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PulmoStent: *In Vitro* to *In Vivo* Evaluation of a Tissue Engineered Endobronchial Stent

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Abstract—Currently, there is no optimal treatment available for end stage tumour patients with airway stenosis. The PulmoStent concept aims on overcoming current hurdles in airway stenting by combining a nitinol stent with a nutrient-permeable membrane, which prevents tumour ingrowth. Respiratory epithelial cells can be seeded onto the cover to restore mucociliary clearance. In this study, a novel hand-braided dog bone stent was developed, covered with a polycarbonate urethane nonwoven and mechanically tested. Design and manufacturing of stent and cover were improved in an iterative process according to predefined requirements for permeability and mechanical properties and finally tested in a proof of concept animal study in sheep for up to 24 weeks. In each animal two stents were implanted, one of which was cell-seeded by endoscopic spraying *in situ*. We demonstrated the suitability of this membrane for our concept by glucose transport testing and *in vitro* culture of respiratory epithelial cells. In the animal study, no migration occurred in any of the twelve stents. There was only mild granulation tissue formation and tissue reaction; no severe mucus plugging was observed. Thus, the PulmoStent concept might be a step forward for palliative treatment of airway stenosis with a biohybrid stent device.

Keywords—Tissue engineering, Stent development, Airway stenting, Animal trial.

INTRODUCTION

Lung cancer is the most frequent cancer in terms of mortality in Europe.[3] As palliative treatment for severe stenosis of the larger airways, which occur frequently with lung cancer, stent placement is the method of choice.[4] To inhibit tumour ingrowth and restenosis, silicone or covered metal stents are used. Still, these stents have major disadvantages like frequent migration and mucus plugging of the airways due to the absence of mucociliary transport[4]: In healthy airways, epithelial cells develop motile cilia on the luminal surface which transport mucus to the laryngopharynx for the mucus to be swallowed or expectorated. Thus, an optimal treatment for severe malignant stenosis of the larger airways is still lacking.

The BioStent concept—a combination of tissue engineering and a metallic stent—is a promising approach to overcome current hurdles such as in-stent restenosis and thrombosis of vascular stents.[16] The BioStent concept is shown in Fig. 1a. A warp-knitted nitinol stent provides mechanical stability. It is embedded in a fibrin matrix with vascular smooth muscle cells. Seeded endothelial cells completely cover the luminal side of the stent. With this setup, the

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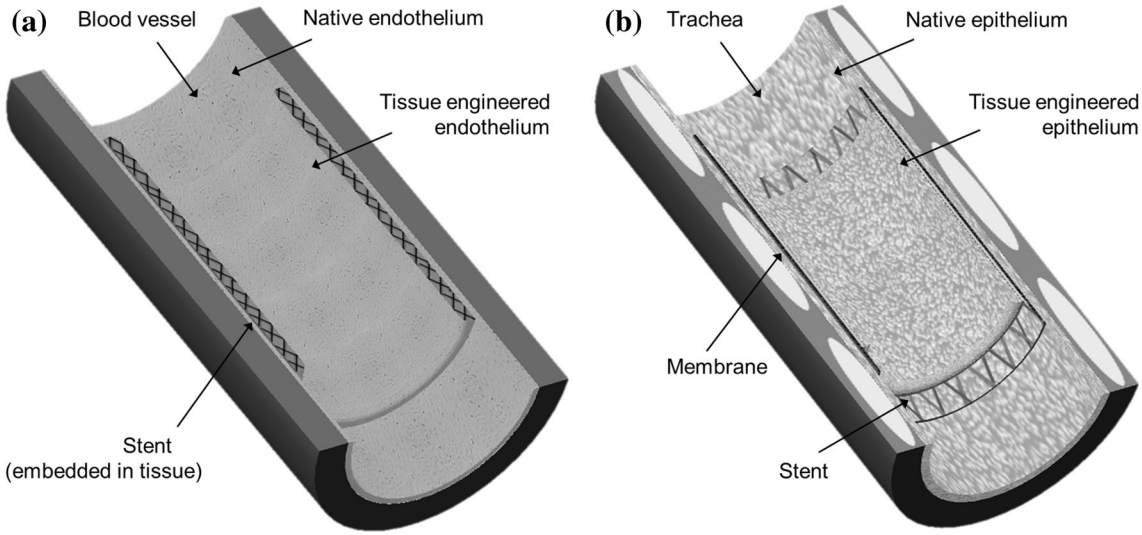

FIGURE 1. Novel tissue engineering stent concepts. a BioStent for atherosclerotic plaques in blood vessels. The stent is embedded in a cell-seeded fibrin matrix and coated with endothelial cells. b PulmoStent for airway stenosis. The stent is covered with a permeable membrane which allows nutrient transport but inhibits tumour ingrowth. The membrane is seeded with respiratory epithelial cells to restore mucociliary clearance.

nitinol is neither in contact with blood nor the surrounding tissue and therefore prevents thrombus formation and neointima development.

To transfer the BioStent concept to an airway stent (as shown by Thiebes *et al.*[15]), two adjustments are made resulting in the PulmoStent concept (Fig. 1b):

(1) As main feature, the stent is covered with a membrane on which respiratory epithelial cells are seeded to restore mucociliary clearance. This membrane acts as a mechanical barrier to prevent tumour ingrowth. To allow nutrition of the epithelial cells on the lumen, the membrane needs to be permeable for glucose and other nutrients and growth factors. However, it still has to provide sufficient mechanical strength to inhibit tumour ingrowth and restenosis.
(2) Fully covered stents often migrate, as they cannot be anchored in the airway tissue, thus our stent has uncovered ends to provide enough tissue anchorage to prevent stent migration.

Additionally, tumour-specific therapeutics can directly be incorporated into the membrane to locally treat the bronchial tumour and suppress its growth.

In this study, we show technical, *in vitro* and *in vivo* evaluation of a novel stent. Basic requirements from the mechanical and clinical point of view were predefined for this new stent concept. The requirements involved factors concerning implantation and explantation (small crimping diameter, radiopaque, removable), influence on the bronchial and peribronchial tissue (radial force, design of the edges, biocompatibility) and long-term stability (durability of the cover).

Thus, a tailor-made hand-braided stent with dog bone structure was developed to meet the requirements and suit the anatomic features of sheep bronchi (see supplementary data). As membrane material, polycarbonate urethane (PCU) has been chosen because of its biocompatibility and routine use for respiratory stents.[13] A set of processing techniques and parameters have been tested to produce an optimal membrane (data not shown). To provide a suitable cover which prevents tumour ingrowth and supports nutrient transport for respiratory epithelial cells, a spray processed nonwoven has been chosen.

In this study, the suitability of the final membrane for cell growth was proven by glucose transfer tests and respiratory epithelial cell culture on the PCU membrane; covered stents with the final design were consequently mechanically tested and compared to a benchmark. Finally, the stent was tested in a sheep model for suitability and functionality. Cells were applied with a novel spray device and embedded in a thin layer of fibrin gel as previously described.[15] We propose that the epithelium can withstand shear stresses and therefore use the sheep's respiratory system as bioreactor for *in vivo* tissue engineering as proposed by Jungebluth *et al.*[7]

MATERIALS AND METHODS

PulmoStent Fabrication

Production of Braided Stents

For this study, hand-braided dog bone stents were produced from nitinol with a wire diameter of 200 μm (Fort Wayne Metals, USA). The basic geometry data

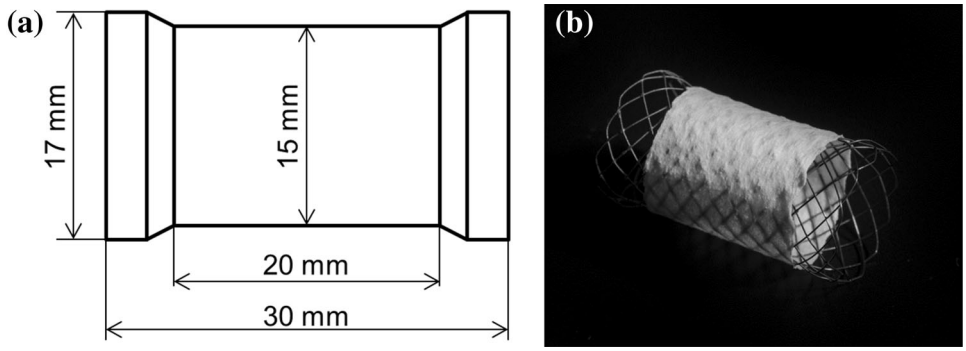

FIGURE 2. PulmoStent. a Dimensions of the core. b Final spray coated hand-braided stent.

include a central diameter of 15 mm and a length of 30 mm. At both ends, the stent was flared to a diameter of 17 mm over a length of 5 mm (Fig. 2a) to prevent stent migration. Using a core with dog bone structure, the stent was produced by a manual braiding process. The wire was guided on a helical curve alternating over and under the already braided structure around the core such that a stent structure from one wire without open wire ends could be produced. The stent has 24 crossing wires and twelve deflection points of the wire at each stent end.

Membrane Fabrication (Covering of Stents)

To achieve a proper adhesion between the stents' nitinol surface and the polymeric nonwoven membrane, the stent underwent a 3-step pre-treatment (patent pending). Two layers of adhesion mediators were applied onto the stents in a reproducible manner. Functionalized polyurethane was then thinly coated on the stents. The proximal flared parts of the stents were not pre-treated. After this pre-treatment, the PCU membrane was sprayed onto the stents. The spray atomization procedure with dissolved PCU (7.5 wt% (Carbothane PC-3575A, Lubrizol Corp., USA) dissolved in chloroform) A1585, AppliChem GmbH, Germany)) was described by Nadzeyka *et al.*[11] For stent coating, a pressure of 0.8 bar, a material flow rate of 2 ml/min and a spraying duration of 5 min were adjusted to achieve a thickness of 250 μm. During spray coating both flared ends of the stents were protected with adhesive tape from PCU covering. The stents were dried at 30 °C for at least 2 h to let the chloroform evaporate from the coating. Then they were carefully cut off the spraying spindle and the adhesive tape with the cover was removed from the flared ends. Figure 2b shows a spray coated hand-braided stent.

Cleaning and Loading

The stents and delivery systems were prepared for sterilization in a pressure controlled cleanroom environment. The delivery systems were flushed with deionised water and then wiped with Virkon and 70% industrial methylated spirits using lint free wipes from the proximal to distal end. The stents were placed in deionised water in an ultrasonic bath for 10 min at 40 °C. After drying, the stents were then loaded into the delivery system and placed in a heat sealed pouch. Ethylene oxide sterilisation was undertaken by Osypka AG, Rheinfelden-Herten, Germany.

Stent and Material Testing

Mechanical Testing

Stent mechanical testing was performed on the covered hand-braided dog bone stent and the commercially available fully silicone covered Nitinol aerstent® (Leufen Medical GmbH, Germany). The aerstent® had a length of 30 mm, a diameter of 16 mm in the central part and a diameter of 18 mm at the proximal and distal ends. Radial testing was performed to measure stent radial resistive force (RRF) during stent loading at the stent crimp diameter. The RRF is a measure of stent radial force when it is loaded to its crimped position. Chronic outward force (COF) was measured during stent unloading at the target vessel diameter. The COF is a measure of stent radial force when it is unloaded and deployed into a bronchus.

For the mechanical testing the stents were loaded to a diameter of 7.5 mm and unloaded to their original diameter using eight radially aligned parallel plates on radial testing machine (RCM-H60, Machine Solutions Inc., MPT, Netherlands) mounted on a single axis tensile tester with a 100 N load cell (Zwick Roell, GmbH & Co. KG, Germany) at a crosshead displacement of 0.1 mm/s). The machine uses an iris mechanism that closes/opens to radially load/unload the stent. A heater control unit attached to the radial testing machine ensured a constant temperature of 37 °C during testing. Frictional forces between the plates of the radial tester were accounted for by

performing an initial test with no stent present. This data was deducted from the stent radial tests in order to obtain accurate radial force measurements related to the stent alone.

Glucose Transfer Testing

Glucose transport across the PCU membrane was determined in an in-house prepared diffusion cell system with two stirred compartments separated by the PCU membrane. The volumes of donor and receptor compartments were 4 ml each. The area of PCU membrane for diffusion was 0.78 cm^2. The donor compartment was filled with 5% glucose in PBS (pH 7.4, 0.049 M NaH_2PO_4, 0.099 M Na_2HPO_4, 0.006 M NaCl). The receptor compartment was filled with blank PBS. Glucose transport was studied at room temperature by analysis of the glucose concentration in 20 μl aliquots from donor and receptor compartments using a commercial glucose meter (Accu-Chek Sensor Comfort®, Roche, Netherlands).

Cell Culture on PCU

Ovine respiratory epithelial cells were isolated and cultured as previously described.[14] Cells in passage 1 were sprayed with a fibrinogen solution (20 mg/ml, Calbiochem, Germany; according to Thiebes *et al.*[14]) onto PCU fixed in CellCrown™ inserts (Scaffdex, Finland) with a concentration of 8×10^4 cells/cm^2 to provide a procedure comparable to the animal trials. The cells were cultured for 7 days in submersed culture with Airway Epithelial Cell Growth Medium (PromoCell, Germany) and consequenty for 21 days with modified Airway Epithelial Cell Growth Medium with retinoic acid (Sigma-Aldrich, Germany) in air–liquid interface. Cells were fixed with paraformaldehyde (Fischar, Germany) for 30 min, the nuclei stained with DAPI and visualized with Zeiss Zoom.V16 and ZEN pro software (2012, blue edition).

Animal Study

The procedures used conform to the ''Guide for the care and use of laboratory animals'' published by the US National Institutes of Health (National Research Council Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). The animal trial was evaluated and approved by the North Rhine Westphalian State Agency for Nature, Environment and Consumer Protection under number 84-02.04.2013.A452. Six healthy female Rhön sheep with a body weight of 45–75 kg were deployed in this study.

Study design

Aim of this study was the proof of principle for the newly developed stent in a large animal model without statistical evaluation of stent behaviour. On the one hand, the suitability of the novel stent in terms of migration behaviour and formation of granulation tissue should be shown, on the other hand, cell-seeded with unseeded stents should be compared. Therefore, there were three groups ($n = 2$) with implantation durations of 6, 12 and 24 weeks. A stent was implanted in each left and right main bronchus, one cell-seeded stent and one without cells. The stents were cell-seeded *in situ* after implantation to prevent damage on the tissue engineered epithelium during the deployment process. At the time of explantation, we analysed migration, formation of granulation tissue and epithelial growth on the cover.

Cell Harvest and Culture

For cell harvest, sheep were pre-medicated with intramuscular 1% atropine sulphate (10 mg, Dr. Franz Köhler Chemie, Germany) and 2% xylazine (24 mg, Vexylan, Ceva, Germany) and endotracheally intubated. Anaesthesia was maintained by ventilation with isoflurane (1–2 vol%). An endoscope with an outer diameter of 8.5 mm was used (60512VG, Karl Storz, Germany). Ten mucosa biopsies were taken with biopsy forceps (Karl Storz, Germany) from the main carina and upper lobe carinae. No relevant bleeding occurred. The biopsies were directly transferred into sterile transport buffer (see Thiebes *et al.*[14]) and stored at 8 °C until further processing. To isolate epithelial cells, the biopsies were transferred to protease solution (1.8 U/ml, Sigma-Aldrich, Germany) supplemented with gentamicin (40 μg/ml, Refobacin, Merck, Germany) for 12 h. Digestion was stopped with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 1% antibiotic/antimycotic (agents: Amphotericin B, Penicillin, Streptomycin; medium and supplements from Gibco, Germany). After centrifugation at 200 g for 5 min, cells were resuspended in DMEM and seeded into cell culture flasks and incubated at 37 °C with 5% CO_2 and 95% humidity. After 2 days, medium was switched to Airway Epithelial Cell Growth Medium and consequently changed every 2–3 days. After 3 weeks of culture, cells were prepared for implantation. First cells were washed with Dulbecco's phosphate buffered saline (PBS, Gibco, Germany), then incubated with trypsin (PAN-Biotech, Germany) for 5 min in the incubator. Digestion was stopped by addition of DMEM. Cells were centrifuged

and dissolved in tris-buffered saline at a concentration of 1.6×10^6 cells/ml.

For spray implantation cell suspension and fibrinogen solution were drawn into different syringes and stored at 37 °C until application.

Stent Implantation

For stent implantation, sheep were anesthetized and intubated as described above. Before stent implantation, a computed tomography (CT) scan was performed and the airways were visually inspected by bronchoscopy to exclude any pre-existing pulmonary ailment. The stents were implanted with a custom-made deployment catheter and fluoroscopical guidance. The application device was made from PE (stabilizer tube) and PTFE (outer sheath) with a total outer diameter of 8.5 mm (Fig. 3).

Two stents were implanted per animal in the right and left main bronchus. Then the cells were spray-coated onto one of the stents (randomly chosen) as described by Thiebes *et al.*[15]. Anaesthesia was terminated and animals extubated as soon as they started breathing spontaneously. Sheep were monitored for 8–24 h after extubation to register choking or coughing complications. Analgesia (4 mg/kg carprofen, Rimadyl, Pfizer, Germany) and antibiotics (2.5 mg/kg enrofloxacin, Baytril 10%, Bayer Vital, Germany) were injected intramuscular for 3 days after the implantation procedure.

Examination of Animals

The animals were examined daily by auscultation. Additionally, every 2–4 weeks stents were bronchoscopically inspected in general anaesthesia.

Evaluation of Results

Euthanasia and Autopsy

On the last experimental day, sheep were anesthetized and intubated as described above. CT scan and bronchoscopy were performed and the above described routine examinations accomplished. Animals were euthanized with pentobarbital (50-80 mg/kg, Narcoren, Merial Germany) and directly dissected. The lungs were macroscopically inspected and examined for abscesses. From both lower lobes biopsies were taken for histology. Afterwards the bronchi with the stents were exposed to document the positioning of and granulation around the stents. Stents and surrounding tissue were stored in sterile transport buffer until further processing.

Histology

For histology, stents were cut into a distal, medial and proximal part and fixed in Carnoy's solution (60% ethanol (VWR, Germany), 30% chloroform (Merck Millipore, Germany), 10% acetic acid (Sigma-Aldrich, Germany)) for 2 h, washed twice with ethanol and dehydrated consecutively in increasing isopropyl solutions. The stents were embedded in polymethyl methacrylate (Technovit 9100, Heraeus Kulzer, Germany) and polymerized at −18 °C.

Embedded stents were processed to 10–30 μm thick sections by sawing-grinding technique and stained with haematoxylin and eosin (H&E) at 60 °C. The lung tissue was fixed in paraformaldehyde (4%, Fischar, Germany) and cut to 10 μm thick sections with a microtome. Images were acquired with a bright field microscope (Imager.D1, Zeiss, Germany) and a CCD-camera (Axiocam MRc, Zeiss, Germany).

To measure the tissue reaction, the area of mucosa and submucosa was measured with GIMP (Version 2.8.2). The length of the analysed tissue was measured with ImageJ (1.44p). With these values, the average thickness of mucosa and submucosa was calculated. As control, we used native bronchi tissue, where no stent was implanted. Results are shown as mean value ± standard deviation.

For scanning electron microscopy (SEM), a piece of the stent with PU-membrane was fixed in 3% glutaraldehyde, washed, dehydrated consecutively in increasing acetone concentrations and critical point-dried with CO_2. Afterwards the samples were sputtered with a 12.5 nm thick gold–palladium layer (Leica EM SC D500). Samples were analysed with a scanning electron microscope (ESEM XL 30 FEG, FEI, Philips, The Netherlands) with acceleration voltage of 7.5–10 kV in a high vacuum mode.

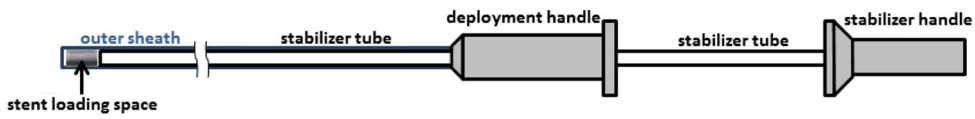

FIGURE 3. Custom-made application system.

RESULTS

Stent and Material Testing

Mechanical Testing

The radial mechanical testing results are shown in Fig. 4a for the covered hand-braided dog bone stent and a commercially available endobronchial stent as control (Leufen aerstent®). A maximum RRF of 86 and 43 N were measured for the covered dog bone stent and aerstent® respectively at a crimp diameter of 7.5 mm. A COF of 5.2 and 11.5 N were measured for the covered dog bone stent and aerstent® respectively during unload at a stent diameter of 12 mm.

Glucose Transfer Testing

Cells growing at air–liquid interface on the luminal side of the stent cover will depend largely on the transport of nutrients *via* the stent cover. To demonstrate that the PCU membrane was fully permeable for transport of nutrients, we studied the diffusion of glucose through the PCU membrane. Transport of glucose over the PCU membrane is fast, resulting in complete equilibrium in glucose concentration between donor and receptor compartment within 1 h, which indicates that the PCU did not represent a hydrophobic barrier for small-molecule nutrients like glucose (Fig. 4b).

Cell Culture on PCU

To proof the suitability of the PCU membrane for cell growth in air–liquid interface and the possibility of long-term culture, ovine respiratory epithelial cells were spray-seeded in a thin layer of fibrin gel and cultured for four weeks on PCU membranes. Cells build up a confluent layer on the PCU. This proves that the nutrient transport through the membrane is sufficient for cell survival and growth (Fig. 4c).

Animal Study

In this study, the stents were implanted in sheep with $n = 4$ stents for 6, 12 and 24 weeks. One of the animals had to be euthanized after 6 weeks due to a severe tracheal stenosis (distance between stenosis and stents was more than 15 cm and therefore unrelated) and dyspnoea which developed probably due to an implantation trauma. As the cause for euthanasia is considered to be not stent-related, the stents are still included in the evaluation.

In general, the stents were well tolerated by the sheep. After implantation, the sheep showed an increased cough reflex which declined after several days. Compared to animals without stents, they coughed more often under effort, but no distress was observed.

Stent Migration

As the stents were newly developed and had not been tested *in vivo* yet, the migration behaviour was of high interest. With bronchoscopy every other week and a CT scan before stent explantation, the location of the stents was checked. The stent shown in Fig. 5 remained at the same location for 24 weeks which can be seen by the bronchus branching at the proximal end of the stent. In A the stent is shown directly after implantation when it is not fully expanded. B shows the same stent after 24 weeks, the stent is nicely lining the bronchus wall. Similarly, no stent dislocated or migrated in any of the animals as can be seen in Fig. 6 with bronchoscopy images of all stents on the day of explantation. The stent after 6 weeks without cells is not presented as the proximal end was stenosed and the stent could not be visualized by the bronchoscope anymore due to implantation in a segmental bronchus branch.

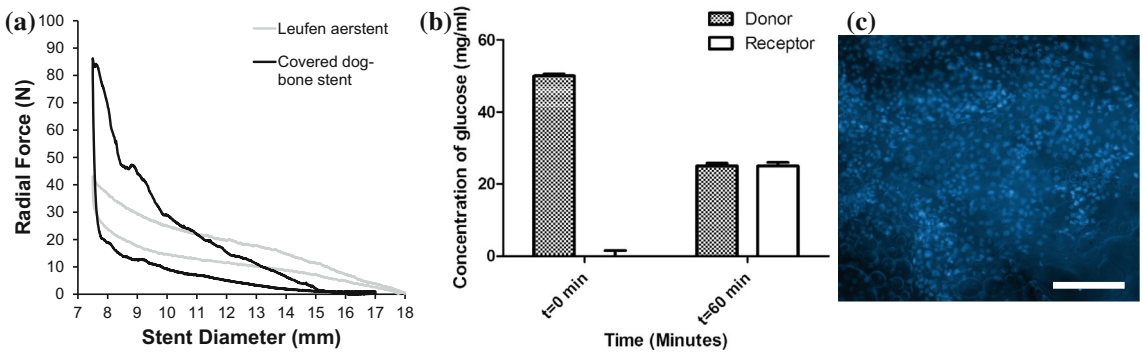

FIGURE 4. a Radial force mechanical testing. Results for the covered dog bone stent and commercially available Leufen aerstent®. b Glucose transport across the polycarbonate urethane membrane. Complete equilibrium is reached after 1 h. c Cells grown on polycarbonate urethane for 21 days in air–liquid interface. Cell nuclei are DAPI-stained. Scale bar 200 μm.

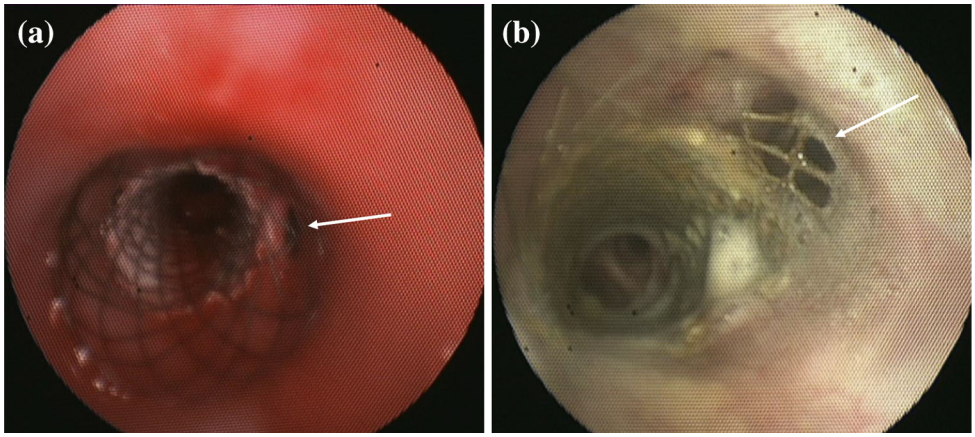

FIGURE 5. Evaluation of stent migration. Stent directly after implantation (a) and 24 weeks after implantation (b). The stent is at the same location as it was implanted as shown by the branching bronchus (white arrows).

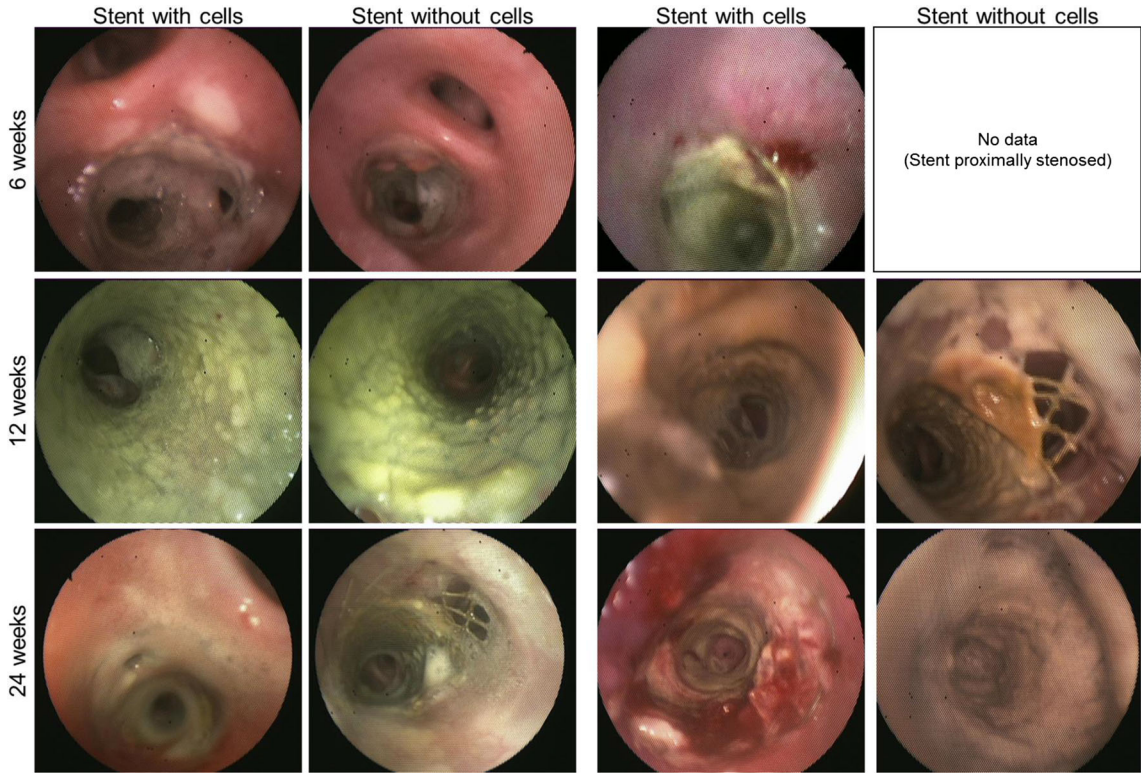

FIGURE 6. Bronchoscopy images before stent explantation.

Tissue Reaction and Granulation

Development of granulation tissue is one of the main complications especially for (bare) metal stents.[8] Granulation is a mass of fibrous connective tissue which develops in the airways after stimulation of the mucosa by pressure or injuries. The stents did not cause any severe granulation in the medial part (see Figs. 7 and 8). In the distal area, increased granulation of about 151% could be seen only in the longest implantation period of 24 weeks. In the proximal area, the stents caused increased tissue thickness of up to 145% at 24 weeks. Especially in one stent granulation was observed proximally as it was implanted in a smaller segmental bronchus. However, no statistical

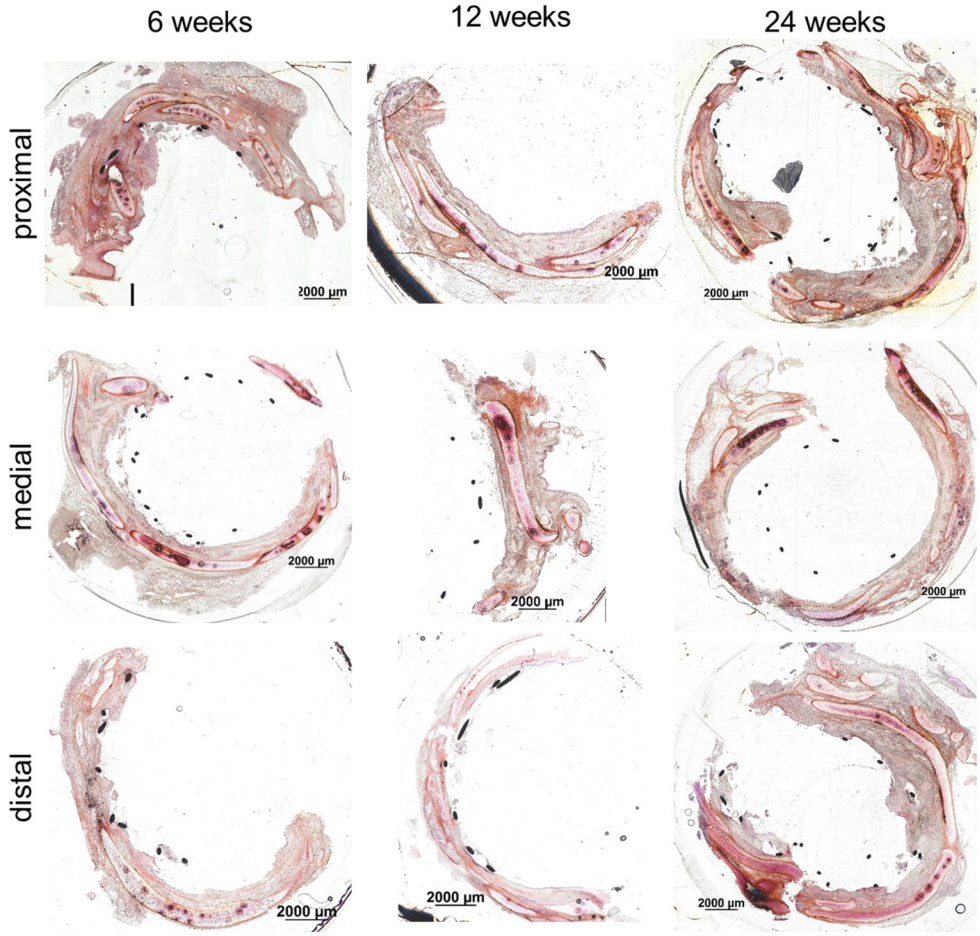

FIGURE 7. Haematoxylin and eosin staining of bronchi tissue with stents.

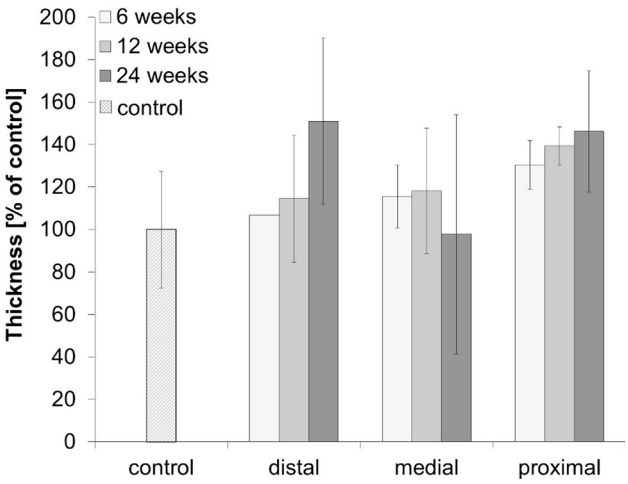

FIGURE 8. Tissue reaction relative to native bronchi tissue. The thickness of mucosa and submucosa as reaction to stent implantation is shown for the distal, medial and proximal part of the stent for implantation durations of 6, 12 and 24 weeks.

significance was reached in the evaluation due to high standard deviations.

Respiratory Epithelium

As one important feature of the PulmoStent concept, we evaluated the presence of respiratory epithelial cells which were seeded onto one of the stent covers *in situ* after implantation. Figure 9 shows SEM images of the PCU membranes. In the left column, the covers of cell-seeded stents are shown. No differentiated respiratory epithelium with cilia can be seen. Still, there are signs of cells on the cover, especially as compared to covers without seeded cells in the right column. On the cover of one cell seeded stent after 24 weeks there is a confluent layer of cells while on all other pre-seeded stents only cell patches are found.

When looking at lung histology and CT data, no significant difference can be found between lungs with a cell-seeded stent and stents without cells. Thus, there

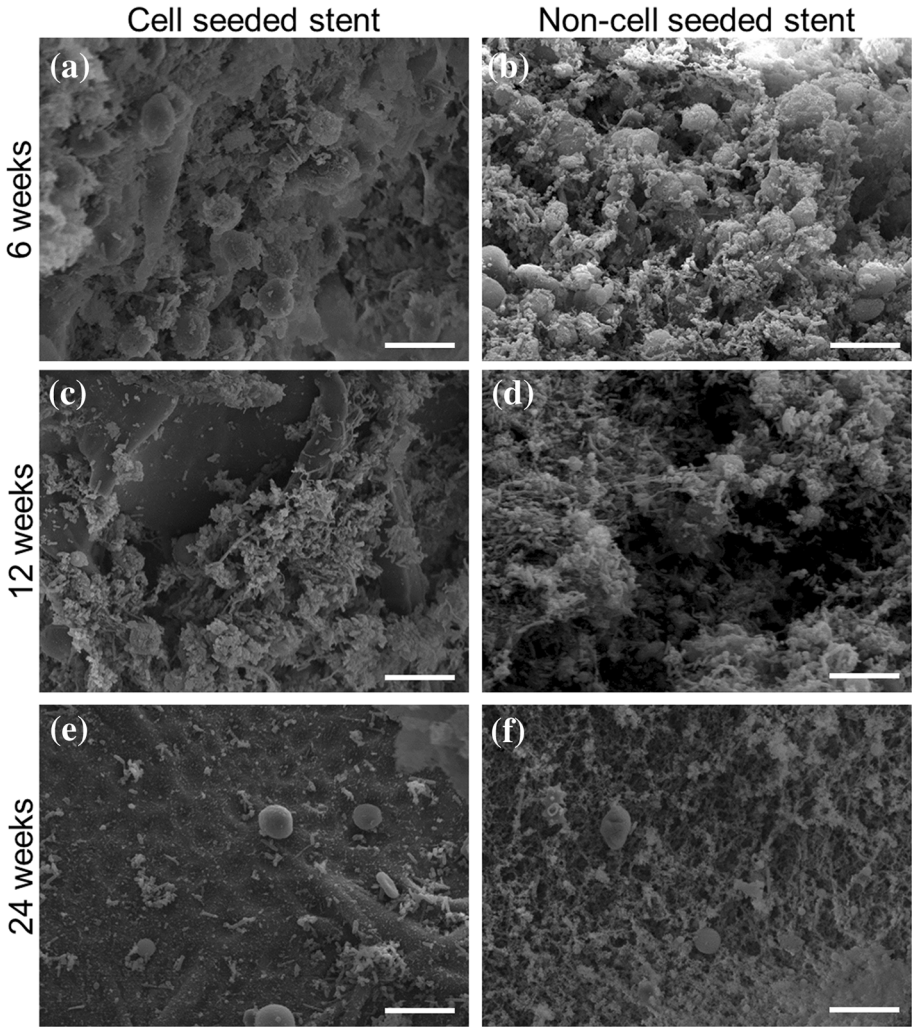

FIGURE 9. Tissue reaction relative to native bronchi tissue. The thickness of mucosa and submucosa as reaction to stent implantation is shown for the distal, medial and proximal part of the stent for implantation durations of 6, 12 and 24 weeks.

is no proof of differentiated respiratory epithelium which allows mucociliary clearance. Nevertheless, in none of the sheep severe mucus plugging distally of the stents was observed in the bronchoscopic controls (see Fig. 6).

DISCUSSION

In this study, we designed a novel airway stent for palliative treatment of bronchial cancer patients. The stents have a permeable cover, which allows epithelial covering to restore mucociliary clearance and a dog-bone structure and open ends to prevent migration. Our studies in sheep demonstrate that the PulmoStents is well tolerated for at least 6 months after implantation, without signs of irritation or mucus plugging.

In a first step, the hand-braided dog bone stent coated with a PCU membrane was developed. Glucose diffusion through the membrane was proven *in vitro*. Glucose transport studies showed rapid glucose transport over the hydrophobic PCU membrane. Most likely, the nonwoven PCU behaves like a porous membrane with water-filled channels through which nutrients can equilibrate over both sides of the membrane. The nonwoven structure of the PCU thus will allow transport of nutrients towards the stent's luminal side to support growth of functional respiratory epithelial cells facing the bronchial lumen. Consequently, long-term culture of respiratory epithelial cells on the membrane was possible in an air–liquid interface and proven by DAPI staining.

Subsequently, an animal study was conducted in which we aimed to show a proof of principle for the developed PulmoStent. In six sheep each two stents

were implanted and one of the stents was spray-seeded with respiratory epithelial cells.

In all sheep, the stents remained at their site of implantation, i.e. no stent migration was observed. This is especially noteworthy as the stents were shown to have a much lower COF than the commercially available Leufen aerstent® with comparable dimensions. Radial testing captured the stent crimp forces during loading and deployment forces during unloading. Based on the testing performed, the covered dog bone stent has a higher RRF and a lower COF than the Leufen aerstent®. COF and RRF parameters are essential for stent design and performance evaluation. Low COFs could potentially lead to stent migration *in vivo*. In contrast, excessively large COFs could lead to airway damage. However, the optimum expansion forces (RRF and COF) are unknown for airway stents.[5] In this study stents were implanted in healthy animals without bronchial stenoses. It remains to be tested, if the lower COF will still suffice to keep a stenosed airway open. In another study in healthy sheep by Puma *et al*. silicone covered stents (Polyflex) showed a migration rate of 50% after 6 months and 100% after twelve months. However, Polyflex stents are known to have a high migration rate. For Ultraflex™ stents (Boston Scientific) which are more similar to the stent presented migration rates between 5 and 13% are described in the literature.[1,12] The report of McGrath *et al*. mentions migration of the stents within the first 24 h after implantation with the need for re-situation of the stent,[10] which was not seen for any of the stents in our study. Thus, being able to show that none of the stents migrated is a very good result. We consider especially the flared and uncovered ends to be important to allow tissue anchorage and prevent stent migration.

The low COF of our stent may still have contributed to the positive results regarding granulation and tissue reaction observed in our animal trial. No significant differences were found in bronchial tissue thickness as compared to native bronchi. The mild granulation we observed occurred mainly on the proximal ends. As it is described that shearing can cause granulation in the airways,[2] this can be a possible reason for the higher tissue thickness at the proximal end of the stents. The increased tissue thickness at the distal stent end after 24 weeks could be induced by higher radial forces to the smaller bronchus diameter.

Still, compared to clinically available stents, the comparably mild formation of granulation tissue observed for our hand-braided stent seems a remarkable result. If the COF is found to be too low for malignant stenoses, the stent can still be highly feasible for patients with tracheal defects that do not require high COFs.

In the animal study, we could not proof differentiated respiratory epithelium with cilia on the stent cover. Still, at least cell patches were found on the cell seeded stents. If the observed cells are the cells from the initial spray coating process or if they have grown in after implantation has to be shown in a separate study. As proven in the *in vitro* experiments, the membrane was suitable for culture of respiratory epithelial cells. A reason for the lack of differentiated epithelium could be a lower nutrition level on the stent *in situ* as compared to *in vitro*, where defined culture medium nourished the cells. Additionally, the membrane could be a suitable scaffold for proliferation of the cells, but not for differentiation. Respiratory epithelial cells can differentiate on collagen-coated substrates or decellularised tracheae, because molecules from the extracellular matrix are present which is different for the PCU membrane.[6,9]

Even though it was proven that the body can be used as a bioreactor for airway tissue development by Jungebluth *et al*.,[7] the shear stresses in the airways directly after cell seeding might have influenced growth and differentiation of epithelial cells as well. The cells were embedded in a thin layer of fibrin gel to protect them from shear stress. Still, very high shear stresses exerted by coughing after stent implantation might have decreased the number of cells on the stent cover and hampered proliferation and differentiation. This could be prevented in future studies by delaying the cell seeding until the cough reflexes due to airway irritation by the stent subside. Increasing the cell number could be supportive as well.

In a clinical setting, the stents will be customized based on CT-data of the patient's respiratory system. For this, a bronchus model would be produced by rapid-prototyping, the stent braided onto this model, covered with PU and sterilized. The overall production time will be approximately 5–6 days. Biopsy, cell isolation and cultivation need 2–3 weeks. Because the cell processing can be done in parallel and is the limiting time factor, the whole production time in a clinical setting will be maximum 3 weeks.

Thus, we here present a newly developed endobronchial stent which was successfully tested *in vitro* and *in vivo*. The stent showed very good migration and granulation behaviour in a healthy sheep model. Further studies will include optimization of the PCU membrane regarding epithelial proliferation and differentiation to be able to show restored mucociliary transport over the stent length.

In this study, a hand-braided single-wire stent with dog bone structure and PCU membrane as cover was developed and tested. The membrane was proven *in vitro* for glucose transport and feasibility for epithelial cell culture in air–liquid interface. In an

animal study in sheep, twelve stents were successfully implanted in sheep for up to 24 weeks and seeded with epithelial cells. No stent migration occurred and only mild granulation was observed.

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