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Investigation of auditory neuronal survival and outgrowth using a cochlear implant in an artificial cochlear model

Abstract: Cochlear implants (CI) can restore hearing to people suffering from sensorineural hearing loss. The CI uses an array of electrodes inserted in the scala tympani to stimulate the spiral ganglion neurons (SGN), which are located in the bony axis of the inner ear. The spatial distance between the electrodes and the SGN results in spread of excitation and unfocused stimulation. This distance could be bypassed by a neurite outgrowth towards the CI. In order to establish a culture system for the development of novel strategies for CI optimization, a neurite outgrowth chamber (NOC) allowing clinically relevant electrical stimulation paradigms was developed using fused deposition modelling. It was made out of biocompatible UV-curing silicone. The NOC was equipped with a slot to insert the electrode array into a scala tympani compartment whereas SGN isolated from postnatal rats were cultured in a neighbouring region mimicking the Rosenthal's canal. Sound delivered through loudspeakers playing a radio program was captured by the CI sound processor, positioned outside the NOC, for 17 hours daily during a four-day period. The NOCs were tightly sealed and the electrode array could be positioned in the scala tympani compartment. The experimental setup allowed cell cultivation and the stimulation resulted in a significantly increased neurite length of around 36% while explant area and neurite number did not differ to the negative control (NC). A new in vitro testing system which considers the anatomy of the cochlea and clinic stimulation conditions has been developed and validated. The validation experiments resulted in increased neurite length. This indicates a progress in bridging the anatomical gap between electrode and stimulated neurons.

Keywords: Cochlear implant, electrical stimulation, spiral ganglion neuron, inner ear, 3D-printing, neurite outgrowth, electrode neuron interface, neuritogenesis

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1 Introduction

According to the World Health Organization around 6.1% of the world population is suffering from hearing loss. 980 Billion U.S. Dollars are spent for medical, educational and social support every year due to untreated hearing damage [1]. Nearly 2.5 billion people are expected to be suffering from some kind of hearing loss by the year 2050 and at least 700 million patients will need hearing rehabilitation [2]. Taking these numbers and circumstances into consideration, the development and advancement of more efficient medical hearing devices are inevitable. Sensorineural hearing loss is a common form of hearing damage. The hair cells in the inner ear are no longer able to transform acoustic signals into electrical signals, which could be interpreted by the brain. CI replace them, by digitalizing and transforming recorded sounds and sending electrical pulses to the auditory nerve [3]. The spatial distance between the stimulating electrodes of the CI and the SGN, located in the bony axis of the inner ear, decreases the stimulation focus significantly. Orientated neurite outgrowth would be a solution in order to prevent spread of excitation and bypass the distance. Therefore, a culture system for the development of novel CI optimization strategies including a CI for electrical stimulation as influencing factor of novel therapy options was established. A NOC made out of biocompatible UV-curing silicone was fabricated using fused deposition modelling. A setup considering clinical relevant stimulation paradigms and conditions was developed and validated by stimulating SGN isolated from early postnatal rats.

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2 Methods

2.1 Fabrication of the NOC

The NOC (shown in **Fig. 1**) is supposed to be a simplified model of the inner ear. It consists of two compartments. The bigger one is representing the scala tympani whereas the neighbouring region serves as model for the Rosenthals' canal. The chamber is equipped with a slot that allows a CI insertion into the scala tympani compartment. The 2 mm long connecting canal is as long as the anatomic spatial distance in reality. This concept design was realised with a CAD model (**Fig. 1a**) using the Software Autodesk Inventor Professional 2021 and 3D-printed (**Fig. 1b**) with the Envisiontec 4th Generation 3D-Bioplotter of the Manufacturer Series equipped with an UV-laser.

Figure 1: a) CAD model of the NOC with a scala tympani compartment (ST) and a Rosenthal's canal compartment (RC), separation indicated by the blue line b) 3D-printed NOC filled with medium and with an inserted electrode array.

A line hatch type with continuous strands was selected. The material consisted of 98% UV-curing silicone and 2% catalyst (both from Envisiontec) and was mixed for two minutes (SpeedMixer DAC 150.1 FVZ by Hausschild SpeedMixer) at 3500 rotations per minute. The CAD model was virtually positioned and sliced using the Perfactory RP software and then stored as a BPL file for the printing process. The printing parameters selected in the machine setup of the software VisualMachines are given in **Table 1**.

Table 1: The printing parameters of the machine setup allowing to adjust the material, the pattern of the print and the UV-laser curing the material.

Parameter	Value
Temperature [°C]	27
Needle Offset [mm]	0.400

Parameter	Value
Pressure [bar]	5.0
Speed [mm/s]	6.0
Pre-Flow [s]	0.13
Post-Flow [s]	0.05
Minimum Length [mm]	0.4
Distance From Contour [mm]	0.10
Distance Between Strands [mm]	0.20
Period [mm]	2.00
Amplitude [mm]	1.00
Available Apertures	365 nm – 17 nm – 14.5 nm
UV Beam Brightness [mW/cm^2]	5.0
Calculated Height [mm]	96.3
Calculated Spot Diameter [mm]	26.9
Height from last Layer [mm]	15.0
Exposure Time [s]	10.0
Wavelength Combination [nm]	365

2.2 Experimental stimulation setup

A CI (Mi1000 Series CONCERTO, MED-EL, Innsbruck, Austria) was used as a stimulation device. It was programmed with a most-comfort-level (MCL) of 24.2 nC in each of the 12 electrode contacts. This value was obtained as the average MCL from 25 CI users from the German Hearing Center of the MHH. The threshold-level was 1.94 nC and the pulse duration 28.75 μs . The CI electrode array was inserted in a way so that the array writhed as shown in **Figure 1b**. The NOC was placed on a pedestal inside of a petri dish and was fixed with vaseline while the implant housing was positioned next to the pedestal. The petri dish was filled with a physiological saline solution. The petri dish was covered and put in an incubator. A transmitter coil was adhered to the bottom of the dish due the magnetic attraction of the receiver coil of the implant. The transmitter coil was connected to a speech processor outside the incubator. The SONNET processor recorded acoustic signals produced by radio loudspeakers for 17 hours daily for four days. This was supposed to simulate sleeping phases and active phases of a patient. The time regulation was realised with help of a 15GD-3A SCHUKO timer switch by EMOS. The batteries of the speech processor were replaced every one and half day.

2.3 Cell culture and stimulation

The fabricated NOCs, cover glasses, petri dishes, vaseline and the pedestals were sterilized by an UV radiation of $1800 \mu\text{J}/\text{cm}^2$ for 1800 seconds emitted inside of the Spectrolinker XL-1000 UV. The bottom of the NOCs were coated with vaseline to fix a smaller cover glass underneath the RC compartment while a larger cover glass sealed the whole NOC to prevent medium leakage. Air bubbles were smoothed out carefully and the cover glasses of the NOCs were coated for increased cell adhesion and neuroprotection. First they were filled with a 0.1 mg/ml ornithine solution (Sigma Aldrich, Taufkirchen, Germany) for one hour at room temperature and rinsed with PBS, meaning phosphate buffered saline, (Invitrogen, Karlsruhe, Germany) afterwards. Each NOC was then filled with a 0.01 mg/ml laminin solution (Invitrogen, Karlsruhe, Germany) and incubated at 37°C for another hour. The solution was then suctioned off and the NOCs were rinsed with PBS. For the cell experiments, panserin (PAN-Biotech, Aidenbach, Germany) was supplemented with insulin ($8.7 \mu\text{g/ml}$; Biochrom, Berlin, Germany), penicillin (30 U/ml ; Biochrom, Berlin, Germany), glucose (0.15% ; B. Braun, Melsungen, Germany), PBS (0.172 mg/ml), HEPES buffer solution ($23.43 \mu\text{mol}$; Invitrogen, Karlsruhe, Germany) and N2 supplement ($0.1 \mu\text{l/ml}$; Invitrogen, Karlsruhe, Germany) as medium. The NOCs, each filled with $150 \mu\text{l}$ of complemented medium, were stored overnight in an incubator set at 37°C , $5\% \text{ CO}_2$ content and 95% humidity. This allowed a final control for leakage in the vaseline seal.

SGN were isolated from five days old rats. After decapitation, the skull was opened and the temporal bones were removed and cooled in a petri dish filled with PBS on ice. The cochlea was then exposed under a microscope (CKX41 microscope from OLYMPUS), so the spiral ganglia could be isolated and cut up into five cell explants (SGE) each. The explants were positioned on the smaller glass slides inside of the NOCs as shown in **Figure 2**. Fetal calf serum (FCS) was added to a concentration of 10% in the medium of the NOCs. After positioning of the SGE, the NOCs were left for 30 minutes without vibrations at room temperature and then transferred and precultivated for 30 hours in the incubator for better attachment of the tissue. The two best prepared NOCs were chosen for the evaluation experiment. One NOC included a stimulating CI while the other NOC did not, serving as a negative control.

During the four days of stimulation, as described in section 2.2, the NOCs were controlled daily for cell adhesion and infections by microscope. After the end of each stimulation day, the medium in both NOCs was replaced by fresh medium in order to remove cell-damaging metabolic products such as

oxygen radicals that might have been caused by the stimulation stress. At the end of the stimulation, the explants were fixed in the chamber with 4% paraformaldehyde for 10 minutes at room temperature which was followed by a triple PBS rinse. The larger glass slide was removed and the smaller one with the explants carefully peeled off with tweezers and transferred for staining.

Figure 2: Microscopic image of five spiral ganglion cell explants (SGE) placed on a cover glass slide underneath the RC compartment and the 2 mm long (red line) canal region of the NOC.

For this they were first permeabilized with 0.5% Triton X-100 (Sigma Aldrich, Taufkirchen, Germany) for 15 minutes and rinsed three times with PBS. The cells were then blocked for one hour with a solution composed of 10% FCS, 5% bovine serum albumin (Sigma Aldrich, Taufkirchen, Germany) and 0.1% Triton X-100. The anti-neurofilament chicken antibody (ab4680; Abcam, Cambridge, United Kingdom) diluted $10,000$ -fold in the blocking solution was added to the cells as the primary antibody for one hour at room temperature in order to specifically stain the neuronal cells. After rinsing with PBS three times again, the secondary antibody Goat Anti-Chicken IgY H&L (DyLight 488, 1: 200; ab96947; Abcam, Cambridge, United Kingdom) and DAPI (1:100; AppliChem GmbH, Darmstadt, Germany) were diluted in blocking solution and incubated at room temperature under protection from light. After one hour, the antibody solution was removed and the PBS rinsing was repeated three times. The stained explants were then stored in a light-protected manner in PBS. Next, the glass slides were imaged using a $4\times$ -objective (Keyence BioRevo BZ-9000E; Keyence, Neu-Isenburg, Germany) and the BZ II Viewer and Analyzer software. Green (neurofilament) and blue (DAPI) fluorescence images were taken and then stitched to form an overview image (**Fig. 3**).

The software FIJI was used to evaluate the images for neurite outgrowth by measuring the explant area, the neurite expansion as well as their number and lengths. The collected data of the cell culture chamber with electrical stimulation and

the NC were statistically evaluated with a paired samples t-test. A Wilcoxon test was used for the statistical analysis of neurite lengths, after testing for normal distribution with D'Agostino and Pearson. Statistical analysis was performed with GraphPad Prism 5.

Figure 3: Microscopic fluorescence image of the spiral ganglion cell explants (light blue areas), cell nuclei (blue spots) and neurites (green lines) after the staining process

3 Results

The designed and 3D-printed NOC made out of UV-curing silicone is suitable for a simplified model of the inner ear that can be used for cell cultivation. It considers anatomic dimensions of the human cochlea and has a 2 mm distance between the electrode array and the SGN, just as in reality. A CI electrode array can be inserted mimicking a cochlear implantation. Since no medium leakage was observed, we conclude, that the NOCs were tightly sealed. An experimental stimulation setup that allows a spatial separation between the NOC with the implant inside an incubator and the speech processor as well as the noise source outside of it was established. The electrical stimulation paradigms are clinically relevant, the setup and fitting to assess cellular processes and neurite outgrowth could be successfully implemented. A significant increase of around 36% of neurite length was observed induced by electrical stimulation. Neurites reached lengths from 119.6 μm up to 4498 μm , whereas lengths from 97.8 μm to 1824 μm were measured for the NC. On average, the lengths of the stimulated cell explants were 755.5 μm , which is a third longer than the average length of 566.6 μm without stimulation. Explant and neurite area as well as neurite number did not differ between stimulation and the NC (**Fig. 4**).

Figure 4: Column chart comparing neurite data of the NOC with CI stimulation (CI) to the negative control (NC). Only the neurite lengths differed significantly. Data given as mean ± SEM, * $p=0,012$ ($N = 1$ with $n = 5$ per condition).

4 Discussion and Conclusion

A new form of in vitro testing system which considers the anatomy of the cochlea and clinical stimulation conditions was presented in this study. It is simple, inexpensive and can be incorporated easily in hearing research. NOCs can be produced in large quantities within a short period of time in an automatic 3D-printing process once they were properly designed. It was shown that electrical stimulation could be delivered successfully and that electrical stimulation has a positive effect on neurite outgrowth using this setup. This is a further step in bridging the spatial distance between the stimulating electrodes and the SGN. It may help pave the way to make CI stimulation more focused and therefore much more effective. The NOC could become even more realistic by implementing an outgrowth matrix that mimics the tissue of the inner ear. Future applications such as a drug delivery system could be added. The combined stimulation may be investigated using our NOC to develop novel CI based drug delivery strategies to improve the hearing outcome of CI patients.

Author Statement

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