded and relaxed by a pulse of medium (pulse medium) produced by a peristaltic pump, which is connected to one end of the system. The pulse medium flows through a closed system of tubes, and never comes in contact with the medium in the bioreactor. The incoming pulse to the silicon tube is monitored by a pressure sensor (Edwards Lifesciences™). The temperature in the reactor is kept at 37ºC by a heater which is regulated with the help of a temperature probe that is connected to a control unit.

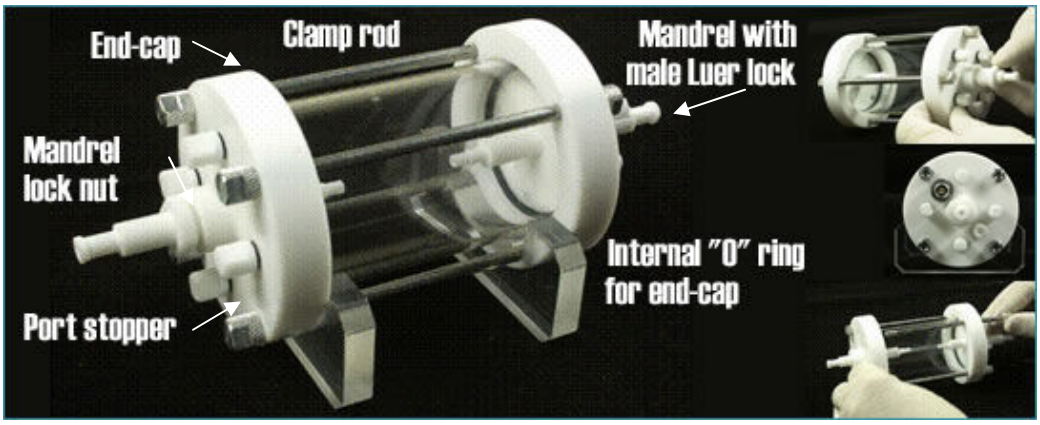


Figure 4. A one-way bioreactor. A silicon tube connects the two mandrels. A pulse is sent through the mandrels, which makes the silicon tube expand and relax.

2.5.1. Dynamic study

Three hydrolysed knitted fabrics were threaded on a 4 cm long silicon tube. The silicon tube with knittings was placed in the bioreactor, and the reactor was then autoclaved and left to dry. $1x10^5$ cells were prepared in a volume of 80 µl medium. By using a syringe and needle, cells were seeded on the knitting as shown in figure 5. After seeding, the bioreactor was incubated for four hours, to allow cell attachment to the

fabric surface. Once the cells were attached, the bioreactor was filled with 230 ml 37ºC complete medium, and then connected to the pump and pressure sensor. The setup of the bioreactor is shown in figure 6. Approximately 120 ml of medium in the bioreactor was exchanged with new medium after two weeks of culturing. Cell viability was measured at time 0, and subsequently after 1 day, 3 days, and 1, 2, 3, 4, and 5 weeks. Every bioreactor experiment was made in triplicate and evaluated with an MTT assay.

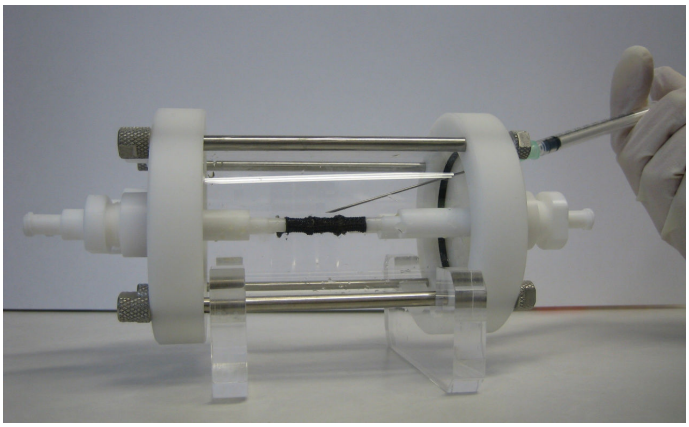


Figure 5. Seeding procedure by a syringe and needle.

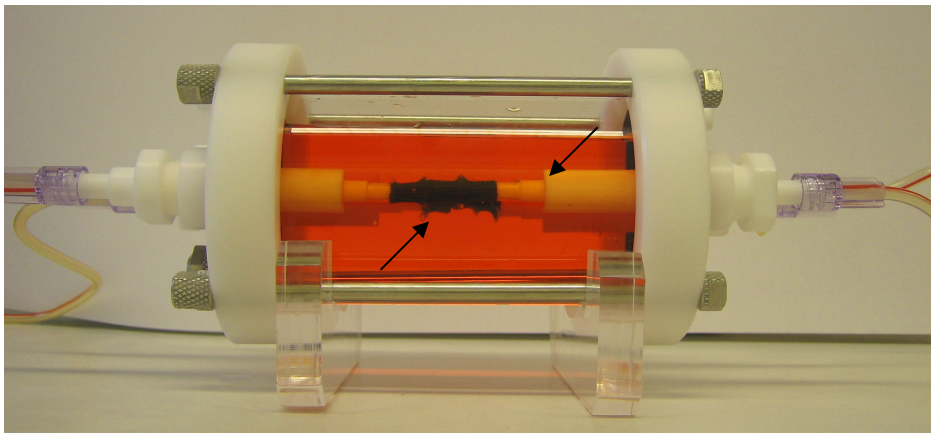


Figure 6. A two-way bioreactor complete with knitting and medium. The reactor has the capacity to run two silicon tubes (arrows) at the same time. The mandrels are connected to tubes through which pulse medium is pumped. This reactor is run with same pulse rate and temperature as a one-way reactor.

2.6. ECM production

The cells were prepared and seeded in the bioreactor as described previously. $2x10^6$ cells were seeded and grown for two weeks. For the ECM production study, 100 µl/ml ascorbic acid (Sigma) was added every day in the reactor medium. The pH value was checked twice a week, and if it was less then 6.9, 5% CO_2 was added directly into the reactor for 10 minutes.

2.6.1. Scanning electron microscopy, SEM

The samples were fixated with 2.5% glutaraldehyde in PBS for at least 1 hour. They were then rinsed in PBS three times and dehydrated in a series of ethanol solutions with

increasing concentration (50% ethanol 10 min, 75% ethanol 30 min, 90% ethanol 90 min, and 2x100% ethanol 60 min), before a supercritical carbon dioxide extraction was performed. For the SEM examination the samples were sputtered with Au/Pd 90/10.

2.6.2. Scanning laser confocal microscopy

The PET fabrics were rinsed with PBS a few times to remove any debris, and were then kept in PBS until examined with confocal microscopy. For the examination, a small piece of the sample was cut out and the cells stained with acridine orange. Acridine orange binds to DNA and RNA, and makes the cells fluorescent.

2.6.3. Histology

The samples were fixated in formaldehyde before being dehydrated in a series of ethanol solutions with increasing concentration. They were then embedded in paraffin and cut into sections 14-16 µm thick. The sections were left to dry at 37ºC overnight before being stained with hematoxylin and eosin and mounted under a cover glass.

2.6.4. Immunohistochemistry

To identify the presence of collagen type I—the most common ECM protein—a peroxidase-based immunohistochemistry with monoclonal anti-collagen type I antibodies was performed. The primary antibody was monoclonal Anti-Collagen Type I developed in rabbit. This antibody recognizes the native (helical) form of collagen type I. The secondary antibody, which binds into the primary antibody, was anti-mouse lgG, conjugated to peroxidase by protein cross-linking. The methods used to find the optimal staining are listed in table 2. Every set included the sample as well as a negative control that was only treated with secondary antibody.

Table 2. Setup for finding the optimal immunohistochemistry analysis. Each set included three samples: a negative control, and a 2-week and a 10-day sample.

Set	Methanol/H_2O_2 50:1 treatment	Serum 10% treatment	Enzyme treatment	1nd ab dilution	2nd ab dilution
1 - 3	No	No	No	2000x	200x
4 - 6	Yes	Yes Fetal bovine	No	2000x	200x
7 – 9	Yes	Yes Fetal bovine	No	100x	200x
10 – 12	Yes	Yes Fetal bovine	No	500x	200x
13 - 15	Yes	Yes Fetal bovine	No	1000x	200x
16 - 18	Yes	Yes Mouse	No	1000x	200x
19 - 21	Yes	Yes Mouse	Yes	1000x	200x

In the first stage, the samples were treated like the histology preparation, with the addition of 30 minutes in methanol/H_2O_2 50:1 in the rehydration step. The samples were washed with PBS, treated for 20 minutes in 10% mouse serum (SIGMA), and then washed again with PBS. The samples were then incubated with the primary antibody for three hours at 37ºC, washed 2 times with PBS, and incubated with the secondary antibody for 30 minutes. The incubation was interrupted with another round of PBS washing.

For the coloring, SIGMAFAST™ DAB/Cobalt Tablets were used. The preparation was done according to the given protocol. The substrate was applied on the sections, and the reaction was monitored every minute in a microscope. The reaction was stopped by gently washing the slides in PBS and deionised water, before mounting them under a cover glass. The negative control was treated with secondary antibody and DAB.

3. Results

3.1. Attachment study

To know how many cells are seeded, the volume cannot be bigger than what the knitting can absorb. If any applied cell suspension drips off the number of seeded cells will be unknown. Experiments showed that for a 1cm long knitting, a seeding volume of 80 µl was suitable. Every hour, counted from the seeding moment, one knitting was rinsed with 5 ml complete medium. Evaluation was done by an MTT assay. The results are shown in table 3.

Table 3. $1x10^6$ cells in 80 µl complete medium were seeded on seven hydrolyzed knittings. The right column shows the number of attached, viable cells on the knitted fabric after 1-6 and 12 hours. The left column shows the number of cells that were rinsed from the knitting by medium refill, after 1-6 and 12 hours.

Knitting		**Wells**	
Seeding of $1x10^6$ cells		Seeding of $1x10^6$cells	
Time (h)	**Abs 570 nm**	**Time (h)**	**Abs 570 nm**
1	1,19	1	0,61
2	1,18	2	0,31
3	0,73	3	0,09
4	0,38	4	0,03
5	0,41	5	0,03
6	0,37	6	0,02
12	0,05	12	0,02

The results indicate a high amount of living cells in the knitting after 1 and 2 hours, but the number of cells that have been washed from the knittings is also fairly high. The cell activity in the wells is hardly measurable after 3 hours, whereas the cell activity in the knittings showed 50% of a seeded cells surviving after 3 hours, and 30% after 4 hours.

3.2. Growth study

3.2.1. Standard curve

An MTT assay with various known cell density was performed for later estimation of the cell proliferation in the static and dynamic cultures. The results of the standard curve can be seen in figure 8.

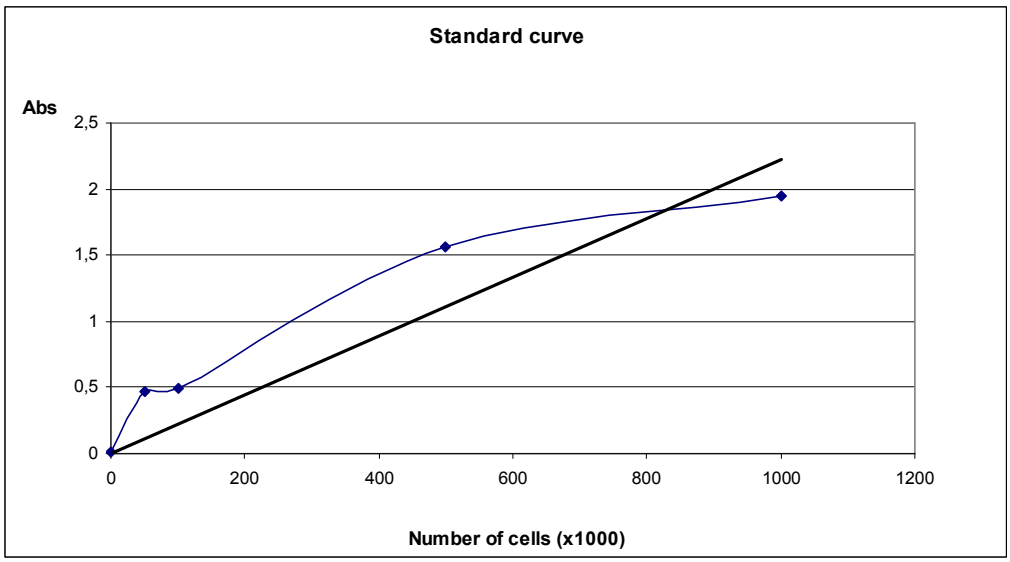


Figure 8. Standard curve (absorbance 570 nm). The data show the mean of the duplicate samples.

3.2.2. Static culture

The static cultures were performed in triplicate at time 0, and subsequently after 1 day, 3 day, and 1, 2, 3, 4, and 5 weeks. Figure 9 shows the result of the static culture growth.

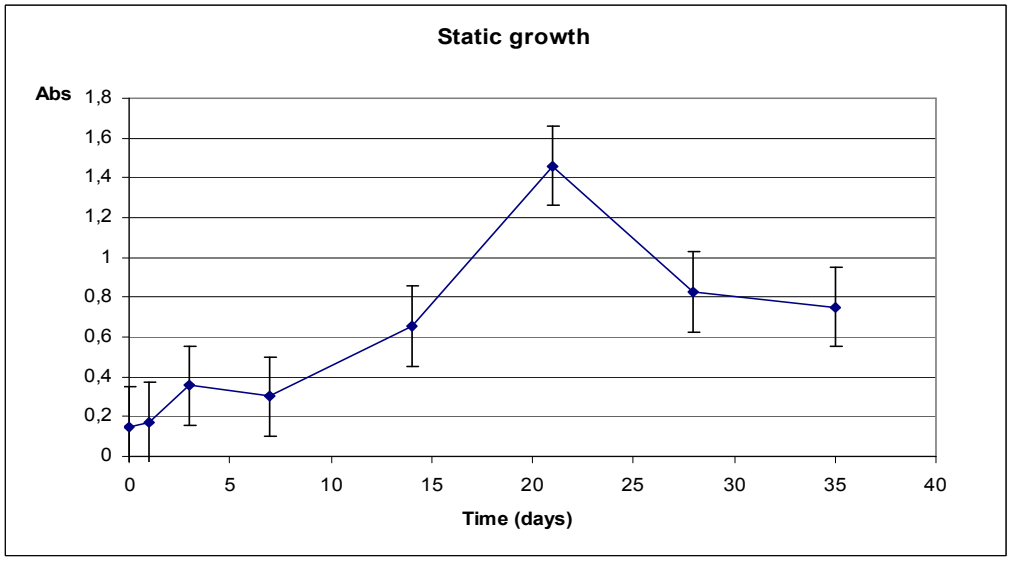


Figure 9. Result for static growth. 100 000 cells were seeded, and each setup was made in triplicate.

The proliferation increases after 1 week from the seeding moment. After cultivation for 3 weeks, a maximum cell proliferation occurred, and a quite substantial decrease takes place from week 3. According to the present table the highest value (around 1.5 absorbance units) corresponds to a cell density of 6.7×10^5, and occurs at 21 days (3 weeks).

3.2.3. Dynamic culture

The given time points were 0, 1, and 3 days, and 1, 2, 3, 4, and 5 weeks. The analysis of the cell proliferation was done by an MTT assay, and the results are shown in figure 10.

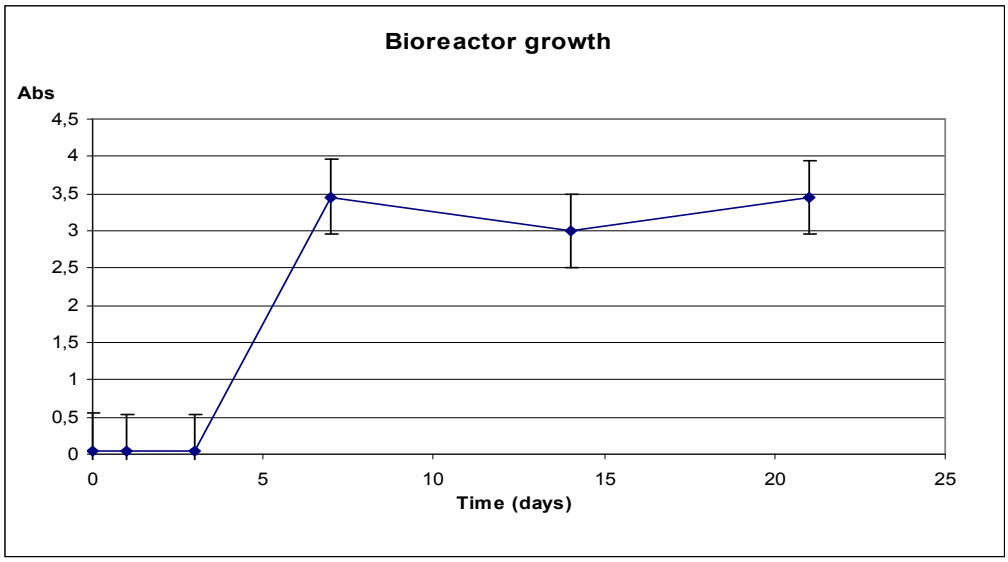


Figure 10. Graph of dynamic growth. The data for weeks 4 and 5 are not shown.

The graph shows a substantial growth after one week. The absorbance of 3.5 units corresponds to a cell density of $1.6x10^6$. Data from day 1 and 3 indicate that the cells do not survive the seeding procedure. Unfortunately, there was no growth in the 2-way reactor for weeks 4 and 5. No cell activity could be measured (data not shown).

3.3. ECM production

The distribution of the cells' ECM production and morphology was analysed by histology, SEM, scanning laser confocal microscopy, and immunohistochemistry.

3.3.1. Histology

The fibroblasts cells and ECM were examined by light microscopy. The fibroblast cells were stained with hematoxylin and were visible as dark blue spots. The matrix was stained by eosin to a purple colour. Figure 11 shows a 10-day dynamic culture sample. In the right upper corner are PET fibres. The cells are evenly distributed throughout the knitting and have deposited ECM that is aligned along the PET fibres. In areas containing clusters of fibroblasts, a denser matrix is seen. The parts of the knitting that had been facing the reactor medium showed a matrix constitution that was denser and were compact than the parts that had been facing the silicon tube. The matrix was deposited in layers, forming a porous network as seen in figure 11.

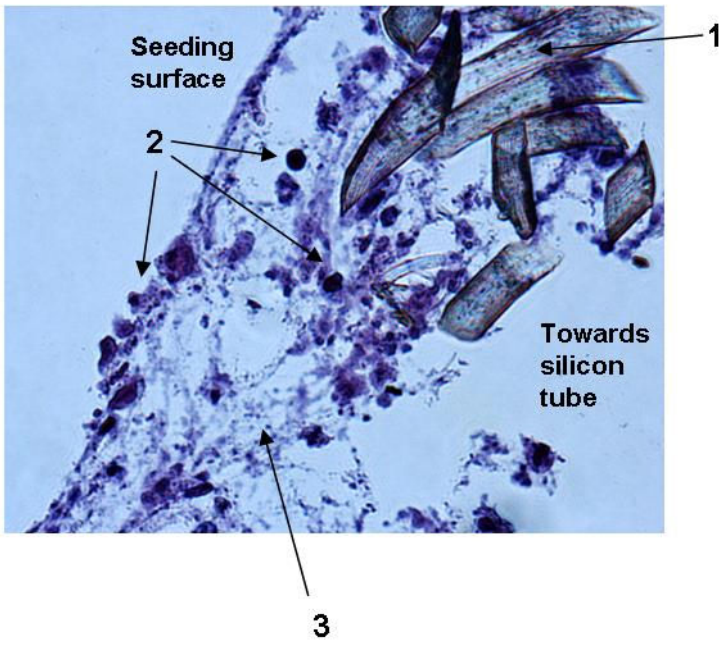
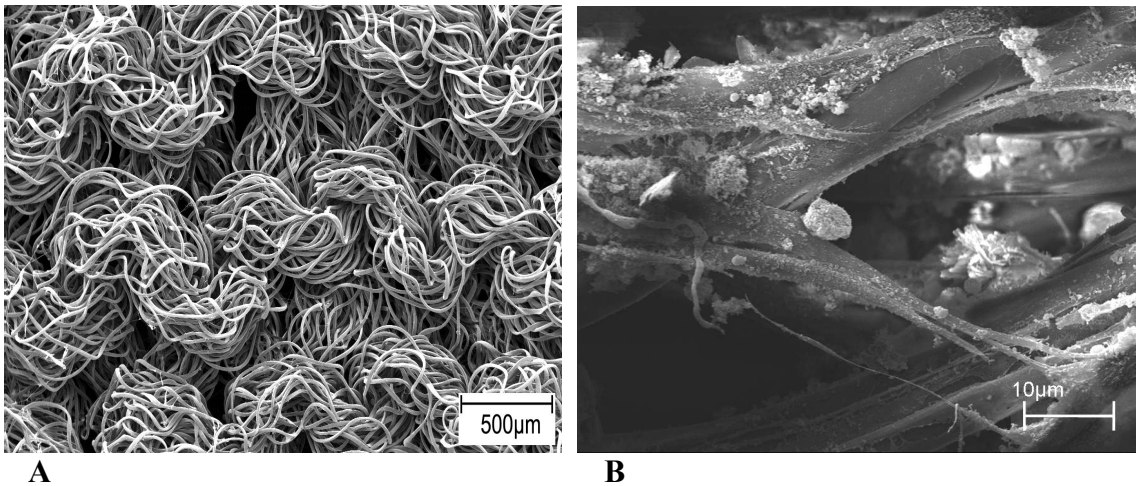


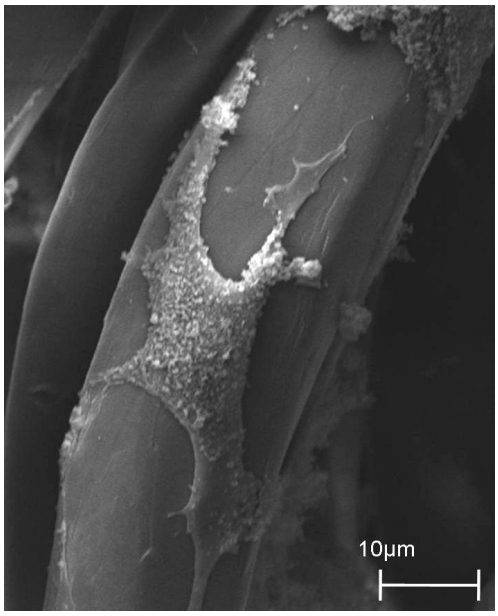
Figure 11. 10-day sample in 40x magnification. **1.** Fibre residues from PET. **2.** Fibroblast cells. **3.** Extracellular matrix. Note the onion-like shape of the ECM. A suggestion for this arrangement is cell response to the dynamic stimulation.

3.3.2. Scanning electron microscopy, SEM

Scanning electron microscopy gives a closer, more detailed picture of the cell morphology, spreading, and cell-fibre interaction on the knitting. Figure 12A shows knitting without any cells. Figure 12B shows how the produced ECM matrix has grown along the fibre and started to create a bridge between two fabric fibres. The linking construction initiates from the upper-left fibre, down to the lower-right fibre. Picture12C shows how a cell is attached to a fibre.



A **B**

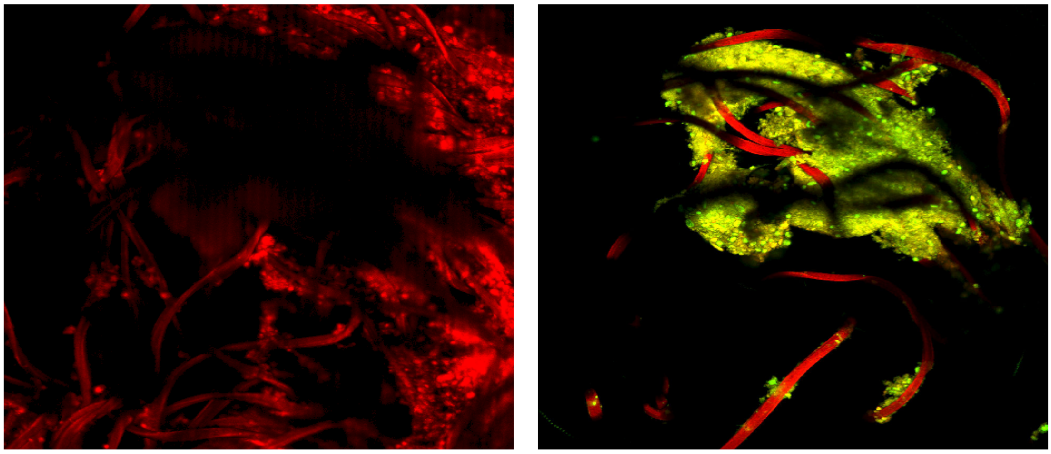


C

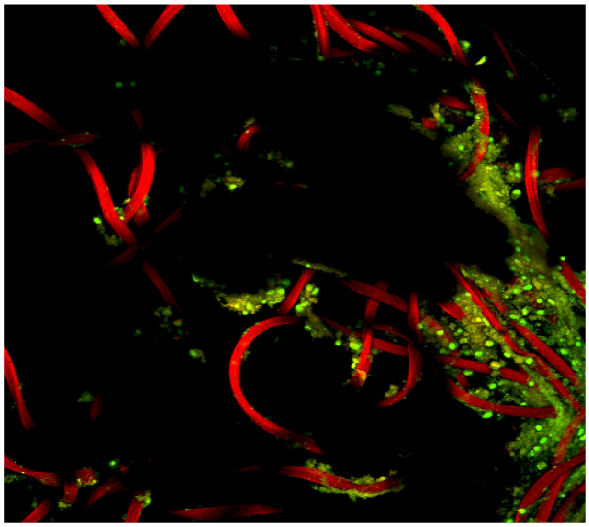
Figure 12. (A) An electron microscopy image of knitted PET without any cells. **(B)** Cells and ECM distribution on the fibre surface. ECM can be seen bridging the gap between two PET fibres. **(C)** A single cell attached to and stretched out over a fibre surface.

3.3.3. Scanning laser confocal microscopy

The cell and ECM arrangement can be further visualized by the use of confocal microscopy. By scanning many thin sections through a sample, a three-dimensional image of the sample can be constructed. Figure 13A shows a section scanned in red light. Cells stained with acridine orange are not visible in this wavelength. Figure 13B shows same section scanned in green light, where cells and PET fibres are visible. Figure 13C shows the two previously scanned sections merged into a single image, which makes it more obvious how cells and fibres interact. The fibroblasts (green spots) are mainly distributed along the fibres (red). The green haze is a result of either unspecified binding, or of a surplus of the acridine staining that is caught in the ECM. The picture illustrates that the cell distribution is tightly bound to the PET scaffold, and that the ECM production works as a bridge to overcome the distances within the scaffold.



A B

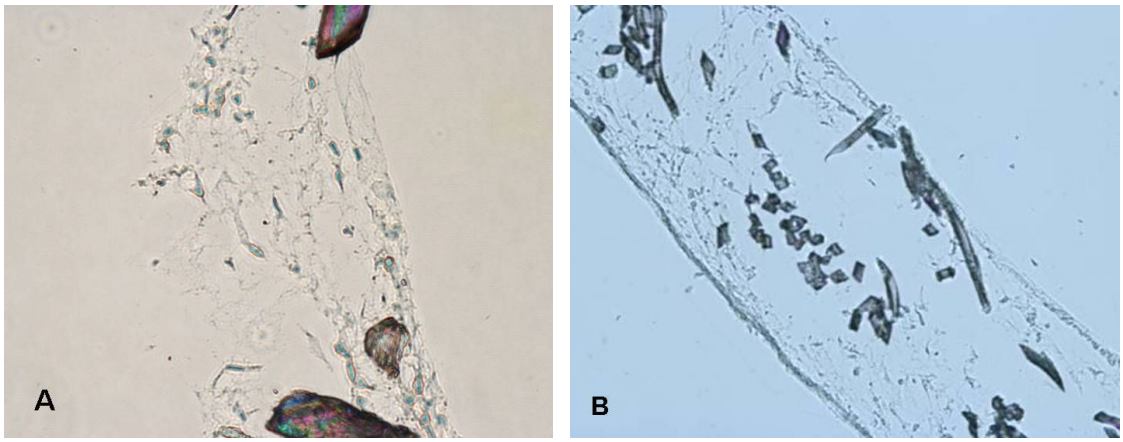


C

Figure 13. Confocal microscopy is a light microscopic technique where only a small part of the sample is illuminated and observed at a time. An image is constructed through point-by-point scanning of the field. **(A)** A section scanned in red light. **(B)** A section scanned in green light. **(C)** Fusion of the two scanned sections (A and B). The fibroblasts can bridge the distances within the knitted PET scaffold, through the production of ECM.

3.3.4. Immunohistochemistry

First, the dilution concentrations for the primary and secondary antibody were established. The evaluation of samples treated in different concentrations showed satisfactory results, with the primary antibody diluted 1000 times, and the secondary antibody diluted 200 times. Peroxidase was detected by a SIGMAFAST™ DAB with Metal Enhancer Tablet Set. Peroxidase activity precipitates a substrate which stains the sample dark blue. A check for this colored substrate was made at three different time points—1 minute, 2 minutes, and 5 minutes. A good result was obtained with 2 minutes. The colored samples and the control can be seen in figure 14.



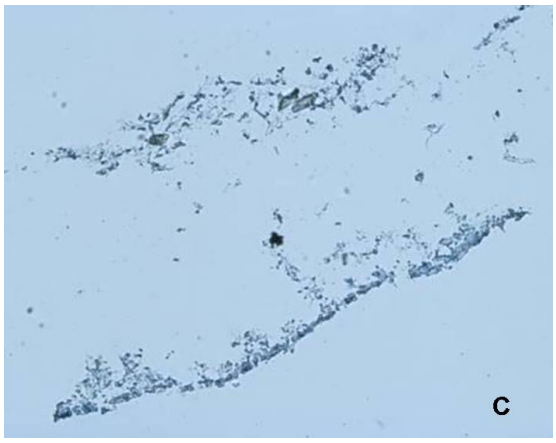


Figure 14. (A) Negative control 40 x magnification. **(B)** 2-week sample 40 x magnification. **(C)** 10-day sample 40 x magnification. None of the three samples were enzyme-treated. Evaluation of enzyme-treated and non-treated samples showed no significant difference.

The 10-day and 2-week sample showed a blue-colored network. The negative control shows a network that is much less blue. These divergences in color indicate that collagen proteins are present in the dynamic culture samples.

4. Discussion

Three-dimensional scaffolds have been shown to have an effect on cellular regulation, such as proliferation and differentiation. This project has examined how cells seeded on a synthetic 3D scaffold attach, distribute, and proliferate under dynamic stimulation. The standard curve that was obtained showed poor results at high cell density, due to a manufacturing fault of the cell culture dishes. For that reason, results from $1x10^6$ cells are not included in the standard curve.

The effect of dynamic stimulation on ECM distribution has also been investigated and given a preliminary characterization. To establish a convenient time for cell adherence, an attachment study was done. The finite possibility of PET fabrics to retain a volume and the cell spreading at seeding are both factors that can affect cell survival. A test made with a volume of 100 µl showed that a fairly big droplet was produced. A droplet on the knitting contains a number of cells that have no possibility to adhere to the surface, and the cells risk dying through lack of nourishment before the knitting has absorbed the applied volume. Too small a volume, on the other hand, increases the risk for uneven spreading of the cells, which also has a negative impact on cell survival. The performed attachment study showed that 50% of the seeded cells were attached after three hours from seeding, and 30% after 6 hours. Therefore, it could be assumes that a time of 3 to 6 hours would be adequate for appropriate cell attachment to the PET scaffold, with sufficient cell survival and no cells being rinsed from the knitting.

A way to improve cell survival and facilitate the attachment would be to immobilize collagen on the knitting. At seeding cells are exposed to stress—partly from the trypsination, but also during the attachment, when the cells are without nourishment for a couple of hours. Collagen immobilization improves attachment and cell survival, which gives a faster proliferation (Atthoff *et al.*, 2006; Attoff & Hilborn, 2006).

The graph of the dynamic stimulation shows that cells from day 0 (seeding), day 1, and day 3 did not survive. According to the standard curve, the value for these measure

points ought to be around 0.5 absorbance units when $1x10^5$ cells are seeded. This cell death might be due to poor spreading of the cell suspension during seeding. Again, this shows how important it is to improve cell survival at seeding. When the cells survive the seeding and manage to attach to the knitting, the growth study showed that cells grow better in a dynamic culture than a static culture. Cells in a dynamic culture showed a significantly faster cell proliferation than those in a static culture. According to the graph (figure 10), the cell density reached its peak after 1 week. The proliferation for a dynamic culture at that time was 10 times larger than that of a static culture at the same time. The dynamic growth was twice as high as the static growth when comparing the highest proliferation of both cultures. Dynamic stimulation affects the expression of genes and the expression of cell regulatory elements, which together enhance the cell metabolism, which results in the production of ECM. The decrease from week 1 to week 2 could be the result of poor dissolving of crystals at the MTT assay. One of the main problems when doing an MTT assay is the difficulty of dissolving the precipitated crystals in the knitting, which can explain the slight decrease after one week's growth in a static culture.

The static culture showed a maximum proliferation after 3 weeks. For longer culture periods such as 4 and 5 weeks, the viable cell density decreased. A possible reason for this might be that a cell layer had been created on the knitting surface, which would inhibit of the proliferation. Because of there is accessible medium, cells on the knitting surface can proliferate faster and generate a dense cell and matrix layer. Nutrients can be halted as a result of contact inhibition or enhanced cell-cell interactions. Earlier studies show that below or above a certain cell density, the growth rate is reduced because of the absence of cell-to-cell feeding or because of crowding (3T3 growth in monolayer) (Todaro & Green, 1963). This denser cell layer may also prevent the diffusion of medium into the knitting's centre. Histology analyses show that fibroblasts are gathered on the external seeding surface and produce a denser matrix compared with the knitting's centre. More experiments must been done to evaluate whether it is impossible to keep cells in an *in vitro* culture longer than 3 weeks.

The ECM production was examined under dynamic stimulation. Cells were grown in the reactor for 10 days and 2 weeks. Analysis of the histology samples in figure 11 showed how a porous network of ECM was formed. SEM and confocal microscopy confirmed a good interaction between the cells and the PET fibres. The topography analysis with SEM showed a good cell distribution over the surface, and that ECM proteins were associated with the scaffold and had started to link between fibres. The porosity of the knitting plays an important role in the regulation of cell distribution and matrix deposition, since cell bridging or ECM linking cannot occur if the fibre distance exceeds a certain critical value (Ma *et al.*, 1999). Confocal microscopy visualized cell allotment and ECM arrangement deeper in the sample. The examination confirmed how ECM production takes place in the PET scaffold, and how cells interact with the scaffold and the matrix.

Immunohistochemistry was used to verify the production of collagen type I. The first analyses with monoclonal antibodies indicated the presence of collagen type I proteins. This was a first verification of matrix production but the definition is still not clear.

When the negative control was treated with DAB substrate, it appeared that the substrate accumulated on the surface, or that it had affinity to the sample. The bluish coloring of the section might arise from insufficient washing. Additional staining or testing is needed to verify the right protein. The difficulty of antibody labelling might arise from the collagen epitope sensitivity to routine formaldehyde fixation and paraffin embedding. An alternative to formaldehyde fixation could be ethanol treatment only. Ethanol has a stabilizing impact on the cell structure and its components. This might maintain the samples quality for antibody labelling.

5. Conclusions

A comparison between the static and dynamic conditions shows that cells cultured in knitted 3D fabric have a significantly higher growth rate and proliferation. The results show that the ECM production starts after approximately 1 week of culturing. In static culture conditions, the cell growth was poor, and it does not seem possible to culture enough cells in the knitting before proliferation inhibition occurs. The seeding is a very crucial issue for cell survival. The cells are exposed to a lot of stress and further studies are necessary to find the optimal cell survival and attachment conditions. It has been shown that matrix is produced after 2 weeks of culturing, and the preliminary results indicate that most of the ECM consists of collagen type I. The inconclusive results from the histology and immunohistochemistry test makes it necessary to develop the method further.

6. Future outlook

A good interaction between the substrate and the living cells is fundamental in tissue engineering. Future applications of ECM scaffolds will likely depend upon the ability to stimulate cell adherence, proliferation, and migration. These are the most essential factors for successful tissue reconstruction. This project studied the impact of dynamic, mechanical stimulation on cells cultured in a 3D scaffold. A detailed comparative study concerning matrix production of cells cultured in static and dynamic cultures on a complete stretched fabric would yield interesting results and could show how the matrix porosity varies between the different elements. Further analysis of long-term culturing would be relevant to implement. It needs to be confirmed whether it is possible to keep a continuous cell proliferation going after 4 weeks of culturing. One of the future goals is to achieve a rapid start of the matrix production after seeding. The results of this study indicate that the cells start to produce ECM after reaching a certain cell density in the knitting. This calls for supplementary studies to see whether a higher cell seeding number would lead to a more rapid ECM production.

To optimize the immunohistochemistry, another fixation and/or embedding could be applied. For instance, cryo-sectioning could be used as an alternative to paraffin embedding. This minimizes the destruction of the epitope recognized by the primary antibody, and might result in better and more convincing antibody labelling. Staining with antibodies for other ECM proteins—such as elastin, laminin, and fibronectin—would be an additional step to take, as would staining for GAGs like hyaluron acid. In

order to specifically quantify the amount of produced collagen, high pressure liquid chromatography (HPLC), could be applied to measure the content of proline in the samples. Compared with other proteins, collagen, with its triple helix, has a higher quantity of proline than other proteins in their primary structure, which makes it a good marker for collagen.

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