Funktionalisierung des Trägermaterials von Cochlea-Implantaten mittels Dexamethason und Hydrogel: Effekt auf Bindegewebe in vitro und in vivo im Meerschweinchen
Wissenschaftliche Betreuung:
1. Prof. Dr. Ingo Nolte,  
Klinik für Kleintiere, Tierärztliche Hochschule Hannover

2. Prof. Dr. Günter Reuter,  
Klinik und Poliklinik für Hals-, Nasen-, Ohrenheilkunde, Medizinische Hochschule Hannover

1. Gutachter: Prof. Dr. Ingo Nolte
2. Gutachter: Prof. Dr. Manfred Kietzmann

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<thead>
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<th>Symbol</th>
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<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
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<tr>
<td>µm²</td>
<td>square micrometer</td>
</tr>
<tr>
<td>2nd</td>
<td>second</td>
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<tr>
<td>aABR</td>
<td>acoustically evoked auditory brainstem response</td>
</tr>
<tr>
<td>abs.</td>
<td>absorption</td>
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<td>AFM</td>
<td>atomic force microscopy</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>ca.</td>
<td>circa</td>
</tr>
<tr>
<td>CI</td>
<td>cochlear implant</td>
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<tr>
<td>CLSM</td>
<td>confocal laser scanning microscopy</td>
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<tr>
<td>cm</td>
<td>centimeter</td>
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<td>cSt</td>
<td>centistokes</td>
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<td>d</td>
<td>day</td>
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<td>dB</td>
<td>decibel</td>
</tr>
<tr>
<td>Dex</td>
<td>dexamethasone</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid-disodium salt</td>
</tr>
<tr>
<td>et al.</td>
<td>et alii</td>
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<tr>
<td>ext.</td>
<td>extinction</td>
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<td>fig.</td>
<td>figure</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
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<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>HE</td>
<td>hematoxylin and eosin</td>
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<td>HPLC</td>
<td>high performance liquid chromatography</td>
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<td>Hz</td>
<td>Hertz</td>
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<td>kilogram</td>
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<tr>
<td>kV</td>
<td>kilovolt</td>
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<td>l</td>
<td>liter</td>
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SGZ    Spiralganglienzellen
sPEG    star shaped polyethylene glycol prepolymer
SPL    sound pressure level
tab.    table
THF    tetrahydrofuran
U      units
UV/Vis ultraviolet-visible spectroscopy
V      Volt
v/v    volume fraction
W      watt
µA     mikroampere
µg     microgram
µl     microliter
µm     micrometer
µs     mikrosecond
µV     mikrovolt
1 EINLEITUNG

Behandlungsbedürftige Schwerhörigkeit tritt heute in den Industrienationen bei ca. 15% der Bevölkerung auf. Als Ursache liegt bei der großen Mehrheit davon eine Innenohrschwerhörigkeit mit einer Schädigung der äußeren, bei höhergradigem Hörverlust auch der inneren Haarzellen vor. Heutige Hörsysteme beim Menschen basieren auf entweder akustischer oder mechanischer Stimulation über den Eingang durch das ovale oder runde Fenster (Hörgeräte, implantierbare Hörsysteme) oder auf elektrischer Reizung (Cochlea-Implantate, CIs). CIs werden chirurgisch in die Cochlea inseriert, um den darin verlaufenden Hörnerv zu stimulieren. Daher werden sie bei Patienten angewendet, deren Taubheit durch nahezu kompletten Verlust der Sinneszellen im Corti-Organ bedingt ist, die jedoch einen unbeschädigten Hörnerv aufweisen. Aufgrund des Ausfalls der für die frequenzspezifische Hörwahrnehmung notwendigen Filter- und Modulierungsfunktion der äußeren Haarzellen wird nur eine unvollständige Kompensation dieser Funktionsverluste erreicht. Es kommt zu einer verstärkten, jedoch wenig frequenzselektiven Anregung, was vor Al lem bei Anwesenheit von Störgeräuschen das Sprachverstehen erschwert.


In der Regel besitzen Implantate eine Silikonbeschichtung. Deren biochemische Funktionalisierung und die Auswertung dessen war ein Teilaspekt dieser Arbeit (Kapitel 2). Die Funktionalisierung geschah in zweierlei Hinsicht. Erstens wird das Silikon als Arzneimittelträger genutzt, um mit Hilfe eines Einbettungsverfahrens in die Polymermatrix die Langzeitabgabe von Dexamethason direkt ins Innenohr zu ermöglichen. Durch die lokale Applikation kann eine systemische Gabe samt ihrer Nebenwirkungen vermieden werden. Zweitens soll die für die Zelladhärenz verantwortliche unspezifische Proteinadsorption verringert werden. Dies geschieht mittels einer Beschichtung des Silikons mit nicht abbaubarem Hydrogel (Polyethylenglycol Prepolymer „sPEG“). Die Zielstellung dieser Arbeit war es, die


Bei der CI-Versorgung schwerhöriger Patienten gibt es immer noch große individuelle Unterschiede hinsichtlich der erreichbaren Erfolgsraten. Die Spiralganglienzellen (SGZ) unterliegen im Zuge einer Ertaubung einer fortschreitenden Degeneration (OTTE et al. 1978). Da das Implantat über die Stimulation dieser Neuronen die Funktion der beschädigten Haarzellen übernimmt,


Diese Arbeit untersucht eine neue Form des Drug Delivery Systems in Verbindung mit implantierbaren Systemen. Herstellung, Oberflächeneigenschaften, Freisetzungs muster, sowie der Einsatz dieser in vitro (Kapitel 2) und in vivo beim Meerschweinchen (Kapitel 4) sind hier beschrieben. Im Kapitel 3 sind die Auswertung der SGZ-Untersuchungen und ein Vergleich der histologischen Methoden beschrieben, Kapitel 4 befasst sich zudem mit der Anwendung der CLSM auf die in vivo Experimente.
2 PUBLICATION 1:
Dexamethasone released from cochlear implant coatings combined with a protein repellent hydrogel layer inhibits fibroblast proliferation

Antonina Wrzeszcz a,1, Barbara Dittrich b,1, Daniel Haamann b, Pooyan Aliuos a, Doris Klee b, Ingo Nolte c, Thomas Lenarz a, Günter Reuter a

a Department of Otolaryngology, Hannover Medical School, 30625 Hannover, Germany
b Institute of Technical and Macromolecular Chemistry, Chair of Textile and Macromolecular Chemistry, RWTH Aachen University, 52074 Aachen, Germany
c Small Animal Clinic, University of Veterinary Medicine Hannover, 30559 Hannover, Germany

1 The two authors contributed equally.
Antonina Wrzeszcz is the sole author and experimentator of the following chapters:
2.1 – 2.2
2.3.3.2 – 2.3.4.4
2.4.2 – 2.6 (end of article)
Figures 5 – 10, Table 3

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2.1 Abstract

The insertion of cochlear implants into the inner ear often causes inflammation and fibrosis inside the scala tympani and thus growth of fibrous tissue on the implant surface. This deposition leads to the loss of function in both electrical and laser-based implants. The design of this study was to realize fibroblast growth inhibition by dexamethasone (Dex) released from the base material of the implant (polydimethylsiloxane, PDMS). To prevent cell and protein adhesion, the PDMS was coated with a hydrogel layer (star shaped polyethylene glycol prepolymer, sPEG). Drug release rates were studied over 3 months and surface characterization was performed. It was observed that the hydrogel slightly smoothened the surface roughened by the Dex crystals. The hydrogel coating reduced and prolonged the release of the drug over several months. Unmodified, sPEG-coated, Dex-loaded and Dex/sPEG-equipped PDMS filaments were co-cultivated in vitro with fluorescent fibroblasts, analysed by fluorescent microscopy and quantified by cell counting. Compared to the unmodified PDMS, cell growth on all modified filaments was averagely 95% ±SD less, while cell growth on the bottom of the culture dishes containing Dex-loaded filaments was reduced by 70%±SD. Both, Dex and sPEG prevented direct cell growth on the filament surfaces while drug delivery was maintained for the duration of several months.
2.2 Introduction

Patients with sensorineural hearing loss can benefit from cochlear implants (CI), one of today's most successful neuroprosthesis. Therefore, the electrode arrays of CIs are surgically inserted into the scala tympani of the cochlea to stimulate the auditory nerve. In conventional cochlear implants, electrical neural stimulation is used to bypass the non-functional cochlea. Since laser light can be better focused in a clear watery environment compared to electrical current, selective activation of residual functional inner hair cells may be achieved through laser-induced vibration of the basilar membrane. A new concept presents laser-based implants with light-conducting fiber optic components that stimulate the inner ear with laser pulses (WENZEL et al. 2004; WENZEL et al. 2009; ZHANG et al. 2009). For this approach, maintaining the optical properties of implant coatings for unhampered passing out of the laser light through the light-conducting fibers is necessary.

The insertion of CI electrode array into the scala tympani causes mechanical tissue damage and a host response consisting of a chronic inflammatory reaction, fibrosis and new bone formation inside the scala tympani (FAYAD et al. 1991; KAWANO et al. 1998a; NADOL u. EDDINGTON 2004; SOMDAS et al. 2007) and most adversely, growth of fibrous tissue on the implant surface (DUCKERT 1983; SOMDAS et al. 2007). These effects may negatively affect performance of subsequent surgeries such as explantation and reimplantation as well as residual hearing and psychophysical percepts (KAWANO et al. 1998a).

As a result of the fibrous encapsulation, the functionality of a laser-based implant would be influenced significantly as the pass of the laser light could be affected resulting in scattering the focused light emission. In addition, higher pulse energies are required that account for a shortened battery life. Due to higher electrical impedance, also electronic-based cochlear implants suffer from function loss at the nerve-electrode-interface. A biocompatible implant material like silicone will be surrounded by a thin, dense layer of fibroblasts, collagen, and macrophages, which forms resistive interstitial pathways for the current to pass through (GRILL u. MORTIMER 1994). The insufficient voltage might provide non-charge-balanced
stimuli lacking the required amplitudes, potentially causing electrochemical reactions and spiral ganglion cell loss. The more powerful electrical stimuli required to excite the neuronal cells might also lead to irreversible electrochemical reactions endangering both tissue and implant (SHEPHERD et al. 1991). Therefore, reduction of the impedance is desirable, also in order to reduce power consumption and spread of current for a more focussed neural excitation.

One of the clinically widely used methods for reducing the foreign body reactions is the application of glucocorticoids. They not only reduce neovascularization and collagenase production, but also inhibit the synthesis of fibroblastic DNA and proliferation-associated proteins (PARRILLO u. FAUCI 1979; OIKARINEN et al. 1988; RAMALINGAM et al. 1997). In numerous studies, a one-shot application of steroids proved to reduce the electrical impedance growth and stimulation threshold, even with effects three to four years after implantation (PEETERS et al. 1998; DE CEULAER et al. 2003; PAASCHE et al. 2006; PAASCHE et al. 2009). Other studies, with acute and chronic implantation of dexamethasone eluting electrodes, showed positive effects up to 10 years (WISH et al. 1990; ANDERSON et al. 1991; MOND u. STOKES 1996). Well suited for this purpose, the artificial glucocorticoid dexamethasone (Dex) impedes fibroblast growth in vitro already at concentrations of 0,01µg/ml (RUHMANN u. BERLINER 1965; PARRILLO u. FAUCI 1979; FAGOT et al. 1991) without inhibiting neurite outgrowth (FURZE et al. 2008). Due to its long pharmacologic action and stability in vitro, among the glucocorticoid steroids Dex is suitable for use in long-term drug delivery. With glucocorticoid receptors distributing widely in the cochlea (HARGUNANI et al. 2006), Dex therapy in the inner ear is broadly used to decrease the level of hearing loss resulting also from electrode insertion trauma (HIMENO et al. 2002; TAKEMURA et al. 2004; ESHRAGHI et al. 2007; VIVERO et al. 2008).

In general, medical silicone is used as a carrier material for CI electrodes. It is therefore aimed to use the silicone as a drug carrier and to enable the long term, evenly distributed delivery of Dex directly into the inner ear. Choosing local application, typical steroidal side effects after systemic administration (SLATER et al. 1959) are avoided and a sufficient drug level is ensured at the required location.
In a previous study we developed a silicone carrier (polydimethylsiloxane, PDMS), which allows the intracochlear release of anti-inflammatory substances over long time periods using an embedding process in the polymer matrix (DITTRICH et al. 2010). Silicones, in particular PDMS, are virtually chemically inert and physiologically harmless. The goal is to achieve a permanent growth inhibition of fibroblasts through the release of Dex from the PDMS matrix. For the release of steroids from silicones numerous examples have been described, in particular long-term release devices for contraception (CORNETTE u. DUNCAN 1970; ROSEMAN 1974; BRANNON-PEPPAS 1993). However, there are few examples of Dex release from PDMS. For example, the effect of Dex released from a PDMS-based local drug release system on the thickness of intima and adventitia in the carotid artery after implantation of a metal stent (MULLER et al. 1994). Release of Dex from a PDMS-based collar on the tip of pacemaker was described (MATHIVANAR et al. 1990) as well as local drug delivery systems, which use the silicone encapsulant of cochlear implants as a drug delivery matrix (FARAHMAND GHAVI et al. 2010).

Following implantation a dynamic protein adsorption phenomenon occurs initially, triggering inflammatory cell interactions. In addition, an increase in electrode impedance might also be induced by changes to the composition of the perilymph and deposition of proteins on the implant surface during the immediate postinsertion inflammatory response (COLEMAN et al. 1974; HUANG et al. 2007a). One possibility to minimize the attachment of cells is the reduction of the unspecific protein adsorption (RATNER 2004). Although showing less biofouling than other materials, PDMS with its highly hydrophobic surface still has a significant tendency to protein adsorption (ANDERSON et al. 1995). Therefore, we tested a protein repellent nano-scale polymer coating of the PDMS with non-degradable hydrogel (star shaped polyethylene glycol prepolymer “sPEG”). In previous studies, it was proved to prevent both adhesion of various proteins and adhesion of cells including human fibroblasts (GROLL et al. 2004; GROLL et al. 2005a; GROLL et al. 2005b; GROLL et al. 2005c). Poly(ethylene glycol) (PEG) is widely used as coating material due to its known biocompatibility and potential to minimize unspecific protein adsorption (HARRIS 1992; HARDER 1998; HALPERIN 1999; OSTUNI 2001). It has been shown that high
grafting densities resulting in high surface coverage of the polymer is a determining factor for protein resistance of PEG (ADEMOVIC et al. 2002; KINGSHOTT et al. 2002). Furthermore, it has been predicted that branched polymer architectures should be superior compared to linear ones for the prevention of unspecific protein adsorption (IRVINE et al. 1996; IRVINE et al. 1998). Earlier, we investigated the protein repelling properties of linear and star-shaped PEG using isocyanate-terminated, star-shaped poly(ethylene-co-propylene) (NCO-sP(EO-stat-PO)) statistical copolymer showing the great potential of the NCO-sP(EO-stat-PO) prepolymer for coating applications (GROLL et al. 2005a; GROLL et al. 2005b). Due to the unique reactivity of the isocyanate end-group, this system does not only undergo a self-condensing reaction during the coating resulting in high surface covering but furthermore offers the possibility to immobilize proteins for the specific adhesion of cells (GROLL et al. 2004; GROLL et al. 2005c; GASTEIER et al. 2007). The results will serve the prevention of the connective tissue induced function loss of the opto-acoustic implants and can also be adopted for electronic-based implants. Thus, this study examines a new local long term drug delivery system in use with implantable systems. Fabrication, surface characteristics, release pattern and in vitro evaluation are reported here.
2.3 Materials and Methods

2.3.1 Generation and characterization of the local drug delivery system

2.3.1.1 Production of the local drug delivery system

Dexamethasone (Sigma-Aldrich, Taufkirchen, Germany) was precipitated in situ in nanoparticulate form from a supersaturated solution. For this purpose, 0.075mg of Dex was dissolved in 3.3ml tetrahydrofuran (THF, analytical grade [Merck, Darmstadt, Germany]). The solution was then dropped into 1.2955mg of the base component (vinyl-terminated poly(dimethylsiloxane, PDMS) of the silicone (Siliconkit Sylgard ® 184 [Sasco, Dortmund, Germany]). THF was added again if necessary to achieve a clear solution. Subsequently, the THF was removed slowly while stirring. PDMS is a non-solvent and Dex precipitated in fine particles. Last remnants of THF were removed under high vacuum. The crosslinker (0.1296mg) was added to the mixture and then transferred into a tuberculin syringe (Braun, Melsungen, Germany). Through a 30G cannula (Braun, Melsungen, Germany), the mixture was injected into a 5cm long piece of Teflon tube with an inner lumen of 0.3mm (VWR, Langenhagen, Germany). The silicone was cured at 60°C for 20min. After curing, the completed drug delivery system was removed from the tube. The final Dex concentration was 50µg per mg PDMS (5% weight percentage).

2.3.1.2 Release experiments

For the determination of the release behaviour, three samples of 2cm length were incubated in 1 ml PBS buffer (10 mM, pH 7.4) at 37°C C and a shaking speed of 200rpm. The medium was changed at defined time points and the concentration of released Dex in the medium was measured by high performance liquid chromatography (HPLC). The evaluation and fit of the data was done with Origin 8G SR4.
2.3.1.3 Determination of solubility

The solubility of Dex was determined in PDMS oil with a viscosity of 1,000cSt. For this purpose, a calibration line at 242nm was created using solutions with concentrations of 0.005 to 0.5mg/l. A saturated solution of Dex was prepared by stirring and heating. The solution was centrifuged and the supernatant was diluted by half. The concentration of the solution was also determined.

2.3.1.4 High performance liquid chromatography

The measurement of drug concentration was measured using a HPLC system (Agilent, Series 1100/1200, Böblingen, Germany) consisting of quaternary pump, degasser, autosampler and column oven. For detection, a wavelength variable UV-detector (VWD, Agilent Series 1100) was used. The evaluation was performed with the device's own software. For calibration, Dex solutions were prepared with concentrations between 0.05 and 50µg/ml. A 150µl sample was separated at a flow rate of 1 ml/min with a mixture of SDS buffer (0.01M) and acetonitrile (55:45 (v/v)) on a C-18 column (Nucleosil-100, 5µm, 0.3x250mm, CS chromatography, Langerwehe, Germany) at 40°C. The detection was performed at 242nm. Dex was eluted after 2.9min.

2.3.2 Generation and characterization of the hydrogel coating

2.3.2.1 Hydrogel coating of dexamethasone loaded silicon filaments

For the introduction of the reactive amine groups on the surface of the samples, the cross linked PDMS filaments with and without embedded Dex were treated with ammonia plasma at 400W and 0.4mbar for 1min. Afterwards, the filaments were immersed to a solution of 10mg NCO-sP(EO-stat-PO) (synthesized according to literature (GOETZ H 2002)) per ml for 10min and then let dried over night at room temperature.

2.3.2.2 Determination of unspecific protein adsorption

For the determination of unspecific protein adsorption, Dex loaded and native silicone filaments with approx. 1cm length were prepared and coated as previously
described. After incubation of the samples in a solution of 50µg/ml
tetramethylrhodamine-labeled bovine serum albumin (BSA [Invitrogen, Darmstadt,
Germany]) in PBS (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) for 20min,
the samples were washed twice with PBS and thereafter twice with water. The
remaining adherent protein was determined using a fluorescent microscope
(Axioplan 2 Imaging, Zeiss, Oberkochen, Germany). As reference, uncoated samples
were treated in a similar manner.

2.3.3 Characterization of the surface topography

2.3.3.1 Atomic force microscopy

The surface topography of PDMS filaments was mapped using an atomic force
microscope (AFM) (NanoWizard II, JPK, Berlin, Germany). All of the AFM
investigations were done in contact mode and in fluid (Hank’s balanced Salt Solution,
Biochrom, Berlin, Germany) using ultra sharp cantilevers (CSC21/AIBS, MikroMasch,
Tallin, Estonia) and a pixel number of 512*512. Two filaments from each group were
investigated on randomly selected areas. The (nano-) roughness of the surfaces was
measured using the JPK data processing software (vers. 3.4.18). For statistics
(GraphPad Prism 5), four different areas of 10µm² were scanned on every single
sample. The mean over these four roughness values and the related standard
deviation were used for the roughness characterization of a sample.

2.3.3.2 Scanning electron microscopy

For further characterization of the surface, scanning electron microscopy (SEM) was
used (FEI Quanta 400F, acceleration voltage 20kV; Hitachi S-3000N SEM, 1.7kV)
with a working distance of 10mm. The filaments were mounted on aluminium stubs
with double-sided adhesive carbon tape. After 10 minutes of gold sputtering (15µm
layer), scanning was performed under high vacuum using a 20,749 fold
magnification. An average area of 10µm² of each filament was scanned.
The surface of the cross and longitudinal section was also examined in a filament
with and without Dex at 50x, 100x and 250x magnifications.
2.3.4 Cell culture studies

2.3.4.1 Cells and samples

The study was performed in two different settings: in the first setting, all cells in the well were counted, i.e. the cells growing on the filaments and the cells on the bottom of the well. In the second setting, only the cells growing on the filaments were counted. For the experiments, green fluorescent protein expressing mouse fibroblasts (3T3/eGFP) were used. They were pipetted into wells containing PDMS filaments of 6 mm length with 5% weight percentage of dexamethasone.

In the first experimental setting, 8 different types of filaments were studied:

1. PDMS-Dummy, 300µm (PDMS 300)
2. PDMS-Dummy, 500µm (PDMS 500)
3. PDMS-Dummy with hydrogel layer, 300µm (sPEG 300)
4. PDMS-Dummy with hydrogel layer, 500µm (sPEG 500)
5. Dex-loaded PDMS, 300µm (Dex 300)
6. Dex-loaded PDMS, 500µm (Dex 500)
7. Dex-loaded PDMS with hydrogel layer, 300µm (Dex+sPEG 300)
8. Dex-loaded PDMS with hydrogel layer, 500µm (Dex+sPEG 500)

In the second experimental setting only the 4 types with 500 µm diameter were studied.

2.3.4.2 Cultivation

In both settings, n=30 filaments of each type (30x8 and 30x4 types) were placed inside 96-well microtiter plates (TPP, Trasadingen, Switzerland) using one well for each sample. Subsequently, 200µl/well cell suspension in DMEM (Dulbecco’s modified Eagle medium [Biochrom, Berlin, Germany] containing 10% fetal bovine serum [Biochrom], 100 U/ml penicillin [Biochrom], 10mg/ml streptomycin [Biochrom]) with a start cell concentration of 2000 cells/µl was applied onto the filaments. Furthermore, n=30 wells without filaments containing only fibroblasts were used as seeding controls.

In the two experimental settings, the plates were incubated at 37°C and 5% CO₂ for 5 (setting I) and 7 (setting II) days. In the second setting, the filaments together with the
cells growing on their surface were transferred on day 2 and 7 into new wells with fresh medium to exclude cellular proliferation on the bottom of the well. After the transferring procedure on day 2, cell numbers were too low for counting after 5 days, so the incubation time of setting II was extended to 7 days.

2.3.4.3 Microscopy

After the incubation period, the proliferation activity and the GFP expression of the cells grown on the filaments were detected qualitatively by transmission light and fluorescence microscopy (Olympus IX81, Olympus Deutschland GmbH, Hamburg, Germany; filter set U-MF2 F41 054 Alexa 488, abs. 490nm/ext. 520nm) using a 40x objective.

2.3.4.4 Cell counting

To determine the proliferation activity, the cells were detached from the filament and the bottom of the wells using 40µl (setting I) or 30µl (setting II, because of lower cell numbers) of 0.25% trypsin/EDTA solution (Biochrom, Berlin, Germany) after previous washing with 200µl HBSS (Hank’s balanced Salt Solution [Biochrom, Berlin, Germany]). Prior to the cell count in the Neubauer-chamber, the cells were resuspended in 200µl (setting I) or 60µl (setting II, because of lower cell numbers) DMEM and stained with 0.25% Trypan Blue buffer (Biochrom, Berlin, Germany). Cell numbers were expressed as the mean ± SEM value of counted cells.

For statistical assessment (GraphPadPrism 5) of the proliferation activity, one way Anova nonparametric analysis and Tukey’s (setting I, Gaussian distribution) and Dunn’s (setting II, no Gaussian distribution) multiple comparison test was used.
2.4 Results

2.4.1 Characterization of the local drug delivery system

2.4.1.1 Release

To observe the sink-condition throughout the experimental period, the samples were transferred into fresh buffer according to defined times. The concentration of Dex in PBS was determined photometrically at 254nm in the UV/Vis detector of the HPLC system.

Figure 1 shows the released Dex amount based on the total quantity of release system used over a period of 3 months. The release of the embedded agent reached after a short initial phase of higher release an approximately constant release rate. Here, the filaments with hydrogel coating showed a lower total release than the filaments without hydrogel coating. These filaments showed a release rate of 2.21µg/mg (± 0.22µg/mg) Dex after a period of 90 days, while the rate for hydrogel-coated filaments was 1.68µg/mg (± 0.10µg/mg). The released amounts of embedded Dex were 4.4% and 3.4%, in the case of the non-coated filaments and the hydrogel-coated filaments, respectively.

Examining the released amounts of Dex during the first three days, there is also a clear difference between the uncoated and coated release systems. Figure 2 (small graph) depicts the released rates of Dex over the first three days. They decreased from 5.87ng/mg*d (± 0.07ng/mg*d) to 3.58ng/mg*d (± 0.06ng/mg*d) in the case of the uncoated and from 2.88ng/mg*d (± 0.06ng/mg*d) to 2.29 mg/mg*d (± 0.01ng/mg*d) in the case of the coated release systems.

The rate, at which Dex was released, decreased also in the further course of the release (Figure 2). After 90 days of release, the rate decreased from the uncoated filaments to 1.02ng/mg*h (± 0.10ng/mg*h) and from the coated filaments to 0.78ng/mg*h (± 0.05ng/mg*h). The highest decrease of the release rate was measured in the first week.

Plotting the released amount of Dex against the root of time, it behaves linearly with the square root of t after an initial lag phase (data not shown). This dependence on
the square root of time indicates a diffusion-controlled release of encapsulated Dex and can be described by the 2nd Fick's law.

There are numerous equations that describe the diffusion-controlled release of a solid from a polymeric matrix, taking into account the geometry. For a simplistic view, the semi-empirical equation (1) can be used (RITGER 1987):

\[
\frac{M_t}{M_\infty} = k t^n
\]

(1)

Here, \( M_t \) is defined as the mass of drug released at time \( t \), and \( M_\infty \) is the mass of drug released as time approaches infinity. \( n \) is the diffusion exponent, whose value should be in the case of Fickian (diffusion controlled) release 0.5. With a cylindrical geometry, this only applies to the early period of the release (below 15% of the encapsulated amount). The constant \( k \) includes the description of matrix and drug and may be set for the early period of release from a matrix with a cylindrical geometry with \( 4 (D_e/\pi a^2)^{1/2} \). Here \( D_e \) is the effective diffusion coefficient of the encapsulated drug and \( a \) is the radius of the cylinder. Therefore, to determine the diffusion coefficient, \( M_t/M_\infty \) was plotted against \( t \) and the equation (1) adjusted to the measured values by varying the parameter \( k \). The values obtained are summarized in Table 1, wherein the effective coefficient of determination \( R^2 \) of 0.95087 and 0.92457 shows a good fit of the model function to the data.

Taking into account the radius of the cylinder-shaped sample (0.150mm), the effective diffusion coefficient can be calculated from the obtained values of \( k \). For the uncoated delivery system, an effective diffusion coefficient of \( 9.29 \times 10^{-19} \text{m}^2/\text{s} \) (±9.5\*10\(^{-20}\) m\(^2\)/s) and for the coated delivery system, an effective diffusion coefficient of \( 4.87 \times 10^{-19} \text{m}^2/\text{s} \) (±6.6\*10\(^{-20}\) m\(^2\)/s) is found.

An alternative interpretation is possible with equation (2). Likewise, the cylindrical geometry of the system is taken into account, wherein the front between the agent containing and agent-free matrix migrating into the interior of the cylinder of radius \( a_0 \) is specified in the radius \( a' \). Considering the solubility of the drug in the matrix (\( c_0 \)) and in the medium (\( c_\infty \)) the effective diffusion coefficient \( D_e \) of the drug in the matrix can be calculated (ROSEMAN u. HIGUCHI 1970).

\[
\frac{a^2}{2} \ln \frac{a'}{a_0} + \frac{1}{4} (a_0^2 - a'^2) = \frac{c_\infty D_e t}{c_0}
\]

(2)
Therefore, to determine the effective diffusion coefficients, the term on the left side of equation (2) was plotted against $t$ and the slope $m$ and intercept $b$ of the straight line were determined by linear regression. The values obtained are summarized in Table 2. Taking into account the solubility of Dex in the matrix $c_s$ and the release medium $c_0$, the diffusion coefficient can be calculated from the slope. The solubility of Dex in the PDMS matrix was not determined directly but assumed on the basis of solubility in a highly viscous PDMS oil. This results in a solubility of 89 mg/kg ($\pm 7$mg/kg). The solubility of Dex in the release medium (10mM PBS buffer, pH=7.4) results in 79 mg/kg ($\pm 2$mg/kg).

As the effective diffusion coefficient for the uncoated release system, $6.52 \times 10^{-19}$m/s ($\pm 1.8 \times 10^{-20}$m/s) and for the coated system release $3.77 \times 10^{-19}$m/s ($\pm 1.6 \times 10^{-20}$m/s) was obtained. The determined effective diffusion coefficients are thus in a similar order of magnitude as determined by the simplified evaluation of effective diffusion coefficients.

Figure 3 compares the measured release of Dex with the calculated released of Dex based on equation (2) for the uncoated release system over a period of 90 days (small graph) and two years. Both release curves correlate predominantly quite well, the amounts are, however, in the period between 10 and 30 days in the calculation somewhat overestimated. After two years, the calculated value of released amount adds up to 14% of the encapsulated drug. The extrapolation of the released amount indicates a decreasing but sustained release over the considered two years. One can assume a further release of the remaining amount for several years, provided that no changes in the coating characteristics occur.

2.4.1.2 Hydrogel coating

Both Dex loaded and native PDMS filaments could be successfully equipped with a hydrogel layer based on NCO-sP(EO-stat-PO). The protein repelling properties of the layer were determined by incubation of different samples with fluorescently labeled bovine serum albumin as shown in Figure 4. When compared to non-coated samples, both samples equipped with a hydrogel layer showed no protein adsorption on the surface.
2.4.2 Characterization of the surface topography

Modification of PDMS with sPEG, Dex and sPEG+Dex resulted in a significant increase of the nano-roughness of the surface topography compared to native PDMS (p < 0.05) with the highest roughness in Dex loaded filaments (p < 0.001) (Figure 5). There were no significant differences in nano-roughness between the modifications (p > 0.05). Where sPEG was used as a coating material, very fine fissures were found to be distributed over the surfaces (Figure 6 arrows). We observed very small cavities on the Dex-modified PDMS surface with a mean diameter of 323.25 ±136.80 nm (mean ± SD) and a mean depth of 29.79 ± 18.92 nm (mean ± SD) (Figure 6C). The surface seems to become more porous because of the inclusion of Dex crystals in the PDMS. In all cases, the porous surface of Dex-modified PDMS was fully covered by the sPEG coating (Figure 6). In all filaments, linear grooves with a thickness of roughly 1 µm and a depth of approximately 250 nm in intervals of approximately 9 µm from each other were observed (Figure 6 asterisks, here not visible on native PDMS).

The surface of the cross and longitudinal section of the filament with Dex showed quite evenly distributed cavities (Figure 7 B,C arrows), partially containing crystal shaped structures which we consider as Dex crystals (Figure 7 B,C arrowheads). In contrast, the sections of the filament without Dex show solely a smooth surface without cavities or crystals (Figure 7 D).

2.4.3 Cell culture studies

2.4.3.1 Setting I

Murine 3T3/eGFP fibroblasts were cultivated in wells containing 4 different types of PDMS filaments to determine the cell numbers inside each well within the cultivation period. After 5 days of cultivation, the PDMS dummies were largely covered with cells while samples loaded with Dex remained nearly completely cell free, independent from the hydrogel coating. Filaments with only hydrogel showed a varying, mostly minimal, cell covering. The surfaces of the bottoms in all the wells were equally fully grown and showed no discernible differences in cell numbers.
The comparison of total cell numbers in the wells (Figure 8) between the filament pairs with different diameters showed no significant difference ($p > 0.05$). In the hydrogel-coated and uncoated PDMS containing wells, no significant difference in total cell number was found. Also, the difference in total cell number between the Dex-loaded samples, both hydrogel-coated and uncoated was not significant. In wells without drug-loaded samples (PDMS 300, PDMS 500 and sPEG 300), cell numbers were slightly, but not significantly, increased when compared to the control (112%, 114% and 118%) except sPEG 500, which showed a slight, but not significant decrease (89%). In contrast, all Dex-loaded filaments showed a highly significant ($p < 0.001$) decrease by an average of 70% in cell number when compared to the unloaded samples including the control (Table 3).

2.4.3.2 Setting II

Murine 3T3/eGFP fibroblasts were cultivated for 7 days in wells containing 4 different types of silicone filaments. Under fluorescence microscopy, the PDMS dummies were almost completely covered with cells, while filaments loaded with Dex and/or coated with hydrogel remained nearly completely cell free (Figure 9).

When comparing the counts of cells grown on the filament surfaces (Figure 10), we found a highly significantly reduced cell growth between the PDMS-dummies ($5.854 \pm 1.12 \times 10^4$/ml cells, $n = 30$) and the other filament types ($p < 0.001$). There was no significant difference ($p > 0.05$) between the cell numbers observed on sPEG ($0.417 \pm 0.109 \times 10^4$/ml cells, $n = 30$) and on Dex ($0.488 \pm 0.134 \times 10^4$/ml cells, $n = 30$) filaments. Nevertheless, significantly lower cell numbers in comparison with both sPEG ($p < 0.05$) and Dex ($p < 0.01$) filaments were revealed on Dex+sPEG filaments ($0.05 \pm 0.021 \times 10^4$/ml cells, $n = 30$). Thus, compared with PDMS-dummies sPEG filaments reduced cell growth by 93%, Dex by 92% and Dex+sPEG even by 99%.
2.5 Discussion

The artificial steroid dexamethasone is known for its potent glucocorticoid effects: anti-inflammatory, immunosuppressant and causing remarkable inhibition of fibroblast growth (RAMALINGAM et al. 1997). However, many studies on steroidal action on the proliferation of fibroblasts in vitro have led to contradictory results with either an inhibition of the proliferation or a stimulatory effect. Durant et al. found that most discrepancies may be due to the use of different experimental models, schedules, culture conditions, to the various methods of proliferation monitoring and to the choice of different cell lines (DURANT et al. 1986). Still, most in vivo studies or such with primary cultures have led to similar conclusions of glucocorticoids inhibiting both fibroblast proliferation and collagen synthesis. Thus, the effect seems to be proved for the application conditions of cochlear implants. Also, many findings in human studies imply that steroids like Dex are considerable agents for optimization of cochlear implant applications (PAASCHE et al. 2006; PAASCHE et al. 2009). However, most application methods, such as one-shot injections via syringe or cannula, are based on short term release without the possibility of affecting the long-term tissue reactions like fibrosis. Even polymer or hydrogel formulations providing a prolonged drug release cannot last longer than several days (ENDO et al. 2005; LEE et al. 2007; DINH et al. 2008; PAULSON et al. 2008; SALT et al. 2011). Applied intratympanically, the rapid loss of the drug through the Eustachian tube and the high concentration gradient between basal and apical cochlear turns are further limitations (PLONTKE et al. 2008; SALT u. PLONTKE 2009). Currently available long term release methods like mini-osmotic pumps, microcatheters and the MicroWick™ give direct access to the inner ear tissues for active drug application with lower concentration gradients, but also for no more than several weeks (KOPKE et al. 2001; RICHARDSON et al. 2006; SWAN et al. 2008). Additionally, since the drug reservoir has to be refilled frequently, it increases the risk of infection of the inner ear. The invasiveness of the therapy, the required time, and the costs should also come to consideration.
Using the cochlear implant itself as a drug delivery device, no additional surgery is required and direct access to the cochlear structures is granted without the limitations of the basal-apical concentration gradient of round window applications (RICHARDSON et al. 2008). Several research groups developed a modified cochlear implant with incorporated drug delivery channels (PAASCHE et al. 2003a; HOCHMAIR et al. 2006; STOVER et al. 2007), but the connection to the delivering pumps still provides a potential risk of infection and fluid leakage. In another approach, Dex eluted from a silicone collar is still released quite rapidly, with 50% and 65% of total Dex already released within one and four weeks, respectively (MATHIVANAR et al. 1990).

Combining both, advantages of local application and long term release, we developed a local drug delivery system based on a polymeric encapsulant of cochlear implants. PDMS was chosen for its good biocompatibility and high permeability for lipophilic agents (particularly steroids) (BAKER 1987). Crystalline Dex enclosed in the PDMS-matrix was shown to be released over a time period of 3 months with the potential for even longer time periods referring to the diffusion kinetics, which revealed the release of only 4.4% (non-coated) and 3.36% (hydrogel-coated) of the embedded drug amount within 3 months. Our results are consistent with the findings of Ghavi et al., who studied Dex release from silicone rubber CI coatings with different weight percentages up to 2% w/w (FARAHMAND GHAVI et al. 2010). A release similar to our findings (5% w/w) was revealed over a time period of 21 months, indicating an analogous long term release for our system. Assuming a volume of the human cochlear perilymph of 160µl (BUCKINGHAM u. VALVASSORI 2001) and a delivery system weight of 1mg, from a delivered dose of 5.87ng per day results a concentration of 36.7µg/ml achieved at the target location directly after implantation. But this value only applies to a closed fluid system like the cell culture is. Considering the complex mechanisms of drug clearance from the cochlear fluids as well as the perilymph loss due to implantation, further computer simulations (SALT 2005) and in vivo experiments should be performed.

For the second biochemical functionalization to prevent unspecific protein adsorption, encoating of the silicone matrix with sPEG was chosen. It constitutes only a small
diffusion barrier for Dex. However, the coating reduces the release to about 23% by changing the diffusion coefficient (Figure 1). As it is also the case in other implantation surgeries, the acute wound caused by the insertion of CI electrode into the inner ear is suggested to undergo a healing process with a time course of maximally 30 days, divided into four time dependent stages (coagulation/haemostasis, inflammation, proliferation, wound remodelling)(VELNAR et al. 2009). A high release rate during the first weeks after implantation is therefore beneficial for the initial healing process and although the initial release from coated PDMS is lower and more constant, it is still recognizably increased (Figure 2). The retardation of release in the coated filaments provides a slower and longer drug delivery with sufficient concentration levels (for exceeding 0.03-0.04µg/ml (SALT et al. 2011), weight percentage of Dex or size of the delivery system could be adjusted) for long term therapy, with positive effects over several years (WISH et al. 1990; ANDERSON et al. 1991; MOND u. STOKES 1996; PEETERS et al. 1998; DE CEULAER et al. 2003; PAASCHE et al. 2006; PAASCHE et al. 2009). The protein repelling effect is due to increased hydrophilicity and degree of hydration (OSTUNI 2001) and could be proved through adsorption of TAMRA marked Bovine Serum Albumin on untreated and hydrogel coated silicone filaments (Figure 4). Compared with non-coated filaments both filaments with and without Dex equipped with a hydrogel layer showed no unspecific protein adsorption onto their surfaces. This is an indication that the incorporation of Dex inside the PDMS matrix does not influence the protein repelling properties of the hydrogel layer.

Electron microscope pictures show a homogeneous distribution of the drug throughout the matrix (Figure 7), while simultaneously flexibility and mechanical stability of the material are not restricted. Nano-roughness of the filament surfaces detected by AFM was significantly affected by different loadings and coatings compared to untreated PDMS (Figure 5). Dex-loaded filaments showed the highest nano-roughness as well as the most uneven surface with small cavities. This may be due to the enclosed Dex crystals, while the crystals in PDMS+sPEG+Dex samples are covered with hydrogel, which therefore smoothen the surface but is still rougher than PDMS alone. The fine fissures in the hydrogel-coated filaments (Figure 6) could
be caused by desiccation or mechanical manipulation. The manufacturing process is assumed to be the reason for the linear grooves found in all filament types. Our in vitro model examined eGFP fibroblast growth in contact with Dex-loaded, unloaded, sPEG-coated and uncoated PDMS filaments. Within experimental setting I, growth of fibroblasts on the surfaces of the filaments and of the bottom of the wells was evaluated (Figure 8). Since only in the wells with Dex and Dex+sPEG filaments the cell growth on the filament surfaces as well as in the vicinity of the filaments (well bottom) was affected (reduced by 70%), we proved the release and diffusion of Dex from PDMS as well as from the sPEG coated PDMS. Hydrogel itself has an antiproliferative effect (GROLL et al. 2004; GROLL et al. 2005a; GROLL et al. 2005b; GROLL et al. 2005c), however, without contact to the cells on the well bottom it had no influence on them. Through diffusion into the cell culture medium, only Dex accounts for the reduced cell growth on the well bottom. Transferred to the circumstances in vivo, the diffusion of the drug into the perilymph would not only affect the cells in direct contact with the electrode, but also the connective tissue cells surrounding the electrode inside the scala tympani. Additionally, this benefit also applies to the platinum electrode-nerve interfaces, which are not coated by PDMS. The slightly higher cell number in wells with PDMS and sPEG-coated PDMS than in the control wells could be explained through the additional growth area on the filaments presented to the cells. The surface of the well bottom including the filaments’ surface was increased by nearly 17% (300µm diameter) and 29% (500µm diameter) compared to the well bottom surface alone. Considering variations in cell distribution and different positions of the filaments, the increase of cell number by 12-17% matches these circumstances. Considering only the surfaces of the filaments (setting II), we found a cell number reduction of over 90% on all treated filaments in comparison to the untreated PDMS (Figure 10). There, not only the Dex-loaded filaments were nearly cell free, but also the sPEG-coating alone showed a remarkable cell repelling effect. Still, highest reduction (up to 99%) of cell proliferation was achieved by combining Dex and sPEG.
2.6 Conclusions

Enclosing dexamethasone in a PDMS-matrix and adding a protein repelling hydrogel layer does not affect the usability of the polymer. Moreover, the release of the steroid was proved to be feasible over a long time span, from months probably up to years. The diffusion of the released Dex into the medium achieves an effective reduction of the fibroblast proliferation in the whole cavity filled with medium while the hydrogel reinforces the effect by preventing unspecific protein adsorption and direct cell seeding on the material surface. Both modifications reduce the cell proliferation separately or combined, from which the latter provides the best results.

Still, since our cell culture studies covered an interval of a week, questions addressing longer time periods of drug administration to achieve and maintain inhibitory effects on connective tissue growth as well as the affection of hearing processes following implantation, may only be clarified in *in vivo* experiments. Also, the durability of the material under the physiological conditions inside the inner ear should be investigated. Therefore, the effectiveness and functional biocompatibility of the experimental approach will be tested in long-term studies in the inner ear of guinea pigs.

In summary, we present a successfully *in vitro* tested long-term local drug application system for the efficient delivery of dexamethasone into the inner ear to reduce implantation-caused inflammatory reactions equipped with a protein and cell repelling hydrogel coating. It represents a suitable method to improve the functionality of cochlear implants.
2.7 Figures and tables

**Figure 1:** Released amount of dexamethasone from coated and uncoated filaments over three months. The samples were incubated at 37°C in PBS.
Figure 2: Release rate of dexamethasone over the entire study period and release rate of dexamethasone over the first three days (small graph)
Figure 3: Comparison of the measured release of Dex with the calculated released of Dex based on equation (2) for the uncoated release system over a period of 90 days (small graph) and two years
Figure 5: Fluorescent images of native (left) and hydrogel coated (middle), dexamethasone loaded PDMS filaments after incubation with tetramethylred-labeled BSA. For comparison, a similar treated, hydrogel coated PDMS filament without dexamethasone is shown (right).

Figure 4: Nano-roughness of the filament surfaces; *** highly significant (p<0.001), * significant (p<0.05) difference; n.s. not significant (p>0.05)
Figure 6: Filament surfaces, 10µm²: (A) Scanning electron microscopy, 20.749x magnification (B) Atomic force microscopy, surface topography in two-dimensional and (C) three-dimensional presentation; arrows: fissures in hydrogel layer; asterisks: linear grooves caused by the manufacturing process.
Figure 7: Filament surface, scanning electron microscopy: (A) filament from the side, magnification 50x; (B) longitudinal section of a filament with Dex, magnification 100x and (C) magnification 250x, shows cavities (arrows), partially containing Dex crystals (arrowheads); (D) cross section of a filament without Dex shows a smooth surface without cavities or crystals, magnification 100x.
Figure 8: Growth of eGFP-fibroblasts in contact with different PDMS filaments with 2 different diameters after 5 days (starting cell number 2000 cells), cells on filament surface and well bottom counted; Mean ± SEM; *** highly significant reduction of cell proliferation compared to PDMS, PDMS + sPEG and control (p<0.001); n.s. not significant (p>0.05)
Figure 9: Growth of eGFP-fibroblasts (green) in contact with different PDMS filaments after 7 days in 40x magnification (A): Fluorescence microscopy; (B): Fluorescence microscopy, black/white; (C): Light microscopy
Figure 10: Growth of eGFP-fibroblasts in contact with different PDMS filaments after 7 days (starting cell number 2000 cells), cells on filament surface counted; Mean ± SEM; *** highly significant reduction of cell proliferation compared to PDMS (p<0.001); ** highly significant (p < 0.01), * significant (p < 0.05), n.s. not significant (p>0.05)
**Table 1:** Parameters $k$ and $n$ for the release systems with and without hydrogel coating and the coefficient of determination $R^2$. The values were obtained by adjusting the function (1) to the measurement results after varying the parameter $k$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Without hydrogel layer</th>
<th>With hydrogel layer</th>
</tr>
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<tbody>
<tr>
<td>$k$</td>
<td>1/s$^{1/2}$</td>
<td>1.45E-5 (±7.43E-07)</td>
<td>1.05E-05 (±7.12E-07)</td>
</tr>
<tr>
<td>$n$</td>
<td>-</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>$R^2$</td>
<td>-</td>
<td>0.95087</td>
<td>0.92457</td>
</tr>
</tbody>
</table>

**Table 2:** Slope $m$ and intercept $b$ of the linear regression for the release systems with and without hydrogel coating and the coefficient of determination $R^2$.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Without hydrogel layer</th>
<th>With hydrogel layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>$m$</td>
<td>m²/s</td>
<td>7.34$^{-19}$ (±2.04$\times$10$^{-20}$)</td>
<td>4.25$\times$10$^{-19}$ (±1.78$\times$10$^{-20}$)</td>
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<tr>
<td>$b$</td>
<td>m²</td>
<td>-1.73$\times$10$^{-13}$ (±6.95$\times$10$^{-14}$)</td>
<td>-1.40$\times$10$^{-13}$ (±6.07$\times$10$^{-14}$)</td>
</tr>
<tr>
<td>$R^2$</td>
<td>-</td>
<td>0.992</td>
<td>0.983</td>
</tr>
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</table>
Table 3: Setting I, growth of eGFP-fibroblasts in contact with different PDMS filaments with 2 different diameters after 5 days (starting cell number 2000 cells); Mean ± SEM

<table>
<thead>
<tr>
<th>Sample type n=30</th>
<th>Counted cells x 10^4/ml</th>
<th>Percentage of control</th>
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<tr>
<td>PDMS 300</td>
<td>32.621 ± 2.282</td>
<td>112%</td>
</tr>
<tr>
<td>PDMS 500</td>
<td>33.213 ± 3.352</td>
<td>114%</td>
</tr>
<tr>
<td>sPEG 300</td>
<td>34.346 ± 3.714</td>
<td>118%</td>
</tr>
<tr>
<td>sPEG 500</td>
<td>25.867 ± 2.657</td>
<td>89%</td>
</tr>
<tr>
<td>Dex 300</td>
<td>8.521 ± 0.805</td>
<td>29%</td>
</tr>
<tr>
<td>Dex 500</td>
<td>9.338 ± 0.698</td>
<td>32%</td>
</tr>
<tr>
<td>Dex+sPEG 300</td>
<td>8.642 ± 0.655</td>
<td>30%</td>
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<tr>
<td>Dex+sPEG 500</td>
<td>10.279 ± 0.614</td>
<td>35%</td>
</tr>
<tr>
<td>Control</td>
<td>29.167 ± 1.782</td>
<td>100%</td>
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</table>
3 PUBLICATION 2:

Spiral Ganglion Neuron Quantification in the Guinea Pig Cochlea using Confocal Laser Scanning Microscopy compared to Embedding Methods

Antonina Wrzeszcz a,1, Günter Reuter a,1, Ingo Nolte b, Thomas Lenarz a, Verena Scheper a

a Department of Otolaryngology, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany
b Small Animal Clinic, University of Veterinary Medicine Hannover, Bünteweg 9, 30559 Hannover, Germany

1 The two authors contributed equally.
Antonina Wrzeszcz was the author and experimentator of all chapters and experiments except of the animal usage for and the generation of the paraffin slides.

3.1 Abstract

Neuron counting in the cochlea is a crucial but time-consuming operation for which various methods have been developed. To improve simplicity and efficiency, we tested an imaging method of the cochlea, and based on Confocal Laser Scanning Microscopy (CLSM), we visualised Rosenthal’s Canal and quantified the spiral ganglion neurons (SGN) within.

Cochleae of 8 normal hearing guinea pigs and one implanted with a silicone filament were fixed in paraformaldehyde (PFA), decalcified, dehydrated and cleared in Spalteholz solution. Using the tissue’s autofluorescence, CLSM was performed at 100-fold magnification generating z-series stacks of about 20 slices of the modiolus. In 5 midmodiolar slices per cochlea the perimeters of the Rosenthal’s Canal were surveyed, representative neuron diameters were measured and the neurons first counted manually and then software-assisted. For comparison, 8 normal hearing guinea pig cochleae were embedded in paraffin and examined similarly.

The CLSM method has the advantage that the cochleae remain intact as an organ and keep their geometrical structure. Z-stack creation is nearly fully-automatic and frequently repeatable with various objectives and step sizes and without visible bleaching. The tissue shows minimal or no shrinking artefacts and damage typical of embedding and sectioning. As a result, the cells in the cleared cochlea reach an average diameter of 21µm and a density of about 18 cells/10,000µm² with no significant difference between the manual and the automatic counts. Subsequently we compared the CLSM data with those generated using the established method of paraffin slides, where the SGN reached a mean density of 9.5 cells/10,000µm² and a mean soma diameter of 13.6µm.

We were able to prove that the semi-automatic CLSM method is a simple and effective technique for auditory neuron count. It provides a high grade of tissue preservation and the automatic stack-generation as well as the counter software reduces the effort considerably. In addition this visualisation technique offers the potential to detect the position and orientation of cochlear implants (CI) within the cochlea and tissue growing in the scala tympani around the CI and at the position of
the cochleostomy due to the fact that the implant does not have to be removed to perform histology as in case of the paraffin method.

3.2 Introduction

The treatment of profoundly deaf patients has been revolutionised in the past decades by the introduction of cochlear implants (CI). Meanwhile cochlear implantation has become widely accepted as routine treatment for patients with complete and incomplete sensorineural hearing loss. However, there are still large individual differences in the level of success achieved with a CI. Since the implant takes over the function of damaged hair cells by means of direct electrical stimulation of the spiral ganglion neurons (SGN), it was assumed that the success of CI's is also dependent on the number, survival and responsiveness of SGN available for electrical stimulation. After onset of deafness these neurons undergo degeneration, which progresses with ongoing deafness (OTTE et al. 1978). Accordingly, in the literature the number of SGN was viewed as one of the crucial elements for the success of cochlear implantation (LOUSTEAU 1987; INCESULU u. NADOL 1998).

In current publications this hypothesis could not be confirmed distinctly in human patients. The high number of variables that can influence CI performance aggravate the comparisons between conditions of the cochlear tissues (nerve and hair cell survival, fibrosis and new bone) and implant function, particularly for complex functional measures such as speech recognition. Nevertheless, interpretation of correlations in animal models and within-subject across-site comparisons in humans suggest that conditions near each stimulation site have a strong influence on cochlear implant function (PFINGST et al. 2011). It is therefore still crucial to evaluate SGN number and survival. One focus of various research groups all around the world is the protection or even regeneration of SGN to improve the cochlear implants' outcome. Nowadays it is widely accepted that neurotrophic factors such as glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) as well as electrical

To verify if a suggested therapy has an effect on the SGN survival, next to electrophysiological measurements, histology has to be performed to evaluate the number of surviving SGN. Therefore the counting of primary auditory neurons in the cochlea is crucial to establish novel therapies for inner ear treatment. Different histological methods such as embedding the cochlea into paraffin (DE FRANCESCHI et al. 2011), celloidin (HINOJOSA u. NELSON 2011) or Araldite resin (ZILBERSTEIN et al. 2012) to subsequently generate histological sections (Fig.11A) are used. Embedding the cochlea into matrixes like OCT (SLY et al. 2012) or epoxy resin and sectioning it on a cryostat or grinding it (Fig.11B) with documentation of every slice, respectively, are common methods for SGN evaluation. Electron microscopy provides high resolution images of the detailed cell morphology (KELLERHALS 1967). All those methods are time-consuming and elaborative, moreover, they are disruptive to the three-dimensional structures of the organ and result in severe shrinkage and damage artefacts of the delicate tissues. The cochlea has a complex spiral-shaped structure, consisting of large fluid-filled spaces and a wide variety of tissue types ranging from dense bone to fragile membranous structures and the delicate organ of Corti containing many different cell types.

To improve simplicity and efficiency, we tested a semi-automatic method based on Confocal Laser Scanning Microscopy (CLSM) (Fig.11C, D) and the free software ImageJ and XuvTools (EMMENLAUER et al. 2009) to visualise and automatically quantify the SGN in a whole mount cochlea sample without the destruction of the spiral geometry and without the necessity of staining. To visualise the tissues inside the undestroyed otic capsule, we chose the approach of clearing the whole cochlea to transparency using Spalteholz solution (SPALTEHOLZ 1914; HAQ 1988). Since the tissues within the cochlea were not physically touched during the preparation and
imaging process, the risk of damage was minimised. Subsequently, we compared the
CLSM data with those generated in our lab using the established method of paraffin
embedding and sectioning (SCHEPER et al. 2009; WARNECKE et al. 2010) and
grinding of epoxy resin embedded cochleae, a method used to perform histology on
tissue where the implant may stay in situ (STOVER et al. 2005). The grinding method
is suitable to detect nuclei, the soma is difficult to examine because of the limited
staining technique. So far, there are no publications of quantitative studies about the
SGN using this grinding method, so the comparison is based upon personal
communication (CESCHI 2012). Since not only the number of cells, but also their
size is important for definite identification and evaluation of their vitality and therefore
their functional capability, this parameter was included into this analysis as well. A
description of the specimen preparation procedure and imaging method is presented
here.
3.3 Materials and Methods

3.3.1 Animals

For this study 16 approximately 3 months old Dunkin Hartley guinea pigs were chosen from Charles River Laboratories, Sulzfeld, Germany and Harlan Winkelmann GmbH, Borchen, Germany. For the CLSM technique n=8 animals were unilaterally implanted with a silicone filament (in context with another study, article in progress), the normal hearing contralateral control ears were used for the SGN counting study reported here. We also show one of the implanted cochleae to depict the possibility of position examination of cochlear implants. Eight guinea pigs provided the material for the paraffin slide method. The use of animals for scientific purposes was permitted by the regional council (Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany, registration number 09/1666 and 10/0104). The studies have been conducted in accordance with the German “Law on Protecting Animals” and with the European Communities Council Directive 2010/63/EU for the protection of animals used for experimental purposes.

Before animals were sacrificed, the normal hearing status (≤50dB sound pressure level) of the lower hearing pathway (function of the cochlea, the brainstem, and the auditory midbrain) was confirmed by the measurement of the acoustically evoked auditory brainstem responses (aABR) under general anaesthesia using 10mg/kg xylazine (Rompun® 2%, Bayer, Leverkusen, Germany) and 40mg/kg ketamine (Ketamin Gräub®, Albrecht, Aulendorf, Germany).

The aABR measurements were performed in a sound-attenuating chamber using a TDT System (Tucker-Davis Technologies, Alachua, Florida, USA), and data were analysed using the corresponding BioSigRP software. Acoustic tone stimuli were generated and presented by a speaker positioned in the external ear canal with frequencies of 1, 4, 8, 16, 32 and 40kHz from 0 to 90dB in 10dB steps. Subdermal needle electrodes (CareFusion Nicolet, Middleton, US) were placed at the vertex (common positive), left and right mastoid (references), and in the neck (ground). The tone bursts had a duration of 10ms with a square cosine rise and fall time of 1ms.
The recorded signal was bandpass filtered from 300 to 3000Hz to suppress the inclusion of background noise. All recorded neurological signals were sampled 270 times and averaged for analysis. Thresholds were defined as the lowest stimulus required to evoke a visually replicable waveform.

After confirmation of normal hearing, the animals received a second injection of the initial anaesthesia as well as a local application of prilocaine subcutaneously above the sternum. Afterwards they were euthanised through transcardial perfusion with 200ml 0.1 M phosphate-buffered saline (PBS, Invitrogen, Karlsruhe, Germany) followed by 100ml paraformaldehyde (PFA, Merck, Darmstadt, Germany). After perfusion animals were decapitated and temporal bones were removed and dissected under a dissection microscope to expose the cochleae. The apex and oval window were pierced.

3.3.2 Confocal Laser Scanning Microscopy

3.3.2.1 Tissue preparation

The tissue preparation is based upon the proceedings described by MacDonald et al. (MACDONALD u. RUBEL 2008; MACDONALD u. RUBEL 2010). To keep the method simple, we omitted the dissection of the cochleae and kept the organs intact. All preparation steps were carried out with gentle rotation on a platform rotator. Fixation was performed at 4°C, all following steps at room temperature. Cochleae (n=8) were PFA-fixed overnight, rinsed 3 x 10 minutes in PBS and decalcified for 22 days in 10% ethylenediamine tetraacetic acid-disodium salt (EDTA, Sigma Aldrich Chemie GmbH, Steinheim, Germany) in PBS, pH 7.4, with regular EDTA change every 1-3 days. After dehydration with ethanol the cochleae were cleared in Spalteholz solution (methyl salicylate benzyl benzoate, MSBB, 5 parts MS and 3 parts BB):

- 70% ethanol overnight or minimum 2 hours
- 95% ethanol 30 minutes
- absolute ethanol 2 x 2 hours (or overnight)
- MSBB/ethanol (1:1) 4 hours
- MSBB 2 hours, then 4 hours, then overnight
Since we found commercially available chambers usually either made of plastic or glued with non-solvent-resistant adhesives, which do not withstand the MSBB we recommend the use of selfmade chambers: the cleared cochleae were transferred into selfmade round glass chambers (22mm outer diameter, 5mm height, 2mm wall thickness) filled with MSBB, fixed on a 45x71mm and 0.15mm thin cover glass (using a solvent-resistant adhesive: Elastosil E 43, Wacker Chemie AG, Stuttgart, Germany) and covered loosely with a 22x22mm coverslip (Fig.12). Chambers are filled completely with the fluid to avoid air bubbles. The specimens were placed horizontally on the glass with the apex oriented downwards to reduce the distance to the objective lens. If desired, the height of the chamber can be chosen approximately to the height of the cochleae, so that the coverslip fixes them gently in their position. This fixation is rather light, it is therefore recommended to generate all image stacks directly in sequence on the same day to avoid displacement of the specimen (VOTH et al. 1964).

3.3.2.2 Imaging and data processing

For microscopy the Leica TCS SP2 AOBS laser scanning confocal microscope equipped with a Leica DM IRE 2 inverted microscope (Leica Microsystems GmbH Wetzlar, Germany) was used combined with the Leica Confocal Software (Leica Microsystems Heidelberg GmbH). An argon laser provided the excitation line at 488 with the gate for the multiplier opened between 500 and 600nm, pinhole airy unit 1. Using the tissue’s autofluorescence (induced by PFA) about 20 slices of the modiolus were generated in 50µm steps (to ensure enough midmodiolar slices for data generation, smaller steps can be chosen) at 200Hz scanning speed, 8x line averaging (arithmetic mean) and 2x photon accumulation. Confocal images were saved as 8-bit tiff. At 100x magnification (objective Leica HC PL Fluotar 10x/0.30) z-series stacks (a series of images obtained by focusing from top to bottom in the z-axis through the specimen) were obtained from the apex, the middle and the base of the modiolus, with stack sizes varying from 500 to 1000µm. The time required for all 3 stacks took about 3-4 hours. As mentioned above, we generated all stacks directly in sequence on the same day to avoid displacement of the specimen. The 3 stacks were stitched using the freeware XuvTools (EMMENLAUER et al. 2009) (Fig.13),
which requires an overlap of about 1/3 of two adjacent stack regions and a predominantly consistent illumination of all stacks for good results. The stacks must not be rotated against each other to be detected by the stitching software. For stitching, 3 tiff-stacks were generated in ImageJ version 1.43u (RASBAND, W., http://rsbweb.nih.gov/ij/. Last update 12.08.2012, National Institutes of Health, Bethesda, Maryland, USA) from the separate tiff images, after conversion to 8bit and smoothing. The stitched stacks were put out in one data file in a format suited to the image processing software Imaris x64 6.0.2 (Bitplane, Zurich, Switzerland), which created three dimensional optical volumes of the modioli that are able to be rotated in every direction and exported as snapshots (tiff or jpg) or movie files. Subsequently, the stitched image stack was converted by Imaris into a tiff series for further processing in ImageJ. To the present day, XuvTools does not support other output formats than Imaris. This limitation will be overcome in the next software version (http://www.xuvtools.org/).

3.3.2.3 Data collection and neuron quantification

In ImageJ on 5 optical sections per cochlea the perimeters of the Rosenthal’s Canal were surveyed at 6 locations (Fig.14) and the neurons first counted manually and then automatically by the ITCN plug-in (Image-based Tool for Counting Nuclei, Center for Bio-Image Informatics, http://www.bioimage.ucsb.edu/downloads/automatic-nuclei-counter-plug-in-for-imagej) to compare it to the manual count. In general, cells larger than 12µm in diameter were counted. Since most neurons fulfilled this condition, the count included practically every cell. Depending on the image quality, in darker images Rosenthal’s Canal was partially covered with shadows, so every structure discernible as cell was counted. First, as region of interest the Rosenthal’s canal was outlined. For the automatic counter plug-in a medium cell diameter of 20µm was estimated and the quality threshold set at 2-3 (2 for darker images). As minimum distance (approximation of the distance between nuclei centers) 10µm was chosen. The number of vital SGN in correlation to the measured cross-sectional area of the Rosenthal’s canal gives the SGN density, expressed as cells/10,000 µm². The turns were named lower basal turn (lb), upper basal turn (ub), first middle turn (1.m), second middle turn (2.m), third middle turn
(3.m), fourth middle turn (4.m), and apical turn (a). Due to preparation methods the 4.m and a. turns could not always be analysed separately. Therefore the SGN densities of these areas, if available, were added for analysis. Soma diameter measurement was performed by dividing the area of each Rosenthal’s canal in four equal parts. In each part 2 representative neurons were chosen for measurement, providing n=40 diameters per location in the cochlea.

3.3.3 **Paraffin method**

After tissue harvesting the apex and the fenestra ovalis were pierced, the cochleae were gently flushed with PFA and samples were placed in PFA for 2 h for further fixation. Before decalcification, the cochleae were rinsed with PBS three times. Afterwards they were placed in 20% EDTA (Sigma Aldrich Chemie GmbH, Steinheim, Germany) for 4-5 weeks at 37°C. Decalcification medium was changed every 2 or 3 days. Before paraffin embedding, dehydration with 50-100% ethanol (BÜFA Chemikalien, Hude/Altmoorhausen, Germany) was performed. From paraffin blocks 5 µm mid-modiolar sections were generated, mounted on glass slides and stained with hematoxylin and eosin (HE).

For analysis, all cochlea turns were identified with an Olympus CKX41 Microscope at a 200fold magnification using cell^P software (Olympus soft imaging solutions GmbH, Münster, Germany). The appellation of the turns was equal to that described for the CLSM method. The first mid-modiolar section was selected randomly. Every fifth following section was chosen for analysis to ensure a separation of 25 µm between the sections. A total five sections per cochlea were analysed. The SGN density and the perikaryal diameter were manually determined as described in section “CLSM”.

3.3.4 **Statistics**

For statistical assessment of the SGN densities and diameters, one way ANOVA nonparametric analysis and Bonferroni’s (densities, Gaussian distribution) or Dunn’s (diameters, no Gaussian distribution) multiple comparison test was used (GraphPadPrism 5).
3.4 Results

3.4.1 aABR
All animals were normal hearing with hearing thresholds between 20 and 50 dB, with lowest at 8 kHz and highest at 1 and 40 kHz frequency.

3.4.2 CLSM method
Initial observations revealed already that autofluorescence from 4% PFA was sufficient to provide a general tissue label for use with CLSM, requiring relatively high excitation power levels of 60-100% at 488nm. Multiphoton mode was given up after finding heat damage and bubble formation inside the specimen. To describe the physical resolution, the numerical aperture (0.30) of the 10x objective can be referred to. It provides a resolution of approximately 1.1µm for green and 1.2µm for red light. The SGN appeared to emit a particularly intense autofluorescence which provided an excellent possibility to trace them within the modiolus tissue. Appearing as bright oval to round spheres, occasionally with discernible nuclei (Fig.15A), they could be satisfactorily recognised by the analysing person as well as by the counter plugin. Occasionally, the plugin counted some cells twice because of counting the soma and the nucleus as two different cells. Comparing the time required, the procedure using the automatic plugin lasted about 2 hours while the manual procedure took about 2.5 hours for one cochlea.

Counting manually, we found a mean density of 17.49 ± 0.6 cells/10,000µm² with the lowest value of 16.3 ± 0.6 cells/10,000µm² in the lower basal turn and the highest value of 19.41 ± 0.53 cells/10,000µm² in the upper basal region. The automatic plugin provided very similar results with a mean density of 18.13 ± 0.26 cells/10,000µm² ranging from 15.98 ± 0.6 cells/10,000µm² in the lower basal turn to 19.53 ± 0.48 cells/10,000µm² in the upper basal region. The mean values for each cochlear region are plotted in table 4. Although having averaged 3.53% higher values in the automatic counts, our results show that the difference between the manual and the automatic counts is not significant (p > 0.05) (Fig.16).
The soma diameters of the SGN reached an average of $21.76 \pm 0.06\mu m$, with largest cells found in the 4th turn and apex ($22.35 \pm 0.14\mu m$) and smallest in the 3rd turn ($20.99 \pm 0.13\mu m$) (Fig.17, table 5). Comparing the cochlear regions, the mean diameters show no significant difference.

Generation of three-dimensional figures by digital imaging using the Imaris software provides good orientation support for the helical shape of the Rosenthal’s canal and the other structures inside the cochlea, like the basilar membrane, stria vascularis or the cochlear nerve (Fig.11D, 3). The tissue shows minimal or no shrinking artefacts and no damage typical for embedding and sectioning. According to our cell size data, the shrinkage factor appears to be quite minor compared to embedded tissues. After treatment with MSBB, a certain degree of tissue hardening as mentioned by Spalteholz could be observed in our specimens.

### 3.4.3 Paraffin method

Spiral ganglion neurons of paraffin embedded cochleae reached a mean density of $9.55 \pm 0.42$ cells/10,000$\mu m^2$ ranging from $8.85 \pm 0.66$ cells/10,000$\mu m^2$ in the 2nd middle turn to $11.28 \pm 0.26$ cells/10,000$\mu m^2$ in the 4th middle turn and apex. The mean values for each cochlear region did not differ significantly and are plotted in table 4 and figure 16. A mean soma diameter of $13.60 \pm 0.03\mu m$ was found, with data shown in table 5 and figure 17. The mean diameters did not change between the different cochlear regions either.

The comparison between the paraffin and CLSM data shows a highly significant difference ($p < 0.001$) for the densities as well as for the diameters (Fig. 16,17).
3.5 Discussion

In contrast to the embedding methods the CLSM has the advantage that the cochleae remain intact as an organ and keep their geometrical structure (HARDIE et al. 2004). There was no rupture of the delicate tissues, as often happens after paraffin embedding and there was also no material loss as after grinding in the epoxy method. There, without documentation (i.e. photographing) the tissue is completely lost (STOVER et al. 2005). With the CLSM method, the cochlea remained completely unharmed and can be stored for further examinations, also for usage of other methods. It is e.g. possible to embed the specimen Afterwards and to produce histological sections (HARDIE et al. 2004). However, embedding in epoxy resin is limited since the cochlea is already decalcified. Despite the tissue hardening after the treatment with MSBB, deformations could occur during the vacuum degasification.

Using a 5x objective, we were able to scan through an entire cochlea, approximately 5mm in depth. However, the laser beam reaches its limitations with increasing distance from the objective, particularly in areas covered by tissue shadows. Using appropriate image processing software, 3D reconstruction from the optical sections was easily accomplished compared to the reconstructions from histological sections. The cochlear structures could be observed from all directions, even though the scan was performed from only one side (Fig.11D, 13, 14A). However, this caused some restrictions in the display of structures on the objective-averted side of the modiolus, since it casted a shadow. For studying the SGN, the slight shadows casted by the basilar membrane were the only limitations on the side facing the objective. It is conceivable to remove the outer tissue parts around the modiolus to improve image acquisition. Due to their size, the SGN were still detectable despite being covered by shadows. Still, for the visualisation of details and morphology of the cells and their nuclei, the paraffin method produces the best results (Fig.14B). It is also not possible to discern type I and II SGN using our experimental set-up. Nevertheless, it is possible to use one further stain besides the PFA-induced autofluorescence. We had positive experience using red labels as Cy5 or Cy7 antibodies. It is e.g.
conceivable to use it combined with a SGN type II label for peripherin (HUANG et al. 2007b).

Another approach to distinguish the SGN types is the evaluation of cell morphology. To enable a sufficient resolution it will be necessary to dissect the cochlear walls at least at one side to expose the modiolus. Hereafter, a confocal scan with higher magnification is possible, e.g. using a 25x long distance objective.

First experiments with implanted animals showed that areas with nerve damage were clearly detectable. The SGN number was locally (basal region) highly reduced and led to hollow spaces inside the Rosenthal’s canal (Fig.18). This animal exhibited a distinct hearing loss in the higher frequencies, whose perception takes place in the basal region of the basilar membrane.

Compared to the size of SGN in paraffin or epoxy sections, the cells in the optical sections appear considerably more voluminous. The lack of shrinkage artefacts demonstrates the excellent tissue preservation of the CLSM method. Due to the decalcification for CLSM and paraffin, the whole organ undergoes the same degree of shrinkage which is relevant for investigation of the SGN density, based on SGN number and area of the Rosenthal’s canal. This is a problematic aspect for the epoxy method, since the area of the bony canal does not shrink. This could be a reason why the results concerning cell numbers in paraffin and epoxy differ so much from each other. Since the neuron size is crucial, we conclude that the CLSM method leads to a more precise neuronal density. Nevertheless, it must be mentioned that the animals were from two different breeding lines which may have led to these differing results.

It is furthermore possible to estimate total SGNs and the length of Rosenthal’s canal by analysing more than the demonstrated five optical sections. Depending on how exactly the data shall be collected, it requires more time and effort. The step size may be set to 30µm, which exceeds the mean diameter of a SGN by 1.5 times (CLARKE 1992). To exclude the double count of cells, there are several methods with higher accuracy applicable, e.g. serial section reconstructions or the disector method (COGGESHALL 1992). Richter et al. suggests using overlays of already
counted sections projected onto their following section in photoshop to obtain an unbiased cell number (RICHTER et al. 2011).

For estimation of the length of Rosenthal’s canal it is conceivable to use the three-dimensionally reconstructed optical volume of the whole modiolius in Imaris. The software provides the possibility to measure distances in the z-axis, thus into the depth of the volume. Finding the central axis of the canal may, however, prove difficult. Another approach with higher accuracy could be the survey of the canal areas in every section and a subsequent 3D volume rendering reconstruction, similar to Johnson et al. in the mouse model (JOHNSON et al. 2011). That method using the TSLIM provides moreover the storage of the 3D positions of every SGN within the canal and enables an exact detection of neuron numbers. However, TSLIM technology is not as widely accessible as CLSM yet. Besides that study, only one further publication provides total number data based on the count of every SGN (WHITLON et al. 2006). The majority of studies extrapolated from cell density over the entire canal volume.

Scanning not only the modiolius but the whole cochlea with a lower magnification (Fig.11D), the method has also the potential to detect the positions of cochlear implants since, unlike with paraffin, tissue processing with implanted devices inside the cochlea is possible. Our test implant (a silicone filament) appeared as elongated shadow inside the round window and the basal turn (Fig.19). This provides the option of verification of the correct location of the implant as well as the documentation of the tissue reactions after implantation which is beneficial for evaluation of new implant concepts (STOVER et al. 2005). The micro-computed tomography used so far is rather appropriate to depict bony structures.

The embedding methods face the difficulty of the necessary axis determination of the modiolius before embedding, which has to be ensured to produce midmodioliar sections along the axis. Once embedded incorrectly, it is not possible to repeat the procedure without massive destruction of the inner ear tissue. With the CLSM method, the cochlea is placed unfixed in the glass chamber so that its position is correctable at any time. Furthermore, the homogeneous refractive index created by
the MSBB used as medium instead of the embedding materials reduces the spherical aberration and improves image quality considerably (MACDONALD u. RUBEL 2010). The specimen was not rotated about its axis like in other methods, e.g. OPFOS (VOIE et al. 1993; VOIE u. SPELMAN 1995) or SLOT (EICKHOFF et al. 2012; KELLNER et al. 2012), with the purpose of being independent of non-commercially available equipment and to use devices already widely established. The only exception is our small glass chamber serving as specimen container during microscopy, which is simple and inexpensive to produce (Fig.12). Choosing ImageJ and XuvTools, we also preferred the use of free available software for image processing. The current dependence on the Imaris software is worked on by the XuvTool developers. The SLOT is a technique which is quite appropriate to depict the whole cochlea and to detect the position of the implant, but the resolution is not sufficiently high enough to examine the tissue inside the basal turn and on the implant surface.

Using the PFA-induced autofluorescence instead of stains for tissue labelling (HARDIE et al. 2004) saves time and costs. If desired, immunostaining is possible provided that the wavelength range is shifted far enough from the broad range covered by the autofluorescence. In our experiments, we found the label Cy5 (or Cy7) in the far red range suitable for demarcation from the autofluorescence. Z-stack creation is nearly fully automatic and is frequently repeatable for correction of brightness or with various objectives and step sizes. Visible bleaching could not be determined even after several hours (up to about 7h) of high laser beam exposure.

In sum, the time required per cochlea involves for aABR, perfusion, dissection and fixation 2 days, for decalcification 20 days (possibly less). For CLSM, dehydration and clearing takes 3 days, imaging 3-4 hours and data processing 2-2.5 hours. For the embedding process, the time required per cochlea adds up to 6-7 days plus 3 weeks decalcification for paraffin and 3 days for epoxy, including complicated and elaborate procedures, while for CLSM the work steps predominantly consist of simply changing the rinsing fluids. For epoxy, minimum 3 days of grinding and image acquisition come to addition. With manual counting, time for data processing is fairly similar for all methods, the automatic count almost halves the effort.
The advantages and disadvantages of the two methods are summarised in table 6. An analysis of normal hearing guinea pig SGN density and diameter publications shows that the results are quite variable (Table 4), depending highly on the embedding material and the criteria applied to the cells to be examined. Comparison is problematic considering the amount of different methods, protocols, materials and thus variable shrinkage factors of the tissue. Where each neuron was counted, the density usually was found higher. Parameters like visible nuclei and SGN typical morphology lead also to higher results than cell size-limited counting. Due to similar counting criteria, the results for paraffin embedding are quite consistent with our own findings. Our epoxy method is difficult to compare with the literature, since the grinding aspect was new. The CLSM results mostly resemble the data of Webster (WEBSTER u. WEBSTER 1981), who also counted every visible cell. Still, after sectioning and processing for the embedding methods the tissue is influenced by distortion and compression (GARDELLA et al. 2003), which is not present with the CLSM method. This may be a reason why the densities and soma diameters are high compared to the majority of the literature.

3.6 Conclusions

We were able to prove that the automatic CLSM method is a simple and effective technique for auditory neuron count. It provides a high grade of tissue preservation resulting in high SGN densities and large soma diameters. The automatic stack-generation and the count plug-in reduce the processing effort considerably. Compared with methods using embedded materials, it has numerous advantages and is suitable for qualitative and quantitative determination of spiral ganglion neurons as well as for detection of implant positions and tissue examination inside the cochlear structures. First studies with implanted guinea pig cochleae are already in progress. It is also planned to test the method for SGN quantification in feline and human cochleae.
3.7 Figures and tables

Figure 11: (A) Paraffin-embedded cochlear section; (B) epoxy resin-embedded and ground section, (C) one optical section and (D) whole 3D optical volume from CLSM scan, 5x objective, 10µm stepsize (green= autofluorescence)
Figure 12: Schematic drawing of a cochlea in glass chamber affixed on a 1mm thin sheet of glass, filled with Spalteholz solution

Figure 13: (A) Three CLSM generated z-series stacks (optical volumes) of the cochlear modiolus using PFA-induced autofluorescence, from apical (left) to basal (right), 10fold magnification; (B) stacks stitched together. Rosenthal’s Canal with spiral ganglion neurons (asterisks), basilar membrane (arrowheads), cochlear nerve (stars)
Figure 14: (A) Cochlear modiolus overview from three stacks stitched together as 3D optical volume; (B) perimeter of Rosenthal’s Canal (white outlined) surveyed and neurons counted in 6-7 locations on every slice in the lower basal (lb), the upper basal (ub), the 1st (1.m), 2nd (2.m), 3rd (3.m) middle turn and the 4th and apical turn together (4.m+a)
Figure 15: Comparison of detail image of Rosenthal’s Canal of normal hearing guinea pigs. CLSM optical section (A) and paraffin section (B)
Figure 16: Spiral ganglion neuron density (neuron number per 10 000µm²) in the lower basal (lb), the upper basal (ub), the 1st (1.m), 2nd (2.m), 3rd (3.m) middle turn and the 4th and apical turn together (4.m+a) in 5 midmodiolar sections of n=8 cochleae. Comparison of manual and automatic neuron count using the CLSM [difference not significant (p > 0.05)] and the paraffin method [highly significant difference to both CLSM counts (p < 0.001)]. Error bars = SEM
Figure 17: Manually measured soma diameter of the spiral ganglion neurons (n=40 per turn location) in the lower basal (lb), the upper basal (ub), the 1st (1.m), 2nd (2.m), 3rd (3.m) middle turn and the 4th and apical turn together (4.m+a) of n=8 cochleae. Comparison of the CLSM and paraffin method [highly significant difference (p < 0.001)]. Error bars = SEM
Figure 18: Optical volume (left) and section (right) of the basal modiolus of a cochlea with nerve damage (arrows) after insertion of silicone implant. Arrowhead: undamaged area with full SGN density. Star: cochlear nerve.
Figure 19: Optical volume of the basal turn of a cochlea with silicone implant (arrow), inserted through round window (RW). Rosenthal’s Canal with spiral ganglion neurons (asterisks), stria vascularis (arrowheads), cochlear nerve (star). Green: autofluorescence; red: Cy5 labelled vimentin (connective tissue), staining protocol according to MacDonald et al., 2008.
Table 4: Mean and SEM spiral ganglion neuron (SGN) density. The numbers of SGN in 10,000µm² are plotted for each separate cochlear region and each histological method.

<table>
<thead>
<tr>
<th>Method</th>
<th>Cochlear region</th>
<th>lb</th>
<th>ub</th>
<th>1.m</th>
<th>2.m</th>
<th>3.m</th>
<th>4.m + a</th>
<th>total</th>
</tr>
</thead>
<tbody>
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<td>CLSM (plug-in)</td>
<td></td>
<td>15.98</td>
<td>19.53</td>
<td>19.22</td>
<td>18.00</td>
<td>17.84</td>
<td>18.17</td>
<td>18.13</td>
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<td></td>
<td></td>
<td>± 0.60</td>
<td>± 0.48</td>
<td>± 0.62</td>
<td>± 0.67</td>
<td>± 0.70</td>
<td>± 0.24</td>
<td>± 0.26</td>
</tr>
<tr>
<td>CLSM (manual)</td>
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<td>17.62</td>
<td>16.65</td>
<td>17.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.60</td>
<td>± 0.53</td>
<td>± 0.70</td>
<td>± 0.57</td>
<td>± 0.55</td>
<td>± 0.21</td>
<td>± 0.60</td>
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<td>Paraffin</td>
<td></td>
<td>9.08</td>
<td>9.32</td>
<td>10.54</td>
<td>8.85</td>
<td>11.05</td>
<td>9.12</td>
<td>9.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.81</td>
<td>± 0.65</td>
<td>± 0.90</td>
<td>± 0.66</td>
<td>± 1.87</td>
<td>± 0.65</td>
<td>± 0.42</td>
</tr>
</tbody>
</table>

Table 5: The mean and SEM SGN diameter (µm) is shown for each separate cochlear region and each histological method. For both histological methods the measurement was performed manually.

<table>
<thead>
<tr>
<th>Method</th>
<th>Cochlear region</th>
<th>lb</th>
<th>ub</th>
<th>1.m</th>
<th>2.m</th>
<th>3.m</th>
<th>4.m + a</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>± 0.12</td>
<td>± 0.14</td>
<td>± 0.15</td>
<td>± 0.13</td>
<td>± 0.13</td>
<td>± 0.14</td>
<td>± 0.06</td>
</tr>
<tr>
<td>Paraffin</td>
<td></td>
<td>13.36</td>
<td>13.64</td>
<td>13.65</td>
<td>13.68</td>
<td>13.58</td>
<td>13.71</td>
<td>13.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>± 0.06</td>
<td>± 0.07</td>
<td>± 0.07</td>
<td>± 0.06</td>
<td>± 0.07</td>
<td>± 0.06</td>
<td>± 0.03</td>
</tr>
</tbody>
</table>
Table 6: Comparison of the methods

<table>
<thead>
<tr>
<th>Clearing in MSBB and CLSM</th>
<th>Embedding into paraffin and sectioning</th>
<th>Embedding into epoxy resin and grinding</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ good overview</td>
<td>+ good overview</td>
<td>+ good overview</td>
</tr>
<tr>
<td>+ 3D imaging easily feasible</td>
<td>- 3D images not possible</td>
<td>- 3D images not possible</td>
</tr>
<tr>
<td>+ good to sufficient visualisation of the neurons of objective-near side of the modiolus and of midmodiolar parts</td>
<td>+ very good visualisation of the neurons with their nuclei</td>
<td>+ sufficient visualisation of the neurons with their nuclei</td>
</tr>
<tr>
<td>+ simplified semiautomatic procedure</td>
<td>- complicated procedure</td>
<td>- complicated procedure</td>
</tr>
<tr>
<td>+ less time consuming</td>
<td>- time consuming</td>
<td>- time consuming</td>
</tr>
<tr>
<td>- requires decalcification</td>
<td>- requires decalcification</td>
<td>+ no decalcification required</td>
</tr>
<tr>
<td>+ repeatable</td>
<td>- not repeatable</td>
<td>- not repeatable</td>
</tr>
<tr>
<td>+ integrity of geometrical structures remains</td>
<td>- no integrity of geometrical structures</td>
<td>- no integrity of geometrical structures</td>
</tr>
<tr>
<td>+ processing with implanted devices inside the cochlea possible</td>
<td>- the implant has to be removed</td>
<td>+ processing with implanted devices inside the cochlea possible</td>
</tr>
<tr>
<td>+ material reusable for repeated scans or for other methods</td>
<td>+ destruction of the material</td>
<td>+ destruction of the material</td>
</tr>
<tr>
<td>+ no stains required, immunostaining possible</td>
<td>+ most standard stains compatible</td>
<td>- not compatible with most standard stains</td>
</tr>
<tr>
<td>+ no visible bleaching</td>
<td>- relatively expensive materials</td>
<td>+ processing of hard tissues possible</td>
</tr>
<tr>
<td>- shadows of Organ of Corti occasionally cover parts of the Rosenthal’s Canal</td>
<td>+ all cochlear regions visible</td>
<td>+ all cochlear regions visible</td>
</tr>
<tr>
<td>- poor visualisation of objective-averted side of the modiolus</td>
<td>+ all cochlear regions visible</td>
<td>+ all cochlear regions visible</td>
</tr>
<tr>
<td>+ not temperature sensitive</td>
<td>- cutting is not possible at warm room temperatures</td>
<td>+ not temperature sensitive</td>
</tr>
<tr>
<td>+ sufficient results after short teaching of CLSM</td>
<td>- cutting needs extensive training to get sufficient results</td>
<td>- grinding needs extensive training to get sufficient results</td>
</tr>
</tbody>
</table>
Table 7: Comparison of literature: SGN densities and soma diameters in normal hearing guinea pigs

<table>
<thead>
<tr>
<th>Method</th>
<th>SGN per 10,000µm²</th>
<th>SGN soma diameter [µm]</th>
<th>Criteria</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paraffin</td>
<td>~10</td>
<td>not investigated</td>
<td>diameter &gt;12µm</td>
<td>(WARNECKE et al. 2010)</td>
</tr>
<tr>
<td>Paraffin</td>
<td>6.66-8.46</td>
<td>not investigated</td>
<td>visible nucleus and cytoplasm</td>
<td>(YLIKOSKI et al. 1998)</td>
</tr>
<tr>
<td>Paraffin</td>
<td>8</td>
<td>not investigated</td>
<td>diameter 12-25µm, nucleus 5-10µm</td>
<td>(MARUYAMA et al. 2008)</td>
</tr>
<tr>
<td>Paraffin</td>
<td>9.41-10.38</td>
<td>14.31-17.34</td>
<td>diameter &gt;12µm</td>
<td>(CESCHI 2012)</td>
</tr>
<tr>
<td>Celloidin</td>
<td>~12.4-16.9</td>
<td>not investigated</td>
<td>visible nucleolus</td>
<td>(BIXENSTINE et al. 2008)</td>
</tr>
<tr>
<td>Celloidin</td>
<td>~20-25</td>
<td>not investigated</td>
<td>each neuron counted</td>
<td>(WEBSTER u. WEBSTER 1981)</td>
</tr>
<tr>
<td>Epoxy</td>
<td>4.1-4.73</td>
<td>10.43-11.77</td>
<td>visible nucleus</td>
<td>(CESCHI 2012)</td>
</tr>
<tr>
<td>Epoxy</td>
<td>14.25 (~12-15)</td>
<td>~18</td>
<td>each neuron counted</td>
<td>(GLUECKERT et al. 2008)</td>
</tr>
<tr>
<td>Epoxy</td>
<td>not investigated</td>
<td>up to 22</td>
<td>SGN typical morphology</td>
<td>(KELLERHALS 1967)</td>
</tr>
<tr>
<td>Resin (Spurr’s low-viscosity resin)</td>
<td>~12-16</td>
<td>perikaryal area 200µm²</td>
<td>SGN typical morphology</td>
<td>(AGTERBERG et al. 2008; AGTERBERG et al. 2009)</td>
</tr>
<tr>
<td>Resin (Spurr’s low-viscosity resin)</td>
<td>~11-15</td>
<td>perikaryal area 262µm²</td>
<td>each neuron counted</td>
<td>(VAN RUIJVEN et al. 2004)</td>
</tr>
<tr>
<td>Resin</td>
<td>~7-8</td>
<td>perikaryal area ~150µm²</td>
<td>visible nucleus</td>
<td>(SHEPHERD et al. 2005)</td>
</tr>
<tr>
<td>JB-4 plastic</td>
<td>~10</td>
<td>14.7</td>
<td>diameter 14-20µm, nucleus 7-10µm</td>
<td>(FRANSSON et al. 2010)</td>
</tr>
<tr>
<td>EMbed 812 plastic</td>
<td>~19</td>
<td>not investigated</td>
<td>not specified</td>
<td>(MILLER et al. 1997)</td>
</tr>
<tr>
<td>OCT</td>
<td>~7-8</td>
<td>perikaryal area 90-100µm²</td>
<td>visible nucleus</td>
<td>(SLY et al. 2012)</td>
</tr>
</tbody>
</table>
4 **IN VIVO EXPERIMENTS:**

Hydrogel Coated and Dexamethasone Releasing Cochlear Implants: Quantification of Fibrosis in Guinea Pigs

4.1 Abstract

The insertion of cochlear implants (CIs) often causes fibrous tissue growth around the electrode, which leads to attenuation of function of CIs. Inhibition of fibrosis *in vivo* using dexamethasone (Dex) released from the implant base material [polydimethylsiloxane (PDMS)] coated with a protein repelling hydrogel (star shaped polyethylene glycol prepolymer, sPEG) was therefore aim of the study. PDMS filaments with Dex or sPEG were implanted into guinea pigs. The hearing status after implantation did not differ significantly in the treated groups. Using Confocal Laser Scanning Microscopy in transparent whole mount preparations, Dex, Dex/sPEG as well as sPEG were able to reduce formation of connective tissue around the implant. However, due to the limited number of cochleae included in the analysis, these results were not statistically significant.
4.2 Introduction

The treatment of choice for severe to profound sensorineural hearing loss is the direct electrical stimulation of the residual auditory neurons via a cochlear implant (CI) (CLARK 1978). The insertion of cochlear implants into the inner ear often causes inflammation and fibrosis inside the scala tympani and thus growth of fibrous tissue on the implant surface (DUCKERT 1983; FAYAD et al. 1991; KAWANO et al. 1998b; NADOL u. EDDINGTON 2004; SOMDAS et al. 2007). As a result of the fibrous encapsulation, the functionality of a laser-based implant (WENZEL et al. 2009) would be influenced significantly as the pass of the laser light could be affected resulting in scattering of the focused light emission. In addition, higher pulse energies are required that account for a shortened battery life. Due to higher electrical impedance, also electronic-based cochlear implants suffer from function loss at the nerve-electrode-interface and from increased power consumption (SHEPHERD et al. 1991; GRILL u. MORTIMER 1994). The aim of this study was to realize connective tissue growth inhibition by dexamethasone (Dex) (WISH et al. 1990; ANDERSON et al. 1991; MOND u. STOKES 1996) released from the base material of the implant (polydimethylsiloxane, PDMS). Following implantation, a dynamic protein adsorption phenomenon occurs initially, triggering inflammatory cell interactions. To prevent cell and protein adhesion, the PDMS was coated additionally with a hydrogel layer (star shaped polyethylene glycol prepolymer, sPEG). In previous studies, it was proved to prevent both adhesion of various proteins and cells including human fibroblasts (GROLL et al. 2004; GROLL et al. 2005b; GROLL et al. 2005c). Poly(ethylene glycol) is widely used as coating material due to its known biocompatibility and potential to minimize unspecific protein adsorption (HARRIS 1992; HARDER 1998; HALPERIN 1999). The protein repelling effect is due to increased hydrophilicity and degree of hydration (OSTUNI 2001). It has been shown that high grafting densities resulting in high surface coverage of the polymer is a determining factor for protein resistance of PEG (ADEMOVIC et al. 2002; KINGSHOTT et al. 2002). In our previous publication, we could prove an inhibition of fibroblast growth for both Dex and sPEG equipped PDMS filaments in vitro (WRZESZCZ et al. 2013a). Crystalline Dex enclosed in the
PDMS-matrix was shown to be released over a time period of 3 months with the potential for even longer time periods referring to the diffusion kinetics, which revealed the release of only 4.4% (non-coated) and 3.36% (hydrogel-coated) of the embedded drug amount within 3 months. But these values only apply to a closed fluid system like the cell culture. In vivo, the complex mechanisms of drug clearance from the cochlear fluids as well as the perilymph loss due to implantation have to be considered. To reproduce the results in vivo, the same types of model implants were implanted for four weeks into guinea pigs. Our findings are presented here.

The cochlea has a complex spiral-shaped structure, consisting of large fluid-filled spaces and a wide variety of tissue types ranging from dense bone to fragile membranous structures, which often fall victim to shrinking and damage artefacts after using embedding methods like paraffin. To avoid these, we used a method based on Confocal Laser Scanning Microscopy (CLSM) to visualize and quantify the fibrotic tissue in a transparent whole mount cochlea sample without the destruction of the spiral geometry. The method has also the potential to detect the positions of cochlear implants since, unlike with paraffin, tissue processing with implanted devices inside the cochlea is possible. The micro-computed tomography used so far is rather appropriate to depict bony structures only. The CLSM method is described in detail in recent publication (WRZESZCZ et al. 2013b).
4.3 Materials and Methods

4.3.1 Production of the local drug delivery system and hydrogel coating
The generation processes have been described previously (WRZESZCZ et al. 2013a).

4.3.2 Animals, aABR and implantation
For this study approximately 3 months old Dunkin Hartley guinea pigs (n = 24) from Charles River Laboratories, Sulzfeld, Germany, were chosen. The use of animals for scientific purposes was permitted by the regional council (Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany, registration number 09/1666 and 10/0104). The studies have been conducted in accordance with the German “Law on Protecting Animals” and with the European Communities Council Directive 2010/63/EU for the protection of animals used for experimental purposes.
Prior to implantation, the normal hearing status (≤50dB sound pressure level) of the animals was confirmed by the measurement of the acoustically evoked auditory brainstem responses (aABR) under general anaesthesia using 10 mg/kg xylazine (Rompun® 2 %, Bayer, Leverkusen, Germany) and 40 mg/kg ketamine (Ketamin Gräub®, Albrecht, Aulendorf, Germany).
The aABR measurement procedure has already been described elsewhere (WRZESZCZ et al. 2013b).
The animals were divided into four treatment groups (Table 8, n = 6 each) and implanted unilaterally with 6 mm long PDMS filaments with a diameter of 300 µm.
After confirmation of normal hearing, the animals received a local application of prilocaine at the surgical site. Tymanotomy was performed and the round window membrane incised to insert the PDMS filament into the scala tympani. The bulla tympanica was then sealed using carboxylate cement (Durelon, ESPE Dental AG, Seefeld, Germany) and the wound closed in two layers.
After 28 days of healing, a second aABR was conducted to compare hearing thresholds to day 0 ante operationem. Still under general anesthaesia, the animals were euthanized immediately through transcardial perfusion with 200 ml 0.1 M phosphate-buffered saline (PBS, Invitrogen, Karlsruhe, Germany) followed by 100 ml paraformaldehyde, 4% (PFA, Merck, Darmstadt, Germany). After perfusion, animals were decapitated and temporal bones were removed and dissected under a dissection microscope to expose the cochleae. The apex and oval window were pierced to enable rinsing.

4.3.3 Tissue preparation, immunolabeling and clearing

The tissue preparation is based upon the proceedings described by MacDonald et al. (MACDONALD u. RUBEL 2010). To keep the method simple, we waived the dissection of the cochleae and kept the organs complete. All preparation steps were carried out with gentle rotation on a platform rotator. Fixation and immunolabeling steps were performed at 4°C (unless stated otherwise), all following steps at room temperature. Cochleae were PFA-fixed overnight, rinsed 3 x 10 minutes in PBS and decalcified for 22 days in 10 % ethylenediamine tetraacetic acid-disodium salt (EDTA, Sigma-Aldrich, Steinheim, Germany) in PBS, pH 7.4, with regular EDTA change every 1-3 days.

The decalcified inner ears were incubated in Image-iT fx (Invitrogen Molecular Probes, Eugene, OR) for 30 min and then transferred to blocking solution (PBS containing 10 % normal goat serum [Vector Laboratories, Burlingame, CA], 0.5 % bovine serum albumin [A-3059-50G, Sigma-Aldrich], 0.1 % Triton X-100 [Sigma-Aldrich]) for 4 h at room temperature. The primary antibody (Monoclonal Mouse Anti-Vimentin Clone V9, Dako, Glostrup, Denmark) was applied diluted in blocking solution and incubated for 3 days. At the end of incubation, the samples were washed three times for 2 h each with PBS. The secondary antibody (Goat polyclonal anti-mouse Cy5, Abcam, Cambridge, UK) was applied afterwards the same way with subsequent PBS washing.

As next step, the cochleae were dehydrated and cleared in Spalteholz solution (methyl salicylate benzyl benzoate, MSBB, 5 parts MS and 3 parts BB) (Table 9).
Since we found commercially available chambers usually either made of plastic or glued with non-solvent-resistant adhesives, which do not withstand the MSBB, we recommend the use of self-made chambers: the cleared cochleae were transferred into self-made round glass chambers (22 mm outer diameter, 5 mm height, 2 mm wall thickness) filled with MSBB, fixed on a 45x71 mm and 0.15 mm thin cover glass (using a solvent-resistant adhesive: Elastosil E 43, Wacker Chemie AG, Stuttgart, Germany) and covered loosely with a 22x22 mm cover slip. Chambers were filled completely with the fluid to avoid air bubbles. The specimens were placed horizontally on the glass with the apex oriented downwards to reduce the distance to the objective lens. If desired, the height of the chamber can be chosen approximately to the height of the cochleae so that the cover slip fixes them gently in their position. This fixation is rather light, it is therefore recommended to generate contiguous image stacks directly in sequence on the same day to avoid displacement of the specimen.

4.3.4 Imaging and data processing

For microscopy, the Leica TCS SP2 AOBS laser scanning confocal microscope equipped with a Leica DM IRE 2 inverted microscope (Leica Microsystems GmbH Wetzlar, Germany) was used combined with the Leica Confocal Software (Leica Microsystems Heidelberg GmbH). An argon laser provided the excitation lines at 488 (for PFA-induced autofluorescence) and 633 nm (for Cy5) with the gate for the multiplier opened between 500 and 600 nm and 650 and 720 nm, respectively. Using pinhole airy unit 1, the slices were generated in 10 µm steps at 400 Hz scanning speed, 8x line averaging (arithmetic mean) and 2x photon accumulation. Confocal images were saved as 8-bit tiff. At 50x magnification (objective Leica HC PL Fluotar 5.0x0.15 BD) z-series stacks (a series of images obtained by focusing from top to bottom in the z-axis through the specimen) were obtained from the apex and the basal turn containing the implant, with stack sizes varying from 1000 to 1700 µm. The time required took about 5-8 hours. Z-stack creation is nearly fully automatic and is frequently repeatable for correction of brightness or with various objectives and step
sizes. Visible bleaching could not be determined even after several hours (up to about 9 h) of high laser beam exposure.

Using the image processing software Imaris x64 6.0.2 (Bitplane, Zurich, Switzerland), three dimensional optical volumes of the cochleae were created that are able to be rotated in every direction and exported as snapshots (tiff or jpg) or movie files.

4.3.5 Data collection and tissue quantification

Choosing a stepsize of $h = 40 \, \mu m$ (every fourth section) a minimum of 20 or more optical sections per cochlea were analyzed to quantify fibrosis. In ImageJ version 1.43 u (Wayne Rasband, National Institutes of Health, Bethesda, Maryland, USA, http://rsbweb.nih.gov/ij/) the areas of the connective tissue and the red amorphous substance were surveyed separately. The volumes were calculated by multiplication of the summarized areas with $h$.

In sum, the time required per cochlea involves for final aABR, perfusion, dissection and fixation 2 days, for decalcification 20 days (possibly less), for staining 6 days, for dehydration and clearing 3 days, for imaging 5-7 h and for data processing about 1 h.

4.3.6 Statistics

For statistical assessment of the hearing status univariate ANCOVA (analysis of covariance) and Bonferroni’s post test was performed in SPSS 21. For fibrotic tissue volumes, one way ANOVA nonparametric analysis and Dunn’s multiple comparison test was used in GraphPadPrism 5.
4.4 Results

Before implantation, all animals showed normal hearing with hearing thresholds between 20 and 50 dB, with lowest at 8 kHz and highest at 1 and 40 kHz frequency. This status did not change in the unimplanted ears 28 days after operation. In contrast, the implanted ears showed a significant hearing loss of about 30 dB in the higher frequencies (16-40 kHz), belonging to the basilar membrane regions near the implant location. The hearing status after implantation in both implanted and non-implanted ears did not differ significantly in the 4 treatment groups (Fig. 20).

In some animals, the implants got lost or displaced during the tissue preparation or the rinsing procedures. These animals were excluded from the study, which led finally to different animal numbers in the treatment groups (PDMS: n = 5, sPEG: n = 5, Dex: n=4; Dex/sPEG: n = 6).

Our earlier study revealed that autofluorescence from the 4 % PFA fixation solution was sufficient to provide a general tissue labeling for use with CLSM, requiring relatively high excitation power levels of 60-100 % at 488 nm. Immunostaining is possible provided that the wavelength range is shifted far enough from the broad range covered by the autofluorescence, otherwise the desired stain would be overlaid. In our experiments, we found the label Cy5 (or Cy7) in the far red range suitable for demarcation from the autofluorescence. Using a 5x objective, we were able to scan through the entire cochlea, approximately 5 mm in depth. However, the laser beam reaches its limitations with increasing distance from the objective, particularly in areas covered by tissue shadows.

Generation of three dimensional figures by digital imaging provided good orientation support for the helical shape of the structures inside of the cochlea, like Rosenthal’s canal, the basilar membrane, stria vascularis or the cochlear nerve (Fig. 21). The implants appeared as elongated shadow entering the cochlea through the round window and lying slightly curved inside the basal turn (Fig. 21-24 arrows). Predominantly in the region of the round window, connective tissue was found on the implant surfaces or in their vicinity (Fig. 22 arrowheads), often forming a tissue envelope. As a second finding, many animals showed basal turns filled with vimentin-
positive red cloudy substance, which we referred to as amorphous substance, possibly containing collagen, other proteins and cell detritus resulting from the inflammation and healing processes. Also blood and ichor could have penetrated the scala after lancing the round window membrane and inserting the implant, leaving remnants after decomposition. In test animals implanted post mortem, these red clouds could not be detected, which indicates that they are associated with wound healing. Their volume was measured separately from the green structures discernible as tissue. Often, the whole basal turn and rarely the scalae of the upper turns (possibly due to the rinsing procedures during the staining and clearing processes) were filled completely. These red clouds seemed to appear predominantly in the animal groups without Dex treated implants (Fig. 23). The two Dex groups showed more frequently a lower grade of connective tissue as well as red substance (Fig. 24).

Regarding the means, quantitative volume analysis (Table 10 and Fig. 25) showed the lowest amounts of connective tissue inside the scala tympani and on the CI surface for Dex/sPEG and Dex implanted animals. The Dex/sPEG group showed a slightly higher amount than Dex alone: compared to PDMS, tissue was reduced by about 80 % and 90 %, respectively. Hydrogel alone caused also a tissue growth reduction (55 %), although not as high as with Dex. Similarly, also the amount of amorphous substance was much lower in the two Dex groups (reduction by 83 and 73 %) than in the other two (20 % in sPEG), with resembling order like in the tissue amounts. Summarizing the tissue and the red substance (="total"), a similar distribution appeared with highest amounts in PDMS, followed by sPEG, Dex/sPEG and Dex (reduction by 30, 75 and 85 %). However, despite the visible differences appearing between the averages, statistically significant differences could not be found, presumably due to the low numbers of analyzed animals and the high variability.
4.5 Discussion

Anti-inflammation, immunosuppression and inhibition of fibroblast growth are well known effects of the artificial steroid dexamethasone (DURANT et al. 1986; RAMALINGAM et al. 1997), which is consistent with our own findings in vitro. We could confirm the Dex release from PDMS filaments (similar to the ones used in vivo here) and a successful growth-inhibiting effect on fibroblasts (WRZESZCZ et al. 2013a). The application of Dex to the inner ear should at least be warranted over the first few weeks after implantation when fibrous tissue formation around the electrode develops. Most application methods such as one-shot injections via syringe or cannula, provide only short term release of active substances. Currently available long term release systems like mini-osmotic pumps and microcatheters cover also no more than several weeks and increase the risk of fluid leakage and infection (KOPKE et al. 2001; PAASCHE et al. 2003b; HOCHMAIR et al. 2006; RICHARDSON et al. 2006; STÖVER et al. 2007; SWAN et al. 2008). Even though they can be incorporated in modified cochlear implant electrodes, the risk of infection still persists. By contrast, using the implanted electrode itself as a carrier of drugs, a local long-term release may be warranted without the requirement of additional surgery. In addition, a direct access to the cochlear structures is granted without the limitations of the basal-apical concentration gradient of round window applications (RICHARDSON et al. 2008). Our local drug delivery system combines both advantages of local application and long term release. To consider the complex mechanisms of drug clearance from the cochlear fluids as well as the perilymph loss due to implantation, in vivo experiments have been performed.

In our in vivo model, connective tissue growth after implantation of Dex-loaded PDMS and sPEG-PDMS filaments showed the lowest tissue growth on the filament surfaces in implanted guinea pigs (Fig. 25). These results were consistent with our previous findings in vitro: In the cell culture studies, we proved an all-over inhibition of fibroblast growth on the implant surface and its vicinity (bottoms of the well plates) through the release and diffusion of Dex from PDMS and sPEG coated PDMS into the cell culture medium. The reduced effect observed in the Dex/sPEG group could
be due to the slower Dex release rates caused by the hydrogel, as already observed previously (WRZESZCZ et al. 2013a). While the antiproliferative effect of the unloaded sPEG worked well on the implant surface, it had no influence on the substances inside the surrounding scala. This may be due to the lack of physical contact to the tissues that shall be inhibited. Due to the diffusion of Dex throughout the whole cochlea, fibrosis would also be reduced at the platinum electrode-nerve interfaces (electrical CI) and at the endings of the light guiding fibers (optical CI), even though they are neither coated by PDMS nor by sPEG.

The lack of differences in hearing loss between the treatment groups indicates that the differently modified implanted materials had no influence on hearing performance. Thus, the damage and trauma induced by implantation was not influenced by the modification of the implants and was similar to the one observed for the regular electrodes. However, it is possible that differences will appear after a longer time period, since application of steroids showed also long term effects in other studies (PAASCHE et al. 2009).

Analyzing the cochleae using the CLSM method has the advantage that the organs remain intact and keep their geometrical structure (HARDIE et al. 2004). There was no rupture of the delicate tissues, as often happens after paraffin embedding and there was also no material loss as after grinding in the epoxy method (STÖVER et al. 2005), which demonstrates the excellent tissue preservation of the CLSM method. The scanning laser optical tomography (SLOT) (EICKHOFF et al. 2012; KELLNER et al. 2012) is a technique, which is also quite appropriate to depict the whole cochlea and to detect the position of the implant. However, the resolution is not sufficiently high to examine the tissue inside the basal turn and on the implant surface. A description and comparison of the methods is discussed in detail in our previous publication (WRZESZCZ et al. 2013b).

Using appropriate image processing software, 3D reconstruction from the optical sections was easily accomplished compared to the reconstructions from histological sections. The cochlear structures could be observed from all directions, even though the scan was performed from only one side (Fig. 22-24). Nevertheless, this caused some restrictions in the display of structures on the objective-averted side of the
cochlea, since the modiolus and the implant casted shadows. For estimation of morphometric measurements, it is conceivable to use the three-dimensionally reconstructed optical volume of the whole basal turn in Imaris. The software provides the possibility to measure distances in the z-axis and thus into the depth of the volume. Only a limited number of the animals were used for the quantification of fibrosis with the CLSM method. Thus, a high variability in the PDMS and sPEG groups was observed and no statistically significant differences could be detected. However, a tendency was observed indicating strong evidence for the fibrosis inhibiting effect of Dex.

4.6 Conclusions

We demonstrated that the CLSM in combination with the Spalteholz-technique is a simple and effective method to examine the implant position inside the cochlea and to quantify the amount of fibrotic tissue four weeks after implantation. In contrast to the in vitro studies, until now no significant effect of Dex and sPEG on tissue growth could be measured, although a tendency is visible. These results provide an adequate basis for further investigations regarding coated electrodes to prevent fibrous tissue growth.
4.7 Figures and tables

Figure 20: Hearing loss in the non-implanted (none) and implanted ears (about 30 dB), 28 days after implantation. Hearing performance in both sides does not differ significantly between treatment groups.
Figure 21: 3D optical volume from CLSM scan of the apical region of a cochlea with silicone implant (arrow), 5x objective. RC=Rosenthal’s canal, OC= Organ of Corti, CN= Cochlear nerve, SV= Scala vestibularis, SM= Scala media, ST= Scala tympani, RM= Reissner’s membrane. Green: autofluorescence, red: Cy5 labelled Vimentin (connective tissue)
**Figure 22:** (A) Optical volume of the basal turn of a cochlea with silicone implant (arrow) surrounded by connective tissue (arrowheads), 5x objective. (B) Optical section, 5x objective. (C) Optical section, 10x objective. RW=Round window, BM=Basilar membrane, OC= Organ of Corti, M= Modiolus, SV= Scala vestibularis, ST= Scala tympani.
Figure 23: (A) Optical volume and (B) optical section of the basal turn of a cochlea with sPEG coated implant (arrow). The basal turn is filled with amorphous substance (red clouds).

Figure 24: (A) Optical volume of the basal turn of a cochlea with Dex loaded and (B) Dex/sPEG implant (arrows). Amorphous substance (red clouds) is minimal.
Figure 25: Volume of connective tissue and amorphous substance found in the four treatment groups. Error bars = SEM
Table 8: Animal treatment groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
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<tbody>
<tr>
<td>Group A</td>
<td>no coating</td>
</tr>
<tr>
<td>Group B</td>
<td>sPEG</td>
</tr>
<tr>
<td>Group C</td>
<td>Dex</td>
</tr>
<tr>
<td>Group D</td>
<td>Dex / sPEG</td>
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Table 9: Spalteholz solution time protocol

<table>
<thead>
<tr>
<th>Solution</th>
<th>Incubation time</th>
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<tbody>
<tr>
<td>70% ethanol</td>
<td>overnight or minimum 2 h</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>30 min</td>
</tr>
<tr>
<td>Absolute ethanol</td>
<td>2 × 2 h (or overnight)</td>
</tr>
<tr>
<td>MSBB/ethanol (1:1)</td>
<td>4 h</td>
</tr>
<tr>
<td>MSBB</td>
<td>2 h, then 4 h, then overnight</td>
</tr>
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</table>

Table 10: Volume of connective tissue (ct) and amorphous substance (as) found in the four treatment groups, in mm³. Lower rows: Mean ± SEM; percentage of PDMS.

<table>
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<tr>
<th></th>
<th>PDMS</th>
<th>sPEG</th>
<th>Dex</th>
<th>Dex/sPEG</th>
<th>ct</th>
<th>as</th>
<th>total</th>
<th>ct</th>
<th>as</th>
<th>total</th>
<th>ct</th>
<th>as</th>
<th>total</th>
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<td>0.265</td>
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<td>0.037</td>
<td>0.841</td>
<td>0.878</td>
<td>0.087</td>
<td>0.0</td>
<td>0.087</td>
<td>0.084</td>
<td>0.499</td>
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<td>0.010</td>
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<td>0.050</td>
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<td>0.385</td>
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<td>0.027</td>
<td>0.055</td>
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<td>0.026</td>
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<td>0.026</td>
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<td>0.101</td>
<td>0.142</td>
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<td>0.163</td>
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<td>±0.167</td>
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<td>±0.115</td>
<td>±0.195</td>
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<td>±0.017</td>
<td>±0.093</td>
<td>±0.058</td>
<td>±0.029</td>
<td>±0.110</td>
<td>±0.073</td>
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<td>100%</td>
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5 ÜBERGREIFENDE DISKUSSION

Indem sie dieses Konzept durch die Möglichkeit einer Langzeitfreisetzung über möglicherweise mehrere Jahre ergänzte, zielte die vorliegende Arbeit auf die Herstellung eines neuen Drug Delivery Systems, das leicht einzusetzen und bei verschiedenen Implantattypen verwendbar sein könnte. Durch die im Vorfeld durchgeführten Freisetzungsversuche (Kapitel 2.4.1.1.) konnte gezeigt werden, dass eine Langzeitabgabe des Dex aus Silikon möglich ist und dass weder die physikalischen noch die Oberflächeneigenschaften des Implantats beeinträchtigt werden.

Als weitere biochemische Funktionalisierung der Implantatoberfläche wurde eine proteinabweisende Hydrogelbeschichtung hinzugefügt, um die Wirkung des Dex zu unterstützen und die Oberfläche direkt vor Zellbewuchs zu schützen (OSTUNI 2001). Das eingesetzte sPEG erlaubte dank seiner Permeabilität nicht nur die Freisetzung des Dex, sondern führte durch die Verlangsamung der Diffusion auch zu einer Verlängerung des Freisetzungszeitraumes. Über die Wahl einer passenden Wirkstoffkonzentration im Silikon lässt sich die Abgabe eines ausreichenden Dosierungslevels in der Perilymphe steuern.


Nicht medikamentös behandelbare Schwerhörigkeit und Taubheit spielen in der Tiermedizin vor Allem im Zusammenhang mit dem Alter oder Vererbung bei meist weißen oder mehrfarbigen Tieren eine Rolle. Kongenitale Taubheit ist bei einer ganzen Reihe von Hunderassen bekannt (u.a. Dalmatiner, Bullterrier, Dogo...

Die histologische Untersuchung der implantierten Meerschwein-Cochleaen hat eine gewisse Herausforderung dargestellt, da es galt, eine zeitefektive, aufwandsarme Methode zur Analyse sowohl der Gewebeweichteile als auch der in der Scala

Im Rahmen der Analysen der gescannten Cochleae hat sich schnell herausgestellt, dass mittels der CLSM eine hervorragende Darstellung der im Modiolus befindlichen Spiralganglienzellen (SGZ) erreichbar ist. Da die Quantifizierung von SGZ als häufige Fragestellung in der CI-Forschung auftritt (STAECKER et al. 1996; YLIKOSKI et al. 1998; GILLESPIE u. SHEPHERD 2005; SCHEPER et al. 2009), erschien dies als ein verfolgenswerter Ansatz. Zwar bietet der konfokale Scan in einer ungeschnittenen Cochlea keine detailreichen Abbildungen der Zellmorphologie

Um das technische Set up simpel zu halten, wurde auf einen 360°-Scan einer drehenden Cochlea verzichtet und das Präparat nur auf der dem Objektiv zugewandten Seite gescannt. Zwar lässt sich durch die Bildbearbeitungssoftware der später zusammengesetzte einseitige Scan in alle Richtungen drehen und von allen Seiten betrachten, jedoch bleiben manche Regionen auf der dem Objektiv abgewandten Seite durch größere Strukturen wie den Modiolus oder das Implantat verschattet. Zwar kann die Cochlea umgedreht und von der anderen Seite gescannt werden, dadurch wird aber kein zusammenhängender Bilderstapel für quantitative Analysen erhalten. In dieser Hinsicht bieten Methoden wie die Light-sheet-based Fluorescence Microscopy (REYNAUD et al. 2008) oder die Scanning Laser Optical Tomography (EICKHOFF et al. 2012; KELLNER et al. 2012) einen Vorteil, benötigen jedoch einen speziellen Apparataufbau dazu, der nicht so weit verbreitet und zugänglich ist wie die Konfokalmikroskopie. Für die vorliegenden Fragestellungen erwies sich der einseitige Scan dank der hohen Leuchtkraft der SGZ, der guten Trefferquote der Zählsoftware und der objektivnahen Lage der Implantate als
durchaus ausreichend. Für eine Verbesserung der Detaildarstellung von Aufnahmen höherer Vergrößerungen als 10x wäre je nach Fragestellung die graduelle Abtragung der äußeren knöchernen Cochleawand zu empfehlen.


6 ZUSAMMENFASSUNG

Antonina Wrzeszcz (2013)

Funktionalisierung des Trägermaterials von Cochlea-Implantaten mittels Dexamethason und Hydrogel: Effekt auf Bindegewebe in vitro und in vivo im Meerschweinchen


In vivo fanden sich keine signifikanten Unterschiede der Hörschwellen in den implantierten Tiergruppen. Dennoch waren sPEG, Dex sowie deren Kombination (Dex/sPEG) in der Lage, die Bildung von Bindegewebe um die Implantate herum um jeweils ca. 55, 90 und 80 % zu reduzieren. Ähnlich der in vitro Ergebnisse erreichten Dex-beladene Proben durch Diffusion des Wirkstoffs eine stärkere Wachstumsinhibition auch in der umgebenden Scala tympani. Die in den Mittelwerten sichtbaren Tendenzen erwiesen sich jedoch als statistisch nicht
signifikant, was vermutlich mit einer höheren Anzahl auszuwertender Tiere verbessert werden könnte.

7 SUMMARY

Antonina Wrzeszcz (2013)

Functionalisation of the cochlear implant base material using dexamethasone and hydrogel: effect on connective tissue in vitro and in vivo in the guinea pig

The insertion of cochlear implants into the inner ear often causes inflammation and fibrosis inside the scala tympani and thus growth of fibrous tissue on the implant surface. This leads to function loss and increased energy consumption of the device. The design of this thesis was to realize fibroblast growth inhibition by dexamethasone (Dex) released from the base material of the implant (polydimethylsiloxane, PDMS).

To prevent cell and protein adhesion, the PDMS was coated with a hydrogel layer (star shaped polyethylene glycol prepolymer, sPEG).

Drug release rates were studied over 3 months and surface characterization was performed. It was observed that the hydrogel slightly smoothened the surface roughened by the Dex crystals. The hydrogel coating reduced and prolonged the release of the drug over several months. Unmodified, sPEG-coated, Dex-loaded and Dex/sPEG-equipped PDMS filaments were co-cultivated in vitro with fluorescent fibroblasts which were quantified after an interval of 5 or 7 days. The release of Dex from the drug delivery system was proved to be feasible over a long time span, from months probably up to years. Compared to the unmodified PDMS, cell growth on all modified filaments was averagely 95% less, while cell growth on the bottom of the culture dishes containing Dex-loaded filaments was reduced by 70% due to the drug diffusion into the culture medium. Both modifications Dex and sPEG reduce the cell proliferation separately (92-93 %) or combined, from which the latter provides the best results (99 %). As second step, the same filament types were implanted unilaterally into guinea pigs. After 4 weeks the hearing status of the animals was measured and the implant position as well as volume amount of fibrosis at the implantation site were analyzed using Confocal Laser Scanning Microscopy (CLSM) in transparent whole mount preparations. This modified version of the method has the advantage to be nearly
fully-automatic and frequently repeatable with various objectives and step sizes between sections and without visible bleaching. The cochleae keep their geometrical structure while the tissue shows minimal or no shrinking artefacts and damage typical of embedding and sectioning in other histological techniques. The CLSM proved also to be a highly effective technique to visualize Rosenthal’s Canal and to quantify the spiral ganglion neurons (SGN) within. Here to, the neurons were first counted manually and then software-assisted. For comparison, guinea pig cochleae embedded in paraffin were examined similarly. The SGN analyzed by CLSM reached an average diameter of 21 µm and a density of about 18 cells/10,000 µm$^2$ (n=8), whereas the paraffin SGN reached only a mean soma diameter of 13.6 µm and a mean density of 9.5 cells/10,000 µm$^2$ (n=8). No significant difference between the manual and the automatic counts was found. This indicates that the semi-automatic CLSM method is a simple and effective technique for auditory neuron count, providing a high grade of tissue preservation. In addition, it offers the potential to detect the position and orientation of cochlear implants within the cochlea, as well as tissue growing around the implant and at the the cochleostomy site.

In vivo, the hearing status after implantation did not differ significantly in the treated animal groups. Dex, sPEG as well as their combination Dex/sPEG were able to reduce formation of connective tissue around the implant by about 90, 55 and 80 %, respectively. Similar to the in vitro results, through diffusion the implants containing Dex led to higher reduction also in the surrounding scala tympani. However, although tendencies were visible in the mean values, these results were not statistically significant, probably due to the limited number of cochleae included in the analysis. Nevertheless, both studies provide evidence that the investigated drug delivery system enables a successful long term release of dexamethasone, achieving in vitro a very good reduction of fibrous tissue, in vivo by tendency. This provides a promising basis for further studies. Furthermore, the modified version of the CLSM method combined with the Spalteholz-technique proved to be highly effective for semi-automatic histological and morphometric analysis of whole mount cochleae with implants. It is an attractive alternative to the traditional methods.
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9 ABBILDUNGSVERZEICHNIS

Figure 1: Released amount of dexamethasone from coated and uncoated filaments over three months. The samples were incubated at 37°C in PBS.

Figure 2: Release rate of dexamethasone over the entire study period and release rate of dexamethasone over the first three days (small graph)

Figure 3: Comparison of the measured release of Dex with the calculated released of Dex based on equation (2) for the uncoated release system over a period of 90 days (small graph) and two years

Figure 4: Fluorescent images of native (left) and hydrogel coated (middle), dexamethasone loaded PDMS filaments after incubation with tetramethylred-labeled BSA. For comparison, a similar treated, hydrogel coated PDMS filament without dexamethasone is shown (right).

Figure 5: Nano-roughness of the filament surfaces; *** highly significant (p<0.001), * significant (p<0.05) difference; n.s. not significant (p>0.05)

Figure 6: Filament surfaces, 10µm²: (A) Scanning electron microscopy, 20.749x magnification (B) Atomic force microscopy, surface topography in two-dimensional and (C) three-dimensional presentation; arrows: fissures in hydrogel layer; asterisks: linear grooves caused by the manufacturing process

Figure 7: Filament surface, scanning electron microscopy: (A) filament from the side, magnification 50x; (B) longitudinal section of a filament with Dex, magnification 100x and (C) magnification 250x, shows cavities (arrows), partially containing Dex crystals (arrowheads); (D) cross section of a filament without Dex shows a smooth surface without cavities or crystals, magnification 100x.

Figure 8: Growth of eGFP-fibroblasts in contact with different PDMS filaments with 2 different diameters after 5 days (starting cell number 2000 cells), cells on filament surface and well bottom counted; Mean ± SEM; *** highly significant reduction of cell proliferation compared to PDMS, PDMS + sPEG and control (p<0.001); n.s. not significant (p>0.05)
Figure 9: Growth of eGFP-fibroblasts (green) in contact with different PDMS filaments after 7 days in 40x magnification (A): Fluorescence microscopy; (B): Fluorescence microscopy, black/white; (C): Light microscopy

Figure 10: Growth of eGFP-fibroblasts in contact with different PDMS filaments after 7 days (starting cell number 2000 cells), cells on filament surface counted; Mean ± SEM; *** highly significant reduction of cell proliferation compared to PDMS (p<0.001); ** highly significant (p < 0.01), * significant (p < 0.05), n.s. not significant (p>0.05)

Figure 11: (A) Paraffin-embedded cochlear section; (B) epoxy resin-embedded and ground section, (C) one optical section and (D) whole 3D optical volume from CLSM scan, 5x objective, 10µm stepsize (green= autofluorescence)

Figure 12: Schematic drawing of a cochlea in glass chamber affixed on a 1mm thin sheet of glass, filled with Spalteholz solution

Figure 13: (A) Three CLSM generated z-series stacks (optical volumes) of the cochlear modiolus using PFA-induced autofluorescence, from apical (left) to basal (right), 10fold magnification; (B) stacks stitched together. Rosenthal’s Canal with spiral ganglion neurons (asterisks), basilar membrane (arrowheads), cochlear nerve (stars)

Figure 14: (A) Cochlear modiolus overview from three stacks stitched together; (B) perimeter of Rosenthal’s Canal (white outlined) surveyed and neurons counted in 6-7 locations on every slice in the lower basal (lb), the upper basal (ub), the 1st (1.m), 2nd (2.m), 3rd (3.m) middle turn and the 4th and apical turn together (4.m+a)

Figure 15: Comparison of detail image of Rosenthal’s Canal of normal hearing guinea pigs. CLSM optical section (A) and paraffin section (B)

Figure 16: Spiral ganglion neuron density (neuron number per 10 000µm²) in the lower basal (lb), the upper basal (ub), the 1st (1.m), 2nd (2.m), 3rd (3.m) middle turn and the 4th and apical turn together (4.m+a) in 5 midmodiolar sections of n=8 cochleae. Comparison of manual and automatic neuron count using the CLSM [difference not significant (p > 0.05)] and the paraffin method [highly significant difference to both CLSM (p < 0.001)]. Error bars = SEM
Figure 17: Manually measured soma diameter of the spiral ganglion neurons (n=40 per turn location) in the lower basal (lb), the upper basal (ub), the 1st (1.m), 2nd (2.m), 3rd (3.m) middle turn and the 4th and apical turn together (4.m+a) of n=8 cochleae. Comparison of the CLSM and paraffin method [highly significant difference (p < 0.001)]. Error bars = SEM

Figure 18: Optical volume (left) and section (right) of the basal modiolus of a cochlea with nerve damage (arrows) after insertion of silicone implant. Arrowhead: undamaged area with full SGN density. Star: cochlear nerve.

Figure 19: Optical volume of the basal turn of a cochlea with silicone implant (arrow), inserted through round window (RW). Rosenthal's Canal with spiral ganglion neurons (asterisks), stria vascularis (arrowheads), cochlear nerve (star). Green: autofluorescence; red: Cy5 labelled vimentin (connective tissue), staining protocol according to MacDonald et al., 2008.

Figure 20: Hearing loss in the non-implanted (none) and implanted ears (about 30 dB), 28 days after implantation. Hearing performance in both sides does not differ significantly between treatment groups.

Figure 21: 3D optical volume from CLSM scan of the apical region of a cochlea with silicone implant (arrow), 5x objective. RC=Rosenthal’s canal, OC= Organ of Corti, CN= Cochlear nerve, SV= Scala vestibularis, SM= Scala media, ST= Scala tympani, RM= Reissner’s membrane. Green: autofluorescence, red: Cy5 labelled Vimentin (connective tissue)

Figure 22: (A) Optical volume of the basal turn of a cochlea with silicone implant (arrow) surrounded by connective tissue (arrowheads), 5x objective. (B) Optical section, 5x objective. (C) Optical section, 10x objective. RW=Round window, BM= Basilar membrane, OC= Organ of Corti, M= Modiolus, SV= Scala vestibularis, ST= Scala tympani.

Figure 23: (A) Optical volume and (B) optical section of the basal turn of a cochlea with sPEG coated implant (arrow). The basal turn is filled with amorphous substance (red clouds).

Figure 24: (A) Optical volume of the basal turn of a cochlea with Dex loaded and (B) Dex/sPEG implant (arrows). Amorphous substance (red clouds) is minimal.
Figure 25: Volume of connective tissue and amorphous substance found in the four treatment groups. Error bars = SEM

10 TABELLENVERZEICHNIS

Table 1: Parameters $k$ and $n$ for the release systems with and without hydrogel coating and the coefficient of determination $R^2$. The values were obtained by adjusting the function (1) to the measurement results after varying the parameter $k$.

Table 2: Slope $m$ and intercept $b$ of the linear regression for the release systems with and without hydrogel coating and the coefficient of determination $R^2$.

Table 3: Setting I, growth of eGFP-fibroblasts in contact with different PDMS filaments with 2 different diameters after 5 days (starting cell number 2000 cells); Mean ± SEM

Table 4: Mean and SEM spiral ganglion neuron (SGN) density. The numbers of SGN in 10.000µm² are plotted for each separate cochlear region and each histological method

Table 5: The mean and SEM SGN diameter (µm) is shown for each separate cochlear region and each histological method. For both histological methods the measurement was performed manually.

Table 6: Comparison of the methods

Table 7: Comparison of literature: SGN densities and soma diameters in normal hearing guinea pigs

Table 8: Animal treatment groups.

Table 9: Spalteholz solution time protocol.

Table 10: Volume of connective tissue (ct) and amorphous substance (as) found in the four treatment groups, in mm³. Lower rows: Mean ± SEM; percentage of PDMS.

Table 11: Mean insertion forces for 16 mm insertion dept and maximal reached insertion depth
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