Characterizing and optimizing poly-L-lactide-co-ε-caprolactone membranes for urothelial tissue engineering

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Different synthetic biomaterials such as polylactide (PLA), polycaprolactone and poly-L-lactide-co-ε-caprolactone (PLCL) have been studied for urothelial tissue engineering, with favourable results. The aim of this research was to further optimize the growth surface for human urothelial cells (hUCs) by comparing different PLCL-based membranes: smooth (s) and textured (t) PLCL and knitted PLA mesh with compression-moulded PLCL (cPLCL). The effects of topographical texturing on urothelial cell response and mechanical properties under hydrolysis were studied. The main finding was that both sPLCL and tPLCL supported hUC growth significantly better than cPLCL. Interestingly, tPLCL gave no significant advantage to hUC attachment or proliferation compared with sPLCL. However, during the 14 day assessment period, the majority of cells were viable and maintained phenotype on all the membranes studied. The material characterization exhibited potential mechanical characteristics of sPLCL and tPLCL for urothelial applications. Furthermore, the highest elongation of tPLCL supports the use of this kind of texturing. In conclusion, in light of our cell culture results and mechanical characterization, both sPLCL and tPLCL should be further studied for urothelial tissue engineering.

Keywords: urothelial tissue engineering; poly-L-lactide-co-ε-caprolactone; PLCL characterization; urothelial cell characterization

1. INTRODUCTION

Urothelial tissue engineering may be a future method in the reconstructive surgery of urothelial defects caused by, for instance, strictures, traumas or congenital abnormalities, such as hypospadias [1]. Traditionally, these defects have been repaired surgically using the patient’s genital tissue as a graft but, in more severe cases, additional distant graft tissue, such as buccal mucosa, is needed. These techniques are prone to complications; therefore, the development of novel reconstruction techniques by means of tissue engineering is essential. In future clinical treatments, autologous human urothelial cells (hUCs) obtained, for instance, from bladder washing [1,2] could be used.

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After cell expansion in vitro, the hUCs are seeded on the biomaterial and the graft is used to reconstruct the urothelium that is lacking. Although these techniques would require two separate operations, it would be acceptable because patients with severe hypospadias usually require more than one surgical procedure [3].

The selection of an appropriate biomaterial for urothelial tissue engineering is critical, because the biomaterial should mimic the natural basement membrane of the urothelium as closely as possible and allow the underlying stroma to attach to the biomaterial. The mechanical properties should be adequate to prevent collapse of the constructed urethra, while being elastic enough to form tubular structures. Further, the biomaterial should be biocompatible, and degrade while the urothelium regenerates without excessive inflammation reaction. Also, from
a surgical perspective the biomaterial should be suturable and relatively easy to handle [4,5]. Lately, poly-α-hydroxy acid-based biomaterials, such as polylactide (PLA), polyglycolide (PGA) and polycaprolactone (PCL), have been used in urothelial tissue engineering, with favourable results [6–8]. However, the mechanical properties of PGA or PLA membranes are not optimal because these materials are inelastic and have too high strength for the intended application. Additionally, the degradation rate of PGA is relatively rapid for urothelial tissue engineering [9–11]. Although PCL is highly elastic and strain is more than 700 per cent at breakage [10], the degradation rate of PCL is rather slow for urothelial tissue engineering, taking up to 2 years [10,12]. Various poly-γ-lactide-co-ε-caprolactone (PLCL) compositions have been studied in tissue engineering applications [13–18], but the main focus has been on producing electrospun nanofibrous membranes. In our recent study [19], we showed that compression-moulded smooth PLCL membranes supported the proliferation and differentiation of hUCs better than human amniotic membrane. To the best of our knowledge, apart from our previous study, there are no other publications using this compression-moulded highly elastic PLCL copolymer for urothelial tissue engineering.

The aim of this study was to further develop and characterize the PLCL matrix for urothelial tissue engineering applications. In this study, we compared the mechanical properties of different lactide-based biomaterial membranes: smooth PLCL (sPLCL) and textured PLCL (tPLCL), and knitted PLA mesh with compression-moulded PLCL (cPLCL). In addition, we compared the effects of these materials on hUC morphology, viability and phenotype maintenance in vitro. Furthermore, we hypothesized that the topographically textured membranes tPLCL and cPLCL would enhance the hUCs’ attachment and proliferation in vitro owing to the fact that surface texturing is generally known to facilitate cellular adhesion and proliferation [20–22]. The PLCL membranes are highly elastic and flexible; however, it has not been shown whether plain PLCL has adequate mechanical stability for urothelial applications. Therefore, we also studied the effect of a knitted PLA mesh on the degradation and mechanical behaviour of PLCL, and the usability of this kind of structure for urothelial applications [10].

2. MATERIAL AND METHODS

2.1. Materials

The polymer used for PLCL membranes was manufactured from 70/30 PLCL (Purac Biochem BV, Gorinchem, The Netherlands), with an inherent viscosity of 1.60 dl g⁻¹. The polymer used for knitted composite membranes was manufactured from polylactide (P(L/D)L A 96/4) (Purac Biochem BV), with an inherent viscosity of 2.12 dl g⁻¹.

2.2. Sample manufacturing

The tubular single jersey knitting made of PLA (P(L/D)L A 96/4) was produced on a circular knitting machine ELHA R-1s (Textilmaschinenfabrik Harry Lucas GmbH & Co. KG, Neumünster, Germany) from 16-filament fibres with a single filament thickness of 10–20 μm. The PLCL membranes were produced by compression moulding of PLCL granules using a NIKE hydraulic press (Hydraulics Ab, Eskilstuna, Sweden). Also, the PLA knitting used for composite membranes underwent preliminary heat pressing as described in table 1. The preliminary moulding of PLCL was performed between polytetrafluoroethylene (PTFE)-taped moulds. The preliminary moulding was followed by the final moulding into sPLCL (figure 1) with a final thickness of 190 μm; tPLCL (figure 1) with a final thickness of 120 μm; and cPLCL (figure 1) with a final thickness of 150 μm (table 1). The cPLCL samples were produced in three steps as the two preliminarily moulded components (the PLCL and PLA membranes) were compression moulded together into the composite membranes (table 1). Afterwards, the membranes were cut into samples, washed with ethanol and sterilized at 25 kGy before further in vitro experiments and characterization.

2.3. Material characterization

2.3.1. Hydrolysis

The hydrolysis was carried out at 37°C in a phosphate buffer solution (pH 6.1) to mimic the pH of urine. The size of samples was 10 × 50 mm (see thicknesses in table 1), and the weight was approximately 100 mg. The volume of buffer was above the required minimum (10 ml) for each sample (according to the International Standard, ISO 15814, 1999). The samples (n = 6) were incubated for 0, 2, 4, 6, 8, 10 and 12 weeks. The buffer solution was changed every two weeks, and pH was measured weekly using a SevenMulti pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland). Once the samples were removed from the solution, they were weighed wet and then tensile tested. After tensile testing, the samples were dried first in a fume chamber for a week and subsequently in a vacuum for

<table>
<thead>
<tr>
<th>pressure (MPa)</th>
<th>temperature (°C)</th>
<th>time (s)</th>
<th>used moulds</th>
<th>membrane thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>preliminary moulding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLCL</td>
<td>10</td>
<td>110</td>
<td>90</td>
<td>PTFE</td>
</tr>
<tr>
<td>PLA 96/4</td>
<td>10</td>
<td>95</td>
<td>60</td>
<td>plain steel</td>
</tr>
<tr>
<td>final moulding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sPLCL</td>
<td>20</td>
<td>130</td>
<td>30</td>
<td>plain steel</td>
</tr>
<tr>
<td>tPLCL</td>
<td>20</td>
<td>130</td>
<td>45</td>
<td>PTFE</td>
</tr>
<tr>
<td>cPLCL</td>
<td>20</td>
<td>130</td>
<td>45</td>
<td>plain steel</td>
</tr>
</tbody>
</table>
a week at room temperature, after which the samples were weighed again when dry.

2.3.2. Tensile testing
For wet samples \((n = 6)\), after hydrolysis, the tensile testing was performed—and for dry samples prior to hydrolysis (zero-week samples). The tensile testing was performed with an Instron 4411 materials testing machine (Instron Ltd, High Wycombe, UK) at a cross-head speed of 30 mm min\(^{-1}\). Pneumatic grips were used, and the gauge length was 25 mm.

As controls, sheep bladder samples \((n = 6)\) were also tensile tested. The sheep bladder was washed with physiological saline and cut into 10 \(\times\) 50 mm samples, prior to testing.

2.3.3. Differential scanning calorimetry
A differential scanning calorimeter (DSC Q 1000; TA Instruments, New Castle, DE, USA) was used to determine the glass transition temperatures \((T_g)\) of the samples. Samples (weight 5 mg) were heated from \(-50^\circ\)C to \(150^\circ\)C at a heating rate of 20 \(^\circ\)C min\(^{-1}\) \((n = 2)\).

2.4. Cell isolation
The protocol by Southgate et al. [23] was used for cell isolation with minor modifications, and the isolation was performed as described previously [19]. Briefly, the tissue samples were cleaned and cut into small pieces and incubated overnight, to loosen the urothelial layer, in a solution containing 0.01 per cent HEPES buffer (1 M, \(N^\prime\)-2-hydroxyethylpiperazine-\(N^\prime\)-2-ethanesulphonic acid; Sigma-Aldrich), 4 \(\times\) 10\(^{-3}\) per cent aprotin (1 kIU \(\mu\)l\(^{-1}\); Sigma-Aldrich), 0.1 per cent EDTA (Sigma-Aldrich), 0.01 per cent penicillin/streptomycin (Lonza, Verviers, Belgium) in Hank’s balanced salt solution (Invitrogen, Paisley, UK) without \(Ca^{2+}\) and \(Mg^{2+}\). The next day, 0.1 per cent trypsin (Lonza) was used to detach the cells from the urothelial sheet. The isolated cells were suspended in a defined urothelium medium (EpiLife, Invitrogen) and cultured in CellBIND T75 flasks (Sigma-Aldrich) at \(37^\circ\)C under a humidified atmosphere of 5 per cent \(CO_2\) in air. The hUC passages 3 and 4, from three male donors, were used in the experiments.

2.5. Flow cytometric marker expression analysis of human urothelial cells
The hUCs were harvested and analysed after primary culture by a fluorescence-activated cell sorter (FACSAria; BD Biosciences, Erembodegem, Belgium), as described previously [19]. Monoclonal antibodies (MAbs) against CD44-PE, CD73-PE, CD105-PE, CD133-PE, CD166-PE (BD Biosciences), CD326-APC (Miltenyi Biotech, Bergisch Gladbach, Germany) and keratin8/18 (Cell Signaling Technology, Danvers, MA, USA) were used. MAb keratin8/18 was conjugated with IgG-alexa488 (Molecular Probes, Eugene, OR, USA). The analysis was performed on 10 000 cells per sample, and unstained cell samples were used to compensate for the background autofluorescence levels. The positive expression was defined as more than 50 per cent expression level.

2.6. Cell seeding
Before seeding the cells, biomaterial membranes were attached to the cell crowns (CellCrown48; Scaffdex, Tampere, Finland), after which the samples with cell crowns were attached to a 48-well plate leading to a 0.4 cm\(^2\) cell culture surface area. The membranes were preincubated in urothelium medium at \(37^\circ\)C for 48 h. The cells were seeded onto each membrane at a density of 30 000 cells cm\(^{-2}\) in a medium volume of 30 \(\mu\)l. The cells were allowed to attach for 2 h, which after 0.4 ml of medium was added to each well.

2.7. Scanning electron microscopy imaging
Scanning electron microscopy (SEM) was used to evaluate the attachment and morphology of hUCs after 2 h, 7 days and 14 days of cell culture. After washing with Dulbecco’s phosphate-buffered saline (DPBS), the cells were fixed with 5 per cent glutaraldehyde.
(Sigma-Aldrich) in 0.1 M phosphate buffer (pH 7.4, Sigma-Aldrich) at room temperature for 48 h. Thereafter, the samples were dehydrated through a sequence of increasing concentrations (30%, 50%, 70%, 80%, 90%, 95% and 100%) of ethanol for 5 min. The final dehydration in 100 per cent ethanol was repeated, followed by critical point drying with liquid CO2. A gold coating was sputtered on the sample, and the coated samples were examined with an SEM (Jeol JSM 6335F; Jeol Ltd, Japan) device. The SEM imaging was repeated twice, using two different cell donors.

2.8. Cell viability and proliferation

Live/dead fluorescent staining was used to evaluate cell viability after 7 and 14 days of cell culture, as described previously [19]. Briefly, the cells were incubated at room temperature for at least 30 min with a mixture of 0.25 μM calcine AM (green fluorescence; Molecular Probes) and 0.3 μM ethidium homodimer-1 (red fluorescence; EthD-1; Molecular Probes) in DPBS. A fluorescence microscope (Olympus IX51S8F-2; camera DP71) was used to image the viable cells (green fluorescence) and dead cells (red fluorescence).

The cell proliferation was determined by WST-1 analysis, measuring the mitochondrial activity of viable hUCs, after 2 h, 7 days and 14 days of cell culture. Briefly, the cells were washed with DPBS and incubated with 50 μl of premixed WST-1 (Promix WST-1 Cell Proliferation Assay System; Takara Bio Inc., Otsu, Shiga, Japan) and 500 μl of DPBS at 37°C for 1 h. Absorbance was measured with a microplate reader (Victor 1420 Multilabel Counter; Wallac, Turku, Finland) at 450 nm.

2.9. Phenotype characterization of human urothelial cells using immunostaining

Immunostaining with primary antibodies, cytokeratin (CK) 7 (1 : 400; Epitomics, CA, USA) and CK19 Ab-1 (1 : 500; Lab Vision, Fremont, CA, USA) was used to confirm the phenotype of hUCs after 7 and 14 days of cell culture, as previously described [19]. Briefly, the hUCs were fixed with 4 per cent paraformaldehyde (Sigma-Aldrich) and incubated overnight in primary antibody dilutions. Thereafter, secondary antibodies from donkey (1 : 400; Alexa-488; green fluorescence; Molecular Probes) were conjugated to primary antibodies. Finally, cell nuclei were stained with Vectashield (DAPI; blue fluorescence; Vector Laboratories, Peterborough, UK), and the cells were imaged with a fluorescence microscope (Olympus).

2.10. Statistical analysis

Statistical analysis was performed with SPSS v. 13 (SPSS, Chicago, IL, USA). After verifying normal distribution and homogeneity of variance, the effect of the culturing period and the effects of different materials on cell number were compared using one-way ANOVA. Post hoc (Tukey) tests were performed to detect significant differences between the culturing periods (2 h versus 7 days and 7 days versus 14 days) and between the different materials. Data were reported as the mean ± standard deviation (s.d.); p < 0.05 was considered significant.

3. RESULTS

3.1. In vitro degradation (and tensile testing)

The pH of the samples remained within the limits (6.05–6.15) that are given in the standard, throughout the hydrolysis for 12 weeks.

3.1.1. Dimensional stability during hydrolysis

The samples retained their structural stability during the hydrolysis. Minor variations in their dimensions could be detected after 12 weeks of hydrolysis (table 2). However, the sPLCL and tPLCL samples became fragile to handle after 10 weeks in hydrolysis. Also, both sPLCL and tPLCL were greatly degraded after the 12-week hydrolysis, and the samples became fragile and difficult to handle without complete disintegration. The composite samples (cPLCL) retained their structure best, as the knitted PLA mesh remained stable throughout the hydrolysis for 12 weeks. However, the PLCL membrane component in the composite samples was degraded at the 12-week time point, and fragmentation in the cPLCL membrane was observed between the PLA mesh loops.

Moderate dimensional changes (2–3%) were observed by week 6 (data not shown). Most of the dimensional changes detected during the hydrolysis were due to an increase or a decrease in thickness. After eight weeks of hydrolysis, the samples showed an increase of 7, 11 and 14 per cent from their original thickness for sPLCL, tPLCL and cPLCL samples, respectively. After eight weeks, the thickness started to decrease and, at the end of the hydrolysis at week 12, the thickness of the samples was close to their original values.

3.1.2. Weight change during hydrolysis

The weight of the tPLCL samples started to decrease after six weeks of hydrolysis and, for the sPLCL and cPLCL samples, after eight weeks of hydrolysis (data not shown). After 10 weeks of hydrolysis, the weight of the samples had decreased by 10 per cent for sPLCL, by 30 per cent for tPLCL and by 20 per cent for cPLCL samples. At the end of the hydrolysis, the weight of the samples had further decreased by 10 per cent for all samples when compared with the 10-week values.

3.1.3. Mechanical properties during hydrolysis

As the graphs in figure 2 indicate, the initial mechanical properties of all the samples decreased after sterilization. The sterilization affected the mechanical properties of sPLCL and tPLCL samples to the greatest extent. The stresses at maximum loads of the samples were 21.3, 18.6 and 13.9 MPa for sPLCL, tPLCL and cPLCL samples, respectively, after sterilization (figure 2). During the hydrolysis for 12 weeks, the stresses at maximum loads of the sPLCL and tPLCL samples decreased steadily and, after six weeks of hydrolysis, the maximum loads of the samples had decreased to 5.7 MPa for the sPLCL and 5.9 MPa for the tPLCL samples. Owing to the degree of degradation, 10 weeks...
was the last time point for mechanical testing of tPLCL. The stresses at maximum loads of the cPLCL samples remained steady until week 6, but, after that, the values also started to decrease and, after 10 weeks, the stress was 5.1 MPa. The stress at maximum load of the sheep bladder samples was 0.16 ± 0.03 MPa.

The initial maximum loads of the samples were 38.7, 23.2 and 20.1 N for the sPLCL, tPLCL and cPLCL samples, respectively, after sterilization (figure 2). During the hydrolysis for 12 weeks, the maximum load of all the samples studied followed the same trend as the stress at maximum load values. After 10 weeks, the maximum load values were 2.0 N for the sPLCL and 1.3 N for the tPLCL samples. The maximum load values of the cPLCL samples at week 10 was 7.7 N and remained constant until week 12.

The maximum load of the sheep bladder samples was 3.6 ± 0.6 N.

After sterilization, the modulus values of the sPLCL and tPLCL samples remained steady at 70 MPa throughout the hydrolysis (figure 2). However, the cPLCL samples had a modulus value of 300 MPa until week 8. After week 10, the modulus values of the cPLCL samples also dropped to 137.9 MPa. The modulus of the sheep bladder samples was 0.45 ± 0.12 MPa.

The strain values at maximum loads of sPLCL and tPLCL samples were between 200 per cent and 350 per cent at the beginning of the hydrolysis. Interestingly, the strain values at maximum load decreased after week 4, and, at week 6, the strains were 35 per cent for both samples. After that point, the strain values of sPLCL and tPLCL samples dropped steadily.

Table 2. A summary of mechanical characterization and cell study results. $T_g$, glass transition temperature; minor change, maximum of 5%; ++++, good; ++, average; +, low.

<table>
<thead>
<tr>
<th>sample</th>
<th>dimensional change during hydrolysis</th>
<th>weight change during hydrolysis</th>
<th>$T_g$ change during hydrolysis</th>
<th>mechanical properties (max load; stress at max load) compared with natural model$^a$</th>
<th>strain compared with natural model$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>sPLCL only minor changes during 12 weeks</td>
<td>only minor changes until week 8</td>
<td>steady drop of $T_g$ during 12 weeks</td>
<td>better than natural model until week 10</td>
<td>better than natural model until week 4</td>
<td></td>
</tr>
<tr>
<td>tPLCL only minor changes until week 6</td>
<td>only minor changes until week 8</td>
<td>steady drop of $T_g$ during 12 weeks</td>
<td>better than natural model until week 10</td>
<td>better than natural model until week 4</td>
<td></td>
</tr>
<tr>
<td>cPLCL only minor changes until week 6</td>
<td>only minor changes until week 6</td>
<td>steady drop of $T_g$ during 12 weeks</td>
<td>better than natural model during 12 weeks</td>
<td>lower than natural model even prior to hydrolysis</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>sample</th>
<th>cell adhesion</th>
<th>cell viability</th>
<th>cell morphology and confluency</th>
<th>proliferation</th>
<th>phenotype maintenance</th>
</tr>
</thead>
<tbody>
<tr>
<td>sPLCL</td>
<td>++</td>
<td>+++</td>
<td>small, roundish or angular, confluent</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>tPLCL</td>
<td>++</td>
<td>+++</td>
<td>small, roundish or angular, confluent</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>cPLCL</td>
<td>+</td>
<td>+++</td>
<td>small, roundish or angular, fewer cells on PLA fibres</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

$^a$Tested native sheep bladder.
and, after the hydrolysis, the values were only 3.8 per cent for sPLCL and 0 per cent for tPLCL. On the other hand, the strain values at maximum load of the cPLCL samples remained steady, being 20 per cent during the hydrolysis, except for the zero-week samples, which had mean strain values of 70 per cent. The strain at maximum load of the sheep bladder samples was 85.1 ± 32.7 per cent.

3.1.4. Thermal properties during hydrolysis
The \( T_g \) values of the different sample series were almost identical (data not shown). A minor decrease in \( T_g \) values was seen after sterilization, from 23°C to 22°C. The \( T_g \) values of the samples dropped steadily during the hydrolysis, and, after week 6, the values were dropped to 20°C. After week 6, the \( T_g \) values started to decrease more, and at the end of the hydrolysis at 12 weeks, the \( T_g \) values were 16°C.

3.2. Flow cytometric analysis
Prior to cell seeding, the hUCs were identified using flow cytometry (figure 3). The flow cytometric analysis showed that the population of isolated hUCs was homogeneous with regard to cell size and surface complexity of unstained control samples. The hUCs expressed extracellular matrix adhesion marker CD44, endothelial markers CD73 and CD105, and epithelial markers CD133, CD166 and CD326. Furthermore, the hUCs expressed the intracellular marker keratin 8/18, which is a specific marker for epithelial cells. These studied markers have previously been used to characterize hUCs [19,24–26].

3.3. Attachment and morphology of hUCs
According to the SEM imaging (figure 4), there were no remarkable differences in cell attachment or morphology between the sPLCL and tPLCL at the 2 h time point. However, on the cPLCL, the attachment of hUCs was inferior on the PLA fibres compared with the plain PLCL regions (table 2).

After 7 days of cell culture, the hUCs on the sPLCL, tPLCL and cPLCL were morphologically similar: small and oval or roundish. Further, the hUCs had already formed clusters despite not being spread homogeneously over the whole cell culture area. At the 14 day time point, the hUCs on the sPLCL, tPLCL and cPLCL had formed a confluent cell layer and adhered to the adjacent cells. However, on the cPLCL, the hUCs spread unevenly, preferring the PLCL regions. On all the membranes, the hUCs were small, and the morphology of cells varied from roundish or oval to cubic or angular. Moreover, no substantial differences were detected in the hUCs’ morphology, regardless of culture surface.

3.4. Viability and proliferation of human urothelial cells
The live/dead staining (figure 5a) demonstrated that the cells were viable on all the membranes studied, and the number of dead cells was negligible after 7 days of culture. Furthermore, the hUCs maintained their viability during the 7–14 days culturing period, and no increase in dead cell number was detected (table 2).

The WST-1 measurement revealed significant differences between the materials (figure 5b). The number of hUCs increased significantly from the 2 h time point to the 7 days time point in all the biomaterials studied: sPLCL (\( p < 0.001 \)), tPLCL (\( p < 0.001 \)) and cPLCL (\( p = 0.005 \)). The WST-1 measurement showed no significant differences in cell attachment between the biomaterials studied at the 2 h time point. At the 7 and 14 days time points, however, the sPLCL and tPLCL supported the hUCs’ proliferation better than the cPLCL, albeit that the difference was statistically significant compared with sPLCL at the 7 days time point (\( p = 0.042 \)) and compared with tPLCL at the 14 days time point (\( p = 0.011 \)).
3.5. Phenotype characterization of human urothelial cells using immunostaining

The hUCs expressed CK7 and CK19 (green fluorescence) after 7 days (data not shown) and 14 days of cell culture (figure 6). The CK19 and CK7 expression of hUCs on the sPLCL, tPLCL and cPLCL was considered intensive at both time points; moreover, the intensity of expression did not substantially change during the 7–14 days assessment period, and the hUCs maintained their phenotype (table 2).

4. DISCUSSION

To the best of our knowledge, this is the first study to characterize the mechanical properties of compression-moulded PLCL membranes for urothelial tissue engineering and to compare the effects of these membranes with regard to the viability, morphology, proliferation and phenotype maintenance of hUCs. The influence of topographical texturing of PLCL using sPLCL, tPLCL and cPLCL membranes was also studied, which has not yet been reported with hUCs.

The in vitro degradation studies indicated that the PLCL samples exhibited moderate dimensional stability and mass loss, relatively high elongation and also moderate thermal property changes until week 6. Therefore, these results demonstrate that these highly elastic and pliable membranes theoretically meet the prerequisites to function properly in urothelial tissue engineering applications and also in other tissue engineering applications in which elasticity and pliability are important [15]. Compared with the study by Eberli et al. [27] of mechanical properties of native porcine bladder, i.e. tensile stress at break less than 1 MPa and strain approximately 130 per cent, our results indicate that PLCL possesses similar mechanical properties. Furthermore, our tensile tests on native sheep bladder show that the values of PLCL samples maintain sufficient tensile strengths at least until week 4, after which the plain PLCL samples showed decreased strain values compared with the natural tissue. The modulus values of all of the studied PLCL samples were higher than the respective modulus values of natural sheep bladder, indicating more rigid behaviour of the PLCL samples. The tensile strength and modulus values decreased...
after the sterilization, and it is assumed that the tensile strength and the modulus values of these samples would also highly decrease in vivo when the cells start to affect the PLCL matrix [28].

As a manufacturing method, compression moulding was selected because relatively thin membranes, but possessing adequate mechanical strength, can be fabricated. Also, no solvents are needed for compression moulding; therefore, no solvent residues are involved. The different manufacturing methods of the membranes had an effect on the degradation behaviour of the samples studied. The results showed that the tPLCL samples were the first to start to degrade and also had lower mechanical properties than the sPLCL samples. This may be due to the fact that the tPLCL samples only had 63 per cent of the original thickness of the sPLCL samples. This was due to the processing method in which the structuring of the tPLCL samples between the PTFE moulds led to thinner sample structures. In addition, the tPLCL samples had a textured surface, and therefore the surface area of the samples was greater than in the sPLCL membranes, leading to higher degradation rates [10,29].

The cPLCL samples showed degradation results similar to those of the sPLCL and tPLCL samples within the PLCL matrix; therefore, the difference, for example, in mechanical properties was mainly affected by the PLA mesh in the structure. The PLA mesh in
the composite samples made the samples tougher than expected and with less ductility than the plain PLCL samples [29,30]. In our application, the elastic properties of the samples are preferable to the toughness of the cPLCL samples. Therefore, the cPLCL samples did not give any additional value compared with the plain PLCL samples. Because of the textured surface, the tPLCL samples were the most pliable samples and the easiest to handle before hydrolysis. The texturing was one of the major reasons that these samples degraded first because texturing increases the surface area of the membrane. It also accounted for the mechanical properties of the tPLCL membrane when compared with the other membranes studied [12].

What should be taken into account is that the samples started to degrade more noticeably after six to eight weeks of hydrolysis and thereafter showed a relatively rapid degradation process. The urothelium is known to regenerate rapidly after injury [31,32], thus the degradation rate is probably adequate for the urothelial tissue engineering applications; nevertheless, in the future this also has to be verified in vivo [5,12]. During surgical implantation into a patient, a biomaterial gives the regenerating urothelium mechanical support. Moreover, the biomaterial functions as a basement membrane for urothelial cells. While the biomaterial degrades and the mechanical properties decrease, the urothelial cells secrete extracellular matrix, forming cells supporting the basement membrane. The biomaterial should also attach to the underlying stromal layer, which also gives mechanical support for the urothelium while the biomaterial degrades [10,33].

A pH of 6.1 most probably affected the degradation time of the membranes studied, as PLCL degradation occurs by hydrolysis of an ester bond, forming lactic acid and caproic acid. This is catalysed by acidic conditions [17]. We chose this pH because it mimics the pH in the lumen of the native urothra and therefore it can be assumed that the degradation rate of these membranes most probably is similar to that in the urinary tract. At the beginning of graft implantation, the urothelial barrier has not been completely developed; therefore, the biomaterial will be in contact with acidic urine. In addition, the pH value of 6.1 has previously been used to study the mechanical characteristics of biomaterials for urothelial applications [34].

For urothelial applications, several cell sources have been used. In addition to primary urothelial cells, oral keratinocytes and foreskin epidermal cells have been studied for urethral reconstruction [35–37]. Fossum et al. [33] used urothelial cells on acellular dermis for clinical studies to reconstruct urethras for paediatric patients. In this study, we used hUCs taken from ureters, because these cells are similar to the cells in the proximal parts of the urethra. Although the distal part of the urethra is covered by squamous epithelium, the urothelial cells are a potential cell source for future clinical urethral reconstruction applications, as demonstrated by Fossum et al. [33]. We used flow cytometry to characterize the hUCs after isolation and also to verify the repeatability of our isolation protocol. The hUC characterization data were consistent with our previously reported results [19], which further indicates the reliability of our method. Otherwise, the hUCs have been characterized in only a few studies. These results were in parallel with the earlier results obtained for urothelial cells [24,38], and hUCs expressed all the markers studied as expected.

The attachment of cells was studied 2 h after the initial cell attachment, using SEM imaging and quantitative WST-1 measurement, indicating that the mechanical texturing using tPLCL or cPLCL provided no additional advantage to hUC attachment. This result contradicted our hypothesis, which was that the mechanical texturing would facilitate cell attachment owing to the well-known advantages of surface structuring [20,21]. On the basis of SEM imaging and live/dead staining, the hUCs on the cPLCL samples seemed to prefer PLCL over PLA areas, because the hUCs attached scantily and covered the PLA fibres unevenly, suggesting the weaker adhesion of hUCs to the PLA fibres.

According to the live/dead staining, all the biomaterials supported the viability of hUCs. No significant change in hUC viability was detected between the 7 and 14 days time points, suggesting good biocompatibility of the studied biomaterials as expected, because PLCL with different compositions has previously been studied for tissue engineering applications, with encouraging results [19,39,40]. The morphology evaluated by SEM imaging was consistent with the live/dead staining. The hUCs on the sPLCL, tPLCL and cPLCL samples were morphologically similar, exhibiting the normal shape of UCs (small and roundish or cuboidal), further indicating good biocompatibility for hUCs.

An increase in cell number was detected during the culturing period with both SEM imaging and WST-1 measurement. After 14 days of cell culture, the hUCs were confluent on the sPLCL, tPLCL and cPLCL samples. Additionally, the WST-1 measurement demonstrated that the cell number on the cPLCL samples was lower than on the sPLCL and tPLCL samples. Interestingly, topographical texturing with tPLCL or cPLCL yielded no additional advantage in cell proliferation compared with sPLCL, suggesting that the material selection has more effect on hUC attachment and proliferation than mechanical texturing.

The immunostaining demonstrated the expression of CK19 and CK7 on all the biomaterials studied, as expected, because both CK7 and CK19 are present in all layers of the native urothelium: basal, intermediate and superficial layers [23,41]. These results are in concordance with our earlier results, in which CK19 expression was also demonstrated [19]. The marker expressions remained intensive during the culturing period, which indicates a stable phenotype during the assessment period [2,19,23,42].

The main limitation of this study was that it only demonstrates the in vitro effects of different PLCL-based membranes on urothelial cell response. Furthermore, the in vitro hydrolysis conditions used always differ from the natural environment. Therefore, in vivo studies are needed to verify the potential of PLCL for urothelial tissue engineering. Despite these limitations, our results showed that both sPLCL and tPLCL exhibited suitable properties for urothelial
tissue engineering applications. From a surgical point of view, the texturing of tPLCL made the handling of the membrane easier than that of sPLCL; therefore, tPLCL membrane will be selected over sPLCL membrane for future in vivo studies.

5. CONCLUSION
Complementing our previous results, this study further verifies the potential of PLCL for urothelial tissue engineering applications. As the cell studies indicated, the sPLCL and tPLCL membranes supported the hUCs’ attachment and proliferation better than the cPLCL membranes. Surprisingly, the material itself, rather than the mechanical texturing, appeared to have more effect on hUC growth. Furthermore, the in vitro degradation and mechanical properties of the PLCL membranes, especially tPLCL, showed a capability to function properly in urothelial applications.

Human urothelial tissue samples were obtained from normal ureters of child donors, aged 1, 4 and 12 years, during routine surgery in Tampere University Hospital, with the approval of the Ethics Committee of Pirkanmaa Hospital District (Tampere, Finland, R071609).

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REFERENCES


