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RESEARCH ARTICLE

Compressed collagen gel: a novel scaffold for human bladder cells

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Abstract

Collagen is highly conserved across species and has been used extensively for tissue regeneration; however, its mechanical properties are limited. A recent advance using plastic compression of collagen gels to achieve much higher concentrations significantly increases its mechanical properties at the neo-tissue level. This controlled, cell-independent process allows the engineering of biomimetic scaffolds. We have evaluated plastic compressed collagen scaffolds seeded with human bladder smooth muscle cells inside and urothelial cells on the gel surface for potential urological applications. Bladder smooth muscle and urothelial cells were visualized using scanning electron microscopy, conventional histology and immunohistochemistry; cell viability and proliferation were also quantified for 14 days *in vitro*. Both cell types tested proliferated on the construct surface, forming dense cell layers after 2 weeks. However, smooth muscle cells seeded within the construct, assessed with the Alamar blue assay, showed lower proliferation. Cellular distribution within the construct was also evaluated, using confocal microscopy. After 14 days of *in vitro* culture, 30% of the smooth muscle cells were found on the construct surface compared to 0% at day 1. Our results provide some evidence that cell-seeded plastic compressed collagen has significant potential for bladder tissue regeneration, as these materials allow efficient cell seeding inside the construct as well as cell proliferation. Copyright © 2009 John Wiley & Sons, Ltd.

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Keywords **plastic compressed collagen; bladder smooth muscle cells; bladder urothelial cells; biomimetic scaffold; *in vitro* study; bladder tissue regeneration**

1. Introduction

Bladder diseases affect almost 400 million people around the world and are a significant problem (Yang *et al*., 2005). Congenital malformations of the lower urinary tract, as well as postnatally acquired diseases, such as traumatic neurogenic bladder, chronic interstitial cystitis and even cancer, may result in dysfunctional and poorly compliant bladder tissues. In some cases, these injuries necessitate surgical replacement or repair of bladder tissue. Vascularized gastrointestinal segments are frequently used as donor tissues for augmentation cystoplasty. However, these tissues possess different properties that preclude the entire replacement of bladder functions. In addition, due to the presence of intestinal mucosa, multiple complications, such as infections, metabolic disturbances, increased mucous production and urolithiasis, are common using this approach (Flood *et al*., 2005). It has become increasingly obvious that the replacement of lost or deficient bladder tissue requires an alternative approach. The ability to engineer a tissue substitute with properties similar to the original bladder tissue *in vitro* remains an elusive goal. Consequently,

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several promising tissue-engineering methods based on autologous cells and biodegradable scaffolds have been developed to engineer urological tissue (Atala *et al.*, 2006; Danielsson *et al.*, 2006).

Scaffold materials should have a good biocompatibility, favouring cell attachment and proliferation, providing designable three-dimensional (3D) structures and appropriate mechanical strength. Collagen, the most abundant protein in the body, has been approved by the US Food and Drug Administration (FDA) in several applications. Collagen, easily purified from animal and human tissues, possesses numerous cell-adhesion domain sequences and interacts as well with other adhesion proteins, such as fibronectin, which facilitates cell attachment and allows specific cellular interactions. This feature could play a role in preserving the phenotypes and functional activities of many cell types. The degradation rate of collagen implants can be regulated by controlling the density of the implant and the degree of intermolecular crosslinking (Kim *et al.*, 2000). Collagen gels, as well as collagen sponges or decellularized porcine small intestinal submucosa (SIS), have proved to be adequate scaffolds for tissue regeneration (Cheng and Kropp, 2000). However, apart from SIS, these collagen matrices offer limited mechanical strength, even if intermolecular crosslinking can somehow enhance their mechanical properties. Cells seeded in a collagen gel contract the implant, thus improving the strength of the material, but this is inherently a very time-consuming process (Feng *et al.*, 2003). A much faster way to increase material strength is to apply plastic compression. During this process, the excess fluid presence in collagen gels due to the casting is expelled, resulting in denser and therefore mechanically stronger structures. The tensile properties of plastic compressed (PC) collagen gels approach those of some native tissues (Neel *et al.*, 2006). This cell-independent technique, developed by Brown *et al.* (2005), allows controlled engineering of biomimetic scaffolds. This compression enables the fabrication of 3D nano- and micro-scale structures that allow high cell viability even when cells are integrated inside the gel (Brown *et al.*, 2005).

This report describes the potential of PC collagen gels as scaffolds for bladder tissue regeneration. Human smooth muscle cells (SMCs) and urothelial cells (UCs), the two major cellular components of the urinary tract, were cultured in and on compressed collagen gels. Cell cultures were tested for cell viability, proliferation potential and cellular distribution.

2. Materials and methods

2.1. Cell culture

Excisional samples were harvested at open ureter or bladder surgery for correction of congenital malformations in children, performed by one of the authors (P.F.). Tissue specimens were cut into small pieces and digested with Liberase Blendzymes I (Roche, Basel, Switzerland) at 37 °C for 1.5–2 h. The cell suspension was passed through a 70 μm cell strainer (BD Biosciences, Allschwil, Switzerland) prior to centrifugation (800 rpm, 4 °C, 5 min). The cell pellet was resuspended in α-MEM medium (Cambrex Bio Sciences, Verviers, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco, Invitrogen, Lucerne, Switzerland), 2 mM L-glutamine (Invitrogen) and 1% penicillin–streptomycin (Invitrogen). Urothelial cells (UCs) were isolated by positive selection with antibody-conjugated magnetic beads (Dynabeads®, Invitrogen/Dynal Biotech, Basel, Switzerland). Beads conjugated with anti-human epithelial antigen (clone Ber-EP4; Dakocytomation M0804, Baar, Switzerland) were mixed in the cell suspension at 4 °C for 15 min to isolate UCs. Collected beads were resuspended in keratinocyte serum-free medium (KSFM; Invitrogen) supplemented with bovine pituitary extract (BPE), epidermal growth factor and 30 ng/ml cholera toxin, as previously described (Southgate *et al.*, 1994). Beads were plated in a 25 cm^2 Primaria T-Flask (BD Biosciences) containing 10 ml supplemented KSFM. Contaminating fibroblasts were removed using beads coated with fibroblast-specific antibodies (ASO2, mouse IgG1, CD90, Thy-1; Dianova, Hamburg, Germany). Negative isolation of smooth muscle cells (SMCs) was performed on the remaining supernatant after the positive selection of UCs and fibroblasts. The supernatant was centrifuged (800 rpm, 4 °C, 5 min) and the cell pellet was resuspended in supplemented α-MEM medium and plated in a 25 cm^2 T-Flask. The cells were grown until confluent and a cell bank was generated.

SMCs and UCs were cultivated in supplemented α-MEM and KSFM medium, respectively, in a humidified atmosphere at 37 °C and 5% CO_2. The cells were passaged every 3–4 days. Experiments were performed on SMCs and UCs between passages 6 and 8.

2.2. Plastic compression of collagen gels

Collagen gels and plastic compression were carried out by a modified technique described previously (Brown *et al.*, 2005). 2.4 ml sterile rat-tail collagen type I solution (2.16 mg/ml protein in 0.6% acetic acid; First Link Ltd, West Midlands, UK) was mixed with 0.3 ml 10× Eagle's minimum essential medium (MEM; Gibco Chemicals, Invitrogen). The solution was neutralized with 1 M NaOH prior to addition of 0.3 ml α-MEM medium and cast into rectangular moulds ($22 \times 33 \times 10$ mm). Gel formation and polymerization at room temperature were completed in 30 min. Plastic compression of the collagen gels was achieved by loading with a 120 g weight for 5 min on top of the polymerized gel, with fluid removal from the lower face into a porous paper pad. Compression resulted in expulsion of >95% of the water contained within the gel to give a 50–60 μm thick collagen gel with correspondingly increased mechanical properties (Brown *et al.*, 2005).

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2.3. Cell-seeded plastic compressed collagen gels

Seeding of SMCs inside PC collagen gels was performed by adding 0.3 ml cell suspension (0.5×10^6 cells) into the neutralized collagen solution; in this case replacing the 0.3 ml cell-free α-MEM medium mentioned above. Cell seeding on top of the collagen construct was performed after plastic compression by dropping 0.1 ml cell suspension (0.15×10^6 cells) onto the gel surface and allowing the cells to attach for 3 h in a humidified incubator (37 °C, 5% CO_2) before addition of further medium. Cellularized PC collagen gels were cultured in ultra-low-attachment six-well plates (Costar, Corning Inc. Life Sciences, Schipohl-Rijk, The Netherlands) filled with blend medium (1 : 1 supplemented α-MEM and KSFM medium) and weighed down with a stainless steel ring to hold the construct flat and reduce cell-mediated shape change. Serum concentration in blend medium was decreased stepwise from 5% to 0% during the first 7 days of culture. The plates were incubated in the humidified atmosphere of an incubator at 37 °C and 5% CO_2. Additionally, some cell-seeded collagen gels were placed into cell culture inserts (Falcon™, Becton Dickinson Labware, France) introduced into low-attachment six-well plates. Cell culture inserts prevented sticking of the PC collagen gel to the well bottom, while the PET membrane of the cell culture insert ensured supply of the medium to the bottom surface of the gel.

2.4. Scanning electron microscopy (SEM)

The gels were fixed in 0.25% glutaraldehyde at room temperature for 1 h and thereafter dehydrated in a series of graded ethanol concentrations (50–100% ethanol). The gels were further dried by supercritical carbon dioxide extraction (5×5 min, 1×20 min). The samples were coated with 20 nm platinum and examined under SEM at 5 kV (JEOL 6300-F, Japan).

2.5. Histology and immunohistochemistry

Gels were fixed in 2.5% paraformaldehyde (PFA) for 30 min and then washed in PBS three times. Fixed samples were embedded in OCT Tissue-TEK® (Digitana AS, Yverdon-les-Bains, Switzerland) and frozen in isopentane, using liquid nitrogen. Serial sections were generated at thicknesses of 5 and 10 μm on a Microm HM550 cryostat and stained with haematoxylin and eosin (H&E; Medite, Zullwil, Switzerland) for conventional histology. Samples were analysed by light microscopy (Olympus AX70).

Frozen sections were used for immunohistochemistry. Defrosted samples were fixed in 2.5% PFA for 10 min and then washed with PBS. Permeabilization was performed by 10 min incubation in 0.1% Triton-X100, followed by a washing step with PBS. The samples were incubated in PBS/2% BSA at room temperature for 1 h prior to antibody addition. Mouse monoclonal anti-α-smooth muscle actin antibodies (1 : 70 dilution, ab7817; Abcam, Cambridge, UK) and rabbit polyclonal anti-cytokeratin 7 (1 : 100 dilution, sc-25 721; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added at 4 °C overnight. After washing with PBS, secondary antibodies, respectively FITC-conjugated anti-mouse IgG (Sigma, Buchs, Switzerland) and Alexa Fluor® 546 anti-rabbit IgG (Invitrogen) were added at room temperature for 1 h. Samples for negative control were only incubated with the secondary conjugated antibodies. DAPI (4′,6-diamidino-2-phenylindole4′,6-diamidino-2-phenylindole) staining was performed prior to observation of all samples under an Olympus AX70 microscope.

2.6. Alamar blue assay

Alamar blue assay was used to monitor cell proliferation/activity. The assay incorporates a fluorometric/colorimetric growth indicator based on detection of cellular metabolic activity. The gels were washed with PBS and incubated with 1 ml Alamar blue working solution (10% Alamar blue; Serotec, Oxon, UK) in PBS in a dark, humidified incubator (37 °C, 5% CO_2) for 90 min. Following gentle agitation at 37 °C for 15 min, 200 μl aliquots from each well were transferred into a 96-well plate. The fluorescence was measured using a Safire$^{2™}$ Tecan Microplate Reader (excitation wavelength 530 nm; emission wavelength 590 nm; Tecan AG, Männedorf, Switzerland).

2.7. Live/dead staining

Cell viability was assessed using the Live/Dead® Viability/Cytotoxicity Kit for mammalian cells (Invitrogen). The gel surface was covered with 150 μl solution containing 3 μM ethidium homodimer and 0.8 μM calcein in PBS. Incubation was performed at room temperature for 30 min. The gels were washed with PBS four times, placed on a microscopy slide and covered with a coverslip. Sample examination was performed under a Leica SP2 upright confocal microscope (Leica Micosystems GmbH, Heidelberg, Germany). The images were analysed for cell number and localization (z coordinate) using Lite software (Leica confocal software).

2.8. Statistics

Statistical analysis of the data was performed using R2.7.1 software (Team, 2005). Significant differences between datasets were determined by ANOVA or Kruskal test. $p < 0.05$ was considered significant, with the following nuances: $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

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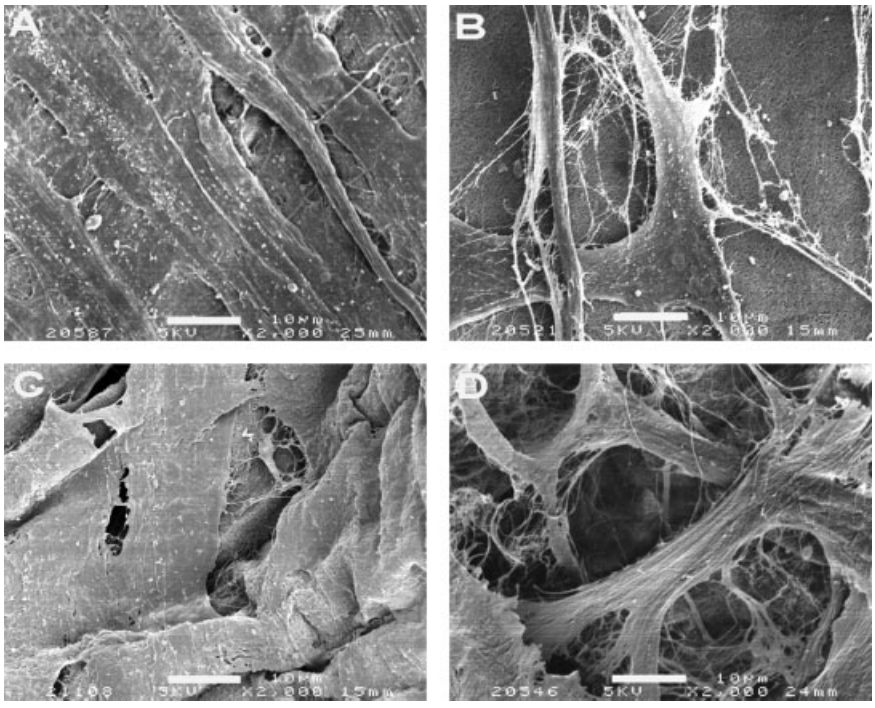

Figure 1. Representative scanning electron micrographs of platinum-coated compressed collagen constructs on day 14 (magnification 2000×), with: (A) SMCs seeded on top of the construct; (B) UCs seeded on top; (C) SMCs seeded inside the gel during the compression process; and (D) SMCs seeded inside and UCs seeded on top of the PC collagen gel. In all panels, the scale bar is 10 μm

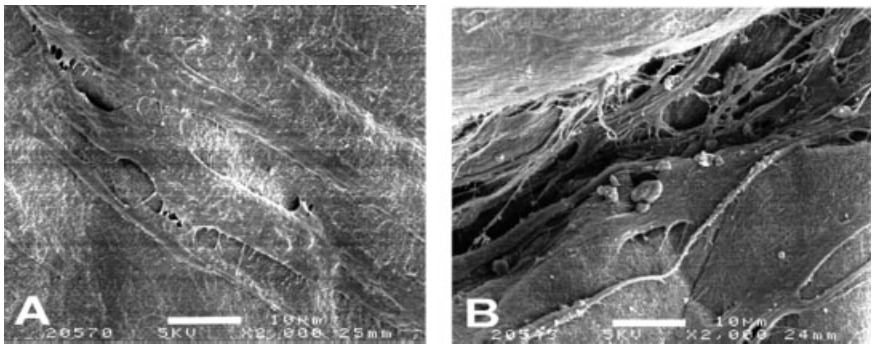

Figure 2. Spreading of UCs seeded at the surface of PC collagen gels with SMCs seeded inside (magnification 2000×). (A) A few UCs were present at day 1. (B) UCs proliferated and formed a dense layer at day 14. In all panels, the scale bar is 10 μm

3. Results

3.1. Morphology of the scaffold surface

At days 1 and 14 after cell seeding, the constructs were imaged by SEM. SMCs as well as UCs could be distinguished on the construct surface at a magnification of 2000×. Cells were observed on all constructs where seeding was carried out onto the top surface (Figure 1A, B, D) as well as on gels with SMCs seeded inside on day 14 (Figure 1C). At day 1, surface cells, SMCs as well as UCs, formed small discrete islands which grew together to form a dense cell layer by day 14 (Figure 2). Cells attached to the collagen surface had a flat, spindle-shaped morphology. Cell proliferation was supported by an increase in density and spreading of the cell layer. UCs were seeded on empty constructs as well as on constructs with SMCs seeded inside (co-culture) to see whether the presence of SMCs influenced the behaviour of the UCs. The morphology of UCs in co-culture experiments looked identical to that with UCs seeded on top of cell-free constructs (Figure 1B, D).

3.2. Histology

Sections stained with H&E clearly showed the presence of bladder cells on top or within the PC collagen gels, according to the seeding strategy on day 1 (Figure 3). Surface-seeded cells formed a layer on top of the scaffold, while inside seeded cells were equally distributed throughout the gel. In contrast, by day 14 nearly all SMCs were found on the construct surface, indicating

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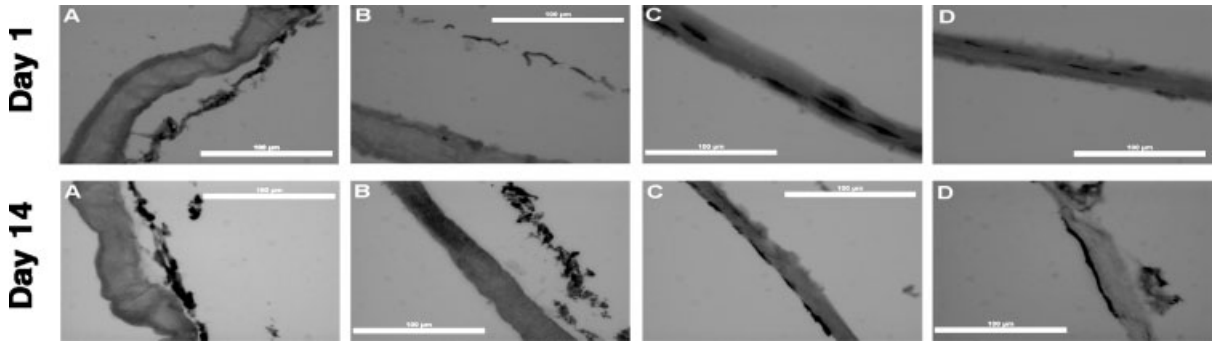

Figure 3. H&E-stained histological sections of PC collagen constructs on days 1 and 14. Sections analysed by light microscopy (magnification 40×), illustrating: (A) UCs seeded on top of PC collagen gels, (B) SMCs seeded on top of PC collagen gels, (C) SMCs seeded inside the gel; and (D) SMCs seeded inside and UCs on top of the construct. The presence of detached cellular layers close to some constructs, in (B), is a sectioning artifact. In all panels, the scale bar is 100 μm

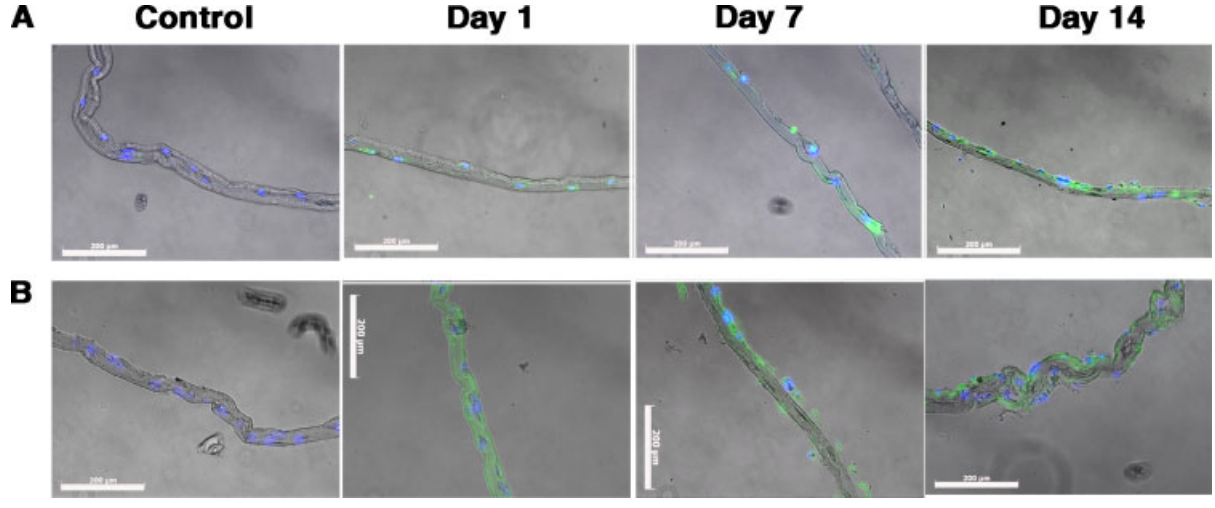

Figure 4. Immunohistochemistry on frozen sections of PC collagen constructs on days 1, 7 and 14. Sections were green-stained by FITC, using anti-α-smooth muscle actin mAb. (A) SMCs seeded inside and (B) SMCs seeded inside and UCs on top of the construct. DAPI staining was performed to visualize the cell nucleus. The control stain was performed with the FITC-conjugated antibody only. In all panels, the scale bar is 200 μm

migration toward the surface. In some sections, cells were detached from the construct and found next to the construct (Figure 3B). This is an artefact that occurred during the processing of PC collagen gels into 5 and 10 μm thin sections.

3.3. Immunohistochemistry

Immunohistochemistry on frozen sections was used to localize SMCs and UCs within the construct and to confirm the observations from H&E staining. Frozen sections were prepared because of the application recommendations of the available antibodies. Expression of α-smooth muscle actin of SMCs seeded inside PC collagen constructs is shown in Figure 4. The anti-α-smooth muscle actin antibody is specific for SMCs.

At day 1, expression of α-smooth muscle actin showed that SMCs seeded inside the gel were mainly localized in the inner part of the construct. With time they tended to migrate to the surface of the construct, independently of the presence of UCs seeded on the surface (Figure 4A, B). Apparently, migration of SMCs is not directed by the presence of UCs. The control stain in Figure 4 demonstrates the specific binding of the conjugated antibody to the anti-α-smooth muscle actin antibody.

In further experiments, seeded PC gels were cultured in cell culture inserts to test whether SMCs would stay inside the construct when medium supply was also provided to the bottom surface of the gel. In this case, the cells tended to migrate to the upper as well as to the lower construct surface (data not shown).

Immunohistochemistry experiments were also performed on UCs seeded on the surface of constructs that were incubated for 14 days in supplemented α-MEM or blend medium, as described above. Expression of cytokeratin 7 was used as a UC-specific cell marker (Figure 5). No cytokeratin-7 staining could be observed for all the time points tested when constructs were cultured in supplemented α-MEM medium (Figure 5C). UCs that had been seeded on top of empty gels and were cultivated in blend medium kept their cytokeratin 7-positive phenotype during the 14 days of culture (Figure 5B). Control stains

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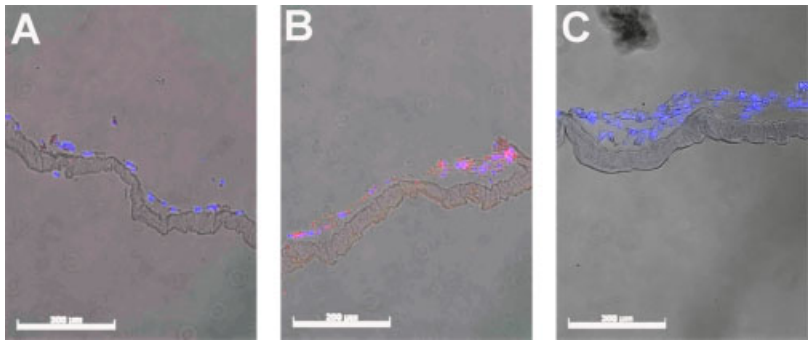

Figure 5. Immunohistochemistry on frozen sections of PC collagen gels seeded with UCs on top. Sections from day 14 were red-stained by Alexa Fluor® 546, using polyclonal anti-cytokeratin 7 antibody. (A, B) Gels cultured in blend medium (1 : 1 supplemented α-MEM and KSFM medium). (C) Gels cultured in supplemented α-MEM medium. DAPI staining was performed to visualize the cell nucleus. The control stain (A) was performed with the Alexa Fluor® 546-conjugated antibody only. In all panels, the scale bar is 200 μm

(Figure 5A) show that the conjugated antibody bound specifically to the anti-cytokeratin 7 antibody.

3.4. Cellular proliferation

Constructs seeded with SMCs and UCs were cultured for 14 days and cell proliferation was measured with the Alamar blue assay at days 1, 7 and 14 (Figure 6).

Cellular proliferation was observed for all tested cell-seeded PC collagen gels, as shown by a constant increase of Alamar blue-specific fluorescence. SMCs seeded on top of constructs showed a significantly higher proliferation during the 14 days *in vitro* culture than those seeded inside the gel. The Alamar blue assay did not reveal any significant differences in cell proliferation between UCs and SMCs that were seeded on top of acellular PC collagen gels. Co-culture constructs had a slightly higher cellular proliferation than constructs with only one cell type.

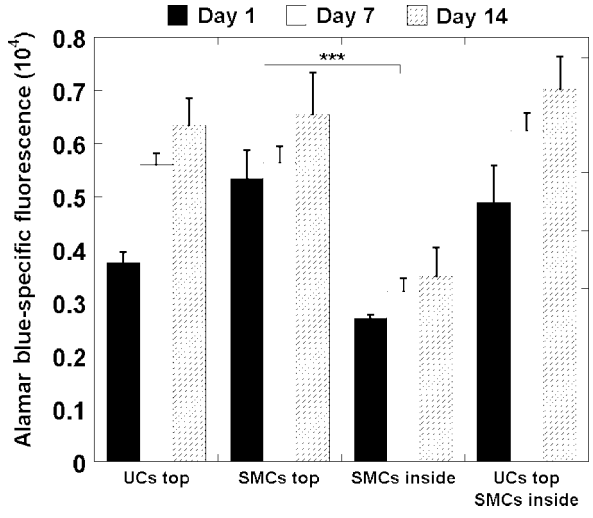

Figure 6. Proliferation of SMCs and UCs in PC collagen constructs. Alamar blue-specific fluorescence was measured at days 1, 7 and 14 for four types of gels: UCs seeded on top of PC collagen gels; SMCs seeded on top of PC collagen gels; SMCs seeded inside; and co-culture gels (SMCs seeded inside and UCs seeded on top of the construct). Statistical analysis (ANOVA) gave $p < 0.001$ for the comparison of SMCs proliferation on and within PC collagen gels for all time points measured. Data are based on three independent experiments

3.5. Cellular distribution inside constructs

Since the Alamar blue assay did not provide any information concerning cell distribution (localization of living and dead SMCs) over the construct thickness, live/dead cell staining was performed on PC collagen gels with SMCs seeded inside, cultured for 14 days in cell culture inserts (Figure 7). Image analysis was done using Leica Lite software. To determine the spatial cell distribution, the construct was subdivided into five regions: surface (relative thickness 0–0.14); top (relative thickness 0.14–0.25); upper half (relative thickness 0.25–0.5); lower half (relative thickness 0.5–0.75); and bottom (relative thickness 0.75–1). The surface region was determined using gels with SMCs seeded on top as a reference. Since there was a slight variation in the total gel thickness, it was decided to use the relative thickness (distance gel surface – cell/thickness of gel) to describe the position of cells. The bottom region was in contact with the PET membrane of the cell culture inserts and the top region was in direct contact with the medium. The overall viability of SMCs seeded inside the constructs ($n = 10$) was around 90% during the 14 days. The spatial distribution of living SMCs (Figure 7, left) and dead SMCs (Figure 7, right) seeded inside PC collagen gels is shown for days 1, 7 and 14. On day 1 no SMCs were found on the gel surface. Living SMCs were more or less equally distributed over the gel thickness, with a minor part in the bottom region. Dead SMCs were mainly found in the upper and lower half of the gel. The cellular distribution changed over the 14 days, as expected from previous data. Living cells appeared at the gel surface, making up 30% of the total cell number on day 14. A constant decrease in cell number was observed in the top part of the gel. From day 1 to day 7, the percentage of cells in the bottom region doubled, but decreased again to 18% on day 14. Interestingly, the percentage of dead cells increased on

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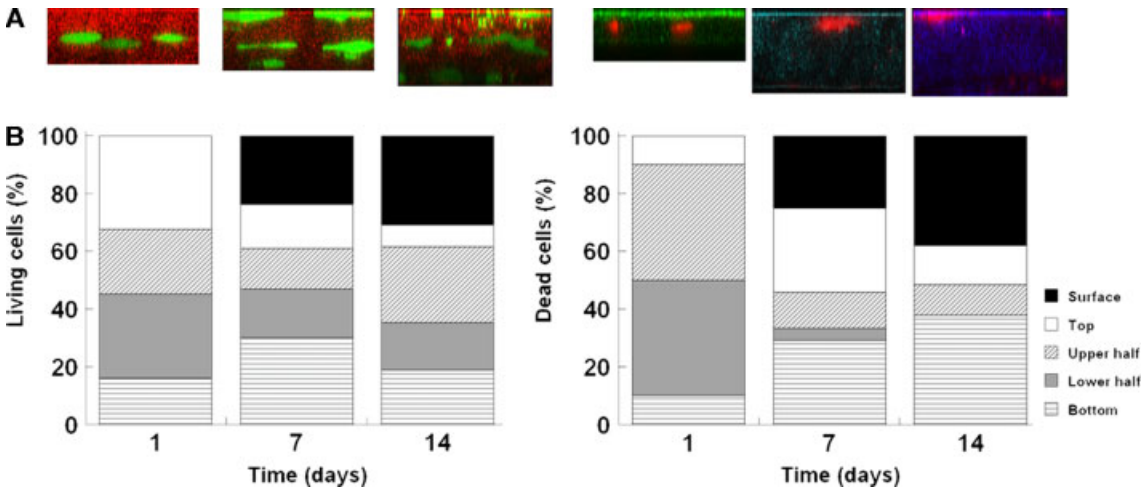

Figure 7. Distribution of living (left) and dead (right) SMCs within PC collagen constructs. Constructs were incubated in cell culture inserts and a live/dead staining was used to assess the percentage of living and dead cells and their distribution within the gels on days 1, 7 and 14. Samples were studied by confocal microscopy, and image analysis was performed using Lite software (Leica). (A) Representative images of SMCs within PC collagen gels; (B) relative distribution of cells over the construct thickness (numbers are based on the positions of around 150 cells)

the surface and the bottom part of the gel up to 37% on day 14 and decreased in the other parts of the gel.

4. Discussion

Apart from functionalized polymer matrices, decellularized small intestinal submucosa (SIS) is used as scaffold for bladder regeneration. SIS is harvested from the jejunum of pigs and consists primarily of a collagen-based ECM; the layer provides structural support, stability and biochemical signals to rapidly regenerate mucosal cell layer. Problems associated with the use of SIS are lack of rapid and efficient epithelialization, inflammation and thrombogenicity of newly implanted grafts (Hodde, 2002; Woods *et al*., 2004). The motivation to use PC collagen constructs as an alternative scaffold comes from the fact that SIS harvesting is a time-consuming process and its collagen architecture and 3D organization is predetermined by the animal tissue (Brown *et al*., 2005). In contrast, PC collagen constructs are made by a simple and fast process with controllable mesoscale structure, are fabricated independently of cell involvement and maintain cell viability in the process. Furthermore, use of PC collagen constructs allows cell integration inside the scaffold, thus increasing cell-seeding efficiency.

In the present study, we tested the potential of PC collagen gels as potential scaffolds for bladder cell growth. The two main cell types of the human urinary tract, SMCs and UCs, were seeded onto this scaffold and tested for cell proliferation, viability and distribution over a period of 14 days. We believe that the *in vitro* period should be kept as short as possible to allow the organism to act as a bioreactor for further cell proliferation and differentiation.

SEM analysis and H&E staining showed that both cell types proliferated well on the construct's surface, forming dense cell layers after 2 weeks. Surprisingly, when SMCs were seeded inside the construct, the cells tended to migrate towards the construct's surface, as shown in Figures 1, 2, 4 and 7. We can speculate that SMCs are able to migrate through the collagen due to matrix degradation (e.g. collagen is sensitive to metalloproteinases) and once they are outside the matrix they do not re-enter the gel. So far the factors influencing the cell migration are not clear. The low percentage of dead cells in the upper and lower halves of the gel allow us to suppose that an oxygen and nutrient gradient throughout the construct is not the cause. This conforms with the limit of passive diffusion to engineered tissues thinner than 2 mm (Griffith *et al*., 2005), depending on cell seeding density and specific oxygen uptake rate. Therefore, diffusion should not be a physical problem in PC collagen constructs having a thickness of around 50 μm. The low percentage of dead cells in the upper and lower halves of the gel goes along with the hypothesis that hypoxia is not the cause for cell migration towards the scaffold's surface.

Cell proliferation and viability were analysed by Alamar blue assay and live/dead staining, respectively. Both assays demonstrated that SMCs remained viable inside the construct over the 14 days of culture. Results from live/dead staining suggest that cell viability was around 90%. The lowest Alamar blue-specific fluorescence was determined for SMCs that were seeded inside the gel (Figure 6), which indicates that the cells were apparently alive but did not proliferate as much as SMCs seeded on top of the gel. A lower proliferation rate for vascular smooth muscle cells in 3D matrices compared to 2D cultures is already reported in the literature (Li *et al*., 2003). Lack of proliferation could also reveal a switch of SMCs to a quiescent contractile phenotype, which usually correlates with a stronger compliant tissue that is able to sustain the function of normal bladder (Adelow *et al*., 2007). However, we cannot exclude the possibility that the matrix structure of the construct and/or UCs cell

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layer on top reduced the ability of the Alamar blue dye to diffuse freely inside the construct; in such a case, the reduced amount of reduced Alamar blue dye would not represent the total amount of living cells inside the gel.

The phenotype of both cell types was verified using antibodies against cell-specific markers: monoclonal anti-α-smooth muscle actin antibodies for SMCs and polyclonal anti-cytokeratin 7 antibodies for UCs. SMCs maintained their phenotype under all tested culture conditions (α-MEM medium and blend medium). In contrast, UCs seemed to lose their cell-specific phenotype in α-MEM medium, as cytokeratin 7 was negative. This suggests that the culture condition used for these experiments could not maintain the UC phenotype *in vitro*. It has been shown that the medium composition influences expression levels of several cytokeratins (Cross *et al*., 2005) and that expression of cytokeratin 7 can disappear in later passages under certain cultivation conditions (Kreft *et al*., 2005). Cytokeratin 7 seems to be constitutively expressed when UCs are cultivated in supplemented KSFM medium (Ludwikowski *et al*., 1999; Sugasi *et al*., 2000). Changing the medium from α-MEM to blend medium allowed the optimized growth and maintenance of a stable UC phenotype, as cytokeratin 7 was still positive after 14 days of culture (Figure 5).

In this *in vitro* study, cell-seeding densities were lower than those reported by other authors using cell-seeded scaffolds in *in vivo* experiments. We seeded only 7.2×10^4 SMCs/cm^2 and 2.8×10^4 UCs/cm^2, whereas previous reports in the literature have used cell seeding concentrations up to 50×10^6 cells/cm^2 (Atala *et al*., 2006). Low cell-seeding densities were used to test cell proliferation, being aware that high cell-seeding densities might inhibit proliferation due to contact inhibition or limited nutrient supply. The data after 14 days of culture suggest that even low starting cell densities can lead to an adequate coverage of the construct surface.

The first *in vivo* data of PC collagen gels reported by Mudera *et al*. (2007) were promising. Cell-seeded PC collagen gels implanted in a test site across the intercostal spaces in a rabbit model showed revascularization, collagen remodelling and recovery of the implant's tensile mechanical strength after 5 weeks *in vivo*.

The excellent cell viability of both cell types and the dense cell layer on the construct surface observed here suggest a potential of PC collagen constructs to be used as scaffolds for bladder tissue regeneration.

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