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Modification of Macroporous Titanium Tracheal Implants With Biodegradable Structures: Tracking In Vivo Integration for Determination of Optimal In Situ Epithelialization Conditions

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ABSTRACT: Previously, we showed that macroporous titanium implants, colonized in vivo together with an epithelial graft, are viable options for tracheal replacement in sheep. To decrease the number of operating steps, biomaterial-based replacements for epithelial graft and intramuscular implantation were developed in the present study. Hybrid microporous PLLA/titanium tracheal implants were designed to decrease initial stenosis and provide a surface for epithelialization. They have been implanted in New Zealand white rabbits as tracheal substitutes and compared to intramuscular implantation samples. Moreover, a basement membrane like coating of the implant surface was also designed by Layer-by-Layer (LbL) method with collagen and alginate. The results showed that the commencement of stenosis can be prevented by the microporous PLLA. For determination of the optimum time point of epithelialization after implantation, HPLC analysis of blood samples, C-reactive protein (CRP), and Chromogranin A (CGA)

analyses and histology were carried out. Following 3 weeks the implant would be ready for epithelialization with respect to the amount of tissue integration. Calcein-AM labeled epithelial cell seeding showed that after 3 weeks implant surfaces were suitable for their attachment. CRP readings were steady after an initial rise in the first week. Cross-linked collagen/alginate structures show nanofibrillarity and they form uniform films over the implant surfaces without damaging the microporosity of the PLLA body. Human respiratory epithelial cells proliferated and migrated on these surfaces which provided a better alternative to PLLA film surface. In conclusion, collagen/alginate LbL coated hybrid PLLA/titanium implants are viable options for tracheal replacement, together with in situ epithelialization.

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Introduction

Respiratory system diseases are one of the most common causes of death, especially in high income countries. For example, tracheal restenosis might become a problem that

can necessitate surgery. Moreover, tracheal damages due to cancer and congenital diseases are quite common and even though end-to-end anastomosis can solve the problem for short lengths (<5 cm) (Roomans, 2010), implants are necessary if the problem is recurrent or the damaged area is above the limits of end-to-end anastomosis. In recent years several commendable efforts in the area of trachea tissue engineering have given very promising results, especially the systems by Macchiarini et al. (2008) and Delaere et al. (2010) have shown possible routes to improve the life quality of the patients. However these systems are dependent on allograft availability and long production periods with high level of expertise, which has also been stated by the authors themselves in their recent reviews (Bader and Macchiarini, 2010). It was also discussed widely whether a more in situ approach might be more beneficial, as trachea is surgically accessible. In such a scheme, some elements of the regeneration were either induced within the body or added after the initial implantation. It has been suggested that the in situ epithelialization might be a more attractive option as the survival of the epithelial cells seeded initially is low (Nakamura et al., 2009; Omori et al., 2008). Thus more widespread and easily transferable solutions are still necessary. Current systems available are either dependent on cartilage production for the airway stability and prevention of collapse or in vivo migration of epithelial cells or epithelial graft for remodeling. Porous titanium technology is an interesting venue to provide biointegrable implants without any concerns on mechanical stability. It is composed of titanium microbeads put together with an electrical arc, which provides a macroporous, low density and yet stable implant system, with an easier production procedure compared to most of porous metal implants (Ryan et al., 2006). We have previously utilized the titanium tracheal implants in mice and sheep with success (Schultz et al., 2002, 2004). Other groups also had success with titanium implants in respiratory area (Janssen et al., 2009, 2010). Trachea is an advantageous area for reconstruction as it is a thin tissue with relatively low oxygen requirements (Kalathur et al., 2010). The main problems encountered in the previous studies were the absence of epithelium for large defects for which there is no possibility of epithelium grafting (skin, mouth, etc.) and an extensive risk of restenosis. Grafting can be a viable route but its availability is a difficulty and more importantly the differentiated state of the graft might cause problems in the target area (such as hair growth or excessive keratinization), so a surface that would facilitate epithelial growth is a preferred option. Utilization of the advantageous properties of two material types either as hybrids or composites is generally necessary in the field of biomaterials (Nicole et al., 2010). Thus utilizing a biodegradable structure, such as a polymer foam, within a biocompatible metallic template is feasible to control cellular movement also in vivo. A prerequisite for colonization of a porous tracheal implant is also to prevent restenosis of the lumen. For example, macropores with a microporous body might slow down

cellular movement and prevent restenosis as we have shown previously (Vrana et al., 2011). However, advantages of a single operation procedure can be overshadowed by the extent of immune response. Also the level of epithelial migration might not be enough for full coverage of the implant surface. For this end, a surface coating which would provide a better medium for epithelial movement would be useful. Layer-by-Layer (LbL) film production methodology can be used for this aim with natural molecules such as collagen to make surface properties more basement membrane-like. LbL films are extremely simple to build up and they are also very versatile, that is, thickness, surface charge, mechanical parameters can be tuned easily by changing the number of deposited layers or the buildup conditions.

In order to decide for the right conditions for in situ epithelialization, monitoring of implant integration is necessary. Also since in trachea, implant failures can be fatal, their strict monitoring is crucial (Grillo, 2002). For this end, rabbits are appropriate models as the shape and relative size of their trachea is a good mimic of that of humans when normalized with respect to their size (ten Hallers et al., 2004). Moreover, rabbits are very susceptible to pulmonary infections, as such infections have been cited as one of the most common causes of death in rabbits (Rougier et al., 2006). This is relevant in the sense that, after implantation due to the unsterile environment of the tracheal passage, the implants might be prone to development of infection. This should be taken into account as the products are developed for clinical trials, for example, abrupt loss of animals has regularly been reported during similar interventions (Tatekawa et al., 2010). Due to these problems, detection of infections and removal of pathogens are extremely important. Determination of pathogen related peptides and monitoring of inflammation/immune system response related molecules by HPLC can thus provide crucial information on post-operational care of the implant. It has been utilized in a wide range of in vivo detection problems such as transplant rejection or cytotoxic material presence (Horuk et al., 2001; Tseng et al., 2005). C-reactive protein (CRP) is a common marker of inflammation and infection which has found clinical applications. Recently Chromogranin A (CGA, a severity marker) has also been shown to be a potent agent for infection and inflammatory response detection. In this study, a hybrid implant for tracheal implantation was monitored in vivo for its effectiveness in cell movement control and for determination of the right time period for secondary seeding of autologous epithelial cells with respect to animal health and tissue integration. The monitoring of animal condition was done by regular blood tests based on CRP levels and characterization of blood samples by HPLC and subsequent peptide sequencing. Also a surface modification of the implant was done with LbL methodology to provide a more basement membrane like structure for migration of respiratory epithelium cells.

Materials and Methods

Materials

Rabbit CRP ELISA kit was obtained from Helica Biosystems (Fullerton, CA) and Triple Express from Gibco/Life Technologies (Saint Aubin, France). Collagen type I (source: Bovine) was obtained from Symatase (Lyon, France) and alginate from Novamatrix (Pronova UPLVG medical grade, FMC Biopolymers AS Novamatrix, Sandvika, Norway). The rest of the products including, poly(L-lactic acid) (PLLA), chloroform, dioxane, and TOX-8 were purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

Animal Model

Adult White New Zealand rabbits ($n = 10$) between 14 and 16 weeks of age and weighing an average of 3 kg were used for this study. Each animal received care in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010) and were housed in our institution at the Faculty of Medicine of Strasbourg (France). They were fed with a standard laboratory diet and tap water ad libitum.

Methods

Implant Production

Porous titanium implants with an inner diameter of 7 mm and an outer diameter of 10 mm were produced by previously defined electric-arc discharge method by using 400–500 μm diameter beads. The final length of the implants was 2 cm. A circular insert of 6 mm was cut out of the implant body with a laser beam (IREPA Laser, Illkirch, France) for posthumous investigations. The body of the implant was filled with a porous PLLA structure as described previously (Vrana et al., 2011). Briefly a 6% PLLA solution in dioxane/water binary mixture (v:v 87/13%) was heated to 60°C and then poured into a Teflon mold that contains the titanium implant. Then the system was left for gelation at room temperature for 30 min and then frozen at -70°C overnight. The system was rendered porous by freeze extraction with an extraction step in cold 80% ethanol (Goh and Ooi, 2008). The outer element of the mold has been previously removed and only the mandrel was left. This creates an open pore gradient from extraluminal side to intraluminal side. Finally a layer of 1% PLLA film was laid into the lumen and then precipitated with the application of EtOH (Fig. 1). The final structure was washed several times with PBS and then sterilized in an antibiotic/antimitotic cocktail as suggested previously (Shearer et al., 2006) followed by UV sterilization.

In Vivo Experiments

Anesthesia. All surgeries were performed under general anesthesia. Anesthesia was induced by intramuscular administration of ketamine (30 mg/kg, Ketamine[®] 500; Virbac, Carros, France) in combination with midazolam (0.2 mg/kg, Mydazolam[®]; Mylan, Saint Priest, France) and xylazine (3 mg/kg, Rompun[®]; Bayer AG; Leverkusen, Germany) and assisted ventilation (O₂:1 L/min). Postoperative analgesia was maintained by fentanyl patch (3 μg , Fentanyl-Mepha[®]; Mepha Pharma, Aesch, Switzerland) for 6 days.

Surgical technique. All operations were performed under sterile conditions. With the animal in the supine position after a vertical midline cervicotomy, the infrahyoid muscles were separated from the tracheal axis. After dissecting to the level of the thyroid, a tracheal segment of three to four rings (15 mm) was resected. Each tracheal extremity was then inserted into the prosthesis, which replaced the defect. A silicone tube was placed in the titanium prosthesis and sutured to the prosthesis with one proximal stitch (vicryl 4.0). The porous titanium prosthesis and the tracheal extremities were joined by four proximal and distal sutures (vicryl 4.0). Before closing the skin and the subcutaneous layers without drainage, a myoplasty was performed to cover the prosthesis and limit possible peritracheal leakage.

A tracheal segment, silicone tubes (stents) were used for the endoluminal calibration of the porous titanium prostheses for the in vivo experiments. They were 40 mm long with an internal diameter of 5 mm. They protruded from each end of the prosthesis by 10 mm, thus providing protection of the tracheo-prosthetic anastomoses.

Implant Follow-Up

A daily clinical follow-up was performed. Data regarding the general well-being of the animals and their weight were recorded throughout the duration of the experiment.

Implantation period was 6 weeks and regular CRP readings were taken weekly by an ELISA kit to determine the level of inflammation as per the instructions of the manufacturer. Blood was obtained from the auricular veins and centrifuged at 5,000 rpm at 4°C for 20 min, the supernatant was obtained and tested for CRP levels. The rest of the plasma was utilized for further characterization.

Plasma Characterization

Purification of proteic material by reverse phase HPLC. Soluble biological material was obtained by acidic extraction with 0.1% of trifluoroacetic acid of rabbit plasma (1/1, v/v). The soluble extract was purified using a Dionex HPLC system (Ultimate 3000; Sunnyvale, CA) on a nucleosil reverse-phase 300-5C18-column (4 mm $\times$ 250 mm; particle size 5 μm ; porosity, 300 Å, Macherey Nagel, Hoerd, France). Absorbance was monitored at 214 and 280 nm, and the solvent system consisted of 0.1% (v/v)

Figure 1. Scheme of the proposed implant, as macroporous structure of titanium implants does not hinder cell movement (a). A hierarchically porous PLLA body with a thin film layer facing the lumen has been introduced (b). c,d: Macroscopic view of titanium only and titanium PLLA implants. SEM images of (e) extraluminal and (f) intraluminal surfaces of the implant (from microporous surface to a smooth surface). g: The perspective view of the final implant model, where the titanium beads are engulfed by the hierarchically porous PLLA body.[Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/bit>]

trifluoroacetic acid (TFA) in water (solvent A) and 0.09% (v/v) TFA in 70% (v/v) acetonitrile–water (solvent B). Elutions were performed at a flow rate of 700 μ L/min using gradients indicated on chromatograms. Each peak fraction was collected and concentrated by evaporation, but not to dryness with the speed-vac.

Western blotting. Samples were loaded on 15% SDS–PAGE and then transferred to PVDF membrane (HyBondTM-P, GE Healthcare). During migration a voltage of 50 V was applied for the first 10 min and then 120 V till end and during transfer 75 V was used for 90–100 min. For immunodetection Millipore Snap i.d. protein detection system was used using 0.5% of bovine serum albumin as blocking agent. Antibodies used were monoclonal anti-CGA (anti-CGA_{47–68}). Secondary antibodies used were goat anti-mouse immunolabeled bands were visualized by using the imager system ChemiDoc XRS (Bio-Rad, Marne-la-Coquette) after addition of ECL reagent (Amersham Bio-Science,

Buckinghamshire, UK). To compare with our previous technique (intramuscular implantation prior to tracheal replacement) (Dupret-Bories et al., 2011), the CGA tests were also done for the samples obtained from animals who received a tracheal titanium implant with or without epithelial cells after 3 weeks of intramuscular implantation. Briefly, the steps were (i) implantation of titanium endoprosthesis tube under the infrahyoid muscles (day 0); (ii) implantation of the prosthesis to replace a resected tracheal segment (day 21). After placing the animal in the supine position, a cervicotomy was performed with a midline vertical incision, allowing access to the infrahyoid muscles, which was separated from the tracheal axis. The prosthesis was placed between the infrahyoid muscles and the trachea. Then a silicon tube was inserted into the porous titanium prosthesis. The skin and subcutaneous layers were then closed with separated stitches of Vicryl 2.0. After 21 days, the cervical cutaneous incision was reopened.

The initial silicon tube was removed from the titanium prosthesis and replaced with a new silicone tube (placed inside the porous titanium prosthesis) that was sutured to the prosthesis with one proximal stitch (Vicryl 4.0). The prosthesis was laterally transposed (with its endoluminal surface covered with epithelium) to replace a 2 cm tracheal segment which was resected during the same operation. The proximal and distal tracheal extremities were inserted into the titanium prosthesis and the junctions of the prosthesis and the tracheal extremities were joined with 6 sutures of vicryl 4.0. Before closing the skin and subcutaneous tissue, a myoplasty was performed to improve coverage and limit future leakage.

Automatic Edman sequencing of peptides derived from the plasma. The N-terminal sequence of purified peptides was determined by automatic Edman degradation on a Procise microsequencer (Applied Biosystems, Courtaboeuf, France). Samples purified by HPLC were loaded to polybrene-treated glass-fiber filters. Phenylthiohydantoin-amino acids (Pth-Xaa) were identified by chromatography on a C₁₈ column (PTH C-18, 2.1 mm × 200 mm) (Gasnier et al., 2004). For the identification of the sequence SWISS-Prot database was used by Blast software.

Histological Analysis

After an observation period ranging from 1 to 6 weeks, the rabbits were euthanized with an intravenous overdose of sodium pentobarbital (120 mg/kg, CEVA Santé Animale, Libourne, France) after intramuscular administration of anesthesia (the same protocol previously described). A block resection, including surrounding tissues as well as the prosthesis, was performed. After explantation, the inserts were removed and observed with scanning electron microscopy (SEM) and confocal microscopy to check the structure and the presence of PLLA respectively. The implants were explanted at 2nd, 3rd, and 6th weeks of implantation and the histological cuts and hemotoxylin and eosin staining was done as reported previously (Schultz et al., 2007). Histological analyses were performed at the IMM (Institut Mutualiste Monsouris, Paris, France), where the blinded analyses were performed by two pathologists not involved in the project.

Collagen/Alginate LbL Production

Collagen type I and Alginate were dissolved at 0.5 mg/mL in 0.15 M NaCl buffer at pH 4. LbL films were constructed on PLLA/titanium implants with an automated dipping robot where for each layer a 1:1 ratio of each polyelectrolyte was applied for equal time periods. The duration of dipping was 8 min for each polyelectrolyte with 5 min washing steps in between until 24 bilayers were reached. During the film construction, all the rinsing steps were performed with a 10 mM citrate buffer containing 0.15 M NaCl at pH 4. Cross-linking of the film layers on the implants was achieved by chemical cross-linking via genipin. Genipin solution (20 mg/mL) was prepared by dissolving 20 mg of the genipin

powder in DMSO/buffer (0.15 M NaCl, pH 4) mixture (1:5). The cross-linking agent solution was let in contact with the coated PEM film for 12 h, followed by several rinsing steps. Formed film layers were characterized by atomic force microscopy (AFM) and SEM imaging.

Epithelial Cell Culture

The use of human tissues was authorized by the French committee "Comité de Protection des Personnes" (CPP-Est III, Nancy), statement number DC-2008-374. Nasal polyps were taken with a written consent from the patients. Human respiratory Epithelial cells were obtained from freshly removed nasal polyps as described before (LeSimple et al., 2007). Cells were seeded on collagen/alginate covered hybrid PLLA/titanium implants as 5×10^4 cells/implant. Cell proliferation was observed by a Resazurin-based proliferation assay ($n \geq 6$) and morphology of the cells by fluorescence microscopy (DAPI and ZO-1 immunostaining). Migration of the epithelial cells was quantified by time lapse microscopy and compared to the positive controls, collagen type IV and collagen type I coatings ($n \geq 40$).

Results

Implant Patency and Fibroblast Movement

The developed method produces a PLLA body within the titanium template with a porosity of 40–60 μm at the extraluminal part to 5–10 μm intraluminally. This structure enables the entry of fibroblasts and macrophages but prevents their migration into the lumen area (Vrana et al., 2011). The addition of the PLLA film layer in the intraluminal side was necessary to provide a surface for epithelial cells to migrate from anastomosis sites. The film layer was a surface visible on the structure of the implant (Fig. 1e). It has been previously shown that such systems can affect the movement of cells in vitro (Matschegewski et al., 2010).

All animals survived the surgical procedure. Implantations went uneventful except removal of the sutures by two rabbits 1 day after implantation. These rabbits were treated with local antiseptics, but one of them was lost at week 4 due to deep infection of the wound site. Another rabbit was lost due to peri-implantitis. Upon explantation, all implants were in contact with the native trachea wall, both in proximal and distal ends. In histological sections, fibroblasts are observed together with occasional multinucleated cells (Fig. 2). For fibroblast migration a similar behavior to that of in vitro conditions was seen in vivo, as there was a distinct decrease in the fibroblast movement in hybrid implants compared to titanium only implants. In the case of hybrid implants the fibroblast were able to get into the polymer body (cells were visible inside the foam after 2 weeks and their depth of movement gradually increased), but they

Figure 2. Histology after explantation. For PLLA/titanium implants cellular movement was controlled by PLLA presence; fibroblasts were restricted to the surrounding area in week 2 (a) then their movement into the implants was more apparent by week 3 (b) and week 6 (c). By week 6 whole implant was covered with the fibrovascular tissue with the tissue ingrowth into the implant, without the presence of fibroblasts in the lumen. On the other hand, only titanium implant was infiltrated with fibrotic tissue after 1 month (d) but the lumen has been started to be colonized which will end up in restenosis. e: The mode of ingrowth was from the extraluminal side. The tissue within the implants had distinct blood vessels. Epithelium movement was restricted to the areas close to the anastomosis sides and even though the amount was higher in PLLA/titanium implants, the middle area of the implants lacked epithelial cells. Thus, after initial integration an in situ seeding of autologous epithelial cells might be necessary. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/bit>]

have not been able to reach the lumen side over a 6-week period (Fig. 2a–c). During this period, fibrovascular tissue surrounded the implant with noticeable growth into the polymer body, which has reached the middle part of the implant after 6 weeks (Fig. 2c, inset). The only titanium implants were colonized totally in 1 month period (Fig. 2d) and there was no way of keeping cell growth away from the lumen, thus fibroblasts in the lumen area were clearly visible, which suggested that longer periods would result in restenosis. Effectively the hybrid system is a temporary obstacle for the fibrovascular tissue infiltration which prevents the overcome of epithelial growth by fibroblasts. In other words, presence of polymer body kept the growth of the fibrous tissue back to give epithelium enough time to grow.

CRP, CGA, and Blood Protein Levels

The change in rabbit health was monitored by CRP levels. CRP is an acute phase protein and it has been established as a good method to monitor inflammation. In this scheme, inflammation might come from two sources: inflammation due to reaction to the implant and inflammation due to infection. When there is no infection symptoms CRP readings dropped after an initial hike in the first week and stayed only slightly over the baseline of healthy animals over 6 weeks, but they were still below the acceptable limit for CRP in rabbits (5 ng/mL) (Fig. 3a). CRP readings for normal animals were around the levels of previously published reports (below 5 ng/mL) (Alvarez et al., 2008). However, when there was an infection, before the symptoms became

Figure 3.

apparent, CRP levels elevated steeply (Fig. 3b). For example, one of the animals had elevated CRP levels (above 20 ng/mL) and then started to show infection symptoms. This animal's trachea was cleared with a small intervention and animal was healthy afterwards and survived up until the pre-determined sacrifice date. This showed that CRP levels are a good early warning method (Löbler et al., 2002). To further check this trend the proteome analysis were done in the serum samples over a timeline of 6 weeks, 1 animal who died of a wound site infection was used as negative control and a healthy animal as positive control. The HPLC profile showed distinct peaks corresponding to alpha and beta hemoglobin $\frac{1}{2}$ as determined by Edman sequencing (Fig. 3c,d). Animals with tracheal implants show an increase in both hemoglobin alpha and beta $\frac{1}{2}$ amount after 1 week of implantation compared to healthy animals, followed by a progressive decrease in 2, 4, and 6 weeks. Similar to the CRP readings, in case of an infection there was a very strong increase in alpha and beta hemoglobin $\frac{1}{2}$ readings.

When the levels of CGA was checked at time points 3, 4, and 6 weeks, the level of unprocessed CGA (72 kDa) stayed stable, but an increase in the processed CGA (20 kDa) was observed (Fig. 4a). This processed CGA fragment (20 kDa) is known to be involved in improving cell attachment (Colombo et al., 2002). As CGA has been previously shown to be an even better indicator of stress (Shooshtarizadeh et al., 2010; Zhang et al., 2009), this check would be robust warnings for the set of infection and subsequent treatment. Moreover, they provide a means to compare different

protocols in their influence in body response. When compared to our previous two-step method (intramuscular implantation and after 21 days, tracheal implantation), the PLLA/titanium hybrid system had less complete CGA. Processed CGA levels of two steps method after 1 week of tracheal implantation were at the level of 6 weeks for PLLA/titanium hybrid system (Fig. 4b). This methodology would open up a venue for checking the implantation behavior in a broader sense and specific to each animal, as tracheal replacement is a debilitating process and such methods for patient-based monitoring are necessary. When collaborated with the histology and CRP results, these results suggested that for initial fibrovascular population of the implants, 3 weeks period was necessary, during this period the initial immune response to implantation decreases but also the animals gradually become more prone to respiratory infections, mainly due to the lack of epithelium. Thus, after in vivo colonization of implant by the surrounding tissue for 3 weeks, the structure will be ready for local antibiotic treatment and subsequent in situ epithelialization.

Epithelialization

The luminal surface of the implant is designed as a smooth film layer to facilitate epithelial cell attachment and migration. SEM imaging of the implant inserts after explantation showed that the film layer was largely intact after 4 weeks of in vivo incubation (Fig. 5). This layer has

Figure 4. Western blots for determination of complete and/or digested CGA during the course of implantation. **a:** Animals which were implanted with PLLA/titanium implants over 6 weeks, complete CGA levels were similar, but the digested CGA (an N-terminal fragment) increased over time. **b:** comparison of PLLA/titanium hybrid implant procedure (3, 4, and 6 weeks denoted 3w, 4w, and 6w respectively) with two steps implantation protocol developed previously (Dupret-Bories et al., 2011) (intramuscular implantation, followed by tracheal implantation denoted 4 and 4c, where c corresponds to addition of epithelial cells. PSG stands for Positive control. The CGA levels were significantly higher in the cases of two steps implantation.

Figure 3. Animal health checks. **a:** CRP readings on healthy and implanted animals over 6 weeks, CRP levels decrease over implantation period after an initial increase. **b:** when there is infection an increase in CRP signal was observed. **c:** HPLC separation of plasma samples obtained from the animals over the course of implantation, where the profile of an healthy animal and a sick animal were used as positive and negative controls respectively. **d:** a representative of the readings where a distinct peak corresponding to hemoglobin beta $\frac{1}{2}$ chain is marked, the comparison of the relative size of the peaks at different time points showed a correlation of the peak with implant integration. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/bit>]

Figure 5. Stability of the film layer. The presence of film layer was monitored (a–c) after 2 weeks, (d–f) after 4 weeks of implantation. The film layer was in place but effects of degradation was apparent, with occasional cracks and an increase in the surface roughness. Confocal images of the film layer acquired at (g) time 0 and (h) after 4 weeks of in vivo implantation. The film layer was mostly intact, but the formation of further pores on the surface was visible (magnification 20×). [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/bit>]

been previously shown to support human respiratory cell proliferation. However in the view of the histological results, in vivo growth of epithelium was still slow (limited to the close proximity of the anastomosis sites). Epithelialization is generally a long process, basically due to the mode of growth of epithelium (Fong et al., 2010) and can only be overcome by surface treatments with growth factor gradients to a certain extent, as the boundary conditions cannot be changed. However, surface coatings can further improve the outcome. Previously in canine specimens the replacement of epithelium was observed on a polycaprolactone (PCL) based lumen coating of a collagen-based scaffold, but only after 18 months following the surgery (Sato et al., 2010). The authors state that there was a distinct improvement in epithelialization in the presence of the film coating. In this

model, no prior autologous cell seeding was done. When decellularized tracheal scaffolds were used, Go et al. (2010) reported a distinct improvement in the case where autologous cells were seeded. Initial attachment of the cells becomes important, which can be very limited depending on the surface properties of the implant.

To further improve the implant design, we coated the lumen of the implants with collagen/alginate polyelectrolyte multilayers cross-linked with genipin. This cross-linking step was necessary to stabilize the film in physiological conditions (pH 7.4). The cross-linking was effective between collagen chains. Genipin acts on collagen molecules with primary amine groups and has the ability to form both intra and intermolecular cross-links (Hwang et al., 2011). However, we demonstrate that, alginate molecules from

Figure 6. Development of collagen/alginate multilayers on the implant lumen. **a:** A thin layer of the collagen/alginate film covers the surface of the implant over the titanium beads and microporous PLLA body after 24 bilayers were deposited **(b)** which showed a strong attachment to the implant body. **c:** Films have on average a thickness around 400 nm as determined by scratch tests **(d)** and they depicted a highly nanofibrillar structure. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/bit>]

the cross-linked film were maintained in physiological conditions (Fig. S1). The exact mechanism of cross-linking is not fully described, even though a mechanism based on nitrogen-iridoid formation between C3 of genipin and the primary amines of lysine, hydroxylysine and arginine (Sundararaghavan et al., 2008) could be considered. When these layer were built at high bilayer numbers (24), a thick layer of film on top of the porous PLLA/titanium hybrid can be obtained (Fig. 6a,b). When the titanium samples are dipped into the polyelectrolyte solutions, the solutions cannot flow into the pore areas and they form thin films even in the absence of PLLA, but these films have wrinkled surface features compared to the smooth surface obtained in the case of PLLA/titanium. The thickness of this film layer determined by AFM was around 400 nm and the components of the film layer had a nanofibrillar structure which is known to improve the cell attachment due to its similarity to basement membrane. This structure had a surface roughness of 20 nm which is also appropriate for epithelial cell culture (Fig. 6c,d).

Human respiratory epithelial cells attached and proliferated on these structures, with near confluent layers obtained by 10 days (Fig. 7a–e). Cells proliferated faster on these structure compared to standard epithelial cell culture on Transwell inserts and also the cell numbers were higher compared to our previous results with PLLA films (Vrana et al., 2011). Transwell inserts were selected as control since they are widely used for in vitro differentiation of epithelial cells to ciliated epithelium by air–liquid interface culture method, which is crucial for respiratory epithelium. Moreover, in long term culture periods which are necessary for differentiation, long-term viability of epithelial cells was higher on collagen/alginate multilayers compared to Transwell. Cells stayed on the implant surface even after 19 days of culture (Fig. 7d), whereas in the case of Transwell and PLLA films, cell numbers saw a decrease after 13 days. To check the rate of migration, epithelial cells were observed with time lapse microscopy and their migration rates were calculated. In general cells migrated slower on collagen type IV than on multilayer counterparts ($P < 0.05$), whereas pure

Figure 7. Human respiratory epithelial cell growth and migration on collagen/alginate coated implants. Collagen/alginate multilayers provided a better surface for cell growth than Transwell. Cell numbers were significantly higher than our previous system with PLLA films too. **a:** Phase-contrast micrographs of confluent respiratory epithelium cells. **b–d:** DAPI/ZO-1 staining of the cells on the implants after days 7, 13, and 19 (magnification 10×). **e:** Epithelial cell proliferation curve on collagen/alginate multilayers versus Transwell and PLLA systems. Cell numbers were significantly higher on collagen/alginate multilayers at days 3, 6, and 13. **f:** Macroscopic view of the coated cell seeded implant. **g:** Rate of migration (μm/h) of respiratory epithelial cells on three substrates: collagen type I, collagen type IV, and collagen/alginate multilayers ($n > 40$). **h, i:** Representative images of migration tests. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/bit>]

collagen type I coating was a better surface for migration. However, collagen type I by itself is not a good substrate for respiratory epithelium proliferation, thus by using the LbL technology a surface that behaves better than pure collagen can be created. An additional coating of a collagen/alginate multilayer would help the epithelialization of implants, however with an average migration rate of 45.6 μm/h, a 2 cm defect can only be covered in about 10 days, if the movement is unidirectional from the both anastomosis sites.

Thus our next step was to seed the epithelial cells to an implant that has already been integrated with the body (after 3 weeks of implantation). This can be done endoscopically with the modification of already available endoscopical intervention equipment. A possible option would be a tubing system that will provide the airflow together with

a cartridge facing the lumen of the surface that needs to be epithelialized filled with freshly isolated autologous epithelium in autologous serum. This might provide an “on-site” method for the epithelialization of the implant. In such a method, the epithelium would not need to deal with the initial inflammation and it can grow on an integrated structure thus the delay in vascularization can be eliminated. As a proof of concept, Calcein-AM labeled respiratory epithelial cells have been seeded on implants just after explantation. These implants were previously remodeled for 3 weeks between the infrahyoid muscles and the trachea. The epithelial cells have attached and spread on these surfaces in an hour (Fig. 8), which indicates that in situ epithelialization is an achievable goal in a clinical setting.

Figure 8. a: Proposed in situ epithelialization system, where the implant was left for integration in vivo and then epithelial cells were seeded in vitro to obtain a functional epithelium. b: Seeding of Calcein-AM labeled respiratory epithelial cells on implant surfaces after 3 weeks of in vivo incubation; the surface created via colonization provided a suitable substrate for epithelial cell attachment and spreading (after 1 day of in vitro incubation). [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/bit/>]

Conclusion

The hybrid PLLA/titanium tracheal implants can stop the initial ingrowth of the fibroblasts into the lumen. The histology and blood characterization results showed that the 3 weeks time-point is the optimal time delay for in situ seeding of epithelial cells. Analysis of CRP and CGA levels are viable methods to check the process of implant integration. A further modification of inner surface of the implants with collagen/alginate multilayers resulted in a 400 nm thick fibrillar film which can support epithelial cell growth and migration. As the next step, these implants will be used for in situ epithelialization experiments.

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