Development of a Polymeric Coating for Cochlear Implant Electrodes to Deliver Dexamethasone into the Inner Ear

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Ai miei Genitori

“Si può sempre fare qualcosa”

G. Falcone
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<tbody>
<tr>
<td>ABR</td>
<td>auditory brainstem response</td>
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<td>aABR</td>
<td>acoustically evoked auditory brainstem response</td>
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<td>ABC</td>
<td>avidin-biotin-complex</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
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<td>CCD</td>
<td>charge-coupled device</td>
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<td>CI</td>
<td>cochlear implant</td>
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<td>d0</td>
<td>day zero</td>
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<td>df</td>
<td>final day</td>
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<td>DH</td>
<td>Dunkin Hartley</td>
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<td>DMEM</td>
<td>Dulbecco's modified Eagle's Medium</td>
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<td>DMS</td>
<td>Dexamethasone</td>
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<td>DNase I</td>
<td>deoxyribonuclease I</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<tr>
<td>EEC</td>
<td>European Economic Community</td>
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<tr>
<td>ENT</td>
<td>ear, nose and throat</td>
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<td>et al.</td>
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<td>Eto</td>
<td>Ethylene Oxide</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>HBSS</td>
<td>Hank's buffered salt solution</td>
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<td>HEPES</td>
<td>2-(4-(2-Hydroxyethyl) - 1-piperazinyl)-ethansulfonsäure</td>
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<td>i.e.</td>
<td>id est</td>
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<td>LAVES</td>
<td>Niedersächsisches Landesamt für Verbrauchsschutz und Lebensmittelsicherheit (Lower Saxony State Office for Consumer Protection and Food Safety)</td>
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<tr>
<td>NH</td>
<td>non-treated normal hearing</td>
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<td>p</td>
<td>probability</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>PLLA</td>
<td>poly-L-lactide</td>
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<td>P(4HB)</td>
<td>poly-(4-hydroxybutyrate)</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>SGC</td>
<td>spiral ganglion cell</td>
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<td>SNHL</td>
<td>sensorineural hearing loss</td>
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<td>SPL</td>
<td>sound pressure level</td>
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<td>TNF</td>
<td>tumor necrosis factor</td>
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PUBLICATIONS

Articles


Oral presentations and posters at scientific conferences

Ceschi P, Roock A, Sternberg K, Schmitz KP, Lenarz T, Kietzmann M, Stöver T, Paasche G. Development of a polymeric coating for cochlear implant electrodes to deliver dexamethasone into the inner ear. 48th Inner Ear Biology Workshop, Lisbon, Portugal, 2011 (Oral presentation)

Ceschi P, Erfurt P, Lenarz T, Kietzmann M, Stöver T, Paasche G. Comparison between two embedding methods for the evaluation of survival of spiral ganglion cells. 48th Inner Ear Biology Workshop, Lisbon, Portugal, 2011 (Poster)


preliminary Study on PLLA as a potential Carrier’s Coating. 34th ARO MidWinter Meeting, Baltimore, USA, 2011 (Poster)


**Other scientific works**


1. INTRODUCTION

The ear is the organ which allows the detection of sound and the maintenance of balance and body position. This organ consists of three compartments, the external, middle, and inner ear. The external or outer ear is made up of the pinna, also referred to as the auricle in human beings, and the external ear canal. The middle part of the ear (tympanic cavity) is divided from the external ear canal by the tympanic membrane (eardrum) and from the inner ear by the oval window. In mammals it is characterized by the presence of three ossicles, the malleus (hammer), incus (anvil), and stapes (stirrup), and by the proximal orifice of the Eustachian tube. The inner ear is embedded inside the temporal bone and has two distinctive anatomical structures: the vestibular apparatus and the cochlea, the organ in which acoustic stimuli are translated into nerve signals.

From a physiological point of view, the external part of the ear collects the sounds and transmits them to the middle ear, where the ossicles couple the movements of the tympanic membrane into vibrations of the oval window. Once inside the cochlea, this mechanical force is converted into excitation of the spiral ganglion cells (SGCs), the nerve cells of the cochlea, and – via the cochlear portion of the eighth cranial nerve – reaches the central nervous system, where sound is actually perceived.

In order to better understand how these processes occur, it is necessary to explore in detail the physiology of the inner ear and, in particular, of the cochlea. This organ derives its name from cochlos, the Greek word for snail, due to its spiral shape. It is characterized by three fluid-filled compartments, also referred to as the scala tympani, scala media (or cochlear duct), and scala vestibuli. These cavities encircle the bony core of the cochlea, the modiolus. The scala tympani, which is in direct connection with the scala vestibuli at the apex of the cochlea (helicotrema), is filled with perilymph. The third cavity, the scala media, is separated from the other two scalae by the Reissner's membrane and by the basilar membrane; it is delimited laterally by the stria vascularis (which is rich in capillaries) and contains endolymph (secreted by the stria vascularis). Along its entire length, the basilar membrane
supports the organ of Corti, where inner and outer hair cells are arranged in several characteristic rows. When auditory input travels from the outer and middle ear into the cochlea, the perilymph moves inside the scala vestibuli and the scala tympani, causing the deflection of the basilar membrane and, consequently, the stimulation of the hair cells. The deflection of these cells is correlated with their depolarization, which generates electrical potentials that are transmitted first to the spiral ganglion and secondly to the central nervous system through the cochlear portion of the eighth cranial nerve (Kandel, 2000). An important component facilitating sound transduction is the endocochlear potential, which is attributed to the differences in chemical composition of perilymph and endolymph. It is known that disturbances of the endocochlear homeostasis may be directly involved in the pathogenesis of severe hearing disorders such as Ménière’s syndrome (Gleich et al., 2008) and sensorineural deafness (Wangemann, 2006; Strenzke et al., 2008).

Hearing impairment can be classified as conductive or sensorineural, and can affect one or both ears. It may be congenital, age-related (presbycusis) or acquired (via disease or trauma, due to exposure to noise or ototoxic drugs, for instance). In the latter case, a distinction is made in humans between pre- and post-lingual deafness. The severity of this condition can vary from mild to profound. While conductive hearing loss is attributable to dysfunctions of the outer and middle ear, sensorineural hearing loss (SNHL) is associated with the impairment of the inner ear or the central nervous system (Boenninghaus and Lenarz, 2007).

Interest in hearing loss, its pathogenesis, and diagnostic and therapeutic approaches is growing in veterinary medicine as well (Strain, 1996; Bianchi and Dondi, 2000). In particular, a pigment-associated hereditary form has been reported in cats and more than 80 dog breeds, and is the subject of many studies and interesting debates (Strain, 1996). Diagnosing deafness in pets, especially those affected by unilateral hearing loss, is not easy. In fact, animals can adapt to this condition so that even the best owners very often do not realize that their pets suffer from it.
Auditory brainstem response (ABR) provides a useful diagnostic tool and is well accepted by animal patients (mainly dogs and cats). The treatment varies depending on the cause of deafness. External hearing aids have been placed inside the external ear of dogs (Dr. E. Bianchi, personal communication). However, there are some limits, in particular the compliance of the patient and of the owner during the post-implantation period, and the costs.

In human patients – specifically, those affected by pre-lingual hearing impairment – deafness can threaten the acquisition of spoken language. Depending on the nature of the disease, some patients may benefit from hearing aids or even implantable devices such as cochlear implants (CIs), which allow electrical stimulation of the SGCs in patients affected by SNHL (Figure 1).

![Cochlear implant (Cochlear Ltd.)](image)

**Figure 1.** Cochlear implant (Cochlear Ltd.)

A retrospective study reports that, as at January 2008, there were 153,000 CI users worldwide (Peters et al., 2010). This device allows patients to understand speech in
quiet environments and, more importantly, efforts are being made to enhance implant users’ speech understanding even in noise (Shafiro et al., 2011). Tangible results have been achieved in terms of level of speech perception, as reflected by the fact that nowadays CI recipients can also be considered effective telephone users (Tan et al., 2011). It is also expected that patients will achieve satisfactory perception of sounds other than speech, such as music (McDermott, 2004).

However, even if these implants have a strongly positive impact on the quality of life of recipients and their relatives, some relevant negative social aspects remain, including the educational outcomes of children with these implants (Punch and Hyde, 2011), as well as economic issues (Colletti et al., 2011). Moreover, from a medical point of view, even if cochlear implantation is widely accepted as a safe surgical procedure with a relatively low complication rate (Ciorba et al., 2011), the increase in the impedance of the electrode contacts – which is usually associated with a foreign-body reaction – may compromise the proper functioning of the CI (Newbold et al., 2010).

In this sense, the surgical procedure for CI insertion has been refined to reduce the surgically induced traumas (Briggs et al., 2006; Addams-Williams et al., 2011). Furthermore, the modification of the electrode array for the delivery of anti-inflammatory drugs has been proposed in order to reduce the post-surgical tissue reaction (Borkholder, 2008; Leary Swan et al., 2008; McCall et al., 2010; Borenstein, 2011).

In order to better interpret the outcomes of any treatment combined with cochlear implantation, the morphology of the inner ear after surgery must be analysed. To obtain a realistic idea of the effects of implantation, the cochlea should be examined with the electrode in situ. Several techniques have been adopted that involved embedding and thus analysing the cochlea subsequent to implantation. However, some of these, such as paraffin embedding and sectioning, enable excellent histological images of the inner ear to be obtained, but do not allow slicing of the metallic portion of the electrode. Other methods (including embedding in methyl methacrylate and grinding) do permit processing of the cochlea with the electrode in situ but cause swelling of the silicone part of the electrode. As any technique has
both advantages and disadvantages, none of them has been accepted as the gold standard for analysing the effects of any treatment associated with a CI.

The *conditio sine qua non* for the development of novel auditory prostheses delivering anti-inflammatory drugs is the evaluation of the effects of the device inside the inner ear. Therefore, one of the chief aims of this thesis was to improve a technique for investigating inner ear morphology in cochleae, including those on which surgery was performed and which received a (modified) cochlear implant, and which need to be analysed together with the electrode *in situ*. Once this method was established (see first paper), it was employed to document the potential *in vivo* effects of two biodegradable polymeric coatings which were developed for CIs (see second paper). On the basis of these studies, it was possible to generate a prototype provided with a polymeric coating releasing dexamethasone (DMS) and, consequently, to evaluate the inner ear response after implantation (additional results).
2. LITERATURE REVIEW

Histological analysis of the inner ear

The discovery of many ear conditions can be attributed to years of laborious histological preparation of human temporal bones (Chole, 2010). Cochlear processing for histological investigation includes paraffin or resin embedding and sectioning (Staecker et al., 1996; Shepherd et al., 2005; Maini et al., 2009). However, due to the metallic core of the CI, the electrode is typically removed, with a consequent lack of information about the device in situ.

Another technique for cochlear analysis is that of embedding in methyl methacrylate. This approach would enable the specimens to be ground with the electrode in situ. In this case, however, any studies on CIs are influenced by the swelling of the silicone body of the electrode array which occurs during processing of the specimens, as can be seen in the data presented by Richter et al. (2005).

Visualization of the CI carrier inside the scala tympani would provide detailed information about the position of the electrode, its interactions with the cochlear structures, and the response of the inner ear to implantation. More importantly, processing of the cochlea together with the electrode would also allow an accurate investigation of the effects exerted on the inner ear by any local treatment combined with a modified implant.

Efforts have been made to facilitate the visualization of the cochlea together with the CI in situ by means of immunohistochemistry and fluorescent stereoscopy (Chikar et al., 2009). However, only a qualitative assessment of the spiral ganglion neurites proved possible.

Bearing this in mind, it would be of particular interest to establish a strategy which allows the specimens to be embedded and sectioned/ground avoiding any alteration of the tissues and materials employed, and which permits a quantitative assessment of the target cells of CI stimulation.

Previously, Stöver et al. (2005) performed a study on implanted temporal bones which involved processing the specimens by epoxy embedding followed by grinding to evaluate the insertion trauma. This technique enabled them to accurately
document the position of the electrode inside the *scala tympani* without occurrence of any swelling artefacts. In the first study included in this manuscript, the embedding procedure established by Stöver et al. (2005) was modified and investigated further. The aim was to evaluate whether this approach would be effective not merely in analysing the position of the implant after surgery, but also in making a quantitative evaluation of the SGCs, i.e. the primary target of CI stimulation. Once this goal is achieved, this technique will offer an alternative means of concurrently investigating the following aspects relating to a cochlea on which surgery was performed: position of the implant, surgical traumas, presence of inflammatory tissue (its amount would be influenced by the removal of the electrode array prior to the analyses), and effects of the surgery and/or intra-cochlear therapies on the SGCs.

**Optimisation of cochlear implants using biodegradable coatings**

Even if the CI is considered one of the most successful of all neural prostheses employed to date (Wilson and Dorman, 2008), some challenges remain. In particular, inflammatory tissue develops around the electrode array as a response to implantation, and this can lead to an increase in electrode impedance (Newbold et al., 2010), which in turn may affect the amount of energy required for effective stimulation of the SGCs.

Efforts have been made to develop anti-inflammatory therapies, both systemic (Quesnel et al., 2011) and local (van de Water et al., 2010), which are designed to minimize the inflammatory reaction after surgery. However, the systemic administration of drugs implies adverse effects which should not be underestimated. Moreover, the presence of the blood-cochlear barrier reduces the efficacy of any systemic treatment (Bird et al., 2011), when compared with local treatments (Parnes et al., 1999). A means of targeting drug delivery inside the inner ear would be the most logical approach for a safe and successful therapy. However, in this case, the anatomical bony barrier which protects the cochlea constitutes the main problem. In fact, the inner ear is embedded in the temporal bone and the cochlea itself is not accessible for invasive pharmacological treatment.
In order to prevent post-surgical complications and to achieve safe and effective local administration of anti-inflammatory agents inside the cochlea, several strategies have been conceived for the application of drugs at the site of implantation.

There are essentially two methods of targeting the cochlea locally: the intra-tympanic and the intra-cochlear approach.

The first route takes advantage of the round window, whose permeability allows the diffusion of therapeutic agents (Banerjee and Barnes, 2004).

For intra-tympanic delivery, several tools have been investigated including biodegradable polymers, hydrogels, nanoparticles, microcatheters, microwicks and osmotic pumps (Leary Swan et al., 2008; McCall et al., 2010). All of these delivery systems represent promising solutions for tailored application of therapeutic agents; however, accurate control of the dosage cannot always be guaranteed.

Intra-cochlear application of therapeutic agents can be achieved by injecting the drugs directly inside the cochlea or by using a micropump (Leary Swan et al., 2008; McCall et al., 2010; Borenstein, 2011), either through the round window or via a cochleostomy in the outer wall of the cochlea.

In CI recipients, surgical access at the time of implantation has also been used as a route for the injection of anti-inflammatory drugs, in order to reduce post-surgical reaction to the implant (Paasche et al., 2006a). In this study a single injection of triamcinolone crystal suspension has been demonstrated to be effective at reducing the impedance of the electrode contacts. However, this approach does not allow sustained delivery of the agents to be achieved. In this sense, the concept of a modified CI designed for intra-cochlear drug delivery is currently one of the most promising prospects, bearing in mind that this approach would provide sustained drug delivery and thus imply better control of drug concentration during treatment (Hendricks et al. 2008; Richardson et al., 2008; Salt and Plontke, 2009). The integration of infusion pumps with the device (Paasche et al., 2003) and the coating of the array with drug-eluting polymers (Richardson et al., 2009) are at present two feasible options that offer a realistic chance of optimising these implants. However, in the first case, the risks of infection are still a matter of debate (Jolly et al., 2010), and equal substance distribution along the electrode array is not achieved (Paasche et
In the second instance, the potential adverse reactions of any coating applied to the CI array need to be assessed.

In this project, the second route was chosen. One of the aims of this thesis was, therefore, the *in vitro* and *in vivo* investigation of the biocompatibility of a novel biodegradable polymeric coating designed for a modified implant (Bohl et al., 2012) which should enable gradual and safe intra-cochlear drug delivery.

Studies have been performed on biodegradable polymeric matrices with a view to developing sustained drug delivery systems for different applications, such as vascular stents (Labinaz et al., 1995), and to better understand the biocompatibility of these materials in several tissues, including the brain (Fournier et al., 2003). Emerging medical applications rely on degradable materials (Griffith, 2000), and particular attention has been paid to polymers such as poly-L-lactide (PLLA) (Anderson and Shive, 1997) and poly-4-hydroxybutyrate (P(4HB)).

PLLA, the L-isomer or biological metabolite of lactic acid, is a thermoplastic aliphatic polyester derived from the bacterial fermentation of renewable sources, such as corn starch. Its role in biomedical application is well known, since it has been used as degradable suture material for over 30 years (Frazza and Schmitt, 1971) and to develop bioresorbable stents (Regar et al., 2001; Fournier et al., 2003; Bünger et al., 2007).

P(4HB) is a polymer produced by microbial biosynthesis, which is considered a promising matrix for bioengineering and had thus been tested for potential medical applications including sutures (Martin and Williams, 2003), vascular stents (Moore et al., 2005) and ophthalmological stents (Löbler et al., 2010), as well as in dermatology (Peschel et al., 2008).

Considering these medical applications, and to facilitate long-term, targeted and safe drug delivery inside the cochlea, it would be of particular interest to take advantage of those matrices that gradually release drugs during their degradation process in creating a modified implant. PLLA and P(4HB) candidates were thus candidates for testing with a view to developing a novel CI coating, designed with the aim of delivering anti-inflammatory drugs along the electrode array during the post-surgical
period. However, despite the fact that drug-eluting stents represent one of the fastest-growing fields in medical research, there is no experimental knowledge of the effects of PLLA and P(4HB) as potential coatings for the delivery of drugs inside the inner ear. This is an environment characterized by low fluid flow (Salt, 2001) in comparison with other body structures, such as the blood vessels in which drug-eluting stents have been successfully placed (Moore et al., 2005).

As outcomes following the implantation of medical devices are mainly related to the post-surgical reaction to the biomaterials which constitute the device itself (Anderson et al., 2008), the first goal was to investigate the intra-cochlear degradation of these polymers and its potential effects on the neuronal cells.

PLLA degradation is known to occur by hydrolytic attack on its ester bond (Griffith, 2000) over a period of months (Lincoff et al., 1997); when considering this polymer, therefore, it is of great value to analyse the effects of this process over a long period. Being biologically produced, P(4HB) does not contain residual metal catalysts used in chemical synthesis of other matrices (Martin and William, 2003), and it undergoes hydrolysis producing 4HB, a natural human metabolite, which is well tolerated in vivo (Bünger et al., 2007) and whose use is approved for the treatment of cataplexy attacks by the US Food and Drug Administration (FDA). Degradation of P(4HB) occurs gradually by surface erosion, the rate depending on the surface area and porosity; it is, however, faster than PLLA degradation (Martin and William, 2003).

**Intra-cochlear delivery of dexamethasone by means of a modified cochlear implant**

Corticosteroids are secreted from the adenohypophysis and may be classified into two categories: mineralocorticosteroids and glucocorticosteroids. Whereas the first group contributes to the electrolyte homeostasis of the body, the second can influence carbohydrate, lipid and protein metabolism and suppress inflammatory processes by inhibiting the formation of pro-inflammatory mediators and influencing the action of receptors on endothelial cells, leukocytes, and monocytes (Lamm and Arnold, 1999).
The beneficial effects of corticosteroids in the treatment of several inner ear disorders, including sudden sensorineural hearing loss (Dallan et al., 2011; Khaimook and Jantarappana, 2011) and Ménière's syndrome (Shea et al., 2012), have been shown. More importantly, their capability of down-regulating the postsurgical inflammatory reaction after cochlear implantation has been demonstrated, and their influence on change over time in impedance at CI electrode contacts subsequent to surgery (Paasche et al., 2009) – which is strongly dependent on the post-surgical reaction (Huang et al., 2007) – has been investigated.

Among other corticosteroids, dexamethasone, a synthetic corticosteroid, was demonstrated to be able to reduce trauma-induced hearing loss following CI insertion (Eastwood et al., 2010). Much effort has gone into more fully understanding the effects of dexamethasone inside the inner ear. There is evidence that, in vitro, dexamethasone – even when incorporated into polymers releasing it – prevents hair cell apoptosis induced by tumour necrosis factor alpha (TNF-α) (Haake et al., 2009), which is associated with trauma-induced hearing loss (Dinh et al., 2008). Moreover, in murine cochlear explants, this drug upregulates glutathione peroxidase 3 (Maeda et al., 2010), which has been shown to attenuate the threshold shift and hair cell loss in noise-induced hearing loss in guinea pigs (Ohinata et al., 2000). These outcomes are consistent with the findings of other authors, who observed in animal models that dexamethasone could prevent hearing loss due to surgical trauma (Eshraghi et al., 2007; Vivero et al., 2008; Maini et al., 2009) and noise-induced trauma (Takemura et al., 2004).

Since the expression of glucocorticosteroid and mineralocorticoid receptors had been observed in the spiral ganglion and the organ of Corti of rats at all stages of neonatal development (Zuo et al., 1995), it must be supposed that the binding of corticosteroids to these receptors can lead to the suppression of multiple inflammatory genes. These findings indicate that corticosteroids, in particular dexamethasone, are promising candidates for reduction of the inflammatory reaction in the inner ear subsequent to cochlear implantation.
In this project, the feasibility of a biodegradable polymeric CI coating releasing dexamethasone was investigated. The candidate molecule was tested *in vitro* on SGC and NIH/3T3 cells. According to the results of this screening and on the basis of the studies conducted previously on the biodegradable polymers, P(4HB) was preferred to PLLA, dexamethasone was loaded inside this polymeric matrix, and prototypes were manufactured for the *in vivo* experiments.
3. FIRST MANUSCRIPT

(Ready for submission)

Evaluation of spiral ganglion cell density and diameter after epoxy embedding of the cochlea

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Short running head: SGC after epoxy embedding
Supported by: German Research Foundation SFB/TR 37 TP C4
ABSTRACT

**Background:** Cochlear embedding followed by sectioning of the specimens are the techniques of choice for the investigation of the morphology of the inner ear and for the evaluation of the success of intracochlear treatments, such as with neurotrophic factors. However, after cochlear implantation, when a detailed histological analysis is required with the electrode array *in situ*, this approach can not be used because of the metal parts of the electrode array. For this reason a method for embedding a surgically implanted cochlea in epoxy followed by micro-grinding was developed earlier.

**Hypothesis:** The aim of the current study was to investigate whether the established epoxy embedding and grinding procedure can also be used for the evaluation of spiral ganglion cell (SGC) density and perikaryal diameters.

**Methods:** Normal hearing guinea pigs of pigmented and albino strains were included in this study. One cochlea of each subject was embedded in epoxy while the contralateral cochlea was embedded in paraffin. Cochleae were sectioned, stained and then analysed for morphology, dimensions and number of surviving spiral ganglion cells per area of Rosenthal’s canal.

**Results:** The number of SGC was higher when the cochleae were embedded in paraffin. Also the perikaryal diameter was larger for paraffin-embedded cochleae. This could at least in parts be attributed to different processing of the specimens and sampling of the information.

**Conclusion:** Despite the differences in the procedures, epoxy-embedding can be used for the investigation of spiral ganglion cell density and diameter.

**Keywords:** Inner ear, cochlea, spiral ganglion cell, epoxy embedding, paraffin, cochlear implant, perikaryal diameter

**Conflicts of interest:**
None to declare
INTRODUCTION

Preservation of residual hearing and reduction of the distance between stimulating contacts and spiral ganglion cells (SGC) gain more and more importance in cochlear implantation. To investigate possible trauma and intracochlear electrode position after cochlear implantation a micro-grinding technique following epoxy embedding of the cochleae was developed earlier (1). With this method, a documentation of the cochlea can be done at distances between grinding planes of only a few micrometers with the electrode in situ. This allows therefore a more detailed analysis of the cochlea with the inserted electrode compared to sectioning at a thickness of 200 to 300 µm as used in other temporal bone studies (2,3). Furthermore, using epoxy embedding, extensive swelling of the silicone body of the electrode array, which occurs when embedding in methylmethacrylate as shown by Richter et al. (4), is avoided. Temporal bones as typically used in insertion studies were fresh frozen, but not fixed. Therefore, any conclusion about preservation of the soft tissue using these bones was not justified.

As another focus of current cochlear implant research is on reduction of tissue growth around the electrode array, reliable methods for evaluation of the tissue formation are necessary. Removal of the electrode carrier before histological evaluation of the cochlea as done by others (5) might compromise the amount of tissue inside the scala tympani as parts of it might be removed with the electrode array. Moreover, as any pharmacological treatment to reduce the tissue growth around the electrode carrier such as with corticosteroids (6,7) or any coating used in connection with cochlear implant electrodes to deliver drugs into the inner ear (8) or to reduce insertion forces (9) could also have an influence on the SGC, the evaluation of the density of surviving SGC is also necessary. Therefore, a method that allows a detailed analysis of the cochlear structures without removal of the electrode carrier is needed.

The aim of the current study was to investigate if the earlier described method of epoxy embedding followed by micro-grinding as used for fresh frozen temporal bones is eligible for the investigation of SGC survival after fixation of the tissue. For this
reason, SGC density and diameters for epoxy and paraffin embedded cochleae are investigated and compared in two normal hearing guinea pig strains.
MATERIALS AND METHODS

Pigmented (BFA) and Dunkin Hartley (DH) guinea pigs (Charles River WIGA GmbH, Sulzfeld, Germany) of both sexes were chosen as model. Six animals of each strain (weight > 250g) were used for this study. The use of animals for scientific purposes had been approved by the Institutional Animal Care and Research Advisory Committee at Hannover Medical School (Germany) and permitted by the regional council (Lower Saxony State Office for Consumer Protection and Food Safety (LAVES) Oldenburg, Germany). The studies have been conducted in accordance with the German “Law on Protecting Animals” and with the European Communities Council Directive 86/609/EEC for the protection of animals used for experimental purposes.

Auditory brainstem response (ABR)

Before animals were sacrificed, the normal hearing status (≤50dB SPL) of all subjects was confirmed bilaterally by the measurement of the acoustically evoked auditory brainstem responses (aABRs). All procedures were performed under general anaesthesia using xylazine (10 mg/kg intramuscular; Rompun® 2%, Bayer, Leverkusen, Germany) and ketamine (40 mg/kg intramuscular; Ketamin Gräub®, Albrecht, Aulendorf, Germany). AABR measurements were performed in a sound-attenuating chamber using a TDT System 3 (Tucker-Davis Technologies, Alachua, Florida, USA) and data were analysed using custom software “HughPhonics” (10). Acoustic tone stimuli of 1, 4, 8, 16, 32, and 40 kHz were generated by the TDT system and presented by a speaker which was positioned in the external ear canal. Subdermal needle electrodes (CareFusion Nicolet, Middleton, US) were placed at the vertex (common positive), left and right mastoid (references), and in the right lower hind limb (ground) to record the aABRs. The tone bursts had durations of 10 ms with a square cosine rise and fall time of 1 ms. The signal was filtered by a high pass of 300 Hz and a low pass of 3000 Hz, to suppress the inclusion of background noise. The recorded neurological
signals from the animals were digitized and averaged at 200-250 cycles per stimulation. These values were collected for all frequencies for each animal. Thresholds were defined as the lowest stimulus required evoking a visually replicable waveform.

**Histological protocol**

After normal hearing was confirmed, subjects were sacrificed by transcardial perfusion with phosphate-buffered saline (PBS, Invitrogen, Karlsruhe, Germany) (100 ml/250 g body weight) followed by Wittmaack fixative solution (11) (50 ml/250 g body weight) modified by Erfurt as follows: 10 % pure acetic acid, 15 % formol and 75 % distilled water under the addition of 5 g potassium dichromate. After perfusion both temporal bones were removed, examined under a dissection microscope for the presence of infections, and placed overnight in the same fixative solution at 4 °C. Consequently cochleae were separated. One cochlea of each subject was further processed for embedding in epoxy and the contralateral cochlea was processed for embedding in paraffin.

**Epoxy embedding and grinding**

One cochlea of each subject was rinsed and dehydrated respectively for one hour in each of the following solutions: PBS, 50 % ethanol (Merck, Darmstadt, Germany), 70 % ethanol, 90 % ethanol, 100 % ethanol. Then it was dried at 65 °C overnight and embedded in epoxy (SpeciFix-40, Struers, Willich, Germany) under vacuum. Titanium oxide (Merck) was mixed into the epoxy to obtain non-transparent (white) samples, in order to avoid mixing the information from the grinding plane with the information associated to the deeper part of the block. Embedded cochleae were ground with a speed grinder-polisher (PowerPro™ 4000, Buehler, Düsseldorf, Germany). Grinding planes were documented every 20 µm. Each plane was stained with toluidin-blue (Sigma, St.Louis, Missouri, USA) for 4 minutes and eosin-orange (Sigma) for 2 minutes and documented by digital microscope and image-analysis software (VHX-600, Keyence, Neu Isenburg,
Germany). Five midmodiolar planes were chosen for evaluation at magnifications of 50x and 200x. From the base to the apex of the cochlea the profiles of Rosenthal’s canal were identified and classified as follows: first basal (b1), second basal (b2), first middle (m1), second middle (m2), third middle (m3), forth middle (m4), and apical (ap).

The cross-sectional area of each Rosenthal’s canal was measured and SGC were counted to determine SGC densities. The perikaryal diameters of 10 cells (if available) of each canal’s cross-sectional profile were measured (Fig. 1a). Inclusion criterion for cell counts was the morphology of the cells (presence of the nucleus). The large diameter of the cell was measured. Sometimes, the apical canals (ap) could not be separated from m4. Therefore, b1 and b2 were averaged to reflect the basal cochlea, m1 to m3 for the middle cochlea and m4 and ap for the apical part.

**Paraffin embedding and sectioning**

Contralateral cochleae were rinsed for one hour in PBS at room temperature on an orbital-shaker (IKA® KS 130 basic, IKA, Staufen, Germany) and then decalcified at 37 °C using a 20 % ethylenediaminetetraacetic acid solution (EDTA, Sigma). EDTA-solutions were changed every twenty-four to seventy-two hours. After fourteen days cochleae were washed with PBS for one hour at room temperature on an orbital-shaker, and consequently they were dehydrated for up to two hours in each of the following solutions: PBS, 50 % ethanol, 70 % ethanol, 90 % ethanol, 100 % ethanol. Then cochleae were placed in methyl benzoate (Merck) overnight at room temperature and embedded in paraffin (Merck).

Each cochlea was sectioned at 5 µm in a midmodiolar plane by a manual rotational microtome (Shandon Finesse 315, Thermo Scientific, Dreieich, Germany). Midmodiolar sections were mounted on glass slides (Superfrost® Plus, Menzel, Braunschweig, Germany) and stained with haematoxylin and eosin (Merck) and every fifth section was microscopically analyzed. As usual, from the base to the apex of the cochlea the profiles of Rosenthal’s canal were identified and classified as follows: first basal (b1), second basal (b2), first middle (m1), second middle (m2), third middle (m3), forth middle (m4), and apical (ap). For each cochlea five
midmodiolar sections (one every 25 µm) were chosen and examined microscopically at a magnification of 200x (Olympus CKX41, Hamburg, Germany). Images were recorded with a CCD camera (Colorview III, SIS, Muenster, Germany) and processed by an image analysis software (CellP, SIS). The cross-sectional area of each cut-through Rosenthal’s canal was measured and spiral ganglion cells were counted (Fig. 1b). Inclusion criteria were the morphology of the cells (presence of the nucleus) and a perikaryal diameter of at least 12 µm, which was measured for 15 cells (if available) in each canal’s cross-sectional profile.

Also in this case sometimes the apical canals (ap) could not be separated from m4. Therefore, b1 and b2 were averaged to reflect the basal cochlea, m1 to m3 for the middle cochlea and m4 and ap for the apical part.

**Statistical analysis**

Data were first analyzed by the Kolmogorov-Smirnov test for normality. Depending on the results of the normality test, paired t-tests or Wilcoxon matched-pairs signed rank tests were used to compare both histological methods. Analysis for differences between the two guinea pig strains was done either by t-test or Kruskal-Wallis tests again depending on the results of the normality test.
RESULTS

During aABR measurements, one BFA guinea pig had hearing thresholds above 80 dB and had therefore to be excluded from the study. All other animals (5 BFA and 6 DH) had thresholds between 20 and 50 dB with an average over all frequencies of $44 \pm 15$ dB for BFA and $46 \pm 14$ dB for DH guinea pigs (mean ± SD). Lowest thresholds were measured at 8 or 16 kHz (depending on the individual animal) and were $25 \pm 8.5$ dB for BFA and $32.5 \pm 7.5$ dB for DH guinea pigs.

Number of SGC

Comparing the number of SGC as counted with both histological techniques, embedding in epoxy followed by grinding and embedding in paraffin followed by sectioning of the cochlea, these numbers were significantly reduced in all parts of the cochlea and both guinea pig strains when using epoxy embedding and grinding (table 1). Differences between the cochlear regions within the different groups were not found. When comparing the numbers of counted SGC per area of Rosenthal’s canal between BFA and DH guinea pigs, no differences were found in apical, middle and basal regions of the cochlea for either histological method (Fig. 2).

Perikaryal diameter

Also the perikaryal diameter of the cells in epoxy embedded cochleae was significantly reduced compared to embedding in paraffin (table 2). This was found again for all cochlear turns as well as both guinea pig strains. Interestingly, when comparing the perikaryal diameters for both strains, differences were seen. When embedded in paraffin, the diameters were in all regions of the cochlea reduced in BFA guinea pigs compared to DH guinea pigs (Fig. 3), whereas after embedding in epoxy, this was only observed for the apical and middle turns. No differences were detected in the basal turn ($p=0.1036$).
Over all, the diameter of all SGC measured in BFA and DH animals after embedding in paraffin was 15.96 ± 2.08 µm (mean ± SD). The diameter of all SGC measured after embedding in epoxy was 11.17 ± 1.07 (mean ± SD). When calculating the ratio of the diameter in paraffin to the 12 µm taken in the literature as lower limit for viable SGC (12,13), the lower limit for viable cells after embedding in epoxy results for 8.4 µm.
DISCUSSION

Embedding of cochleae in paraffin to investigate the effectiveness of treatments with pharmaceuticals such as neurotrophic factors (14) or antioxidants (15) on cochlear hair cells and spiral ganglion cells is a well established technique. Regarding SGC, their number per area of Rosenthal’s canal and their soma diameter are mostly investigated. In this respect, SGC with a diameter of 12 µm or more are considered viable cells by several authors (12,13).

However, paraffin-embedding and sectioning can not be employed with an electrode array being inserted into the cochlear turns due to the metal part of the CI. For this reason a micro-grinding technique after embedding of the cochlea in epoxy is used with implanted cochleae to investigate electrode position and insertion trauma (1). With the latter approach, the morphology of the SGC in Rosenthal’s canal is changed (Fig. 1).

When pharmaceuticals are applied to the cochlea in combination with a cochlear implant it is typically aimed at reducing fibrous tissue growth around the implanted electrode array and/or an enhanced survival of SGC. Therefore it is of great interest to investigate the effects of pharmaceuticals on both the SGC and the fibrous tissue around the electrode array after implantation in the same cochleae. For this purpose the electrode arrays should remain in situ so that the tissue around the implant is not disturbed. Therefore the aim of the current study was to investigate if epoxy embedding can also be used for evaluation of SGC density.

This study was set up such that from normal hearing animals always one cochlea was embedded in epoxy and the contralateral cochlea was embedded in paraffin. Using this approach and by confirming normal hearing by aABR measurements, same numbers and diameters of SGC in Rosenthal’s canal are to be expected. Any differences should then be related to the different histological procedures.

The measured numbers of SGC per area in sections of normal hearing guinea pig cochleae in the literature are typically between 8 and 15 cells per 10.000 µm², as
also found in the current study for paraffin embedded cochleae. In general reported values for pigmented guinea pigs (16-19) seem to be little smaller than for albino guinea pigs (20,21). In recent studies, the perikaryal or soma diameter of SGC in pigmented guinea pigs is given to be between approximately 14 and 17 µm (16,17,22). This can be corroborated by the current findings. In this study, differences between both strains of guinea pigs were not observed regarding the numbers of SGC but for the perikaryal diameter. The average perikaryal diameter was larger for DH guinea pigs. As this was true for both embedding methods we can only speculate that there might be real differences between different guinea pig strains. As no data were found in the literature regarding SGC diameters in albino guinea pigs, the data for both strains were averaged for a direct comparison of both embedding procedures.

As expected the measured perikaryal diameter was smaller in cochleae embedded in epoxy (diameter: 11.17 µm) than in paraffin (diameter: 15.96 µm). This could be explained by shrinking due to the different embedding procedures, especially the drying process at 65 °C that followed dehydration during processing for epoxy embedding. On the other hand the differences in the thickness of the sections and planes and the differences in the documentation have to be taken into account (23,24). In fact, when documenting paraffin slices, the information for the optical image was collected through the full thickness of the slices of 5 µm. And thus, in the optical image, always the largest diameter anywhere within these 5 µm was documented. In contrast, due to the addition of titanium oxide to the epoxy, the information for the optical image during the grinding procedure after epoxy embedding was only collected from the surface and it was not influenced by any other shapes inside the sample.

To get an idea about the possible influence of this effect on the measured cell diameter, the soma of a SGC could be approximated by a ball of a diameter of 15 µm. Due to geometrical considerations (Fig. 4) 70 % of the cells seen in a 5 µm paraffin section should then have diameters of 12 µm or more. When using epoxy embedding, this is reduced to 60 % of the cells. This means that just due to the
reduction of the thickness of the slice to zero (only information from the surface is gathered) a smaller number of SGC in epoxy embedded samples is to be expected. This remains true also if not a minimum diameter but the presence of a nucleus is taken as morphological marker for SGC. When calculating the number of cells with maximum diameter (15 μm) the differences between both methods get even larger. Whereas 25% of the cells in paraffin sections show the maximum diameter, only (approximately) 1 of 15 cells would do so in epoxy. Therefore, smaller SGC diameters in epoxy embedded cochleae are also to be expected. These geometrical considerations do therefore also contribute, together with the different processing of the specimens, to the differences in cell numbers and perikaryal diameters as found for both embedding techniques in the current investigation.

In the literature 12 μm were taken as lower diameter limit for a viable SGC (12,13). As the real amount of shrinkage after epoxy embedding is unknown, the comparable lower perikaryal diameter limit for viable cells was calculated according to the measured diameters. The average diameter for SGC in paraffin-embedded and sectioned cochleae was 15.96 μm. In relation to the 12 μm as the lower limit for viable cells this led to a calculated lower limit for viable SGC in epoxy embedded cochleae of 8.4 μm. Even though this diameter was not based on a real test for SGC viability, it might provide an additional inclusion criterion for counted SGC as the 12 μm are often taken for paraffin embedded cochleae. This lower diameter limit for epoxy embedded SGC seems to be reasonable as all cells with diameters above this value are morphologically classified as SGC. Furthermore, as the shrinking should affect all cell types of the spiral ganglion, it is not expected that other cell types have somata with visible nuclei of this size. Other ways to determine this minimum diameter were in our opinion not justified. As both ears in one animal should have the same numbers of SGC it might be reasonable to count SGC with diameters above 12 μm in paraffin sections, measure the perikaryal diameters of the same number of cells in the other cochlea of the same animal embedded in epoxy and take the lowest diameter as lower limit for viable cells. This procedure would ignore the fact that the number of SGC is reduced in a grinding plane compared to the sections of the
contralateral side due to shrinking and the above described influences of differences in the collection of the information for a microscopic image.

In conclusion, even if tissue shrinking occurs, epoxy embedding followed by grinding can be used to determine SGC densities and diameters. This method provides a tool not just to evaluate the surgical insertion trauma after cochlear implantation and the electrode position but it also permits the evaluation of the influence of treatments on SGC and potentially the investigation of the post surgical inflammatory reaction with CI electrode arrays *in situ.* When investigating SGC survival, a lower minimum diameter has to be taken into account.

**ACKNOWLEDGEMENTS**

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Supported by: German Research Foundation SFB/TR 37 TP C4
REFERENCES


**FIGURE LEGENDS**

**Fig. 1:** Histological images of Rosenthal’s canal. A: paraffin-embedded cochlea. B: epoxy-embedded cochlea. Examples of SG.

**Fig. 2:** Comparison of the numbers of counted SGC in pigmented (BFA) and albino (Dunkin Hartley) guinea pigs. For all regions of the cochlea no differences were detected between both embedding methods. Additionally no differences were found between the different regions of the cochlea. Cells are marked with asterisks.
Fig. 3: Comparison of the perikaryal diameters of SGC in pigmented (BFA) and albino (Dunkin Hartley, DH) guinea pigs. With the exception of the basal region in epoxy embedded cochlea, the measured diameters were always significantly larger in DH animals. Again, no differences were detected between the cochlear regions. * p<0.05; ** p<0.01

Fig. 4: Schematic drawing of the soma of a SGC approximated by a ball of 15 µm in diameter. B: radius of the cell (7.5 µm); A: half the lowest cross-sectional diameter of a viable SGC (6 µm); X: part of the radius of the cell, where the cross-sectional diameter is above 12 µm. With \( \cos \alpha = \frac{A}{B} \), \( \alpha \) is calculated to be about 39°. With \( \sin \alpha = \frac{X}{B} \), X is calculated to be 4.5 µm. During grinding, the information is only collected from the surface. That means in 9/15 of all cases (60 %), this cell would be considered a viable cell. In contrast, when slicing with a thickness of 5 µm, the cell would be visible over a length of 20 µm (cell diameter plus slice thickness). Therefore in 14/20 of all cases (70 %), the measured diameter would be at least 12 µm.
### TABLES

**Table 1**
Comparison of spiral ganglion cell (SGC) counts per 10000 µm² for both techniques, embedding in epoxy followed by grinding and embedding in paraffin followed by slicing. Values are given as mean ± SD. * p<0.05; ** p<0.01; *** p<0.005

<table>
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<th>Guinea pig strain</th>
<th>Cochlear region</th>
<th>Epoxy</th>
<th>Paraffin</th>
<th>Significance</th>
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**Table 2**
Comparison of perikaryal diameters (in µm) of SGC for both techniques, embedding in epoxy followed by grinding and embedding in paraffin followed by slicing. Values are given as mean ± SD. ** p<0.01; *** p<0.005

<table>
<thead>
<tr>
<th>Guinea pig strain</th>
<th>Cochlear region</th>
<th>Epoxy</th>
<th>Paraffin</th>
<th>Significance</th>
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4. SECOND MANUSCRIPT

(Submitted to Journal of Biomedical Materials Research: Part B – Applied Biomaterials)

Biodegradable Polymeric Coatings on Cochlear Implant Surfaces and their Influence on Spiral Ganglion Cell Survival

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ABSTRACT

To improve the electrode nerve interface of cochlear implants (CI), the role of poly(L-lactide) (PLLA) and poly(4-hydroxybutyrate) (P(4HB)) as potential coating matrices for CI was assessed both in vitro and in vivo in terms of degradation behaviour and effects on spiral ganglion neurons, the main target of the electrical stimulation with a CI.

Growth rates of fibroblasts on the polymers were investigated and a direct-contact test with freshly isolated spiral ganglion cells (SGC) was performed. In addition, the effects of the polymer degradation inside the inner ear were evaluated in vivo. The polymer degradation was assessed by use of scanning electron microscopy in combination with an energy-dispersive X-ray analysis.

In vitro, no influence of the polymers was detected on fibroblasts' viability and on SGC survival rate. In vivo, SGC density was decreased only 6 months after implantation in the basal and middle turns of the cochlea in comparison to normal-hearing animals but not between implanted groups (coated or uncoated). The analysis of the electrode models showed that in vivo P(4HB) is characterized by a gradual degradation completed after 6 months; whereas, the PLLA coatings burst along their longitudinal axis but showed only little degradation within the same time frame.

In conclusion, both polymers seem to justify further evaluation as possible coating for CI electrodes. Of the two options, due to its excellent coating adhesion/stability and optimal degradation behaviour, P(4HB) may prove to be the more promising biodegradable polymer for designing a drug delivery system from the surface of CI electrodes.

Keywords
Cochlear implant, coating, biodegradable polymers, poly(L-lactide), poly(4-hydroxybutyrate), spiral ganglion cells
INTRODUCTION

Cochlear implantation is the standard surgical procedure for the treatment of patients suffering from severe to profound sensorineural hearing loss. However, even though the results with cochlear implants (CI) are usually remarkable, there still remain two main factors related to the electrode neural interface that can strongly influence individual outcomes. The first is the post-surgical inflammatory reaction around the electrode array that is reflected by increasing electrode impedances after implantation [1]. As the amount of tissue growth can vary from little loose fibrous tissue to new bone formation [2], optimisation of the interface of the CI electrode is mandatory. The second is the number of surviving spiral ganglion cells (SGC) that are the target cells for electrical stimulation with a CI [3, 4]. There is evidence from animal models that glucocorticoids can preserve acoustic hearing thresholds following cochlear implantation [5-7]. Additionally, it has been shown that in human patients a single intraoperative intracochlear steroid deposition can lower the impedances during the first three to four years after implantation [8]. As this single shot application was easy to apply but difficult to control in terms of the amount of drugs deposited inside the cochlea, a technique that permits a continuous drug release would be more attractive.

Several approaches have been studied in the last decades in order to control the electrode-nerve interface [9, 10] or to deliver anti-inflammatory drugs inside the inner ear [11-14]. Among the approaches under investigation were delivery of drugs by diffusion through the round window [15], fluid based drug delivery through catheters [16] or canals within the CI [17, 18] or the use of reservoirs attached to or inside the CI electrode [19, 20]. All of them generate concentration gradients along the electrode array because of their distinct entry points of the drugs into scala tympani. Currently the only way to achieve an even drug distribution in scala tympani is incorporating the drug (dexamethasone) in the silicone of the electrode array [21, 22]. Incorporating of substances into the silicone matrix potentially alters the mechanical stability or cross-linking of the silicone body of the CI electrode. An alternative option might be coating of the CI electrode array surface.
Biodegradable polymeric coatings have been investigated to improve coronary stents with respect to the reduction of vascular inflammation, in-stent restenosis and stent thrombosis [23-26]. Two examples of biodegradable polymers are the synthetic poly(L-lactide) (PLLA) and the natural poly(4-hydroxybutyrate) (P(4HB)). While PLLA is safely being used as degradable suture material, as well as in urological and coronary stents and drug delivery systems [27-30], P(4HB) is being employed for resorbable meshes (TephaFLEX®) and is currently being evaluated for a number of medical applications [31-33]. Recently, a specially tailored local drug-delivery system for the prevention of fibrosis after CI insertion based on a dexamethasone-containing silicone electrode carrier and a dexamethasone-containing PLLA or P(4HB) coating on its surface has been described [34]. This system is well investigated in vitro regarding its biocompatibility, drug release behaviour and coating stability during a simulated implantation procedure; however, little is known about the potential application of such biodegradable coatings within the inner ear. When coating a coronary stent with biodegradable polymers, degradation products of the polymers are removed by the blood flow. When transferring these degradable coatings to CI electrodes, at least two important factors must be considered. Firstly, the intracochlear fluid flow is normally very low [35]; this will probably influence degradation kinetics and degradation products that can potentially accumulate in the cochlea and secondly, as mentioned above, potential negative effects on survival of SGC must be considered/excluded.

The aims of this study were, therefore, to evaluate the effects of PLLA and P(4HB) on fibroblasts and SGC in vitro and to follow these investigations with studies of the in vivo degradation behaviour in the stationary environment of the cochlea and the biocompatibility as evaluated by the effects on SGC survival.
MATERIALS AND METHODS

Sprague-Dawley rats (three to five days old) were chosen to isolate the SGC and guinea pigs of both sexes served as the model for the in vivo screenings. The use of animals for scientific purposes was approved by the Institutional Animal Care and Research Advisory Committee and permitted by the regional council, respectively. The studies have been conducted in accordance with the German “Law on Protecting Animals” and with the European Communities Council Directive 86/609/EEC for the protection of animals used for experimental purposes.

Biodegradable polymeric coatings

Fabrication of polymeric films
The biodegradable polyesters poly(L-lactide) (PLLA, Resomer® L214, M_w=720000 g/mol, Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany) and poly(4-hydroxybutyrate) (P(4HB), TephaFLEX®, M_w=170000 g/mol, Tepha, Inc., Boston MA, USA) were used for the fabrication of the polymeric films. For film pouring, 1 g of PLLA and P(4HB), respectively, was dissolved in 25 mL chloroform (J. T. Baker Inc., Deventer, The Netherlands) and poured into a glass petri dish (Ø = 9 cm). After pouring, the solvent was allowed to evaporate until a film of 100 µm thickness, analysed by means of a thickness gauge (2109 Mitutoyo, Mitutoyo Europe GmbH, Neuss, Germany), had formed. Subsequently, the films were cut out of the petri dish and washed in methanol for 2 days and then in distilled water for another 2 days. Afterwards, the films were dried for 7 days in a vacuum drier at 40°C and 40 mbar. For the in vitro biocompatibility assays, circular test specimens with a diameter of 6 mm were cut with a cork trepan from the prepared films.

Fabrication of silicone fibres and films
The medical grade, two-component silicone system NuSil MED-4234 (NuSil Technology Europe, Mougins, France) was employed for the fabrication of silicone
fibres. It consists of a silicone base (part A) and a curing agent (part B); part A and part B were mixed in a ratio by weight of 10:1. Subsequently, the mixture was degassed in vacuum in order to avoid the formation of bubbles inside the silicone. The mixture was then put into a plastic syringe and injected into a two-part aluminium mould with a bore hole of 0.4 mm diameter. The silicone was cured at 110°C for 2 h, after which the mould was opened and the silicone fibre was removed. For the fabrication of silicone films, the mixture was poured onto a polypropylene (PP) plate, then a second PP plate was placed on top of it and a slight pressure was applied to achieve a silicone film thickness of approximately 1 mm. The reaction mixture was allowed to cure for 1.5 h at 110°C. After curing, the covering plates were removed and the silicone film was cut into discs of 6 mm diameter.

**Chemical surface activation of silicone by NH₃ plasma**

Prior to plasma-chemical activation the silicone samples were rinsed with ethanol (Sigma-Aldrich, Taufkirchen, Germany) for cleaning. Plasma-chemical activations were run on a plasma system that was equipped with a 300 W radio-frequency generator (Diener electronic GmbH + Co. KG, Ebhausen, Germany). Initially, the chamber was evacuated to a pressure of 0.09 mbar and then an ammonia (NH₃) pressure of 0.30 mbar was applied. The plasma was run for 1 min at 15% generator power; afterwards, the chamber was vented with air.

**Wet-chemical application of polymeric undercoatings**

In order to enhance the adhesion of PLLA- or P(4HB)-based coatings on the silicone surface, chemically coupled PLLA or P(4HB) undercoatings in terms of monolayers were applied according to Bohl et al, 2012 [34]. For this purpose, amino groups on the chemically relatively inert silicone surface were generated using NH₃ plasma. Then the silicone samples were immersed into a solution containing 1.8 g/L of N-hydroxysuccinimide (NHS, Merck KGaA, Darmstadt, Germany), 1.8 g/L of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDC; Merck KGaA), as well as the undercoating polymers in chloroform (CHCl₃, J. T. Baker Inc.) for PLLA and 1,2-dichloroethane (EtCl₂, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for
P(4HB), respectively. A comparative overview for the concentrations of the applied PLLA and P(4HB) solutions, the reaction temperatures and reaction times necessary for the generation of PLLA or P(4HB) undercoatings are shown in Table 1. The different solvents were required because the test polymers have a different solubility in organic solvents; therefore, the reaction time was adapted accordingly. After the application of the chemically coupled PLLA or P(4HB) undercoatings, the silicone samples were rinsed with the corresponding solvent and dried in a vacuum drying cabinet (model UM200, Memmert GmbH & Co KG, Schwabach, Germany) at 40°C for 24 h.

**Spray-coating process for application of polymeric coatings**

After the generation of chemically coupled PLLA- or P(4HB)-based undercoatings, the next PLLA or P(4HB) layer growth was created by means of a spray-coating process for which a specially designed spray-coating device was developed by the Institute for Implant Technology and Biomaterials (IIB e.V., Rostock, Germany). Polymer solutions with concentrations of 1.53 g/L of PLLA dissolved in chloroform and 2.3 g/L of P(4HB) dissolved in 1.2-dichloroethane, respectively, were used. For each silicone fibre, a PLLA or P(4HB) coating with an absolute mass of ~200 µg/cm length was applied. The coated silicone fibres were dried in the vacuum drying cabinet at 40°C for 24 h. The mass of the different dried PLLA and P(4HB) coatings were determined by use of a Mettler Toledo UMX 5 Ultra-micro Balance (Mettler-Toledo GmbH, Giessen, Germany).

**Microscopy**

The surface morphology of the polymer-coated silicone fibres was assessed after the different implantation intervals (1, 2, 3 and 6 months) and compared to the non-implanted controls using environmental scanning electron microscopy (XL30 ESEM, Philips, Eindhoven, The Netherlands) equipped with an energy-dispersive X-ray (EDX) analysis unit. The fibres were fixed with conductive tape on aluminium trays and the scanning electron micrographs were taken at 1.2 mbar pressure in a moisturized atmosphere and an accelerating high voltage of 10 kV at a detector
distance of 10 mm. The presence or absence of the polymeric coatings after the in vivo degradation process was assessed by EDX measurements performed at the beam entrance of the electron microscope. For element (Si, C, O) determination, the spectra of the fibres bombarded with electrons were analysed.

In vitro screenings

Fibroblasts
For the in vitro tests, murine NIH/3T3 fibroblasts (ATCC number CRL 1658) were utilised. Before seeding the cells, the discs of PLLA, P(4HB) and silicone were placed at the bottom of 96-multiwell culture plates (NUNC, Langenselbold, Germany) in order to perform a direct contact test. In addition, cells were cultivated on the bottom of the culture plates (polystyrene) as control. Fibroblasts were sub-cultivated at 80% confluence and seeded in 96-multiwell culture plates at a density of 1x10^4 cells/well in 100 µL supplemented medium, that was prepared using Dulbecco’s Modified Eagle’s Medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (FCS, Gibco, Karlsruhe, Germany), and 1% penicillin/streptomycin (Sigma-Aldrich). To measure survival rates, cells were stained after 48 h incubation at 37°C, 5% CO₂ and 95% humidity using neutral red (4 mg/mL, Merck KGaA) dissolved in pure water and diluted 1:50 in supplemented DMEM to get a final concentration of 0.08 mg/mL. An additional 100 µL of this solution was added to each well. After another 3 h incubation at 37°C, the plates were centrifuged at 1000 rpm for 5 minutes at 4°C, the supernatant was removed and cells were washed with 100 µL of a solution containing distilled water, 1% CaCl₂ and 0.5% formaldehyde. After 5 minutes centrifugation, cells were solubilised using a solution of 1% acetic acid and 50% ethanol in distilled water. Plates were put on a shaker for 30 seconds and stored at 4°C for 10 minutes. Subsequently, 90 µL of the supernatant containing the stain released by the cells were transferred into new wells and photometric measurements were performed at 570 nm by Multiscan Ascent plate reader (Thermo Scientific GmbH, Bremen,
Germany) and compared to the control wells. Survival rates were calculated in percent of untreated control.

**Spiral ganglion cells**

For the dissection of the cochleae and the spiral ganglion, enzymatic dissociation was performed as described previously [36]. Briefly, isolation of the spiral ganglia from neonatal Sprague-Dawley rats was followed by enzymatic dissociation using Hank's buffered salt solution (HBSS, Invitrogen) supplemented with 0.1% trypsin (Serva, Heidelberg, Germany) and 0.01% DNase (Roche, Mannheim, Germany). The process was stopped using FCS. Spiral ganglia were washed in DMEM and gently, mechanically dissociated. Viable cells, including SGC and other cell types were counted in a Neubauer counting chamber using trypan blue (Sigma-Aldrich) and resuspended in DMEM.

The discs of PLLA, P(4HB) and silicone were placed at the bottom of 96-multiwell culture plates before seeding the cells and then the discs and the remaining wells (controls) were coated with 100 µL poly-DL-ornithine (0.1 mg/mL, Sigma-Aldrich) at room temperature for 1 hour. When this solution was removed, wells were rinsed in 100 µL phosphate buffered saline (PBS, Invitrogen) and incubated at 4°C overnight with 100 µL laminin (0.01 mg/mL, Invitrogen). Before use, wells were rinsed first in PBS and secondly in DMEM.

Cells were seeded at a mean density of 1.5 x 10⁴ cells per well and were cultivated in DMEM supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 25 mmol/L; Invitrogen), glucose (6 mg/mL; Braun, Melsungen, Germany), penicillin (30 U/mL; Grünenthal, Aachen, Germany), N2 supplement (3 µL/mL; Invitrogen), insulin (5 µg/mL; Sigma-Aldrich), PBS (550 µL/ 32 mL), 10% FCS and brain-derived neurotrophic factor (BDNF, 50 ng/mL; Invitrogen). In addition, cells were cultivated on the treated bottom of the culture plates as control. After an incubation time of 48 h, SGC were fixed with 1:1 acetone/methanol solution and incubated with the monoclonal mouse 200-kD neurofilament antibody (clone RT97; Novocastra Ltd, Newcastle-upon-Tyne, UK; 1:500 dilution in 1.5% normal horse serum). Thus, wells were rinsed with PBS and incubated with a secondary
biotinylated anti-mouse antibody (Vector Lab, Burlingame, California, USA; 1:2000 dilution in 1.5% normal horse serum). Subsequently, wells were washed and incubated with ABC complex solution (Vectastain Elite ABC-Kit, Vector Lab) according to the manufacturer's protocol. The staining was visualized with dianobenzidine (Peroxidase Substrate Kit DAB, Vector Lab).

To determine the cell survival rates, SGC were counted (mean ± SEM) using an inverted microscope with automated table control (Olympus CKX41, Hamburg, Germany), a mono-coloured camera (Colorview III, SIS, Muenster, Germany) and imaging software (analySIS Version 3.0, SIS).

Each test was repeated at least four times with 5 discs per group and per plate (PLLA, P(4HB), and silicone) and 5 wells as control on each plate. In addition, the experiments were also performed without poly-DL-ornithine- and laminin-coating.

**In vivo screenings**

Pigmented guinea pigs (Charles River WIGA GmbH, Sulzfeld, Germany) of both sexes (weight >250 g) were chosen as a model. The subjects were randomly divided into three main groups. The first group (silicone, n=20) was implanted with uncoated silicone samples, the second group (PLLA, n=43) with samples coated with PLLA and the third group (P(4HB), n=43) with P(4HB)-coated samples. Non-treated normal-hearing guinea pigs (NH, n=6) served as an additional control group. All groups - except from the NH-group - were divided in four subgroups related to the duration of the experimental periods (1, 2, 3 and 6 months).

All *in vivo* measurements and surgical procedures were performed on a heating pad maintained at 37°C and under general anaesthesia using an intramuscular injection of xylazine (10 mg/kg body weight intramuscular; Rompun® 2%, Bayer, Leverkusen, Germany) and ketamine (40 mg/kg intramuscular; Ketamin Gräub®, Albrecht, Aulendorf, Germany). Anaesthesia was maintained using ketamine/xylazine injections intramuscularly, as required. Prilocain (Xylonest®, Astra Zeneca GmbH, Wedel, Germany) was applied as local anaesthetic.
**Auditory brainstem response (ABR)**

Before animals underwent surgery, the normal-hearing status (≤50 dB SPL) of all subjects was confirmed bilaterally by the measurement of acoustically-evoked auditory brainstem responses (aABRs). Additional aABR measurements were performed at the end of the respective trial period.

The aABR measurements were performed in a sound-attenuating chamber using the TDT System 3 (Tucker-Davis Technologies, Alachua, Florida, USA), and data were collected and analyzed using custom software “HughPhonics” [37]. Acoustic stimuli of 1, 4, 8, 16, 32 and 40 kHz were generated by the TDT system and presented by a speaker that was connected to the external ear canal via a tube that had been calibrated for output intensities. Subdermal needle electrodes (CareFusion Nicolet, Middleton, USA) were placed at the vertex (common positive), left and right mastoid (references), and in the right, lower hind limb (ground) to record the aABRs. The tone bursts had a duration of 10 ms with a square cosine rise and fall time of 1 ms. The signal was processed via a high-pass filter at 300 Hz and a low passfilter at 3000 Hz to suppress background noise. The recorded neurological signals were digitized and averaged at 200-250 cycles per stimulation. Thresholds were defined as the lowest stimulus required that yielded a visually replicable waveform.

**Surgical procedures**

All subjects were implanted bilaterally under sterile conditions on day 0 (d0) with silicone fibres as a model for CI electrode arrays consisting of a silicone carrier (400 µm diameter; 5 mm length) uncoated (silicone-group) or coated with the biodegradable polymers (PLLA- and P(4HB)-groups). Samples were sterilized by ethylene oxide gas technique, which has been observed to cause hardly any change to the polymeric coating [38]. To insert the prototype into the cochlea, the middle ear was exposed and the round window membrane was incised under microscopic vision. The prototype was inserted into the cochlea until resistance was met. This typically corresponded to an insertion of 60% of the sample (3 mm) (Fig. 1); then the
bulla was closed using carboxylate cement (Durelon, ESPE Dental AG, Seefeld, Germany) and the wound was sutured.

On day final (df), at the end of the implantation period, a second aABR measurement was performed under general anaesthesia and animals were immediately euthanised by transcardiac perfusion with a pre-wash using PBS (100 mL/250 kg body weight) followed by modified Wittmaack fixative solution (10% pure acetic acid, 15% formol and 75% distilled water under the addition of 5 g potassium dichromate) (50 mL/250 kg body weight).

**Histological protocol**

Both temporal bones were removed after perfusion, opened and examined under microscope for the potential presence of infections. Most electrode prototypes were removed at this stage for further investigation of the coatings. Temporal bones were then placed overnight at 4°C in the modified Wittmaack fixative solution and processed for epoxy-embedding. Briefly, the cochleae were rinsed and dehydrated for one hour in each of the following solutions: PBS, 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol; dried over night at 65°C and then embedded in epoxy (SpeciFix-40, Struers, Willich, Germany) under vacuum. Titanium oxide (Merck KGaA) was mixed into the epoxy to obtain non-transparent samples in order to distinguish the information presented on the polishing plane from the information associated with deeper sections of the epoxy block. Embedded cochleae were ground with a speed grinder-polisher (PowerPro™ 4000, Buehler, Düsseldorf, Germany). Polishing planes were documented every 20 µm. Each plane was stained with toluidin-blue (Sigma-Aldrich) for 4 minutes and eosin-orange for 2 minutes, documented and analysed by digital microscope and image analysis software (VHX-600, Keyence, Neu Isenburg, Germany). Five mid-modiolar sections were chosen for evaluation at magnifications of 50x and 200x. From the base to the apex of the cochlea, the Rosenthal’s canals were detected and classified as follows: first basal (b1), second basal (b2), first middle (m1), second middle (m2), third middle (m3), fourth middle (m4), and apical (ap) (Fig. 2). SGC were counted and the diameters of
at least 10 randomly selected cells (if available) of each canal’s cross-sectional profile were measured to determine the SGC densities. As sometimes the apical canals (ap) could not be separated from m4, b1 and b2 were averaged to reflect the basal cochlea, m1 to m3 for the middle cochlea, and m4 and ap for the apical cochlear region.

Using this procedure, the cells of the fibrous tissue around the electrode carrier will also be stained; therefore, the cochleae (with and without the prototypes in situ) could be investigated for the presence of fibrous tissue, as well.

**Statistical analysis**

The results of 5 wells of a 96-multiwell plate that had been treated in the same manner were averaged and normalized to controls (100%) before averaging different plates. To analyse the in vitro results, Kruskal-Wallis test followed by Dunn’s Multiple Comparison test were used.

Analysis for differences between the groups in vivo was accomplished either by ANOVA followed by Bonferroni test or Kruskal-Wallis test followed by Dunn’s Multiple Comparison test, depending on the results of the Kolmogorov-Smirnov test for normality.
RESULTS

In vitro biocompatibility

When testing the survival rates of NIH/3T3 fibroblasts on the investigated biodegradable polymers in comparison to silicone and to the control (polystyrene culture plates), growth rates of fibroblasts on P(4HB) were comparable to the growth rates on silicone; whereas, cell growth on PLLA was similar to the growth on the control surface even though none of the differences were significant (Fig. 3a). When performing the experiments with freshly isolated SGC, no influence of the biodegradable polymers on cell survival was detected in comparison to silicone, even if on all materials the average cell survival was reduced by 30 to about 40% compared to the cell culture material. When repeating the experiments without ornithin- and laminin-coatings, more SGC survived on the polymer surfaces in comparison to silicone but with larger variations between the tests (Fig. 3b).

In vivo biocompatibility

During the experimental period one subject died after the aABR screening, five guinea pigs died 1 to 3 days after the surgery (two of them received silicone samples, two P(4HB) samples and one a PLLA sample, respectively), and eight animals between one and two months after surgery. Four of these eight animals belonged to the silicone group and four to the PLLA group. Another two animals were euthanized because of being affected by a neurological syndrome or incurable foot necrosis on days 90 (PLLA) and 36 (P(4HB)), respectively. Additionally, in the inner ear of three healthy subjects who had been sacrificed as usual on df, there was evidence of acute inflammation (one P(4HB) and one PLLA after one month and one P(4HB) after 6 months). These cochleae were not included in the histological evaluation; resulting in the following number of animals analysed: silicone: n=14; PLLA: n=36 and P(4HB): n=38. Of these, animals for histological evaluation were chosen randomly: silicone: n=14 (n=5+3+3+3 for 1, 2, 3, and 6
months, respectively); PLLA and P(4HB): n=24 each (n=6 for each survival period) and, additionally, normal hearing: n=6. While removing the samples, in eight subjects (five of them belonging to the 3 months P(4HB) group, two to the 3 months PLLA group and one to the 2 months PLLA group), a visible detachment of parts of the coating was detected.

**ABR thresholds**

One guinea pig belonging to the normal-hearing (NH) group was excluded from the study as the hearing thresholds on d0 were above 50 dB at all frequencies. Average thresholds at a frequency of 8 kHz for NH guinea pigs were 34 dB whereas at the time of implantation thresholds for silicone, PLLA and P(4HB) groups were 35 dB, 32 dB and 40 dB, respectively. Similar thresholds were detected before implantation for all groups at 32 kHz (NH: 42 dB, silicone: 32 dB, PLLA: 34 dB, P(4HB): 41 dB). At the end of the implantation period all treated groups presented an aABR threshold shift (Figure 4a,b). Overall, the hearing loss seems to be largest at 32 kHz. Additionally, the number of animals without a detectable threshold (stimulation up to 100 dB) increased from 7%, 21%, and 4% for silicone, PLLA, and P(4HB) groups at 8 kHz to 14%, 50%, and 42% at 32 kHz, respectively. The threshold shift was independent of the duration of implantation for silicone and PLLA groups, but was more pronounced in the 3 months and 6 months groups with P(4HB) coated fibres. However, no significant differences in threshold shift among the treated groups were detected at both frequencies.

**Histological analyses**

In all groups, a continuous decrease of SGC densities compared to the normal-hearing control group was observed over time, especially in middle and basal turns. This decrease became significant only after 6 months of implantation for PLLA in apical and middle turns and for P(4HB) in middle and basal turns. No differences were detected between coated silicone fibres and uncoated fibres at all time points for both polymers in apical, middle and basal regions of the cochlea (Fig. 5a-c).
The average number of SGC in normal-hearing animals was with 3.94±0.48 cells per 10,000 µm² (mean±SEM), slightly lower in basal turns compared to middle (5.12±0.74 cells per 10,000 µm²) and apical (4.82±0.86 cells per 10,000 µm²) turns. Differences in SGC densities between the different cochlear turns of treated animals were only detected after 3 and 6 months in the P(4HB) group and after 3 months in the PLLA group. In both cases, the SGC density was highest in apical turns compared to middle and basal turns (Tab. 2).

When evaluating the diameter of the SGC, no change over time was detected for silicone and P(4HB) groups in comparison to NH animals (Fig. 6a-c). This was also found for PLLA with the exception of the group being implanted for 2 months. SGC diameters in this group were reduced compared to animals implanted for 2 months with uncoated silicone samples at all cochlear turns. Additionally, its diameters were reduced compared to NH animals in both the apical and middle regions of the cochlea and to the cell diameters in the apical turns of the 1 month and 3 months groups being implanted with PLLA-coated silicone fibres (Fig. 6). Differences between turns were generally very low for all groups at all time points. The only differences seen were in the P(4HB) group after 3 months between the basal and middle or apical turns and in the PLLA group after 6 months between basal and middle turns (Tab. 3).

A systematic quantitative evaluation of the tissue growth around the samples in situ was not justified because in most cases the prototypes were removed bilaterally for the analysis of polymer degradation. Nevertheless, the presence of inflammatory tissue inside the scala tympani was investigated. In general, a thin layer of cells around the implanted sample could be detected (Fig. 7). A strong formation of fibrous tissue was neither observed in explanted cochleae nor in cochleae with the fibre in situ. Sometimes after removal of the samples, some minor tissue pieces were found on the surface of the explanted samples.
Degradation-induced changes of surface morphology of polymer-coated silicone fibres

Microscopic studies were performed to evaluate the in vivo degradation-induced changes in surface morphology of the PLLA- and P(4HB)-based coatings. Previous ESEM studies of the polymer-coated silicone fibre in comparison to the uncoated silicone fibre revealed that the PLLA-coated fibres had rough and pitted surfaces [34]. In contrast, the P(4HB) coatings yielded smooth and complete fibre coverings [34]. During the in vivo degradation process of up to 6 months, it was observed that the PLLA coatings were not fragmented whereas the P(4HB) coatings were degraded completely under these quasi-stationary in vivo conditions (Fig. 8). The PLLA coatings were found to burst along their longitudinal axis (Fig. 8). Furthermore, the presence and absence of the polymeric coatings during in vivo degradation was validated by EDX measurements (Tab. 4). It was confirmed that the P(4HB) coatings were fully degraded after 6 months, because the element Si was determined similar to the uncoated silicone fibres (Fig. 9). No Si signals were found on PLLA-coated silicone fibres after the implantation interval of 6 months (Fig. 9), indicating the presence of slowly degradable PLLA coatings.
DISCUSSION

The electrical stimulation of the hearing nerve with a CI depends, in part, on the number of surviving SGC and the composition of the surrounding of the electrode array in the scala tympani, which can be affected by the traumatic effects of implant surgery but potentially protected by intracochlear drug therapy. However, to date, there is no evidence of an effective and well-accepted technique to deliver drugs inside the inner ear after the surgery in a safe, non-invasive and long-term fashion way [39].

A viable strategy to achieve controlled inner ear drug delivery after cochlear implantation without necessarily generating drug concentration gradients along the electrode array could be represented by the modification of the CI carrier as local drug delivery system by biodegradable coatings. The possible coating of CI electrode arrays with PLLA or P(4HB) has been described recently [34]. However, the development of a CI provided with a polymeric biodegradable coating implies a full understanding of the properties of the candidate biomaterials both in vitro and in vivo. For this reason, and because the response to specific materials may vary from one application site to another [40], in our experiment we first performed a sequence of studies in vitro in order to verify the survival rates of SGC, as the primary auditory neurons of the inner ear, and fibroblasts on the aforementioned polymers.

In vitro results showed that on both biodegradable polymers and on uncoated silicone cell survival was reduced by 30 to about 40% when compared to the control (cell culture plate material). However, cell survival rates were not significantly influenced by the biodegradable polymers in comparison to silicone when established SGC culture methods were used. In order not to mask possible effects of the polymers on SGC by the protein coating with ornithine and laminin usually used for SGC cultures [36], additional experiments were performed without this coating in order to investigate the cell survival in direct contact to the polymers. There were no significant differences, although the data demonstrated that, on average, substantially more SGC survived on the polymers’ surfaces in comparison to silicone, albeit with huge variation between the tests. As the application of silicone on a wide
range of medical devices, including CI, is well documented [41], these data confirmed that PLLA and P(4HB) can also be considered as safe biomaterials for coating of CI with regards to the potential effects on SGC in vitro.

On the basis of these results, an in vivo model was used to analyse the degradation of the polymers inside the inner ear and their potential effects on the spiral ganglion neurons. The biocompatibility of the polymers has been demonstrated earlier for many medical devices as heart valves and intravascular stents [40, 42]; however, the findings cannot be generalised directly to a CI. With vascular stents the success of the device depends strictly on its mechanical functions in a dynamic environment [43]; whereas with a modified CI, the peculiar stationary microenvironment that characterises the cochlea must be taken into account [39, 44]. For this reason, we assessed the degradation behaviour of PLLA and P(4HB) and their effect on SGC inside the inner ear.

After harvesting the cochleae and while removing the samples from the inner ear, it was observed under microscope that the PLLA coatings were not fragmented but the P(4HB) coatings were degraded completely within a period of up to 6 months. The P(4HB) degradation was faster in comparison to Loebler et al. [32] and Martin and Williams [45], where P(4HB) test specimens took up to a year to fragment completely. It can be assumed that the degradation process was potentially accelerated under these quasi-stationary in vivo conditions due to the accumulation of acidic degradation products and their autocatalytic action. As shortcoming of the slowly degradable PLLA coatings was found that they burst along their longitudinal axis. The delaminated PLLA coatings could potentially cause irritations and subsequent foreign-body reactions. Furthermore, in some cases, a new PLLA-coated fibre had to be supplied during the surgery because the coating did not adhere properly to the silicone carrier. In general, P(4HB) coatings performed better during surgical procedures.

In addition to observations under microscope the presence/absence of the polymeric coatings on the silicone surfaces were also confirmed by EDX measurements. The investigation of degradation by determination of degradation-induced mass loss (determined by gravimetry), decrease of molecular weight (analysed by gel
permeation chromatography) and increase of crystallinity (measured by differential scanning calorimetry) was not feasible due to the low coating mass and the resulting measuring inaccuracy.

A systematic, quantitative analysis of the growth of fibrous tissue around the samples in situ was not done in this study, as in most cases the samples were removed for evaluation of the polymer degradation. With the removal of the sample from the scala tympani, it is not possible to control the amount of tissue that might be removed together with the sample or that might remain inside the scala tympani, especially if there is only little fibrous tissue. Nevertheless, we can speculate that if there had been strong tissue growth around the sample, it would have been seen either inside the cochlea during histological evaluation or on the samples after explantation. In our case, only a thin layer of cells was observed when the samples remained in situ; therefore, we can infer that neither PLLA or P(4HB) nor their degradation products induce an enhanced tissue reaction after implantation in the cochlea compared to silicone.

It is well known that perforation and/or surgical manipulation of the ear carries a significant risk of deafness [46]. A significant decrease in SGC density has also been described in vivo following insertion-induced intracochlear damage [47, 48]. In the worst cases, significant loss of residual hearing following cochlear implantation has been reported, which may be immediate or delayed [49]. The aetiology of postsurgical hearing loss is likely multifactorial and in cochlear implantation it may be due to direct trauma to the basilar membrane or lateral cochlear wall, or loss of perilymph [12].

To assess the SGC functionality, aABR responses were measured on d0 before implantation and on df, the final day of live testing. As a response to the surgical trauma a general increase in the hearing thresholds was to be expected [12]. This increase was detected across all frequencies in animals receiving coated or uncoated silicone fibres. As there were no differences between the groups, we can conclude that both polymers and their degradation do not affect hearing thresholds and, therefore, do not cause severe functional damage to the SGC.
Since the numbers of SGC can be reduced significantly without any effects on the hearing threshold [50], a further parameter to assess the effects of polymer degradation inside the inner ear was the density of surviving SGC. In an earlier study, Maini and colleagues demonstrated that all their test animals displayed a reduction in SGC density mainly in the basal turn by 3 months after the surgery in the implanted cochlea when compared to the control (non-treated contralateral ears) [12]. Our data showed a decrease in SGC density in the PLLA and P(4HB) groups by 6 months after the implantation in comparison to NH animals, but not in all cochlear regions. A possible explanation might be the different study methods. They calculated the ratio of SGC numbers of implanted and unimplanted ears before averaging the results within the same group and compared different groups. In the current study, because both ears were implanted, only group mean values could be compared. But importantly, no significant differences in SGC densities between subjects that received the uncoated samples (silicone-group) and the coated samples (PLLA and P(4HB) groups) were detected, indicating that these outcomes might be associated with the surgical trauma but not with the polymers tested.

When comparing cell densities, it is known that SGC densities are similar in all turns of the cochlea [12] and only three months after implantation these authors detected a reduction in SGC density in the basal turn. We corroborated these observations. Differences were only observed after 3 and 6 months and were larger in basal and middle turns compared to apical turns. On the other hand, in a study with poly-vinyl-alcohol as CI coating, all cochlear turns were affected by loss of SGC [48]. It could be speculated that by-products were dispersed inside the perilymph and, consequently, they exerted a negative effect along the entire cochlea. This might also explain the reduced SGC density compared to NH animals in the apical region after 6 months in subjects of the PLLA group but does not explain that in this group no changes were found in the basal region of the cochlea.

Soma diameters of SGC may influence the excitability of the neurons [51] and might provide further information about the health status of the cells. In the literature, the lower limit for viable SGC is considered to be 12 µm [52, 53]. The average SGC diameter in the current investigation was smaller with values between 10 and 11 µm.
This can be attributed to the applied histological method of embedding the cochleae in epoxy followed by a microgrinding technique that was developed to evaluate cochleae with a CI electrode array *in situ* [54]. The smaller diameters may be explained either by some shrinking of the tissue and differences in the optical documentation of the histological images, or by staining only the cell nuclei, and should affect all groups in the same manner. However, the only differences in diameters found in the current investigation were between the 2-months PLLA group and several other groups, predominantly in the apical and middle regions. It remains unknown why just this single experimental group exhibited a lower diameter compared to the other groups. As also differences between cochlear regions were only found anecdotally (Tab. 3), we conclude that both polymers, in general, do not negatively influence the SGC.
CONCLUSION

PLLA and P(4HB) have not previously been used clinically in otolaryngology, and prior to any clinical use their biosafety needs to be established and confirmed for the specific application within the inner ear. Both polymers did not exhibit any negative effect on a standard fibroblast cell line and freshly isolated SGC in vitro compared to a silicone used for commercial CI electrode arrays. Also in vivo, no negative effect of the polymers compared to the silicone could be detected on SGC density and diameter, as well as on hearing thresholds and tissue response to the implant indicating that both polymers may be used in the stationary environment of the cochlea. Furthermore, the rate of any complications for the animals throughout the implantation time was not greater in comparison to uncoated control implants. Since the mechanical stability of P(4HB) was better during implantation and in contrast to PLLA its degradation was finished after 6 months, P(4HB) may provide the basis for the development of a biodegradable coating of CI electrode arrays for local drug delivery to the inner ear.

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FIGURE LEGENDS

Fig. 1: Intraoperative view on the cochlea (*) with the round window (thin arrow) and an implanted silicone fibre (thick arrow).

Fig. 2: Midmodiolar plane of a ground guinea pig cochlea 6 months after implantation of a P(4HB) coated silicone fibre (arrows). Basal (b1, b2), middle (m1 to m3) and apical (ap) turns are marked.
Fig. 3:A: Cell viability of NIH/3T3 fibroblasts on the polymers (mean ± SEM). At least 4 plates with 5 wells each were tested. No significant differences were detected. B: Survival of SGC on the polymers (mean ± SEM). Samples were tested with (+) or without (-) laminin and ornithine coating which is generally taken to optimize conditions for cultured SGC. Again at least 4 plates with 5 wells each were tested.

Fig. 4: Shifts in ABR thresholds between the day of implantation (d0) and the final day (df) at the test frequencies of 8 kHz (A) and 32 kHz (B) (mean ± SEM). Differences were calculated for each individual animal and thereafter group mean values were calculated. No statistically significant differences were detected.
Fig. 5: Spiral ganglion cell density in apical (A), middle (B) and basal (C) regions of the cochlea after implantation with uncoated silicone fibres (silicone), PLLA coated fibres (PLLA) or P(4HB) coated fibres (P(4HB)) in comparison to normal hearing animals (NH) given as means ± SEM. Key: 1m: 1 month of implantation; 2m: 2 months of implantation etc. *: p < 0.05; **: p < 0.01.
Fig. 6: Diameter of SGC in apical (A), middle (B) and basal (C) regions of the cochlea after implantation with uncoated silicone fibres (silicone), PLLA coated fibres (PLLA) or P(4HB) coated fibres (P(4HB)) in comparison to normal hearing animals (NH) given as means ± SEM. Key: 1m: 1 month of implantation; 2m: 2 months of implantation etc. *: p < 0.05; **: p < 0.01; ***: p < 0.001.
**Fig. 7:** Example of a PLLA coated silicone fibre in situ after 6 months of implantation. The coating is still visible, but also some detachment from the silicone (arrow). The dark colour around the fibre comes from the toluidin-blue staining of the fibrous tissue. In the enlargement even single cells can be detected.
Fig. 8: Exemplary electron micrographs of silicone fibres coated with P(4HB) or PLLA after different implantation intervals. An uncoated silicone reference is given for comparison.
**Fig. 9:** Si EDX data (percent by weight) of the coated silicone fibres after different implantation intervals in comparison to the uncoated silicone fibre (data shown as means ± SD); n = 10 samples for each time interval and polymer.
### TABLES

#### Tab. 1: Reaction conditions for application of polymeric undercoatings

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Polymer concentration in g/L</th>
<th>Solvent</th>
<th>Reaction temperature in °C</th>
<th>Reaction time in min</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(4HB)</td>
<td>18.2</td>
<td>EtCl₂</td>
<td>55</td>
<td>480</td>
</tr>
<tr>
<td>PLLA</td>
<td>1.5</td>
<td>CHCl₃</td>
<td>60</td>
<td>960</td>
</tr>
</tbody>
</table>

#### Tab. 2: Comparison of SGC densities between cochlear turns. Only significant differences are listed. Key: a: apical turns; m: middle turns; b: basal turns; *: p < 0.05; **: p < 0.01; ***: p < 0.001

<table>
<thead>
<tr>
<th>Sample</th>
<th>Implantation time</th>
<th>Differences between</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicone</td>
<td>t=1 month</td>
<td>m – a</td>
<td>*</td>
</tr>
<tr>
<td>P(4HB)</td>
<td>t=3 months</td>
<td>b – a</td>
<td>**</td>
</tr>
<tr>
<td>P(4HB)</td>
<td>t=3 months</td>
<td>m – a</td>
<td>*</td>
</tr>
<tr>
<td>P(4HB)</td>
<td>t=6 months</td>
<td>b – a</td>
<td>***</td>
</tr>
<tr>
<td>P(4HB)</td>
<td>t=6 months</td>
<td>m – a</td>
<td>**</td>
</tr>
<tr>
<td>PLLA</td>
<td>t=3 months</td>
<td>b – m</td>
<td>***</td>
</tr>
<tr>
<td>PLLA</td>
<td>t=3 months</td>
<td>b – a</td>
<td>***</td>
</tr>
<tr>
<td>PLLA</td>
<td>t=3 months</td>
<td>m – a</td>
<td>**</td>
</tr>
</tbody>
</table>
Tab. 3: Comparison of diameters of SGC between cochlear turns. Only significant differences are listed. Key: a: apical turns; m: middle turns; b: basal turns; *: p < 0.05; **: p < 0.01

<table>
<thead>
<tr>
<th>Sample</th>
<th>Implantation time</th>
<th>Differences between</th>
<th>Level of significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>P(4HB)</td>
<td>t=3 months</td>
<td>b – m</td>
<td>*</td>
</tr>
<tr>
<td>P(4HB)</td>
<td>t=3 months</td>
<td>b – a</td>
<td>**</td>
</tr>
<tr>
<td>PLLA</td>
<td>t=6 months</td>
<td>b – m</td>
<td>*</td>
</tr>
</tbody>
</table>

Tab. 4: EDX data on surface composition (percent by weight for the relevant elements Si, C and O) of the coated silicone fibres after different implantation intervals in comparison to the uncoated silicone fibre (data shown as means ± SD); n = 10 samples for each time interval and polymer.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Si [%]</th>
<th>C [%]</th>
<th>O [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicone fibre + P(4HB) t=0 months</td>
<td>0.30 ± 0.08</td>
<td>56.33 ± 0.71</td>
<td>42.31 ± 0.59</td>
</tr>
<tr>
<td>Silicone fibre + P(4HB) t=1 month</td>
<td>1.52 ± 0.96</td>
<td>58.68 ± 1.20</td>
<td>37.26 ± 2.75</td>
</tr>
<tr>
<td>Silicone fibre + P(4HB) t=2 months</td>
<td>1.78 ± 0.56</td>
<td>54.76 ± 3.75</td>
<td>29.99 ± 2.97</td>
</tr>
<tr>
<td>Silicone fibre + P(4HB) t=3 months</td>
<td>2.17 ± 1.06</td>
<td>59.96 ± 1.70</td>
<td>30.30 ± 2.11</td>
</tr>
<tr>
<td>Silicone fibre + P(4HB) t=6 months</td>
<td>25.92 ± 2.22</td>
<td>48.12 ± 2.00</td>
<td>24.09 ± 0.82</td>
</tr>
<tr>
<td>Silicone fibre</td>
<td>32.17 ± 5.47</td>
<td>40.33 ± 6.85</td>
<td>26.33 ± 1.69</td>
</tr>
<tr>
<td>Silicone fibre + PLLA t=0 months</td>
<td>1.40 ± 1.02</td>
<td>53.02 ± 2.09</td>
<td>44.52 ± 2.41</td>
</tr>
<tr>
<td>Silicone fibre + PLLA t=1 month</td>
<td>2.25 ± 1.09</td>
<td>56.34 ± 1.69</td>
<td>34.07 ± 4.19</td>
</tr>
<tr>
<td>Silicone fibre + PLLA t=2 months</td>
<td>1.17 ± 0.95</td>
<td>55.64 ± 3.23</td>
<td>40.74 ± 4.90</td>
</tr>
<tr>
<td>Silicone fibre + PLLA t=3 months</td>
<td>1.18 ± 1.08</td>
<td>53.03 ± 1.84</td>
<td>39.05 ± 6.83</td>
</tr>
<tr>
<td>Silicone fibre + PLLA t=6 months</td>
<td>0.61 ± 0.36</td>
<td>51.48 ± 2.21</td>
<td>43.22 ± 5.33</td>
</tr>
</tbody>
</table>
5. ADDITIONAL RESULTS

To investigate the effect of dexamethasone on fibroblasts, NIH/3T3 cells were seeded in 96 multiwell plates at a density of 10,000 cells/well in DMEM (Invitrogen), supplemented as previously described (see second manuscript). Thus, dexamethasone (Fluka) was dissolved in ethanol to reach a final concentration of 0.1 mol/L. This solution was diluted in cell culture medium and added to the cell culture at concentrations between $10^{-4}$ mol/L (the upper limit to avoid toxic effects due to concentrations of ethanol higher than 0.02 mol/L) and $10^{-12}$ mol/L. For the negative control, cells were seeded in five wells containing culture medium without dexamethasone. As the positive control, a solution of tetraethylthiuram (TETD, Sigma-Aldrich, Taufkirchen, Germany) dissolved in ethanol was used, at a concentration of $10^{-2}$ mol/L and subsequently diluted in cell culture medium to achieve a final concentration of $10^{-5}$ mol/L.

After 48 hours of incubation at 37°C, 5% CO$_2$ and 95% humidity, cells were stained by using neutral red as previously described; subsequently, absorption was measured at 570 nm and compared with the control wells. In each experiment, five wells per plate were treated identically and the screening was repeated at least four times. Survival rates were calculated as a percentage of untreated controls.

The results of five wells of a 96 multiwell plate treated in the same manner were averaged and normalized to controls (100%) before taking the mean of different plates. To analyze the in vitro results, the Kruskal-Wallis test was used, followed by Dunn’s Multiple Comparison test.

The effects of dexamethasone on SGCs in vitro were also investigated (a detailed description of the dissection of the spiral ganglion being provided in the second manuscript). Dexamethasone was added to the cell culture at concentrations between $10^{-4}$ to $10^{-12}$ mol/L. After 48 hours, cells were stained with anti-neurofilament antibody as outlined above, and counted. All experiments were repeated at least four times, with a minimum of four samples per experiment for each group and control wells.
For the final screening, dexamethasone-containing P(4HB) coatings were tested in vivo. A solution of dexamethasone in methanol (15 g/L; LiChrosolv, Merck KGaA, Darmstadt, Germany) was added to give a polymer-to-dexamethasone ratio of 70/30% w/w (DMS30 group) or 85/15% w/w (DMS15 group).

The studies were conducted in accordance with the German “Law on Protecting Animals” and with the European Communities Council Directive 86/609/EEC governing the protection of animals used for experimental purposes.

Before dexamethasone (DMS)-loaded samples could be investigated in vivo, the supplier stopped breeding pigmented guinea pigs (BFA) guinea pigs. Therefore, Dunkin Hartley (DH) guinea pigs had to be used for all further experiments.

Animals were divided in three groups as per the experimental design (P(4HB), n=7; DMS15, n=6; DMS30, n=6). A control group (n=7) was included in this study, composed of subjects who received a silicone prototype. Normal-hearing (NH) DH guinea pigs included in the first screening (n=6) were considered as an additional control group. An experimental period of 28 days was chosen.

All in vivo measurements and surgical procedures were performed on a heat pad maintained at 37°C and under general anaesthesia. Before animals underwent surgery, the normal-hearing status (≤50 dB SPL) of all subjects was confirmed bilaterally by the measurement of acoustically evoked auditory brainstem responses (aABRs). All subjects were implanted bilaterally under sterile conditions on day 0 (d0) with silicone fibres as a model for CI electrode arrays consisting of a silicone carrier (400 µm diameter; 5 mm length) uncoated (silicone group), coated with the biodegradable polymers (P(4HB) group) or coated with a dexamethasone-containing P(4HB) coating (DMS30 group and DMS15 group). As is standard practice, additional aABR measurements were performed at the end of the trial period.

After perfusion both temporal bones were removed, opened, examined under microscopic vision for the potential presence of infections, embedded in epoxy and analysed.

Analysis of differences between the groups in vivo was performed either by ANOVA followed by the Bonferroni test, or using the Kruskal-Wallis test followed by Dunn’s
Multiple Comparison test, depending on the results of the Kolmogorov-Smirnov test for normality.

**In vitro screening**

In this study, SGC survival and fibroblast viability were investigated for different concentrations of dexamethasone. At the concentrations tested, dexamethasone exerted no toxic effects on SGC and NIH/3T3 cells (Figure 2). On the contrary, a significantly higher incidence of vital SGCs was observed at a dexamethasone concentration of $10^{-9}$ mol/L ($p<0.05$) [data published in Bohl et al., 2012].

![Figure 2. Effect of dexamethasone on the survival of SGCs and viability of NIH/3T3 fibroblasts after 48 hours incubation compared with the medium control (* $p<0.05$).](image)

**In vivo screening**

During the experimental period, one guinea pig from the DMS30 group died two days after surgery. None of the subjects sacrificed (as is standard practice) on the final day showed evidence of acute inflammatory reactions in the middle and/or inner ear. While removing the samples, no detachment of the coatings was observed.
Auditory brainstem response (ABR)

Normal-hearing status was confirmed for all guinea pigs on day 0. For the additional control group (normal-hearing, non-treated guinea pigs, NH) in this study, the same subjects included in the first study (chapter 4.1) were chosen.

At the end of the study period, a shift in hearing threshold was detected at high frequencies across all groups. However, this was significant at a frequency of 8 kHz only for the group P(4HB) (*p<0.05) (Figure 3). No other significant differences among the different groups were detected at this frequency.

![Figure 3](image)

**Figure 3.** Acoustically evoked auditory brainstem responses (ABR) at a frequency of 8 kHz before implantation (d0) and at the end of the period of study (df). Comparison between non-treated, normal-hearing guinea pigs (NH), animals which received silicone prototypes (S) and animals which received prototypes loaded with two different concentrations of dexamethasone (polymer-to-dexamethasone ratio of 85/15% w/w – DMS15 – and 70/30% w/w – DMS30) (* p<0.05).

Interestingly, at a frequency of 32 kHz, when comparing the single subgroups, a significant increase in the hearing thresholds was seen for the silicone, P(4HB), and DMS-15 groups (p<0.05) (Figure 10), but not for the DMS-30 animals. At this
frequency, a significant threshold shift was also detected for the P(4HB) (p<0.01) and DMS-15 (p<0.05) groups in comparison with the normal-hearing guinea pigs (NH), but – as before – not in the DMS-30 group (Figure 4).

![Figure 4](image)

**Figure 4.** Acoustically evoked auditory brainstem responses (ABR) at a frequency of 32 kHz before implantation (d0) and at the end of the period of study (df). Comparison among non-treated, normal-hearing guinea pigs (NH), animals which received silicone prototypes (S) and animals which received prototypes loaded with two different concentration of dexamethasone (polymer-to-dexamethasone ratio of 85/15%w/w – DMS15 – and 70/30%w/w – DMS30) (* p<0.05).

**Histological analysis**

Significant differences were detected only in the silicone group, as a reduction in SGC density was found in the basal turns when compared with the middle turns (p<0.05) (Figure 5). No significant differences were found along the entire cochlea in the treated groups as compared with the non-treated, normal-hearing animals (basal: 3.6 ± 1.2 cells/10,000 µm²; middle 5.1 ± 0.5 cells/10,000 µm²; apical 3.5 ± 0.9 cells/10,000 µm²) (mean ± SD) (data not shown).
Figure 5. Spiral ganglion cell (SGC) density in the basal, middle and apical turns (averaged) 28 days after implantation. Comparison among animals who received prototypes not loaded with dexamethasone (silicone and P(4HB)) and animals who received prototypes loaded with two different concentration of dexamethasone (polymer-to-dexamethasone ratio of 85/15%w/w – DMS15 – and 70/30%w/w – DMS30) (* p<0.05).

Additionally, perikaryal diameters were measured. No significant differences were observed between treated and non-treated normal-hearing animals (basal: 11.71 ± 1.37 µm; middle 11.60 ± 0.69 µm; apical 11.32 ± 1.01 µm) (mean ± SD) (data not shown). When comparing the treated groups, significant differences in cell size were found in the silicone group – with cell diameters reduced in the middle turns as compared with the basal turns (p<0.05) – and in the DMS-30 group, within which cells in the middle turns were of smaller diameter than those of the apical turns (p<0.05) (Figure 6).
Figure 6. Spiral ganglion cell (SGC) diameter in the cochlear turns 28 days after implantation. Comparison among animals who received prototypes not loaded with dexamethasone (silicone and P(4HB)) and animals who received prototypes loaded with two different concentration of dexamethasone (polymer-to-dexamethasone ratio of 85/15%w/w – DMS15 – and 70/30%w/w – DMS30-) (* p<0.05).

A thin layer of cells around the prototype (Figure 7) was observed in a few cochleae, but no differences were seen between cochleae implanted with prototypes loaded with dexamethasone and unloaded samples.

The gradual degradation process affecting the coatings was confirmed again by the project partners in Rostock (Anne Bohl, personal communication).

Figure 7. Midmodiolar plane of a cochlea embedded in epoxy (DMS-15). The prototype has been introduced through the round window (blue arrow) inside the scala tympani. In this specimen it is possible to detect a layer of cells around the prototype (white arrow).
6. GENERAL DISCUSSION

One of the techniques of choice for histological analyses of the cochlea is paraffin embedding followed by slicing of the specimens (Staecker et al., 1996; Maruyama et al., 2007). When using this method, however, there are certain limitations that have to be considered. In particular, where cochleae were implanted with a CI, the electrode must be removed before the specimens undergo sectioning, so that there is a lack of information regarding the position of the electrode and the presence of a tissue reaction around it.

A comparative study was published by Eshragi et al. (2003) with a view to determining the position of the electrode and the insertion trauma. In this study a cryosectioning technique was employed as an alternative method. However, use of this technique yielded sections at intervals of only 200 µm. Another method which allowed thinner sections and more detailed information to be obtained was investigated by Richter et al. (2005), who embedded the specimens in methyl methacrylate and ground them. The limitations of this technique were the artefacts caused by swelling of the silicone body of the electrode array.

Another approach, consisting of epoxy embedding followed by micro-grinding of the specimens, has been employed by Stöver et al. (2005) in human temporal bones for insertion trauma studies. It was proven to be an effective tool, not only for evaluating damage to the cochlear microstructures resulting from CI insertion and for obtaining detailed information about the position of the electrode, but also in terms of avoiding certain artefacts such as the swelling of the implant’s silicone matrix. As this method allowed a detailed analysis of the inner ear (with specimens obtainable at intervals much lower than 200 µm) – including analysis in combination with the electrode, whose metal part can be ground – this thesis investigated its suitability for evaluating the effects on the inner ear tissue of local treatments combined with a modified CI. Density and diameter of SCGs were the parameters selected to evaluate the impact of this procedure on the specimens as compared with paraffin embedding.
Normal-hearing guinea pigs were chosen as a model. After perfusion, one cochlea from each subject was embedded in paraffin, with the contralateral cochlea processed for epoxy embedding. After analysing the specimens, the results proved that embedding in epoxy leads to reduction of SGC density – in all cochlear regions and in both strains – compared with the outcomes obtained from specimens embedded in paraffin. Differences were also observed when monitoring cell diameter. In fact, while the mean value for SGC perikaryal diameter for specimens embedded in paraffin was 15.96 µm – thus confirming the data presented by other authors, who demonstrated that the mean values can range from 14 and 17 µm (Shepherd et al., 2005; Glueckert et al., 2008) – the mean value for cell somata in epoxy was 11.17 µm.

Several factors have to be taken into consideration to explain these differences. First and foremost, as it is assumed that both ears of one normal-hearing subject should not present differences in SGC density and morphology, it can be speculated that the drying process during epoxy embedding causes some shrinkage of the SGCs. Additionally, the documentation approach may also in part account for the differences documented in perikaryal diameters and cell numbers. In point of fact, while information from paraffin sections was collected through the full thickness of the slices, epoxy images were obtained from the surface of the specimens. This implies that, if epoxy embedding was chosen, a smaller perikaryal diameter was likely.

At the end of this investigation, it was possible to estimate a lower diameter limit for viable SGCs in cochleae processed for epoxy embedding (8.4 µm). This was calculated considering the lower limit adopted in the literature for viable SGCs embedded in paraffin, which is 12 µm (Maruyama et al., 2008; Yagamata et al., 2004), even if this value is still the subject of debate.

Two different breeds of guinea pig were included in this study, pigmented (BFA) and Dunkin Hartley (DH). When investigating SGC density, no differences were found between the strains. Importantly, the perikaryal diameters were larger in DH guinea pigs for both embedding methods.
The difference in cell soma diameter observed between strains may provide evidence for existing diversity between guinea pig breeds; however, further studies are needed to confirm this finding.

In conclusion, although tissue preservation after epoxy embedding is not as effective as with paraffin embedding, and results with both techniques should not be compared directly, the inner ear structures (with particular regard to SGC density and diameter) can be investigated subsequent to epoxy embedding and micro-grinding of the specimens, even if to some degree the shrinkage of the SGCs has to be taken into account. Nevertheless, these results justify the use of this method to perform histological screening of the cochlea, even in cases where a CI has been implanted and needs to be analysed in situ, as this method enabled the electrode to be ground as well. This is especially true when all experimental groups are evaluated using the same procedures. More interestingly, this technique can also be employed to investigate the potential effects on SGCs of sustained intra-cochlear delivery of drugs by means of a modified CI array, which is why it has been chosen in this project for further studies.

Several therapeutic strategies for local treatment of inner ear conditions are under investigation. The inner ear may be locally targeted by means of two routes: the intra-tympanic and the intra-cochlear routes.

In the first case, the permeability of the round window is advantageous in terms of drug diffusion (Banerjee and Parnes, 2004) using biodegradable polymers, hydrogels, nanoparticles, microcatheters, microwicks, or micropumps (Leary Swan et al., 2008; McCall et al., 2010).

In the second case, drugs can be applied by means of a cochleostomy (Leary Swan et al., 2008; McCall et al., 2010). Another option is modification of the CI to obtain a drug reservoir capable of releasing drugs gradually for a long period after surgery (Jolly et al., 2010). The application of drug-releasing biodegradable matrices on prostheses is under investigation in many medical fields, including cardiology (Regar et al., 2001), neurology (Fournier et al., 2003), dermatology (Peschel et al., 2008).
and ophthalmology (Löbler et al., 2011). Coatings such as poly-pyrrole and poly-vinyl-alcohol have also been the target of experimental research in the inner ear (Tykocinski and Cowan, 2005; Richardson et al., 2009). For these reasons, when planning this study, it was assumed that biodegradable matrices were suitable candidates for developing a CI coating for sustained drug delivery inside the inner ear.

Two materials were chosen for the present study: PLLA, whose role in biomedical applications has been confirmed in several studies (Frazza and Schmitt, 1971; Regar et al., 2001; Fournier et al., 2003; Bünger et al., 2007), and P(4HB), as its application in bioengineering is considered promising (Martin and Williams, 2003; Moore et al., 2005; Löbler et al., 2010; Peschel et al., 2008).

The study was divided into two parts. Firstly, the polymeric matrices were tested in vitro; secondly, they underwent in vivo testing, and subsequently testing with dexamethasone loading as well.

Although, in vitro, both SGC survival and fibroblast growth were reduced on all materials, the polymers exerted no negative effects as compared with silicone, the material of choice for manufacturing CI electrode arrays. The cell survival rate was lower on silicone than on the control material, as confirmed by Hansen et al. (2009), who demonstrated that the growth of SGCs in vitro may be influenced by materials and their arrangement.

In order to promote the culture of SGCs (Brors et al., 2002), the bottom of the culture plates was treated with laminin and ornithine. However, cells were also cultivated on untreated surfaces to verify whether the presence of any substrate could mask any potential effect of the polymers on the cell’s growth.

Even if a high variation between samples was found without the laminin/ornithine coating, the results showed that SGCs survive both on treated and untreated surfaces, confirming that the tested polymers did not affect cell growth in comparison with silicone.
The main focus of the study was the investigation of polymer degradation in the quasi-stationary environment of the cochlea and its influence on SGCs. On the basis of the *in vitro* results, therefore, prototypes were designed in collaboration with the University of Rostock’s Institute of Biomedical Engineering and with the Laser Zentrum Hannover, and implanted inside the scala tympani of normal-hearing guinea pigs for different periods of time (i.e. one, two, three and six months). The effects on SGCs were tested by means of the evoked aABR stimuli and in terms of SGC density and diameter.

In animals implanted for only one month, all subjects already exhibited a threshold shift. As there were no significant differences between the treated groups, these outcomes can be attributed to the surgical traumas rather than to a toxic effect on the SGCs exerted by the degradation of the polymers.

Histological analysis of the specimens revealed a decrease in SGC density within the P(4HB) group only six months after implantation – only as compared with the normal-hearing control group – in the basal and middle turns of the cochlea. Additionally, a reduction in SGC numbers was detected in the middle and apical cochlear turns of the PLLA group subjects in comparison with normal-hearing animals six months post-implantation. As no significant differences were detected between subjects which received the uncoated samples (silicone) and animals that were implanted with coated prototypes (both P(4HB) and PLLA), this decline in SGC numbers is unlikely to be solely caused by polymer degradation. Moreover, the differences between cochlear regions in terms of SGC density cannot be explained by polymer degradation as – in the case of PLLA, at any rate – after six months, SGC density was reduced in apical regions but not in the basal part of the cochleae. Neither could this effect be explained by diffusion of degradation products, as the model electrodes are implanted only in the basal region. It is, therefore, assumed that both the threshold shifts and the reduction in SGC density are associated with a general decrease over time in SGC numbers following the insertion trauma.
A quantitative evaluation of tissue growth around the specimens *in situ* was not the aim of this study, as in most cases the prototypes had to be removed bilaterally for further research into the degradation behaviour of the coatings. However, the presence of inflammatory tissue inside the *scala tympani* was investigated in those specimens where the prototypes were not removed, and it was possible to observe a thin layer of cells around the sample in a few cochleae only, confirming that the coatings being researched are well tolerated inside the inner ear and did not cause any additional tissue reaction.

P(4HB) and PLLA did not exert significant negative effects on SGCs either *in vitro* and *in vivo* in comparison with silicone, which is regarded as a well-tolerated implantable material for the human body (Luria, 2003). Additionally, this study also showed that P(4HB) would be preferable to PLLA for use as a CI coating, since prototype analysis revealed that P(4HB) is characterized by more gradual degradation than PLLA is. Furthermore, its mechanical stability during handling and insertion was also superior to that of PLLA. Thus, P(4HB) should be preferred to PLLA as a CI coating.

On the basis of these outcomes, further testing was justified in order to evaluate the potential effects of a P(4HB)-coating loaded with anti-inflammatory drugs inside the inner ear.

Although the intra-cochlear pharmacokinetics of corticosteroids are not fully understood (Lamm and Arnold, 1999), their use in the treatment of inner ear conditions is well accepted (Dallan et al., 2011; Khaimook and Jantarappana, 2011; Shea et al., 2012). More interestingly, the benefits of a one-shot injection into the cochlea at the time of the surgery have been shown in CI recipients (Paasche et al., 2009). In this study, the authors found that a single application of triamcinolone crystal suspension reduced the impedance of the CI for several years after implantation. Even though a long-term treatment effect has been demonstrated for this approach, it has not been possible to genuinely control the amount of anti-inflammatory drugs deposited inside
the cochlea. An effective and safe route for a long-term application of drugs inside the inner ear has been tested in animal models, and consists of hydrogel formulations loaded with dexamethasone (Paulson et al., 2008; Lemke et al., 2009; Maini et al., 2009; Wang et al., 2009; Salt et al., 2011). An even more promising approach for CI patients, however, would be the modification of the implant by silicone loaded with dexamethasone (Farahmand Ghavi et al., 2010). In this thesis, an alternative to this strategy consisting of an electrode model with P(4HB)-coating loaded with dexamethasone was investigated.

First, dexamethasone was firstly tested in vitro on SGCs and fibroblasts. In accordance with other authors, no toxic effects were observed on SGCs (Wefstaedt et al., 2005). Dexamethasone also did not exert any inhibitory or toxic effects on NIH/3T3 fibroblasts or freshly isolated SGCs. The latter was reported by Furze et al. (2009), with these authors also reporting the inhibition of the outgrowth of fibroblasts from spiral ganglion explants. Consequently, a P(4HB) coating was loaded with dexamethasone, and prototypes were implanted in the cochlea of normal-hearing guinea pigs.

Although hearing thresholds shifted at high frequencies across all groups, this was significant only for the unloaded P(4HB)-coated electrode models. As the pattern at 8 kHz was similar to the results of the degradation study reported here, this can again be attributed to the surgical trauma. Most of the threshold shifts – including those in the unloaded groups of the degradation study – were not significant; therefore, in this case, it remains unclear whether dexamethasone exerts a protective effect. At 32 kHz, a frequency that is located where the electrode model is positioned, hearing thresholds were significantly increased at the end of the experimental period in all groups except the DMS-30 group. Moreover, animals which received a silicone or a P(4HB) prototype exhibited a significant threshold shift in comparison with the control group (non-treated, normal-hearing guinea pigs) as well. Here, it may be the case that the higher DMS concentration protected the animals from a significant
threshold shift. This would concur with the findings of Eshraghi et al. (2007) who reported protection from surgical trauma by dexamethasone.

Additionally, no significant differences in SGC densities and diameters were found among implanted groups, confirming that dexamethasone does not exert toxic effects on SGCs inside the inner ear. In other studies, dexamethasone was proven not merely to be well tolerated inside the inner ear but also to prevent hearing damage due to ischemia (Morawski et al., 2009), noise-induced trauma (Takemura et al., 2004), surgical trauma (Eshraghi et al., 2007; Maini et al., 2009) and aminoglycoside toxicity (Himeno et al., 2002), typically as indicated by ABR measurements. Unfortunately, in some of these studies information on SGCs is not given (Eshragi et al., 2007; Morawski et al., 2009) and some authors investigated the effects on other inner ear cells, specifically hair cells (Himeno et al., 2002; Takemura et al., 2004). This makes a direct comparison, including a comparison of the effects of the treatment on SGCs, impossible.

Significant differences in SGC densities were found in the current study only when comparing the basal and the middle turns of the silicone group, and this finding can be explained by the surgical traumas which probably had a negative impact on SGC survival in the basal turns. There was no difference in the group with coated but unloaded specimens, and any differences that existed between turns in the degradation study involved higher cell density in the apical than in basal or middle turns. It is, therefore, highly unlikely – although it cannot be ruled out – that the DMS had a certain positive effect on SGC survival by means of protection against surgical trauma.

In the literature, the debate about the effects of dexamethasone inside the inner ear remains unresolved. Huang et al. (2007) claim that dexamethasone has no significant effects on electrode impedance. Kiefer et al. (2008), however, who designed a dexamethasone-eluting CI array, reported lower threshold shifts in animals who received the loaded prototypes. These findings can in part be explained by the different application routes (injection through the tympanic membrane vs.
delivery by means of a modified CI array), by the presence of a basal-apical concentration gradient in the perilymph which may influence the distribution of drugs along the cochlear turns (Salt, 2008), or by the change in perilymph composition or the composition of the drug, which can reduce the benefits of the treatment (Huang et al., 2007). Conflicting data are also explicable in terms of the small number of patients in clinical trials that focus on the treatment of SNHL, Ménière's disease, tinnitus, noise-induced hearing loss with glucocorticosteroids (Lamm and Arnold, 1999) or by the level of drug applied (Labinaz et al., 1997).

When the investigations into hearing thresholds and SGCs in this study are considered together, it is evident that dexamethasone released by a P(4HB) coating, at the concentration used, did not exert toxic effects on SGCs. Moreover, there might be some beneficial effects of the released DMS on hearing thresholds (although these require verification in further experiments).

One of the aims of this thesis was to investigate the potential effects of dexamethasone on SGCs. Furthermore, most of the samples had been removed from the specimens in order that further tests on the coatings could be performed by the project partners. For these reasons, additional analyses of the presence of inflammatory tissue around the prototypes in situ were not conducted, even though this would be the ultimate aim of dexamethasone delivery into the cochlear in conjunction with cochlear implantation. On the strength of the results of this thesis, however, further studies can now be recommended on specimens embedded in epoxy in order to carry out more effective investigations in situ, including studies of the inflammatory reaction after implantation of polymeric CI coatings loaded with different concentrations of DMS.

In conclusion, as finding a treatment of the inner ear that is both safe and effectively dosed is somewhat problematic, further studies are required to enhance understanding of the mechanisms behind the effects of dexamethasone inside the inner ear. However, on the basis of the investigations presented in this thesis, it has
been shown that a biodegradable coating manufactured with P(4HB) which is loaded with dexamethasone, combined with a modified CI array, is a potential alternative means of sustained intra-cochlear delivery of anti-inflammatory drugs. This strategy would open up a new scenario for the management of post-surgical tissue responses in CI patients.
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8. SUMMARY

Piera Ceschi

Development of a Polymeric Coating for Cochlear Implant Electrodes to Deliver Dexamethasone into the Inner Ear

Cochlear implants (CIs) are increasingly used for the treatment of patients suffering from sensorineural hearing loss (SNHL). Remarkable progress has been made in recent decades, and tangible results have been achieved in terms of speech perception both in quiet and noise, as well as during phone calls. However, some relevant issues remain unresolved, notably a poor electrode-nerve interface. Efforts are being made to improve the surgical procedure, the technical properties of the device, and to develop strategies for intra-cochlear anti-inflammatory drug delivery to reduce the tissue growth around the electrode array after implantation.

To explore the effects of inner ear treatments, morphological analysis of the cochlea subsequent to implantation is essential. Paraffin embedding is well accepted as a technique for the investigation of the inner ear; however, the electrode has to be removed prior to embedding, as the metallic parts cannot be sectioned. This results in a lack of information about the presence of inflammatory tissue around the CI, as part of it is removed together with the electrode. Another strategy involves embedding the specimens in epoxy and subsequent grinding. This technique, which has been used for insertion studies in temporal bones, provides scope for embedding and grinding the cochlea after implantation without removal of the electrode. As it was still unclear whether this approach also allows the effects of any treatment on SGCs to be evaluated, the epoxy-embedding method was explored and compared with the paraffin-embedding technique in respect of SGC density and diameter.

The present study showed that SGC density and diameter were reduced in all cochlear regions for specimens embedded in epoxy. This was probably due to the drying process during epoxy embedding – which can cause shrinking of the SGCs – and to the documentation approach, as information was obtained from the surface of the specimens, while information from paraffin sections was collected through the full thickness of the slices. Finally, it was possible to estimate a lower diameter limit for
viable SGCs in cochleae processed for epoxy embedding (8.4 µm). This was calculated taking into consideration the lower limit for viable SGCs embedded in paraffin as given in the literature, although this value is admittedly still under debate. This study confirmed that, although tissue after epoxy embedding is not as well preserved as with paraffin embedding, the inner ear structures – in particular SGC density and diameter – can be investigated subsequent to epoxy embedding and grinding of the specimens. The technique was hence applied to investigate novel polymeric coatings for CIs.

Two biodegradable polymers, poly-L-lactide (PLLA) and poly-4-hydroxybutyrate (P(4HB)), were chosen as potential platforms for a sustained intra-cochlear anti-inflammatory drug delivery and tested both in vitro and in vivo. **In vitro** SGC survival was reduced on all materials; however, no negative effects were exerted by the polymers in comparison with silicone. **In vivo** prototypes were implanted inside the scala tympani of normal-hearing guinea pigs for one to six months. Although all subjects already exhibited a threshold shift after only one month, no significant differences between implanted groups were found. Moreover, histological analysis showed a decrease in SGC density in animals that received a P(4HB) prototype in the basal and middle turns of the cochlea, and also in the PLLA group in the middle and apical cochlear turns merely six months post-implantation (in comparison with the normal-hearing guinea pigs only). However, no significant differences were detected between subjects which received the silicone samples and animals which were implanted with coated prototypes (both P(4HB) and PLLA). Hence, considering these findings and the fact that the prototypes have been implanted only in the basal cochlear turns, it is more likely that these outcomes are attributable to surgical trauma than to a toxic effect on SGCs exerted by the degradation of the polymers.

P(4HB) showed better mechanical stability during implantation, and prototype analysis after explantation revealed that it is characterized by a more gradual degradation than PLLA is. Hence, P(4HB) would be preferable for use as a CI coating. On the basis of these outcomes, further testing was justified to investigate the effects of P(4HB) coating loaded with dexamethasone inside the inner ear.
As no toxic effects of dexamethasone on SGCs were observed in vitro, a P(4HB) coating was loaded with dexamethasone, and prototypes were implanted in the cochlea of normal-hearing guinea pigs. After one month, hearing thresholds were shifted at high frequencies for all groups; however, a significant shift was detected only at a frequency of 8 kHz for the subjects who received an unloaded P(4HB) prototype, perhaps due to the surgical trauma. Importantly, at 32 kHz – a frequency associated with the anatomical structures close to the site where the specimens were positioned – hearing thresholds were significantly increased in all groups other than the DMS-30 group, suggesting a protective role of dexamethasone from surgical traumas in these animals.

The investigations presented in this thesis allowed the characterization of epoxy embedding for histological evaluation of the effects of cochlear implantation on the soft tissue of the inner ear, with particular regard to SGCs. Using this method, PLLA and P(4HB) were found to be suitable for polymeric coating of these implants, with some advantages for P(4HB), justifying its use for the delivery of dexamethasone into the inner ear as an alternative means of sustained intra-cochlear delivery of anti-inflammatory drugs. This strategy would open up a new scenario for the management of the post-surgical tissue responses in CI patients.
9. ZUSAMMENFASSUNG (German)

Piera Ceschi

Entwicklung Dexamethason freisetzender Polymerbeschichtungen von Cochlea Implantate


Um die Wirkungen der Behandlungen im Innenohr zu untersuchen, muss eine histologische Analyse der Cochlea nach der Implantation durchgeführt werden. Die Einbettung des Präparates in Paraffin ist als Technik für die Untersuchung der Cochlea akzeptiert. Da die Elektrode jedoch metallische Teile enthält, die nicht geschnitten werden können, muss die Elektrode vor der Einbettung entfernt werden. Dadurch können auch Teile des Bindegewebes um den Elektrodenträger entfernt werden, was zu einem Mangel an Informationen über das Bindegewebe um den CI führen kann. Um dies zu vermeiden, können die Proben in Epoxid eingebettet und danach geschliffen werden. Diese Technik wurde bereits für Insertions-Studien in Felsenbeinen verwendet. Jedoch war bisher nicht bekannt, ob diese Methode auch für die Untersuchung der Auswirkung lokaler Behandlungen auf die Nervenzellen des Innenohres, die Spiralanglienzellen (SGZ), geeignet ist. Daher wurden in dieser Arbeit Paraffineinbettung und Epoxideinbettung hinsichtlich Spiralanglienzelldichte und –durchmesser verglichen.

Die Untersuchungen zeigten, dass nach Epoxideinbettung SGZ-Dichte und -durchmesser in allen Windungen reduziert waren. Dies ist wahrscheinlich auf das

Obwohl das Gewebe nicht so gut wie bei Paraffineinbettung konserviert ist, kann das Innenohr – insbesondere Anzahl und Durchmesser der SGZ – nach Einbettung in Epoxid dargestellt und ausgewertet werden. Daher wurde diese Methode gewählt, um neue Polymerbeschichtungen für CI in vivo zu untersuchen.

Zwei biologisch abbaubare Polymere, Poly-L-Lactid (PLLA) und Poly-4-Hydroxybutyrat (P(4HB)), wurden als potentielle Substanzen für die Entwicklung einer CI-Beschichtung zur intracochleären Gabe von Antiphlogistika ausgewählt und in vitro und in vivo getestet.

In vitro war das Überleben von SGZ auf allen Materialien reduziert, jedoch zeigten die Polymere keine toxischen Effekte im Vergleich zu Silikon.

In vivo wurden Modellelektroden für 1 bis 6 Monate in die Scala tympani von normalhörigen Meerschweinchen implantiert.


Allerdings wurden keine signifikanten Unterschiede zwischen unbeschichteten und beschichteten Proben gefunden, was zu dem Schluss führt, dass die Implantation und das damit verbundene Trauma eher einen Einfluss auf das SGZ Überleben hat, als die Degradation der Polymere.
P(4HB) zeigte im Vergleich zu PLLA eine bessere mechanische Stabilität und eine gleichmäßigere Degradation. Daher wurde eine P(4HB)-Beschichtung gewählt, um die Auswirkungen der Freisetzung von Dexamethason im Innenohr zu untersuchen.


In der vorliegenden Arbeit wurde eine Methode zur histologischen Analyse implantierter Cochleae mit dem Elektrodentrüger in situ im Hinblick auf ihre Auswirkung auf das Gewebe des Innenohres, insbesondere die SGZ, charakterisiert. Unter Verwendung dieses Verfahrens erwiesen sich PLLA und besonders P(4HB) Beschichtungen als geeignet für die Anwendung auf CI. Über die Gabe von Dexamethason aus der P(4HB) Beschichtung konnte das Potential dieser Polymerbeschichtungen für die langfristige Gabe von Antiphlogistika im Innenohr gezeigt werden. Diese Strategie könnte für die betroffenen Patienten einen neuen Weg zur Reduktion des Bindegewebebewachstumes nach CI Implantation aufzeigen.
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