ASSESSMENT OF SILK FIBROIN FOR URETHRAL RECONSTRUCTION

Master's Thesis

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Specifications

Number of pages: 38 pages without the appendix, 53 pages with the appendix

Keystrokes total: 55.701

Period: September 1st, 2017- May 31st, 2018

Medicine with Industrial Specialisation, Biomedicine

School of Medicine and Health,

Aalborg University, Denmark

May 31st, 2018

1 ABSTRACT

Background: Urethral strictures have recurrent nature; therefore, treatment techniques often remain limited. Silk fibroin (SF) has shown promising results in the repair of urethral strictures, especially in combination with seeded cells. Still, the best combination of seeded cells on the SF scaffold is yet to be discovered.

Aim: The aim of this project is to investigate a method for the assessment of SF tissue engineered sheets aimed for the repair of urethral defects.

Methods: For the fabrication of the scaffolds, four different methods have been applied; electrospinning, freestanding silk films, freeze drying and stepwise deposition of SF. Afterwards, the scaffolds were seeded with cells; keratinocytes and fibroblasts. Thin polyester inserts were used as a control and transepithelial electrical resistance (TEER) was measured. Lastly, immunostaining of cells was carried out to track cell proliferation.

Results and conclusion:

When it comes to scaffold fabrication, all the methods used in this study, beside free-standing silk films, had good mechanical properties and thickness and were support cell proliferation. SF showed to have desirable properties for cell growth, thus no difference between SF coated and the membranes without coating can be found. Measures of TEER showed inconsistent values over time, which might be due to detachment of the SF coating from the surface and difficulties while performing the method. Also, it was found that co-seeding with fibroblasts had effect on the formation of TJ in keratinocytes only when seeded on SF coated inserts.

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ABBREVIATIONS

JAMs- junctional adhesion molecules

SF- silk fibroin

TJ- tight junctions

TEER- transepithelial electrical resistance

ZO- zonula occludens

1. INTRODUCTION

Birth defects and acquired abnormalities of the urethra are a major focus in tissue engineering. Current therapeutic solution employs a surgical procedure in which buccal mucosa is used as a suitable replacement for the damaged tissue. Yet, the source of the tissue has been limited, especially in case of long and recurrent strictures. Another issue is the assessment of the tissue, which is usually done “in vivo”. Hence, there is a need for development of a method, that would allow “in vitro” valuation of the tissue without affecting the tissue development. Therefore, the goal of this project is to investigate that type of a method for the assessment of the integrity of tissue engineered sheets aimed for the repair of urethral defects. For this purpose, different manufacturing methods using mostly silk fibroin (SF) were used.

Hence, to better understand the issue, it is important to understand the anatomy of the urethra.

1.1 URETHRA, ANATOMY AND HISTOLOGY

The male urethra is approximately 20cm long and has three divisions; the prostatic, membranous and penile (cavernous) urethra. The prostatic urethra connects to the bladder neck and it is the widest part of the urethra that passes through prostate. On the posterior wall of the prostatic urethra, the urethral crest is placed. Also, the prostatic urethra has the openings of the ejaculatory ducts and the numerous openings of the prostatic ducts. Histologically, it is lined with transitional cell epithelium. The membranous urethra is the shortest portion lined with pseudostratified columnar epithelium and is surrounded by the sphincter urethrae membranaceae (external sphincter). The last portion of urethra is the penile urethra which runs through the spongious body and ends at the external meatus. It is lined with stratified columnar, pseudostratified epithelium and distally the stratified squamous

epithelium. The penile urethra is the longest portion of the urethra and it is dilated in the bulbar urethra. The penile urethra is also dilated distally at the glans penis, where it forms the fossa navicularis. The midportion of the penile urethra is tethered superiorly by the suspensory ligament.[1] Histologically, the urethra has three cell layers: the urethral epithelium, fibroblasts, and smooth muscle cells (SMC). As mentioned above in the text, each urethral division has different epithelial lining. [2]

1.2 ANOMALIES OF MALE URETHRA

The need for urethral reconstruction occurs in both male and female, adult and paediatric patients. However, the incidence in female patients is lower due to anatomically shorter urethrae.[3]

Variety of acquired diseases results in need for a urethral reconstruction. One of the most common reasons for a urethral reconstruction are urethral strictures and stenosis.[4] Urethral strictures refers to a narrowing undergoing in parts of the urethra surrounded by corpus spongiosum and has the prevalence of 229-627 per 100,000 males or 0.6% of the at risk population.[3,4] Internal injuries of the epithelium caused by instrumentation or inflammation, penetrating or blunt injuries on the outside of the corpus spongiosum induce scarring in the corpus spongiosum. [4] The initiating factor is partial loss of epithelial lining and the healthy epithelia that surrounds the damaged area is joined together by urethral closure pressure. [4,5] Missing parts of epithelium induce healing processes in the vascular spongy tissue which is place under the epithelial lining. Cross- adhesion and subsequent spongiofibrosis are the healing processes in corpus spongiosum and it is determined by the degree and the etiology of the injury. [4,5] Also, the passage of urine through the injured area enhances inflammation and following spongiofibrosis.[4,5] Urethral strictured by etiology can be divided in iatrogenic, traumatic, inflammatory and idiopathic strictures.[4,5] Urethral stenosis, on the other hand, refers to a compression in the membranous urethra, the prostatic urethra and the bladder neck.[4–6]

Urethral reconstruction in paediatric patients is required due to congenital anomalies, most commonly hypospadias. Prevalence of hypospadias in Europe is 18.61 per 10,000 births. [7] Hypospadias is a condition where during the abdominal development of the penis, meatus of the urethra is proximal from its glanular placement.[8] Based on the position of the urethral opening, hypospadias can be *anterior hypospadias* where the meatus is still placed in the glandular or subcoronal region*; middle hypospadias* in which the opening is on the ventral side of the penis and *the posterior hypospadias* with meatus placed on penoscrotal junction, scrotum, or perineum.[7] (Figure 1.) Stricture disease after hypospadias repair is also a significant problem.[9]

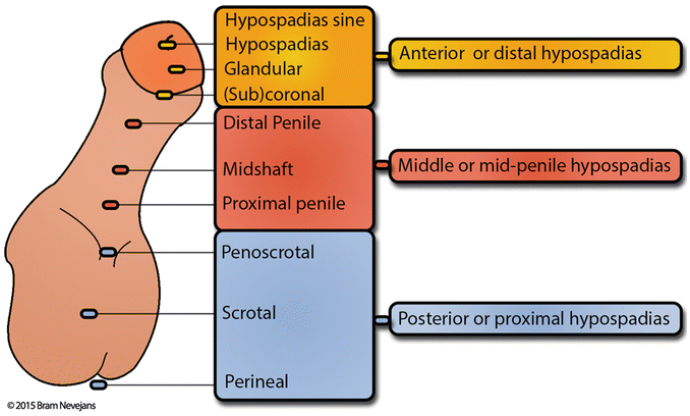


Figure 1.: Classification of hypospadias extracted from Waterloos et al. (2017.) [10]

1.3 CURRENT THERAPEUTIC SOLUTIONS AND FUTURE PERSPECTIVES

There are over 300 techniques used in the repair of urethral strictures, depending on size of the stricture.[11] Also, treatment of the hypospadias is based on the placement of the urethral opening.[12] In both cases, urethroplasty is performed. In case of shorter strictures and distal hypospadias, one stage urethroplasty by joining of the two healthy ends is performed. [4,11,12] However, in case of severe hypospadias and longer strictures, the repair solutions remain limited.[13] In those cases, two staged urethroplasty is performed usually by using the buccal mucosa grafts due

to easy harvesting procedure and low rate of graft rejection. [2,13] Despite, a disadvantage of buccal mucosa grafts is limited source of the tissue, especially in long, recurrent strictures and severe hypospadias often followed by strictures.[2] Therefore, alternative ways for urethral reconstruction have been investigated.

1.4 CURRENT DEVELOPMENTS IN URETHRAL RECONSTRUCTION

An alternative solution for the urethral reconstruction is needed, due to already mentioned limitations of methods in use. An ideal scaffold would be biocompatible, biodegradable and bioresorbable. It should promote the proliferation of the seeded cells and the proliferation of the native cells after the implantation. Also, it should mimic mechanical and physical properties of the repaired tissue.[2] Both, synthetic and natural materials are used for the tissue engineering of urethra.

Synthetic polymers ongoing are polytetrafluoroethylene and poly(ethylene terephthalate), polyglycolide (PGA), poly-l-lactide (PLL), polycaprolactone (PCL) and poly(lactic-co-glycolic acid) (PLGA) [14,15] Although showing good mechanical features and porosity, fabricated scaffold still need a surface treatment to optimise cell attachment.[16]

Natural materials can be divided into natural collagen-based matrices and protein derived scaffold and cellulose. By the decellularization process of heterogenic tissue natural collagen-based matrices are produced. Usually small intestinal submucosa, bladder acellular matrix, acellular corpus spongiosum and acellular dermal matrix are used with a variety of cells seeded onto them. These matrices showed to have molecules which enhance cell growth, despite the high density of the matrix affects metabolism of the cells and the residue of nuclei after decellularization can cause inflammatory respond. Protein derived scaffolds such as silk fibroin and SF in combination with keratin, calcium peroxide and gelatin, showed to have good features for urethral reconstruction, both in combination with cells and acellular. [17,18]Tissue engineered buccal mucosa using cultured fibroblast and keratinocytes in combination with different natural materials shown to have good

properties and promising outcomes shown in clinical trials, especially when using autologous cells. [2,19,20]

1.5 SILK FIBROIN

SF as mentioned before showed to have desirable properties for the scaffold fabrication. It is a protein-based polymer extracted from the silkworm *Bombyx mori* in the process called degumming where sericin, the other component of the silkworms is removed to isolate SF. [21] SF has widespread use in tissue engineering, implant devices and in drug delivery. [22] Mechanical properties is the asset of SF due to great breaking strength and elasticity, which makes it a great material of choice for urethral reconstruction where advanced structural stability is needed at the same time as elasticity. Biodegradability of SF is another advantage, it has low immunogenicity and antigenicity when it is properly degummed and sterilized.[23]

SF has gained a lot of attention when it comes to urethral reconstruction. Different methods are used to fabricate different scaffold types, for instance electrospun fibers, aqueous sponges, tubes, hydrogels and films [22]. Furthermore, acellular scaffolds constructed of SF have been assessed, where SF scaffolds enhanced tissue regeneration reported at a similar level as small intestinal submucosa with reduced immunogenicity.[17] Various cell types seeded onto matrices have been reported. Electrospun SF scaffolds when seeded with urothelial cells in canine models have shown good biocompatibility with the gradual epithelial development into different layers.[18] Another study has used SF matrices to construct tissue engineered buccal mucosa by seeding autologous keratinocytes and fibroblast. Sufficient biocompatibility of seeded cells with SF has been shown in canine models with an efficient epithelial layers development. [20]

1.6 TIGHT JUNCTIONS AND THEIR IMPORTANCE

One of the spotlight of the tissue engineering in urethral reconstruction is to create a scaffold with desired permeability and adequate epithelial development. The key role of epithelium is to form a

physical barrier to prevent the leakage of the body fluids. Therefore, it is important to understand undelaying physiological properties of epithelium essential for those features. Cells in epithelium form tight junctions, a semi-permeable paracellular diffusion barrier, or a closure, that blocks the passive diffusion of molecules. [24] When it comes to keratinocytes, they construct an insoluble network of cross-linked proteins and lipids therefore constructing a barrier that prevents leakage and protects against physical trauma. Tight junctions are primarily formed of four protein groups; claudins, zonula occludens (ZO), junctional adhesion molecules (JAMs) and occludin. [10, 11]. Another feature of tight junctions is size-selective paracellular diffusion of small hydrophilic molecules based on their size and charge. TEER is often used to evaluate ion conductance of formed tight junctions, and is suitable for monitoring a change in resistance over time. [24]

However, the ability of the tissue to form a barrier that prevents leakage is usually investigated after “in vivo” implementation and development. It is desired to develop a method that would be able to track the tissue development “in vitro” without disrupting it. Therefore, the goal of this project is to investigate TEER as a potential method for the assessment of the integrity of tissue engineered sheets aimed for the repair of urethral defects.

2 AIM AND HYPOTHESIS

The aim of this project is to investigate a method for the assessment of SF tissue engineered sheets aimed for the repair of urethral defects. It is hypothesized that the SF scaffolds will encourage proliferation and maturation of keratinocytes and induce the formation of TJ. Also, co-seeded fibroblasts with keratinocytes will enhance the formation of tight junctions.

3 METHODS

3.1 SCAFFOLD FABRICATION

Fabrication of silicone moulds for scaffolds

To fabricate moulds for the scaffolds, silicone (SYLGARD® 184 Sigma-Aldrich-761036) was used. The curing agent to base was mixed in 1:10 ratio and placed into a glass Petri dish with the diameter of 70mm and the depth of the silicon in the end was 1.5mm. Afterwards, silicon moulds were made by carving six round holes with a diameter of 8mm.

Freestanding silk films

Silk films were produced by adding 300µ 6% (wt/vol) SF (Lyophilized Silk Fibroin Protein, Simatech Incorporation, SF used in the whole study) into silicone moulds and left on the bench at room temperature overnight. Afterwards, to make the scaffolds water insoluble for cell culturing, they are incubated in 70% methanol and 30% deionized water for 10min. To remove methanol residues, scaffolds were washed with sterile water and PBS.

Freeze drying of SF scaffolds

300µ aqueous solution of 6% SF was added into each mould and left to dry in the flow bench for 3 hours.

After drying on the air, the scaffolds were placed in the freezer at -18°C overnight. The next day, scaffolds were placed frozen in the freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Alpha 1-2 LDplus) for 24 hours.

Then, to make sure that the scaffolds were sterile, they were soaked in ethanol and left on the bench to dry. Afterwards, they were placed into cell crown inserts (Z742383, CellCrown™ inserts, Sigma-Aldrich) and left under UV light for 2 hours.

Electrospinning of SF

An additional method for fabrication of SF scaffolds was electrospinning according to the study by Xie et al..[18] Prior to electrospinning a solution 5% (wt/vol) polyethylene glycol (PEO) in water was prepared and mixed with silk solution to generate an aqueous solution of 6.4% (wt/vol) silk/1% (wt/vol) PEO. After placing the solution in the syringe electrospinning was performed with a flow rate of 0.01-0.03 mL/min and electrical potential from 8-15kV using Y-flow Electrospinner (2.2.D-500, Nanotech solutions). Glass slides were used as a surface for electrospinning.

Subsequently, scaffolds were washed in ethanol to sterilize them.

PCL scaffolds as a control

PCL scaffolds with 700nm diameter electrospun polycaprolactone (PCL) with random orientation were used (NanoECM™, Nanofiber solutions) as a control to freeze dried scaffolds. Scaffolds were placed into cell crown inserts mentioned before and left under the UV light to ensure sterility.

Transwell cell culture inserts and SF coating

10µm thick transparent polyester membrane (CLS3470, SIGMA, Corning®, Transwell®) with a diameter of 6.5µm and pore size of 0.6µm were used for cell seeding. Two plates were seeded, one coated with SF and the other one without the coating.

Silk coating was performed according to the method described by Wang et al.[25] Each insert was coated in 100µm of SF for 2 min. After 2 min. inserts were washed with methanol and water (1:1 ratio) for 1min and followed by drying using a gentle flow of liquid nitrogen. The process was

repeated 3 times to achieve 3 coating layers. After the entire process, the inserts were washed with PBS to remove residues of methanol.

3.2 CELL CULTURE

At first, to develop the cell culture, both cell types fibroblast (CRL-2429) and Human Epidermal Keratinocytes, adult (HEKa) were cultivated in T-75 polystyrene tissue flasks (CELLSTAR®, Greiner Bio-One™) in the incubators at 37 °C, 20 % O_2 and 5 % CO_2.

Two different media were used. First, for the fibroblast DMEM (Gibco™ DMEM, Dulbecco's Modified Eagle Medium, from now on referred as DMEM-media) supplemented with 10% fetal calf serum (FCS) and penicillin and streptomycin antibiotic solution (P/S). For keratinocytes Gibco™ Keratinocyte-SFM supplemented with Gibco™ Keratinocyte-SFM Supplement: Human Recombinant Epidermal Growth Factor (EGF 1-53) & Bovine Pituitary Extract and P/S antibiotic solution was used. The media with above mentioned supplements will be referred as Keratinocytes SFM-media.

Cell seeding on the scaffolds

When it comes to seeding the cells on the scaffold, first fibroblasts were seeded. Using trypsin, cells were detached from the flask and counted. At the density of 10000 cells per cm^2 cells are seeded on the scaffolds. Scaffolds are placed into a glass, sterile Petri dish with large diameter and cover. The scaffolds were placed upside down and the cells were added and left in the incubator for 3-4 hours to attach to the scaffold. Afterwards, each scaffold was placed in 12 well plate (CLS3513 Corning® Costar® TC-Treated Multiple Well Plates SIGMA) additional DMEM medum is added and left incubated. Every 2 days the media is replenished and in total before adding keratinocytes, cells were cultivated for 7 days.

Before seeding the keratinocytes, fibroblast medium was removed, and the scaffolds were washed with PBS to remove traces of serum. Keratinocytes were detached from T75 flasks using

TrypLE (Gibco™ TrypLE™ Express Enzyme (1X)) and counted. Cells were seeded with the density of 10000 cells per cm^2. Before adding the media, to avoid the cells falling to the bottom of the plate, cells are left to attach for 3-4 hours in the incubator. Later, SFM keratinocytes medum was added and every 48h medium was replenished. Cells are cultivated in the first attempt for 7 days.

Cell seeding on the SF coated inserts

After the coating process, the cells were seeded on the inserts. Fibroblasts were seeded first, at the density of 10,000 cells per insert. The same process of seeding on the bottom side was performed as previously described in the section 2.1. in inserts number B4,B5 and B6. The organisation of the plates can be seen in the Figure 2. Inserts number B2,B5 and B6 in the SF coated plate were coated from both, the bottom and the top, while the rest was coated only from the top. Both plates, containing coated and not-coated inserts had the same organisation. Fibroblast were cultured in DMEM media for 2 days. After that, the inserts were washed with PBS to remove traces of serum and keratinocytes were seeded again with the density of 10 000 cells/insert. Later, cells were seeded SFM keratinocytes media.

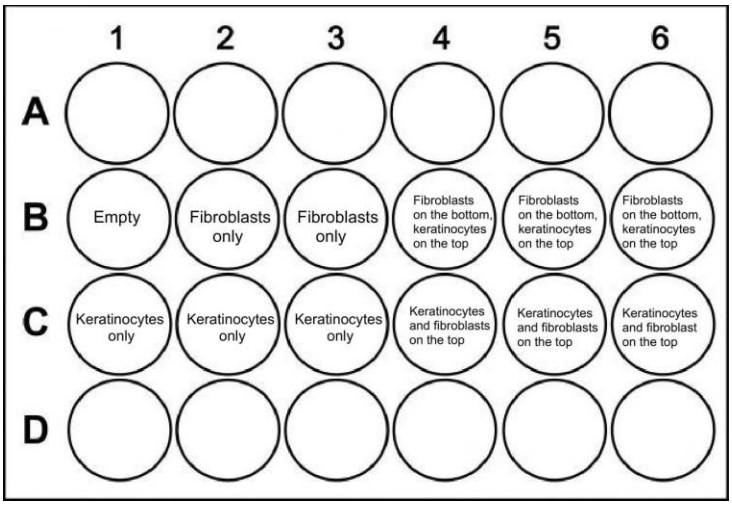


Figure 2.: A representation of the plate organisation containing inserts.

Formalin fixation

Cells and the scaffolds were fixed using formalin by washing them with PBS and adding formalin. Cells and scaffolds were incubated for 15 min at room temperature. Afterwards, formalin was removed, and the cells were washed and left with PBS.

3.3 STAINING OF THE CELLS

Cells were stained with Cytokeratin Pan (green) that detects cytokeratin's 5, 6, 8, 17, and 19 in the cytoplasm of MCF-7 cells. Previously formalin fixed samples were used. First, the cells were incubated the cells were incubated with Hoechst 33342 (Molecular Probes™) to stain the nuclei. To permeabilize the membrane cells were incubated with PBS + TX-100 0.1% (100 ml PBS + 0.46 ml TX-100 20% w/w). Incubation with a blocking agent (PBS + BSA 1%) was performed to avoid unspecific binding of the antibodies. Then, the cells were stained with the primary antibody consisting of monoclonal mouse anti-human Cytokeratin (MNF116, DAKO A/S, Denmark) and the secondary antibody anti-mouse IgG conjugated with Alexa-fluor® 488.

Another staining performed was staining of actin filaments using Alexa Fluor 488® phalloidin by following the same steps as for cytokeratin staining.

3.4 TRANSEPITHELIAL ELECTRICAL RESISTANCE (TEER)

To measure the tight junctions between the keratinocytes, TEER was measured in each well. First, electrodes were soaked in alcohol to ensure sterility, afterwards electrodes were soaked in the medium. TEER was measured using Millicell® ERS-2 Voltohmmeter and STX01 Chopstick

Electrodes (Millipore), first in a well without seeded cells to calculate the referent resistance and subsequently in each well. When it comes to coated inserts, referent insert was only coated, without cells seeded. The measurement is repeated 3 times and the mean of 3 repetitions were included in the final calculation. The equation used is:

$$\text{TEER REPORTED} = \text{TEER}_{\text{MEASURED}}\ (\Omega) \times \text{MAREA(cm2)} - \text{TEER}_{\text{REFERENT}}$$

Inserts area is 0.33cm^2 and referent is an insert without cell seeded. Furthermore, for the graph construction, average of values measured in 3 inserts with the same specifications (same coating or no coating and same cell types) and at the same day was extracted. As an example, (Figure 2.) average values measured in C1,C2 and C3 second day after keratinocytes seeding referred as Day2. Further, growth rate was calculated in compartment with the average from previous day. Starting day has value of 0.

4 RESULTS

4.1 SCAFFOLD FABRICATION AND OPTIMISATION

Free standing silk films

Freestanding silk films fabrication, following the method already described, resulted in clean, transparent films with satisfying thickness of scaffolds. However, films did not have desirable porosity. The surface of the scaffold was completely closed, which would enable cell penetration into the scaffold. (Figure 3. and Figure 4.)

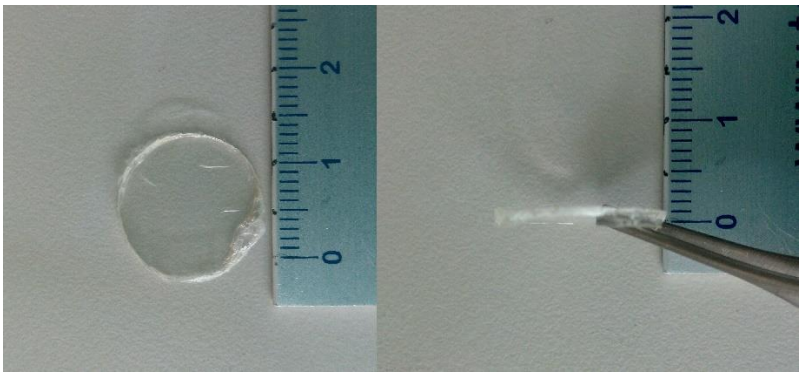


Figure 3. A freestanding silk film

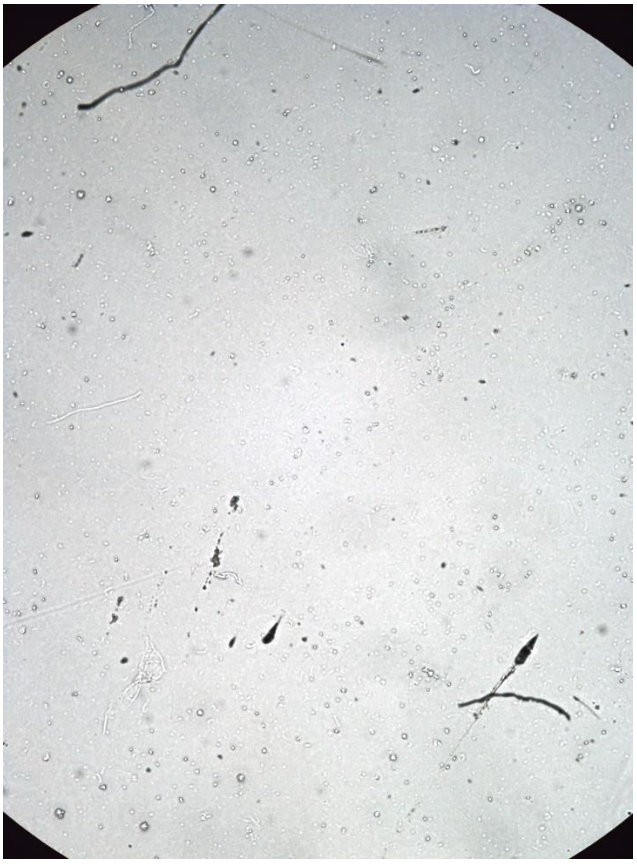


Figure 4.: Figure 9.: A picture taken with inverted phase contrast microscope presenting free standing silk film.

Optimisation of freeze dried and electrospun scaffolds

Before the freeze drying, scaffolds must be air-dried. To assess the optimal time of air drying prior to freeze drying, scaffolds were frozen at different time points. Time points used were 1,2,3 and 4

hours. As it can be seen on the Figure 5., scaffolds dried for 4 hours had no visible surface porosity and formed a stiff scaffold. Also, scaffolds dried for 1 and 2 hours had enlarged pores. Based on the pictures obtained, scaffolds dried for 3h had suitable porosity and thickness, therefore, those scaffolds were selected as appropriate for seeded cell seeding. (Figure 6.)

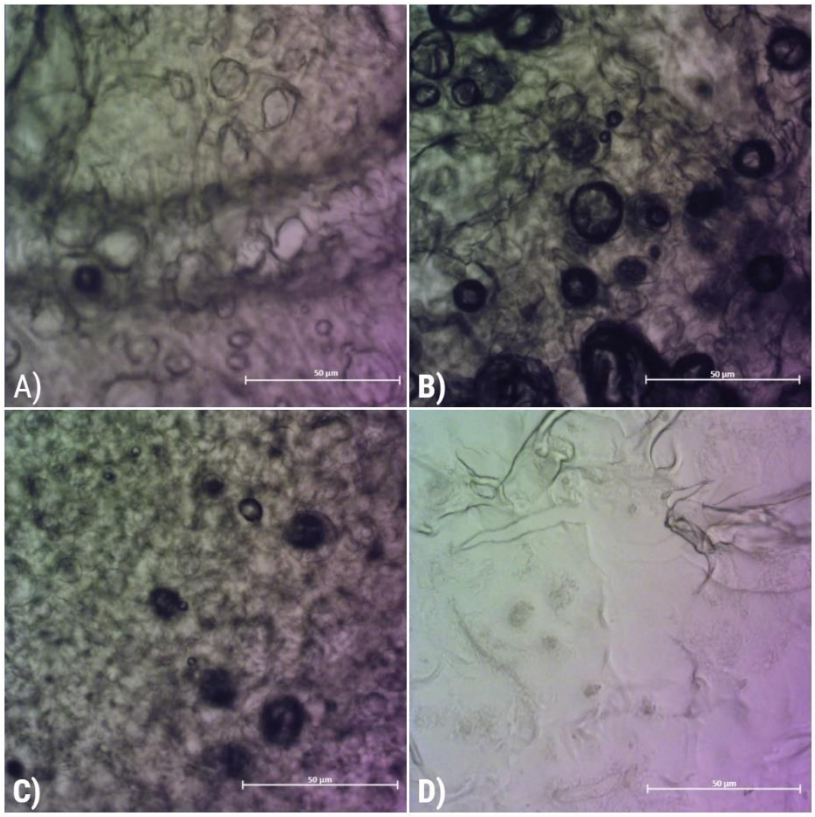


Figure 5.: Pictures of freeze dried SF scaffold taken with inverted phase contrast microscope. Picture A) presents scaffold air dried for 1 hour, picture B) scaffold air dried for 2h, picture C) for 3h and picture D) for 4h.

Figure 6. Pictures of freeze dried SF scaffold.

Other scaffolds types used were electrospun scaffolds. In the process of electrospinning, a thin layer of SF on the glass slides was collected. (Figure 7.) After sterilisation with alcohol, fibroblasts are seeded. Pictures were obtained by phase contrast microscope one day after seeding and has shown good cell attachment. (Figure 8.)

Figure 7.: A picture of glass slide covered with a thin layer of electrospun SF.

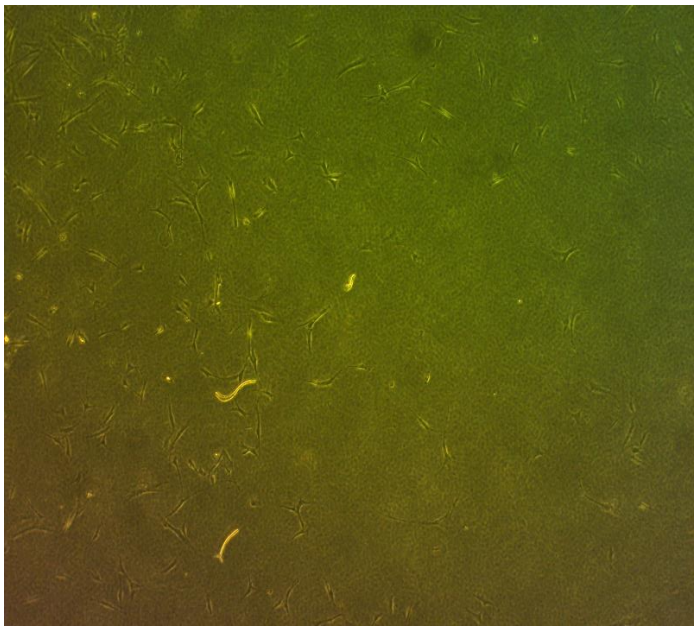


Figure 8.: A picture of fibroblast seeded on a glass slide covered with a thin layer of electrospun SF taken with inverted phase contrast microscope.

Furthermore, to test if SF fabricated by freeze drying and electrospinning promotes the cell attachment and proliferation, scaffolds, that were seeded with fibroblasts for 7 days, were fixed and stained using Hoechst (blue staining) and Alexa-fluor® 488-phalloidin conjugate (orange) to visualise the cells.

As it can be seen in Figure 9., cells proliferated, therefore it can be concluded that electrospun and freeze-dried SF scaffolds have suitable properties when it comes to the cell proliferation.

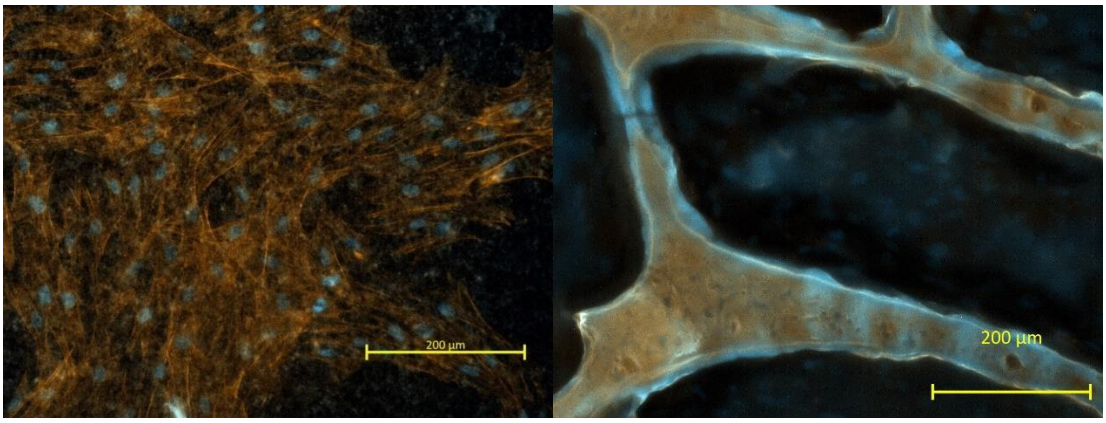


Figure 9.: Pictures of immuno-stained fibroblasts seeded on electrospun SF (left) and freeze dried (right) SF scaffolds obtained by a fluorescence microscope, where orange represents actin filaments of the cells, while blue represents cell nuclei.

Despite, further manipulation of above mentioned scaffolds was not possible because of technical difficulties during the production of freeze dried scaffolds, while electrospinning, the layer of SF fabricated was too thin. Consequently, to assess the properties of SF for urethral reconstruction, SF coating on polyester membrane were obtained.

SF coated polyester membrane

Inserts with polyester membrane were coated with SF. As it can be seen in the Figure 10. a thin layer of coated silk has been achieved. Analysis of the cell proliferation will be presented further in the results.

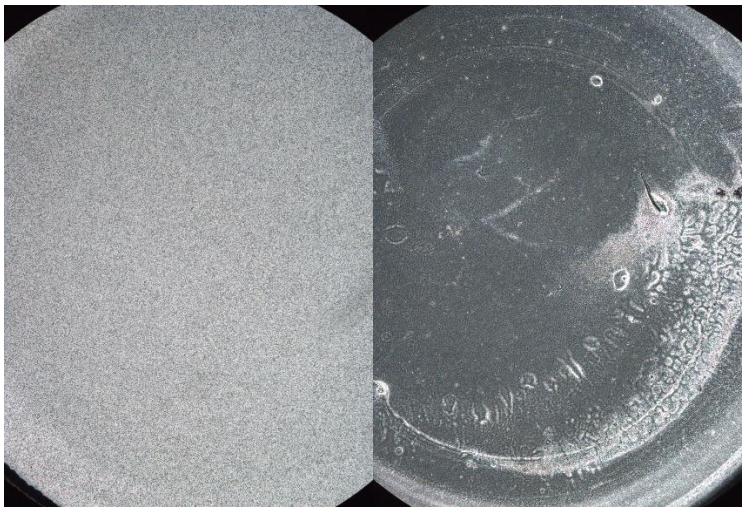


Figure 10.: Pictures taken with inverted phase contrast microscope. The picture on the right presents SF coated polyester membrane and the left is the membrane without coating.

4.2 TRACKING OF THE PROLIFERATION OF THE CELLS SEEDED ON SF COATED POLYESTER MEMBRANE

Cell proliferation was tracked by taking pictures of living cells each day with an inverted phase contrast microscope. In the results, presented pictures are from the fist, fifth and ninth day (last day) after seeding keratinocytes.

On the second day after seeding keratinocytes, pictures were taken to check if the cells are attached to the bottom. As it can be seen in the Figure 11. cells were attached in all cases and no

difference between coated and not coated as well as between differently arranged inserts can be seen.

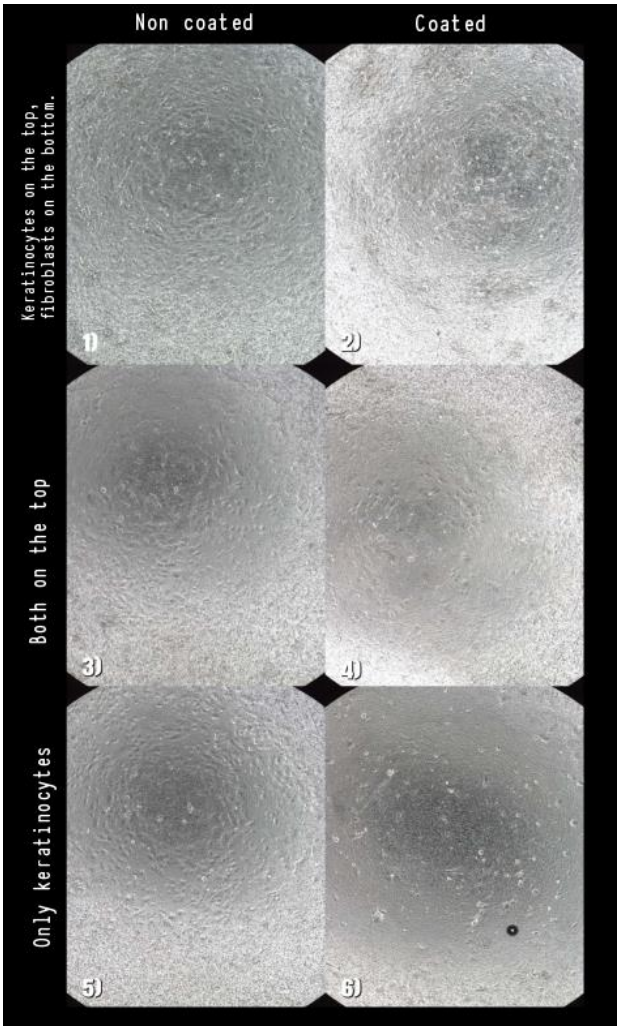


Figure 11.: Pictures taken with inverted phase contrast with 10x magnitude on the day 2. On the left side (odd numbers) are pictures of not coated inserts, right side (even numbers) are pictures of coated inserts. 1) and 2) are seeded with keratinocytes on the top and fibroblasts on the bottom, 3) and 4) both on the top and 5) and 6) only keratinocytes.

Secondly, pictures taken on the fifth day after seeding show that the confluency reached is around 60%. However, it can be noticed that keratinocytes seeded on the coated inserts grew in clusters, while the ones on not coated had more sparse growth. (Figure 12.)

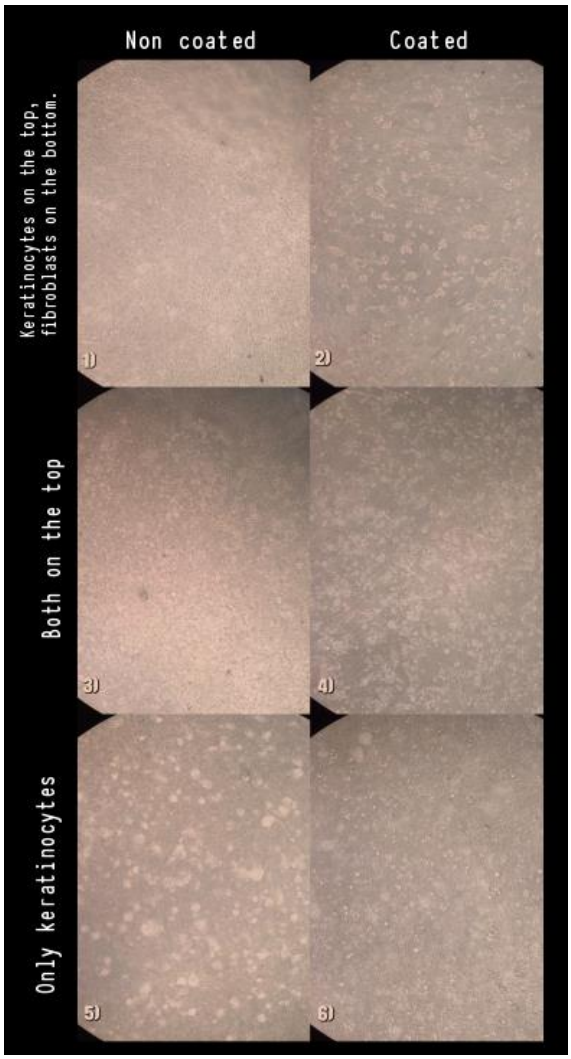


Figure 12.: Pictures taken with inverted phase contrast microscope with 10x magnitude on the day 5. On the left side (odd numbers) are pictures of not coated inserts, right side (even numbers) are pictures of coated inserts. 1) and 2) are seeded with keratinocytes on the top and fibroblasts on the bottom, 3) and 4) both on the top and 5) and 6) only keratinocytes.

Lastly, on the 9th day cells already reached 100% confluency, and, in both cases, cells cannot be identified individually, hence, immunostaining of the cells was performed. (Figure 13.)

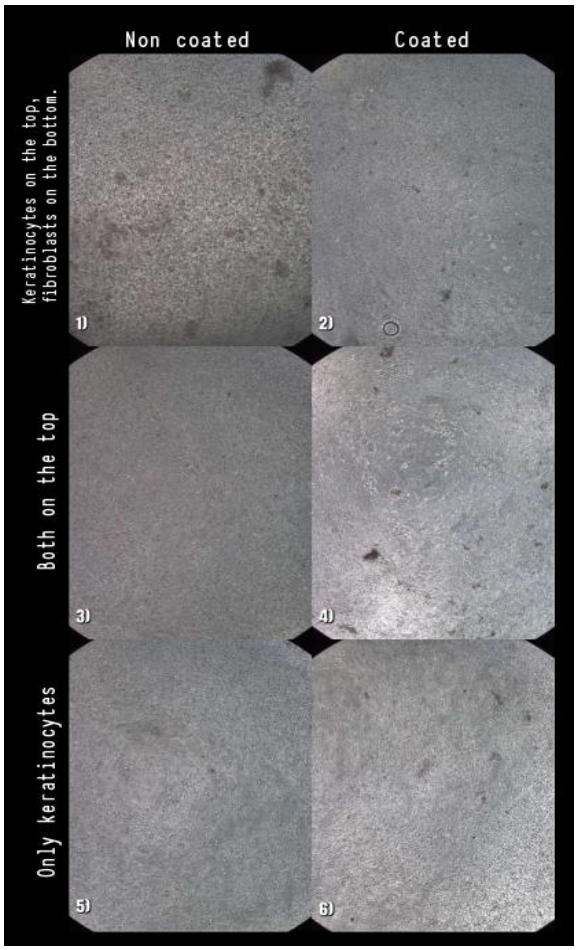


Figure 13.: Pictures taken with inverted phase contrast microscope with 10x magnitude on the day 9. On the left side (odd numbers) are pictures of not coated inserts, right side (even numbers) are pictures of coated inserts. 1) and 2) are seeded with keratinocytes on the top and fibroblasts on the bottom, 3) and 4) both on the top and 5) and 6) only keratinocytes.

4.3 IMMUNOSTAINING OF THE CELLS SEEDED ON SF COATED INSERTS

Immunostaining was carried out after the cells were fixed on the 9th day. Cells were stained using PAN cytokeratin that detects keratins 5, 6, 8, 17, and 19 in the cytoplasm and Hoechst 33342 as a stain for nuclei. The aim was to detect how the cells, specifically keratinocytes proliferated on the SF coated scaffold compared to the polyester membrane. However, on the surface of the scaffolds, no cells stained was detected. Despite, on the bottom of the well plate, a certain number of the cells were stained which indicates that the immunostaining was done properly (Appendix 10.) and the loss of the cells might be due to the repeated washing steps while immunostaining.

4.4 TEER AS A WAY OF TRACKING THE ABILITY OF KERATINOCYTES TO FORM TIGHT JUNCTIONS WHILE SEEDED ON THE SF SOAKED SCAFFOLDS

TEER measurements were performed to measure the integrity of tight junctions of epithelial monolayer. The method was performed every day and depicted in graphs. It was expected that the samples in both cases will have the same or continuously growing values compared to a value measured day before in the inserts with same specifications. Unlike, the results have shown extremely variable values.

The growth rate of inserts without coating, various categories follow the same trend of growth or fall in value, which indicated that the change in value might be due to the measuring process. Despite, growth rate on day 9 in compartment to day 1 reaches up to 8.31 for inserts seeded with both cells on the top, while lowest of 0.68 is measured in inserts seeded with keratinocytes only. (Figure 14.)

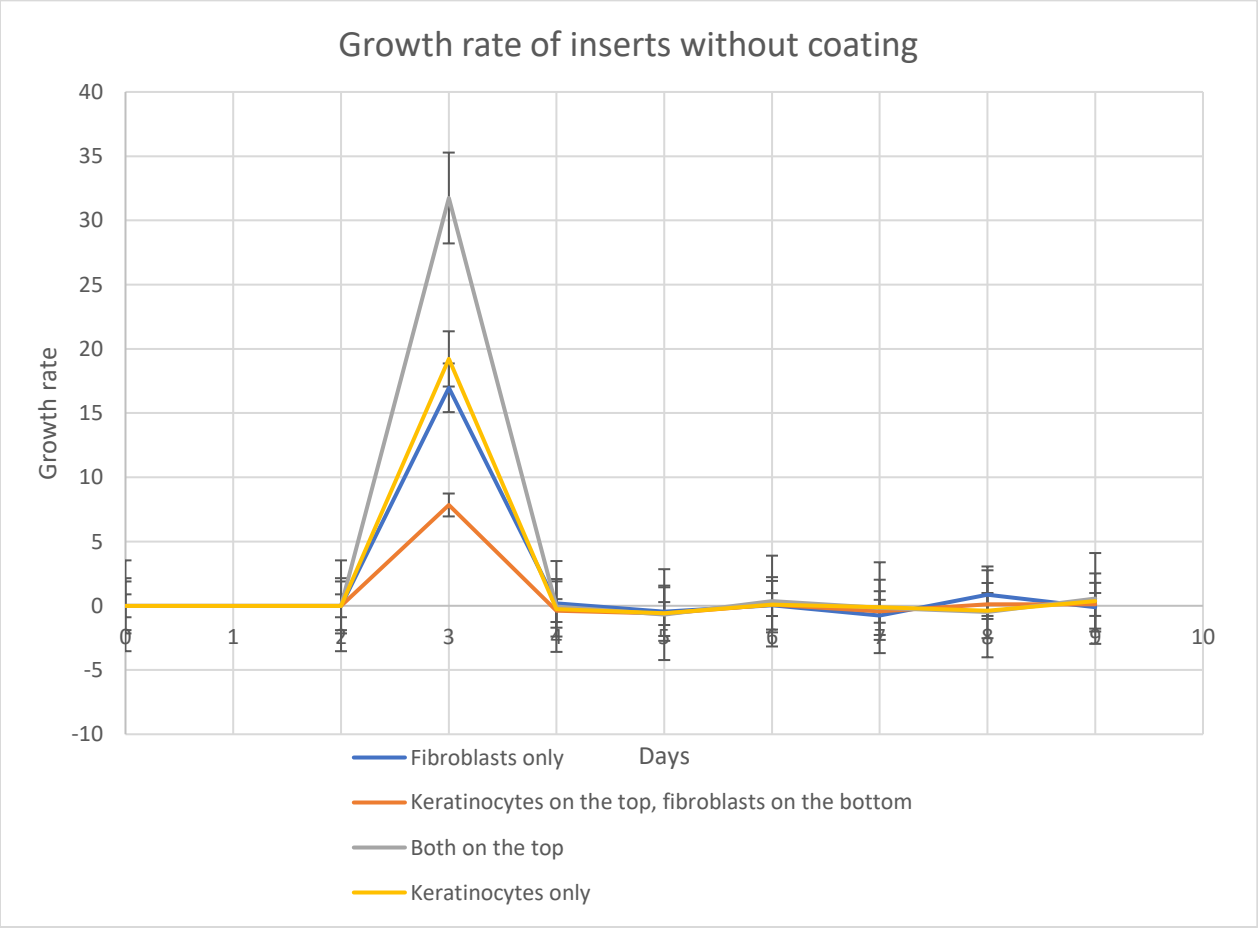


Figure 14.: Growth rate of TEER measurements of not-coated inserts.

On the other hand, results of silk coated are more variable and all the samples have negative growth rates on the last day in compartment to the first one. Probable reason might be the fact that SF coating started to detach slowly during the time, which might affect the measurement. (Figure 15.)

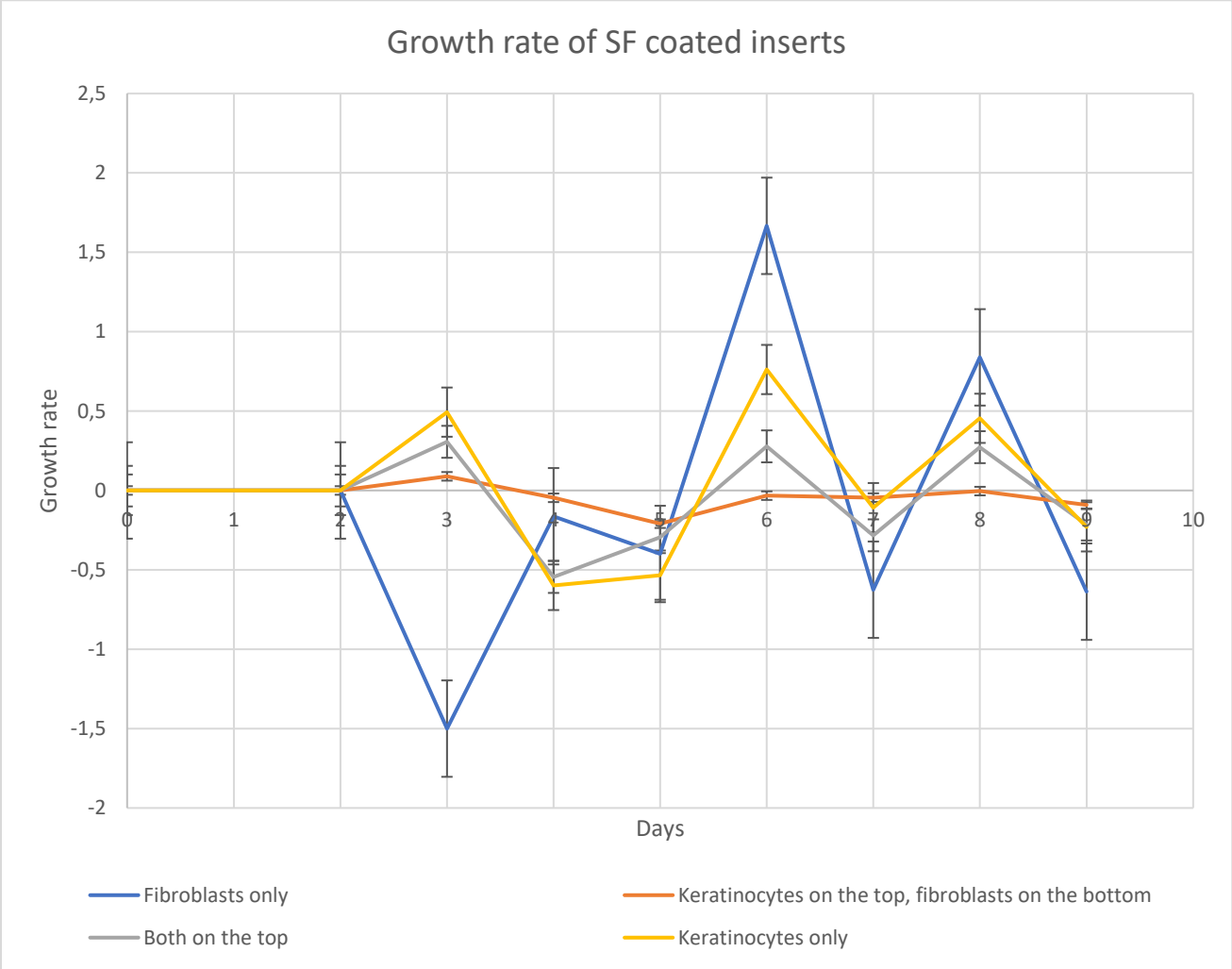


Figure 15.: Growth rate of TEER measurements of SF coated inserts.

Also, to show the influence of fibroblasts on keratinocytes growth and their ability to form TJ inserts seeded with both keratinocytes and fibroblast on the top were compared with only keratinocytes seeded in both SF coated and not-coated inserts. TEER measured in the samples with keratinocytes only have higher resistance in the inserts without SF coating, which is contradictory to our expectations. (Figure 16.) However, SF coated inserts showed higher resistance in the co-seeded

inserts, but for the last two days where the resistance is higher in keratinocytes only inserts. (Figure 17.)

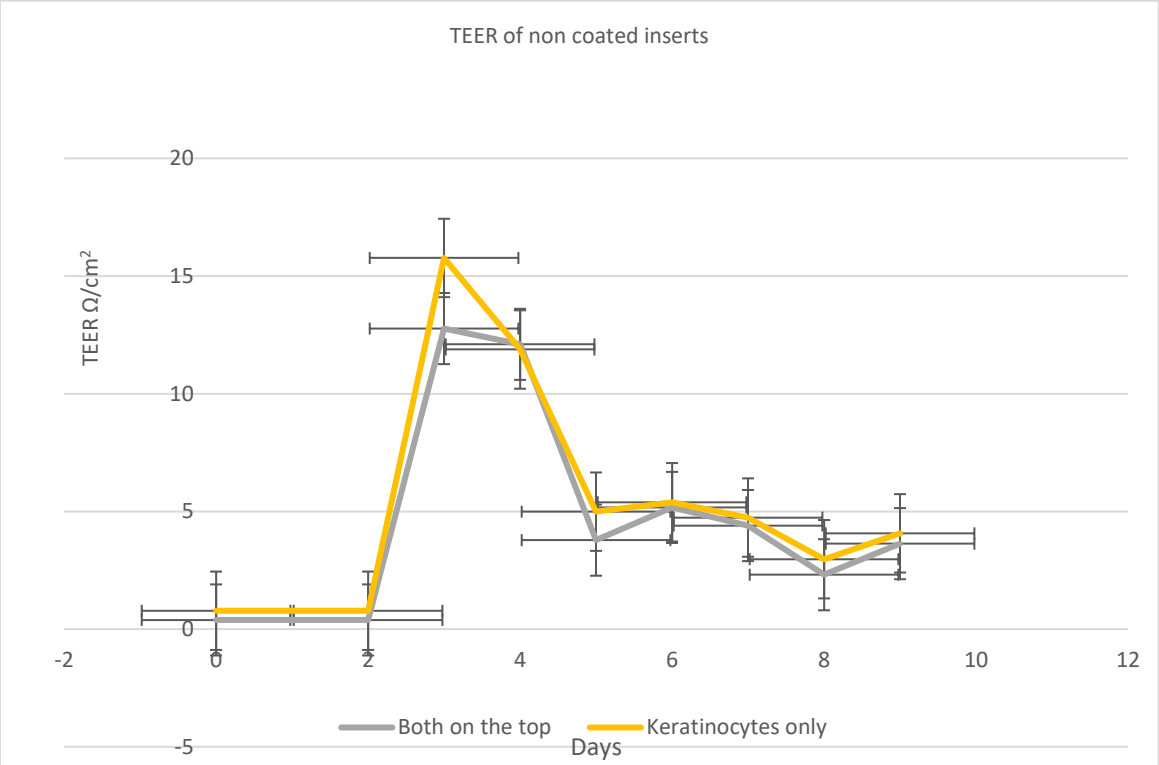


Figure 16.: A graph depicted of true TEER values to compare the difference in inserts seeded with both fibroblasts and keratinocytes on the top to inserts seeded with only keratinocytes in inserts without coating.

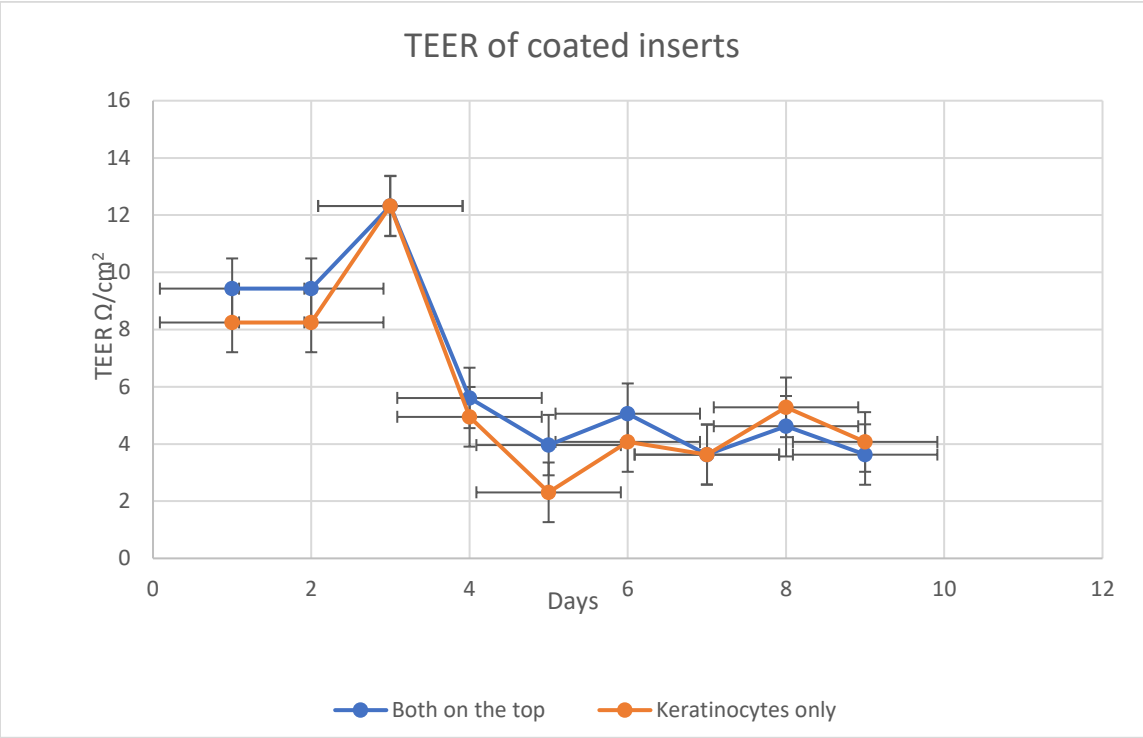


Figure 17.: A graph depicted of true TEER values to compare the difference in inserts seeded with both fibroblasts and keratinocytes on the top to inserts seeded with only keratinocytes in SF coated inserts.

5 DISCUSSION

5.1 SCAFFOLD FABRICATION AND OPTIMIZATION

Several factors are important for the fabrication of the scaffold. Pore size and porosity are important for cell attachment and the proliferation. Pore sizes are important due to the fact that large pores prevent cell attachment while small pore size enables cells to penetrate the scaffold.[26] In this study, one of the goals while fabricating the scaffolds was to achieve the optimal pore size and porosity. First, when it comes to freestanding silk films, optimal porosity was not accomplished. Yet, the porosity in this case can be enhanced by blending SF with biocompatible polymer.[27] Results have shown that freeze dried scaffolds achieved desired porosity, which was expected since recent researches have shown that the freeze drying method efficient to achieve highly porous scaffolds.[28] Also, in the study by Xie et al, elecrospun SF scaffolds formed highly interconnected, porous structure with the desirable pore size, which was in a certain level proved in this study, however, due to an extremely thin layer of electrospun SF, further manipulation was not possible.[18]

5.2 SF FOR SCAFFOLD FABRICATION

SF already showed positive properties for cell attachment and cell proliferation. The aim of this study was to test how the SF scaffolds enhance the ability of keratinocytes to form TJ. As mentioned above, different methods were used to fabricate the SF scaffolds. Yet, due to limitations, only assessment of SF coated polyester membranes was continued. In the study by Huang et al., in vitro and in vivo characterization of SF coated polyester was performed for vascular prothesis and it was shown that SF-impregnated had desirable permeability in vitro and in vivo. Also, in vivo assessment showed SF to be nontoxic and degradable.[29] In this study was shown that SF coated polyester membranes complemented cell attachment and growth. Despite, there was no clear evidence of enhanced properties compared to the membranes without coating. The intention was to perform immunostaining of the cells to visualize the cells and to confirm their epithelial origin, thus to

compare the distribution of cells between coated and not-coated inserts. As mentioned before, the staining of scaffolds in both showed no cells on the surface. Potential implication might be high number of washing steps while performing immunostaining.

5.3 Assessment of SF scaffolds for urethral reconstruction

Buccal mucosa happens to be an adequate replacement for urethral tissue, therefore, in this study properties of a scaffold seeded with keratinocytes and fibroblast were investigated. TJ play key role in permeability of the cells. Different cell seeding groups were made also to investigate the effect of fibroblasts on keratinocytes. In the study by Zhang et al. 2011., it was shown that fibroblast growth factor-peptide (FGF-P) reduced permeability and enhances cell proliferation and the barrier function of keratinocytes. [30] Therefore, it was expected that TEER measurement in the samples co-seeded with fibroblast is going to be higher, thus the expectations were met only in the samples with SF coating.

A study by Rajkhowa et al., showed that keratinocytes seeded on SF membranes showed high expression of occluding which is an indicator of formed TJ.[31] It was also expected in this study that SF coated inserts will induce the formation of TJ and the TEER values will increase by the time. However, inconsistent data might be due to detachment of SF coatings.

5.4 Limitations and future applications

First, scaffold fabrication protocol should be optimized to fabricate scaffolds consisted of SF only to truly investigate its properties. This might be done using electrospinning and freeze drying, regardless the technical difficulties experienced in the study. Secondly, to investigate the formation of TJ on SF, keratinocytes should be grown for a longer period to truly reach confluency. This would also help to see the effect of co-seeded fibroblasts on the formation of TJ. TEER values might be affected due to difficulties of manual assessment where electrodes are not stable. Stabilizing the electrodes and consistent measurement at the same place might contribute to achieve better results.

6 CONCLUSION

When it comes to scaffold fabrication, all the methods used in this study, beside free-standing silk films, had good mechanical properties and thickness and were support cell proliferation. SF showed to have desirable properties for cell growth, thus no difference between SF coated and the membranes without coating can be found. Measures of TEER showed inconsistent values over time, which might be due to detachment of the SF coating from the surface and difficulties while performing the method. Also, it was found that co-seeding with fibroblasts had effect on the formation of TJ in keratinocytes only in SF coated inserts.

7 ACKNOWLEDGMENTS

The author would like to thank Professor Peter Fojan from The Faculty of Engineering and Science, Aalborg University for the provision of the equipment. The author would also like to thank to Petra de Graaf, dr from Utrecht University for providing urothelial cells that were used in preliminary experiments.

8 REFERENCES

1. Levin TL, Han B, Little BP. Congenital anomalies of the male urethra. [cited 2017 Dec 12]; Available from: https://link.springer.com/content/pdf/10.1007%2Fs00247-007-0495-0.pdf
2. de Kemp V, de Graaf P, Fledderus JO, Ruud Bosch JLH, de Kort LMO. Tissue Engineering for Human Urethral Reconstruction: Systematic Review of Recent Literature. Zhao F, editor. PLoS One [Internet]. 2015 Feb 17 [cited 2018 Apr 6];10(2):e0118653. Available from: http://dx.plos.org/10.1371/journal.pone.0118653
3. Alwaal A, Blaschko SD, McAninch JW, Breyer BN. Epidemiology of urethral strictures. Transl Androl Urol [Internet]. 2014 Jun [cited 2018 Mar 20];3(2):209–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26813256
4. Latini JM, Mcaninch JW, Brandes SB, Chung JY, Rosenstein D. ICUD on Urethral Strictures SIU/ICUD Consultation on Urethral Strictures: Epidemiology, Etiology, Anatomy, and Nomenclature of Urethral Stenoses, Strictures, and Pelvic Fracture Urethral Disruption Injuries. 2014 [cited 2018 Apr 5]; Available from: https://pdfs.semanticscholar.org/a879/01417f583aad9e9b2e7a27964ec2deaf49b8.pdf
5. Mundy AR, Andrich DE. Urethral strictures. [cited 2018 Apr 5]; Available from: http://www.imop.gr/sites/default/files/urethralstrictures.pdf
6. Herschorn S, Elliott S, Coburn M, Wessells H, Zinman L. ICUD on Urethral Strictures SIU/ICUD Consultation on Urethral Strictures: Posterior Urethral Stenosis After Treatment of Prostate Cancer. 2014 [cited 2018 Apr 5]; Available from: https://ac-els-cdn-com.zorac.aub.aau.dk/S0090429513011175/1-s2.0-S0090429513011175-main.pdf?_tid=973b6ac4-ed83-454e-b224-18264337aaba&acdnat=1522938825_9094adb9da2d206a42ed5536cfa5d599
7. Bergman JEH, Loane M, Vrijheid M, Pierini A, Nijman RJM, Addor M, Barisic I, Béres J, Braz P, Budd J, Delaney V, Gatt M, Khoshnood B, Klungsøyr K, Martos C, Mullaney C, Nelen V, Neville AJ, O 'mahony M, Queisser-luft A, Randrianaivo H, Rissmann A, Rounding C, Tucker D, Wellesley D, Zymak-zakutnia N, Bakker MK, Hermien ·, De Walle EK, Bergman JEH, Bakker MK, De Walle HEK, Loane M, Vrijheid M, Pierini A, Nijman RJM, Addor M-C, Braz P, Budd J, Delaney V, Gatt M. Epidemiology of hypospadias in Europe: a registry-based study. World J Urol [Internet]. 2015 [cited 2018 Apr 3];33(33). Available from: https://link.springer.com/content/pdf/10.1007%2Fs00345-015-1507-6.pdf
8. Kraft KH, Shukla AR, Canning DA. Hypospadias. Urol Clin North Am [Internet]. 2010 May 1 [cited 2018 Apr 3];37(2):167–81. Available from: http://www.ncbi.nlm.nih.gov/pubmed/20569796
9. DUEL BP, BARTHOLD JS, GONZALEZ R. MANAGEMENT OF URETHRAL STRICTURES AFTER HYPOSPADIAS REPAIR. J Urol [Internet]. 1998 Jul 1 [cited 2018 Apr 6];160(1):170–1. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022534701630830
10. Waterloos M, Hoebeke P, Spinoit A-F. Hypospadias Variants. In: Congenital Anomalies of the

Penis [Internet]. Cham: Springer International Publishing; 2017 [cited 2018 Apr 3]. p. 159–66. Available from: http://link.springer.com/10.1007/978-3-319-43310-3_24

11. Tritschler S, Roosen A, Füllhase C, Stief CG, Rübben H. Urethral Stricture: Etiology, Investigation and Treatments. [cited 2018 Apr 6]; Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3627163/pdf/Dtsch_Arztebl_Int-110-0220.pdf
12. Subramaniam R, Spinoit AF, Hoebeke P. Hypospadias repair: an overview of the actual techniques. Semin Plast Surg [Internet]. 2011 Aug [cited 2018 Apr 6];25(3):206–12. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22851912
13. Springer A, Tekgul S, Subramaniam R. An Update of Current Practice in Hypospadias Surgery. Eur Urol Suppl [Internet]. 2017 Jan 1 [cited 2018 Apr 6];16(1):8–15. Available from: https://www-sciencedirect-com.zorac.aub.aau.dk/science/article/pii/S1569905616301415
14. Xue J-D, Gao J, Fu Q, Feng C, Xie H. Seeding cell approach for tissue-engineered urethral reconstruction in animal study: A systematic review and meta-analysis. Exp Biol Med [Internet]. 2016 [cited 2018 Apr 7];241:1416–28. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4994921/pdf/10.1177_1535370216640148.pdf
15. Abbas TO, Mahdi E, Hasan A, AlAnsari A, Pennisi CP. Current Status of Tissue Engineering in the Management of Severe Hypospadias. Front Pediatr [Internet]. 2018;5(January):1–8. Available from: http://journal.frontiersin.org/article/10.3389/fped.2017.00283/full
16. Xiao Y, Zhang L. Journal of Biotechnology & Biomaterials Biomatrices in Urethral Reconstruction. J Biotechnol Biomater [Internet]. 2016 [cited 2018 Apr 7];6(6). Available from: http://dx.doi.org/10.4172/2155-952X.1000231
17. Goo Chung Y, Tu D, Franck D, Seok Gil E, Algarrahi K, Adam RM, Kaplan DL, Estrada Jr CR, Mauney JR. Acellular Bi-Layer Silk Fibroin Scaffolds Support Tissue Regeneration in a Rabbit Model of Onlay Urethroplasty. PLoS One [Internet]. 2014 [cited 2018 Apr 7];9(3). Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3954771/pdf/pone.0091592.pdf
18. Xie M, Song L, Wang J, Fan S, Zhang Y, Xu Y. Evaluation of stretched electrospun silk fibroin matrices seeded with urothelial cells for urethra reconstruction. J Surg Res [Internet]. 2013 Oct 1 [cited 2018 Apr 7];184(2):774–81. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022480413003594
19. Simsek A, Bullock A, Roman S, Chapple C, Macneil S, MacNeil S. Developing improved tissue-engineered buccal mucosa grafts for urethral reconstruction. Can Urol Assoc J [Internet]. 2018 Feb 2 [cited 2018 May 16];12(5):E234-42. Available from: http://cuaj.ca/index.php/journal/article/view/4826
20. Xie M, Xu Y, Song L, Wang J, Lv X, Zhang Y. Tissue-engineered buccal mucosa using silk fibroin matrices for urethral reconstruction in a canine model. J Surg Res [Internet]. 2014 May 1 [cited 2018 Apr 7];188(1):1–7. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24411303
21. Qi Y, Wang H, Wei K, Yang Y, Zheng R-Y, Kim IS, Zhang K-Q, Hardy JG, Holland C. A Review of Structure Construction of Silk Fibroin Biomaterials from Single Structures to Multi-Level

Structures. [cited 2018 Apr 7]; Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5372488/pdf/ijms-18-00237.pdf

22. Rockwood DN, Preda RC, Yucel T, Wang X, Lovett ML, Kaplan DL. Materials fabrication from Bombyx mori silk fibroin. Nat Protoc [Internet]. 2011 Sep;6(10):1612–31. Available from: http://dx.doi.org/10.1038/nprot.2011.379
23. Kundu B, Rajkhowa R, Kundu SC, Wang X. Silk fibroin biomaterials for tissue regenerations. Adv Drug Deliv Rev [Internet]. 2013 Apr 1 [cited 2018 Apr 7];65(4):457–70. Available from: https://www.sciencedirect.com/science/article/pii/S0169409X12003572
24. Steed E, Balda MS, Matter K. Dynamics and functions of tight junctions. [cited 2018 May 16]; Available from: https://www.researchgate.net/profile/Emily_Steed/publication/281263239_Dynamics_and_functions_of_tight_junctions/links/565edb2908ae1ef92983f505.pdf
25. Wang X, Kim HJ, Xu P, Matsumoto A, Kaplan DL. Biomaterial coatings by stepwise deposition of silk fibroin. Langmuir. 2005;21(24):11335–41.
26. Garg T, Arora S, S Rayasa MR, Singh O, Murthy R. Scaffold: A Novel Carrier for Cell and Drug Delivery Create new project "Analytical method development and Validation " View project Oral Gel for Mucositis View project Scaffold: A Novel Carrier for Cell and Drug Delivery. Crit Rev Ther Drug Carr Syst [Internet]. 2012 [cited 2018 May 18];29(1):1–63. Available from: https://www.researchgate.net/publication/221853404
27. Lu Q, Hu X, Wang X, Kluge JA, Lu S, Cebe P, Kaplan DL. Water-Insoluble Silk Films with Silk I Structure. [cited 2018 May 18]; Available from: http://europepmc.org/backend/ptpmcrender.fcgi?accid=PMC2830340&blobtype=pdf
28. Nazarov R, Jin H-J, Kaplan DL. Porous 3-D Scaffolds from Regenerated Silk Fibroin. [cited 2018 May 18]; Available from: https://pubs-acs-org.zorac.aub.aau.dk/doi/pdf/10.1021/bm034327e
29. Huang F, Sun L, Zheng J. In Vitro and In Vivo Characterization of a Silk Fibroin-Coated Polyester Vascular Prosthesis. Artif Organs [Internet]. 2008 Dec [cited 2018 May 24];32(12):932–41. Available from: http://doi.wiley.com/10.1111/j.1525-1594.2008.00655.x
30. Zhang K, Tian Y, Yin L, Zhang M, Beck LA, Zhang B, Okunieff P, Zhang L, Vidyasagar S. FIBROBLAST GROWTH FACTOR-PEPTIDE IMPROVES BARRIER FUNCTION AND PROLIFERATION IN HUMAN KERATINOCYTES AFTER RADIATION. Radiat Oncol Biol [Internet]. 2011 [cited 2018 May 24];81:248–54. Available from: https://ac-els-cdn-com.zorac.aub.aau.dk/S0360301611002343/1-s2.0-S0360301611002343-main.pdf?_tid=6594a713-f95c-446d-8d4b-577e7860b15b&acdnat=1527153943_8642fd9d383a6bf835e7d4d9a1c2ef49
31. Rajkhowa R. Utilising silk fibroin membranes as scaffolds for the growth of tympanic membrane keratinocytes, and application to myringoplasty surgery. Artic J Laryngol Otol [Internet]. 2012 [cited 2018 May 24]; Available from: https://www.researchgate.net/publication/230670548

APPENDICES

APPENDIX 1

Protocol for cell seeding

Materials:

1. Frozen cells
2. Base Medium for the cell type seeded.
3. Antibiotics solution: Pen/Strep; Pen: 10,000 /mL, Strep: 10 mg/mL
4. Additional supplements based on cell type
5. Sterile 500mL bottle
6. Centrifuge tubes (50mL)
7. T75 culture flasks
8. Counting chamber

Procedure:

1. Prepare the growth medium. Take a sterile bottle with volume of 500 mL and place a filter on the top. Add 450 mL of medium and add 5mL of antibiotics (P/M) and 50mL of FCS and additional supplements and filter under vacuum. Set aside 5 mL for the further centrifugation and 15 ml for the TP75 flask in which cells will be cultured.
2. Place the frozen cells in the water bath with temperature of 37°C.
3. Place 15 mL of the medium in TP75 flask and store it in the incubator to avoid thermal shock of the cells.
4. Place 5 mL of the medium in the centrifuge tube. Centrifuge the unfrozen cells with the 5 mL of medium at 20ºC and 1200rpm for 5 minutes.
5. Perform cell counting if needed.
6. Wash out the medium except the pellet containing the cells. Add 1 ml of the medium and then pipette up and down and move the cells into the T75 flask.
7. Label the T75 flask.
8. Put the T75 flask/s containing the cells into the incubator until the next media change.

Media change:

Every 2 or 3 days, the cells were replenished with new medium until the cells reach 80–90% confluence (approx. 5×10^5 cells per flask) by taking out the previous media and adding another 15 mL of cell culture media.

Cell counting procedure:

Materials:

1. 0.4 % solution of trypan blue
2. Counting chamber

Procedure:

1. Take 10 µl of cell suspension and mix it with 10 µl of 0.4 % solution of trypan blue.
2. Fill both Bürker-Türk counting chambers underneath the coverslip.
3. Put the hemocytometer under the microscope.
4. Focus on the grid lines of the hemocytometer with a 10x objective.
5. Using a hand tally counter, count the live, unstained cells in one set of 16 squares.
6. Move the hemocytometer to the next set of squares and continue counting until all 4 sets of 16 squares are counted.
7. Calculate the average of all 16 square counts, multiply by 10^4.

APPENDIX 2

Cell fixation

Materials:

1. PBS
2. Formalin

Procedure:

1. Remove the PBS from the wells
2. Add 0.5 mL of formalin/well to fix the cells/ECM. Leave for 15 minutes at 37 °C.
3. Remove formalin and wash with cells with PBS.
4. Add PBS (1 mL/well) and store the samples in the fridge.

APPENDIX 3

Cell trypsynisation protocol

Matherials:

1. T-75 flask
2. Trypsin/EDTA solution
3. Media
4. PBS
5. Centrifuge tubes
6. Counting chamber

Procedure:

7. Warm enough amount of medium for whole protocol.
8. Remove the cell medium from the T-75 flask.
9. Mix 10 ml of Trypsin and 10 ml EDTA in a tube.
10. Put 10 ml of PBS to remove all traces of serum.
11. Take 1.5ml of trypsin/EDTA solution previously prepared, add it to the cell culture cells in the T-75 flask and incubate it for 2-3min.
12. Microscope to check if all cells are detached from the surface.
13. Add 5 ml of medium to stop the trypsinization.
14. Aspirate the cell and place them into a new tube and centrifuge (5min, at 1200G, 2oC).
15. Remove the supernatant (in one move) and add 1 mL of fresh medium to resuspend the cells.

Freezing protocol:

Materials:

1. Freezing medium: : normal medium + 10% FCS + 7,5% DMSO (1ML PER VIAL, 2ML FOR TWO)
2. Centrifuge tubes
3. Small tubes for freezing

After the trypsinization process, count cells and the number of the cells to freeze should be 1 million.

Procedure:

1. Centrifuge the cells on 1200RPM, at 20°C for 5min.
2. Resuspend the cells in 1mL of freezing medium.
3. Aspirate the cells and put them into the tube for freezing.
4. Write the name, date, number of cells and passage number on the tube.

APPENDIX 4

Freeze drying protocol

Materials:

- 70% ethanol
- Moulds
- 6% silk fibroin
- Freeze dryer

Procedure:

1. Add silk fibroin in the moulds
2. Leave it to dry on air in the bench, at room temperature for 3h.
3. Put it together with the moulds in the freezer overnight.
4. Turn on the pump on the freeze dryer to warm it up.
5. When the pump is ready, take the samples and leave them for 24h. Note: ensure that the optimal vacuum is reached.

APPENDIX 5

Electrospinning protocol

Materials:

1. Glass scintillation vials (20 ml)
2. Small stir bar
3. Hot plate
4. Syringe (10 ml)
5. Blunt-tip needle

6. Syringe pump
7. High voltage supply
8. Nonstick aluminum foil
9. Reciprocating shaker

Protocol:

Electrospun fibers:

1. Prepare a solution of 5% (wt/vol) PEO in water in a 20-ml glass scintillation vial. Use a small stir bar to ensure proper mixing. NOTE: The PEO will take time to dissolve into solution, leave it to stir at 4 °C at least overnight. This solution can be stored at 4 °C for 1 year.
2. Place the silk on a stirrer at 4 °C and add 5 ml of the 5% (wt/vol) PEO (900,000 g mol−1) solution into 20 ml of 8% (wt/vol) silk solution to generate an aqueous solution of 6.4% (wt/vol) silk/1% (wt/vol) PEO. This may vary, the final aqueous solution should be 6.4. % (wt/vol)silk/1%(wt/vol)PEO. NOTE: A high viscosity, cut the pipette tip for easier pipetting and slowly add drop by drop while it is stirring. This will take few hours.
3. Draw up 10 ml of the silk/PEO solution into a 10 ml syringe.
4. Mount the syringe on the syringe pump.
5. Place the tip of the needle 7–20 cm from a collection surface. Place aluminum foil as a collection surface, also glass slides (optional).
6. Attach the positive voltage lead to the needle on the syringe and the ground lead to the collection surface. Set the current to slightly above 0 A.
7. Turn on the high voltage and syringe pump. NOTE: Be careful while the high voltage is on and do not touch charged surfaces such as the needle or positive voltage lead.
8. Adjust the solution flow rate (0.01–0.03 ml min−1), electric potential (8–15 KV) and the distance between the capillary tip and the collection screen (7–20 cm) to obtain a stable jet. NOTE: This may vary.
9. Collect the silk fibers until the desired thickness is achieved.
10. Immerse the fiber mats in a 90% (vol/vol) methanol/water solvent for 20 min to obtain water-insoluble fiber mats.
11. Incubate the methanol-treated fiber mats in ultrapure water on a reciprocating shaker overnight to remove the PEO from the mats.
12. Dry the fiber mats in a chemical fume hood.

APPENDIX 6

SF coating

Materials:

1. Inserts
2. Silk fibroin (6%)
3. 1:1 methanol/water
4. Liquid nitrogen source

Procedure:

1. Add 100µ of SF on the insert and let dry on air, at room temperature for 2min.
2. Wash with ethanol/water for 1min.
3. Dry it for 2min under gentle flow of liquid nitrogen until dry.
4. Repeat process until desired number of layers is reached.
5. Wash the inserts with PBS and store them in PBS until further use.

APPENDIX 7

Transepithelial electrical resistance protocol

Matherials:

1. Millicell® ERS-2 Voltohmmeter
2. Electrodes
3. 50mL centrifuge tubes
4. 70% ethanol
5. Media

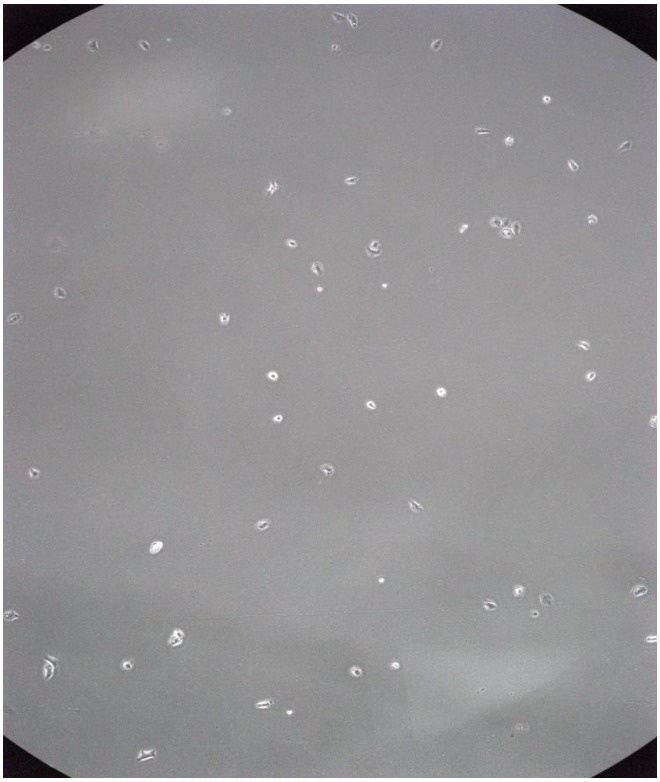
Procedure:

1. Make sure that the voltohmmeter is fully charged.
2. Soak electrodes in 70% ethanol for 15min.
3. Let the electrodes dry and soak them into medium.
4. Measure first the control well without cells.
5. Repeat the measurement 3 times. NOTE: always measure at the same corner/place.

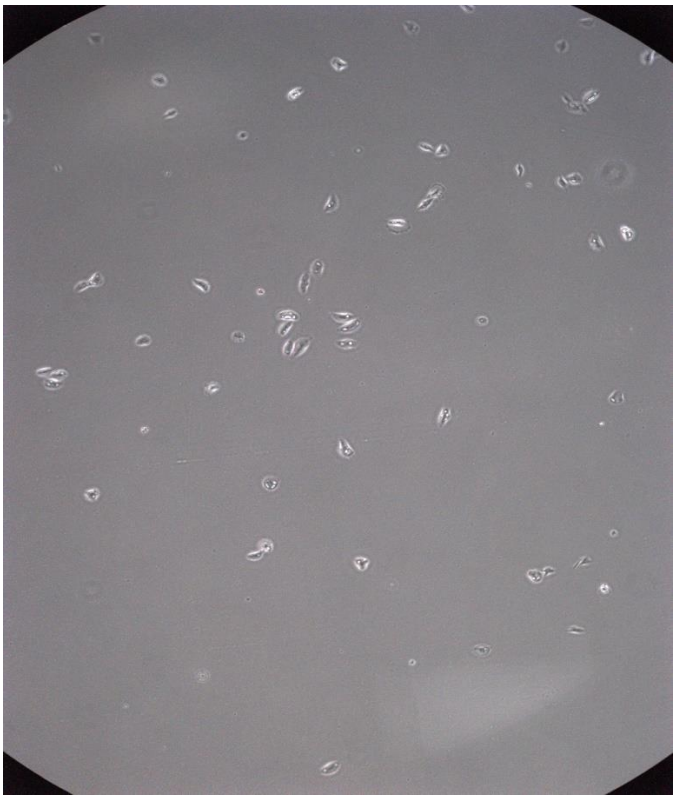
6. Calculate the resistance by using formula: TEERREPORTED= TEERMEASURED (Ω) x MAREA(cm2)

APPENDIX 8

Preliminary results, expansion of urothelial cells



Picture 1.: Picture of urothelial cells line taken with phase contrast microscope, passage 6, day 1.



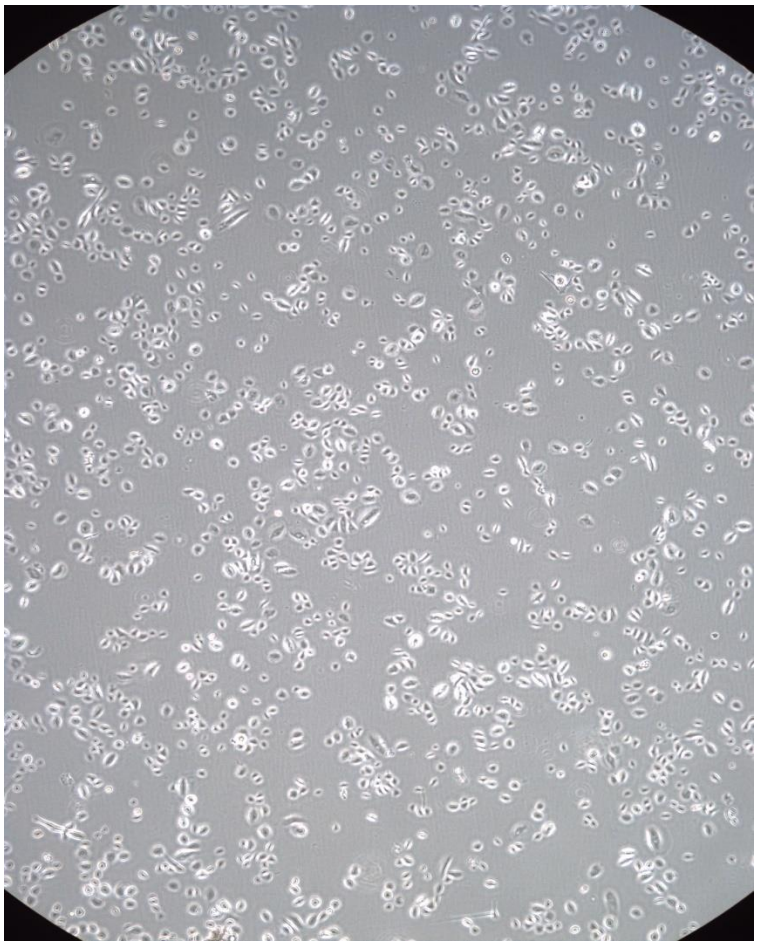
Picture 2.: Picture of urothelial cells line taken with phase contrast microscope, passage 6, day 3.

Picture 3.: Picture of urothelial cells line taken with phase contrast microscope, passage 6, day 6.

APPENDIX 9

Expansion of keratinocytes and fibroblasts

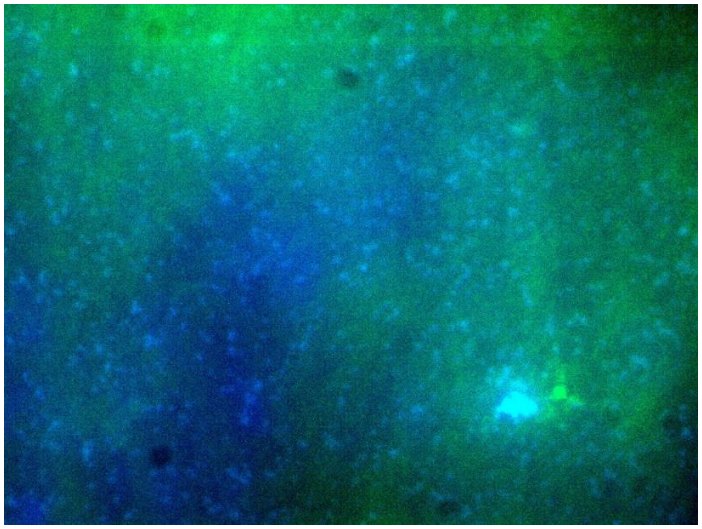
Picture 1.: Picture of fibroblast cells line taken with phase contrast microscope, passage 7, day 5.



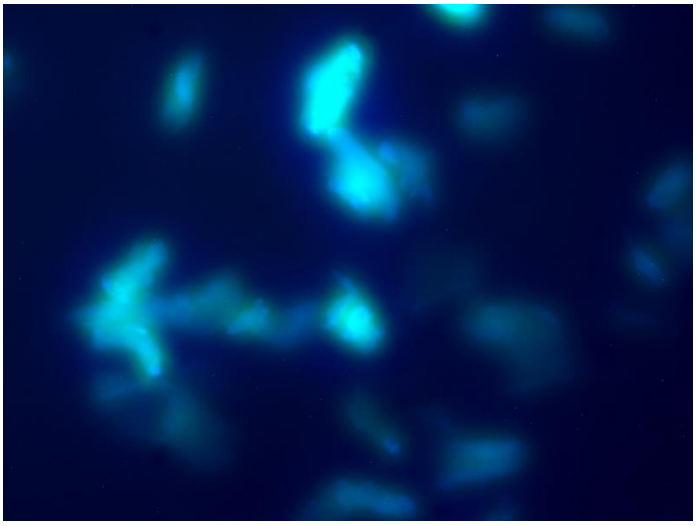
Picture 2.: Picture of keratinocytes cells line taken with phase contrast microscope, passage 3, day 6.

APPENDIX 10

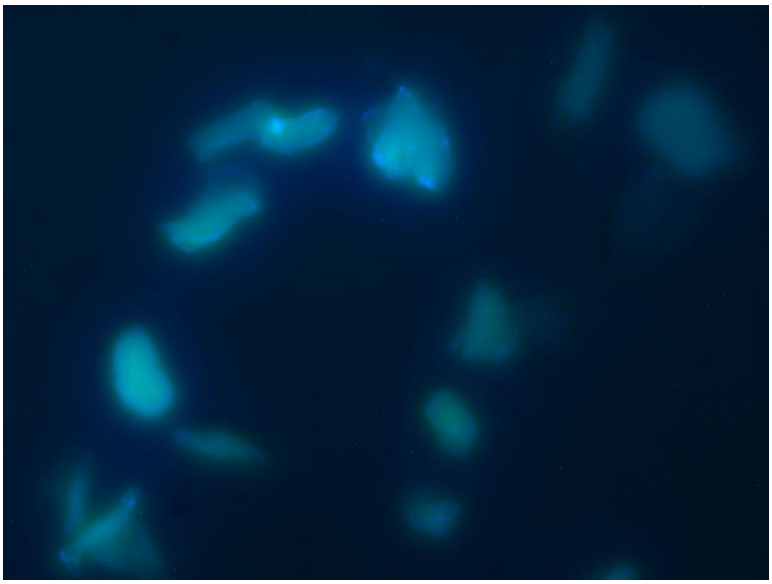
Pictures of immunostaining of inserts



Picture 1.: Immunostaining of the bottom of the well plate that contained SF coated inserts seeded with both keratinocytes and fibroblasts on the top. Green color presents PAN cytokeratin stain while blue presents cell nuclei stained with Hoechst 33342.



Picture 2.: Immunostaining of the bottom of the well plate that contained SF coated inserts seeded with keratinocytes on the top and fibroblasts on the bottom. Green color presents PAN cytokeratin stain while blue presents cell nuclei stained with Hoechst 33342.



Picture 3.: Immunostaining of the bottom of the well plate that contained insert without coating seeded with both keratinocytes and fibroblasts on the top. Green color presents PAN cytokeratin stain while blue presents cell nuclei stained with Hoechst 33342.