

CELL-BASED SENSOR CHIP FOR NEUROTOXICITY MEASUREMENTS IN DRINKING WATER

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Abstract

Our drinking water contains residues of pharmaceuticals. A sub-group of these contaminants are neuro-active substances, the antiepileptic carbamazepine being one of the most relevant. For assessment of the neurotoxicity of this drug at a sub-therapeutic level, a cell-based sensor chip platform has been realized and characterized. For this purpose, a microelectrode array chip was designed and processed in a clean room and optimized in terms of low processing costs and good recording properties. For characterization of the system neuronal cells were plated on microelectrode array chips and electrical activity was measured as a function of applied carbamazepine concentration. We found that the relative spike rate decreased with increasing drug concentration resulted in IC_{50} values of around $36 \mu M$. This value is five orders of magnitude higher than the maximal dose found in drinking water. IC_{50} values for burst rate, burst duration and synchrony were slightly higher, suggesting spike rate being a more sensitive parameter to carbamazepine.

Keywords

Microelectrode array, carbamazepine, neurotoxicity, cell-based biosensor

Introduction

Every year large amounts of human and veterinary pharmaceuticals find their way in our drinking water cycle. With new and improved analytic methods these drugs can be detected – even in very low concentrations. These new options increase the awareness of possible effects correlated to the chronic uptake of drugs within drinking water. Although, it is likely that residues in the water have no adverse effects on the human health, there is no regulation in terms of critical concentration values [1] due to a lack of data regarding chronic exposure at low doses.

The number of pharmaceutically active substances found in drinking water is surprisingly high. More than twenty different active components and their metabolites have been detected in drinking water [1]. Tab. 1 shows some examples of detected drug in drinking water. In this work we focused on neuro-effective drugs as they have hardly been addressed in the literature yet. Therefore, we searched literature for measurements of neuro-effective drugs found in drinking water [1, 2]. Typical compounds are diazepam, diclofenac, and carbamazepine.

Tab. 1: Some examples of drugs detected in drinking water and their maximal concentration c_{max} .

active component	drug group	c_{max} $\mu g/l$	reference
carbamazepine	anticonvulsant	0,03	[3]
diazepam	ataractics	0,01	[3]
paracetamol	analgetic	0,015	[4]
diclofenac	analgetic	0,14	[4]
sotalol	beta blocker	0,005	[5]

The most relevant substance identified was carbamazepine. Carbamazepine (CBZ) is a medication used primarily in the treatment of epilepsy and neuropathic pain and it is well known to reduce electrophysiological activity of the brain by blocking voltage-gated sodium channels [6]. Also, the activation of GABA_A receptors were reported [7]. In Germany, CBZ has been identified at twenty-six different samples with a maximum concentration of $0.03 \mu g/l$

(0.13 nM) [1]. Obviously, the measured concentrations depend strongly on country and the area where samples were taken. Maximum CBZ concentration in efflux of German sewage treatment plants were 6.3 µg/l [8], whereas higher concentrations of up to 27.27 µg/l have been found in the efflux of sewage treatment plants in Cyprus, which leads to the assumption that drinking water concentration in that region could be even higher [9]. Our hypothesis is that CBZ, if incorporated on a regular base – even at sub-therapeutic levels – has the potential to induce unwanted changes in the brain's functionality.

With this background, we propose the application of a cell-based *in vitro* assay to assess the neurotoxicity of CBZ and as a perspective other neurotoxins relevant for drinking water. For this purpose a cell-based sensing platform shall be developed. Microelectrode array (MEA) chips coupled to neuronal cell networks are a common approach to achieve a basic understanding of electrophysiological properties of neuronal networks [10, 11]. Electrical activity of a neuronal network is recorded *in vitro* and changes related to the application of a neuroactive substance are analyzed. With this goal, we fabricated MEA chips with gold electrodes to record electrophysiological signals of a cortical neuronal network and characterized the cell-based sensing platform for the drug CBZ.

Materials and Methods

MEA chip processing

The microelectrodes for recordings of neuronal signals have been processed of various metals or conduction polymers [12]. In this study a MEA chip was processed with gold microelectrodes. Fig. 1 shows the MEA chip and its microelectrode layout. The outer size of the glass chip is 45×45 mm providing 59 microelectrodes with a diameter of 30 µm (and a spacing of 200 µm) and a reference electrode. All electrodes are connected via conducting lanes to large metal pads at the outer boarder of the chip, while insulation and biocompatibility are provided by a thick SU8 resist. To improve the impedance, the electrodes may be coated with nanowires or carbon black to increase the surface area of the electrodes. The glass ring on top of the MEA chip builds a culture chamber of about 1.5 ml.

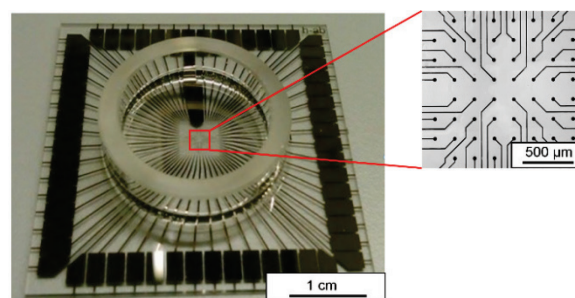


Fig. 1: MEA chip with glass ring (left) and its magnified microelectrode array (right). The microelectrode array consists of 59 microelectrodes. A larger reference electrode is located inside the glass ring area next to the microelectrode array.

Neuronal cell culture

Neuronal cell culturing was performed by using cryo-conserved embryonic cortical rat neurons (E18 and E19) (Lonza Ltd, Basel, Switzerland). Cell culture medium was prepared according to the following protocol: L-glutamine (2 mM) and 2% NSF-1 were added to the PNBM basal medium. NSF-1, a supplement supporting neuronal growth and survival, was aliquoted, frozen and added to the medium immediately before each use. Neurons were thawed and cultured with a density of approximately 5.000 cells/mm² onto each substrate and incubated in a humidified atmosphere for 4 h. Finally, petri dishes were filled with approximately 1.5 ml culture medium. The Medium was fully replaced twice a week.

Electrophysiological recording

Neuronal signals were recorded extracellularly at a sampling rate of 10 kHz with a MEA60 amplifier system (Multichannel System, Reutlingen, Germany) and stored for offline analysis. Recordings were performed outside the incubator. To maintain the sample temperature at 37°C, a temperature-controller (TC01, Multichannel Systems, Reutlingen, Germany) was employed during the recordings. Controlled CO₂ atmosphere (5%) was provided by a custom-made mini incubator and a CO₂ controller (CO2-Controller 2000, Pecon, Erbach, Germany).

Data analysis

Raw data were analyzed using custom-made Matlab (The Math Works Inc., Natick, Massachusetts, U.S.A.) based software tool *Dr. Cell* featuring spike detection using a threshold-based algorithm, with a threshold calculated by multiplying the standard deviation of the noise by a factor of -5 [12]. Fig. 2 shows a typical raw signal and its corresponding spike train which is used for analysis. Shortly successive occurring spikes were defined as burst, if the burst consists of at least three spikes with times between each spike not exceeding 100 ms (“mini-bursts”) [13]. Synchrony of the neuronal network was measured by binary binning each spike train into 500 ms bins, calculating cross-covariance without delay between all pairs of spike trains, and averaging those values [14].

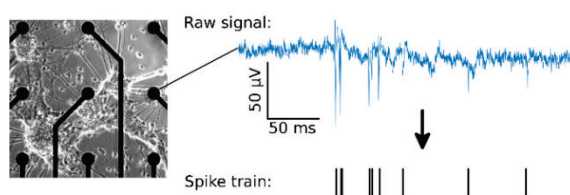


Fig. 2: Nine electrodes are displayed (electrode diameter 30 μm) including neuronal network. Right: Raw neuronal signal recorded from one electrode featuring spikes whose occurring times (spike train) are used for analysis.

Statistics

Data (given as mean $\pm$ standard deviation of N samples) were collected from different cell cultures. Data points of dose-response curves from electrophysiological recordings were fitted using the Hill equation

$$y = A_2 + \frac{A_1 - A_2}{1 + \left(\frac{c}{c_0}\right)^{N_H}} \quad (1)$$

where c donates the concentration of the test substance, c_0 the half maximal effective concentration (designated as EC_{50} for excitation and IC_{50} for inhibition), N_H the Hill coefficient, and A_1 and A_2 the spike rate under control conditions and saturated concentration of the test substance.

Significant changes from control condition were assessed using parameter free Kruskal-Wallis test. Multiple testing was performed using Bonferroni correction.

Drugs

Test substance CBZ (CAS number 298-46-4; C4024-1G, Sigma Aldrich, St. Louis, USA) was diluted in

dimethyl sulfoxide (DMSO) (CAS number 67-68-5; Sotrachem, Saint Denis, France) and was manually applied to the medium. CBZ concentrations of 2 μM , 20 μM , 40 μM , 60 μM , 100 μM , 200 μM and 1000 μM were added accumulatively and recorded for 5 minutes each. Maximum DMSO concentration at 1000 μM CBZ was 0.5%, which has been shown to have no significant effect on neural activity [6].

Results and Discussion

Typical patterns of spike trains in the absence and presence of CBZ are displayed in Fig. 3. Spike rate in the absence of CBZ were around 500 spikes per minute per electrode. Fig. 4 shows CBZ concentration dependent parameter like spike rate (Fig. 4a), spike amplitudes (Fig. 4b), burst rate (Fig. 4c), burst duration (Fig. 4d) and spike synchrony (Fig. 4e) normalized to the control condition (absence of CBZ). Data are displayed as mean $\pm$ standard deviation. Except for spike synchrony, normalized parameter were calculated for every active electrode and subsequently averaged across the chip. Fitting of the spike rate with Hill equation resulted in an IC_{50} value of 38 μM which is in agreement with values reported for primary murine frontal cortex neurons [10]. IC_{50} values for burst rate, burst duration and synchrony were slightly higher suggesting spike rate as the most sensitive parameter to capture the effect of CBZ. Concentrations of 60 μM and higher caused statistical significant effects compared to control condition for spike rate ($p < 0.05$), burst rate ($p < 0.05$), burst duration ($p < 0.01$) and synchrony ($p < 0.01$). Spike amplitude decreased not before 100 μM and was therefore the most insensitive parameter to CBZ.

Compared to concentrations measured in drinking water, IC_{50} values and statistical significant acute effects are five orders of magnitude higher. Therefore, no acute effects on neurons are expectable due to exposure of CBZ in concentrations measured in drinking water. Since CBZ exposure over two weeks showed GABA-receptor upregulation *in vivo* [15] this effect could be also expected at neurons on MEA chips. Since neurons coupled to MEA chips can be cultured for several weeks, long-term exposure is possible and can lead to significant alterations in spontaneous activity like shown for mercury [16]. Even if no alteration in spontaneous activity is observable, acute experiments after long-term exposure may reveal long-term effects. For example an application of the allosteric GABA_A inhibitor bicuculline could change the signal activity differently of control and exposed neurons [17]. Using this strategy, identifying a concentration not causing any long-term effects could be used to define a threshold value for drinking water. Moreover, neurons coupled to MEA chips might be also applied to study acute and long-term effects of

certain mixtures of pharmaceuticals found in drinking water.

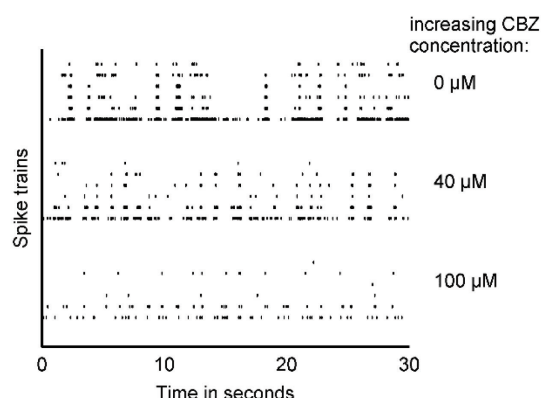


Fig. 3: Spike trains of six electrodes recorded from a MEA chip are shown in the absence of CBZ (0 μM) and the presence of CBZ (40 μM and 100 μM). With increasing CBZ concentration neuronal activity decreases.

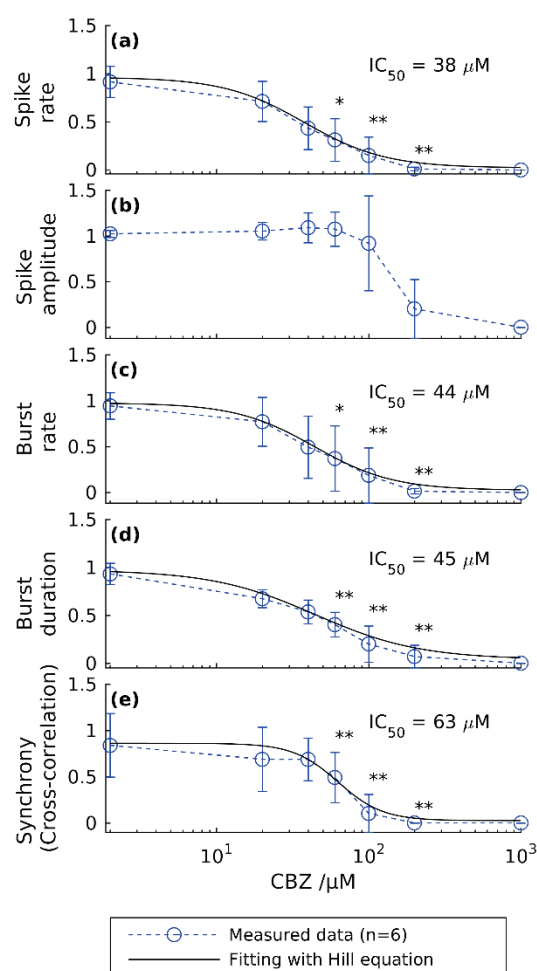


Fig. 4: Effect of increasing concentrations of CBZ on the spike rate (a), spike amplitude (b), burst rate (c),

burst duration (d), and spike synchrony (e) of neurons coupled to MEA chips ($N = 6$, 21–25 div). Data points represent the mean $\pm$ standard deviation and were normalized to the control at zero concentration (not shown in the diagram). Data are fitted using the Hill equation to calculate half maximal inhibitory concentration (IC_{50}). Significant changes from control condition assessed with Kruskal-Wallis test followed by Bonferroni corrected multiple testing, are indicated by *, $P < 0.05$ and **, $P < 0.01$.

Conclusion

We realized an in vitro cell-based sensing platform in order to assess neurotoxicity of active components that are relevant contaminants of drinking water.

Neurons coupled to MEA chips were electrical active and signals could be recorded and analyzed. The acute application of the neuroactive substance CBZ caused a concentration dependent decrease in spike rate with an IC_{50} value of around 36 μM , which was expected [11]. Further experiments will now address the chronic application of CBZ for several days and weeks.

Acknowledgement

Research described in the paper was supervised by Prof. C. Thielemann, biomems lab, UAC Aschaffenburg and financially supported by the Bayerisches Staatsministerium für Bildung, Kultus, Wissenschaft und Kunst in the frame of FH Forschungsschwerpunkt. We would like to thank Dr. CRISTOPH NICK for the making of the MEA chip layout.

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