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High-throughput *in vitro* assay to evaluate the cytotoxicity of liberated platinum compounds for stimulating neural electrodes

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ABSTRACT

Background: It is currently unclear how the platinum (Pt) species released from platinum-containing stimulating electrodes may affect the health of the surrounding tissue. This study develops an effective system to assess the cytotoxicity of any electrode-liberated Pt over a short duration, to screen systems before future *in vivo* testing.
New method: A platinum electrode was stimulated for two hours under physiologically relevant conditions to induce the liberation of Pt species. The total concentration of liberated Pt species was quantified and the concentration found was used to develop a range of Pt species for our model system comprised of microglia and neuron-like cells.
Results: Under our stimulation conditions (k = 2.3, charge density of 57.7 μC/cm^2), Pt was liberated to a concentration of 1 ppm. Interestingly, after 24 h of Pt exposure, the dose-dependent cytotoxicity plots revealed that cell death became statistically significant at 10 ppm for microglia and 20 ppm for neuronal cells. However, in neuron-like cell cultures, concentrations above 1 ppm resulted in significant neurite loss after 24 h.
Comparison with existing methods: To our knowledge, there does not exist a simple, *in vitro* assay system for assessing the cytotoxicity of Pt liberated from stimulating neural electrodes.
Conclusions: This work describes a simple model assay that is designed to be applicable to almost any electrode and stimulation system where the electrode is directly juxtaposed to the neural target. Based on the application, the duration of stimulation and Pt exposure may be varied.
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1. Introduction

Neural electrodes are an important component of many rehabilitative or palliative approaches to restore functionality to patients with neurological deficits. Currently treatable and envisioned conditions range from sensory impairments to motor deficits and organ dysfunction, as well as direct interfaces with prosthetic/robotic limbs and computers (Prochazka et al., 2001; Jezernik et al., 2002; Clark, 2006; Halpern et al., 2008). Neural electrodes can be used to either record from neural tissue, or stimulate excitable structures in an attempt to develop closed-loop rehabilitative strategies.

For the last several decades, electrical stimulation of neural structures has been performed using a wide range of electrode-containing devices, including deep-brain stimulating (DBS) systems, spinal cord stimulators, and cochlear implants (Cogan, 2008). Stimulating electrodes inject electrical charge into or near neural structures in order to induce a physiological response in the surrounding cells. Electrical stimulation induces propagating action potentials that produce a controlled and targeted release of neurotransmitters to elicit the desired functional outcome.

Currently, the safety of a given therapeutic neural stimulation protocol is evaluated using two parameters: charge per phase and charge density per phase (McCreery et al., 1990; Shannon, 1992). These criteria were derived empirically from a limited set of data on disk electrodes in direct contact with the feline cortex (McCreery et al., 1988; McCreery et al., 1990) and have been extrapolated to be

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used across different target tissues. The traditional electrical stimulation safety restrictions for DBS (stimulating at a level $k \leq 1.75$) are based on the Shannon plot and resultant equation (Shannon, 1992), which limits charge and charge density of stimulation devices. The limits imposed by the Shannon plot/equation hinder therapeutic options by creating a heavy regulatory burden for devices that go outside the parameter range. The Shannon limit is not based on a mechanistic understanding of adverse events associated with electrical stimulation, but instead on the observation of *in vivo* tissue damage (Shannon, 1992). Neural implants that employ new stimulation paradigms and locations would benefit from safety criteria that are based on a more fundamental understanding of the mechanism(s) of damage.

Platinum or a platinum-containing alloy is a common construction material for the electrical contacts of current neural stimulating electrodes (Cogan, 2008). It is well known that Pt compounds have cytotoxic effects (Rosenberg et al., 1965) primarily via their intercalation into DNA. Therefore, one leading hypothesis is that Pt ions, which are released from the electrode during stimulation, may play a key role in causing the tissue damage observed *in vivo* surrounding the device (Agnew et al., 1977; Robblee et al., 1983). Past studies have investigated the cytotoxicity of Pt compounds on human pulmonary cells (Waters et al., 1975) as well as animal cell lines (Turnbull et al., 1979; Johnson et al., 1980), observing varying degrees of cytotoxicity and mutagenicity depending on the particular compound, cell type, and incubation time. Platinum compounds have also been investigated for their toxicity in environmental health research (Bunger et al., 1996; Brook, 2006) as well as neurotoxicity as a side effect of cancer treatment (Luo et al., 1999). Pt compounds such as cisplatin have been used extensively to treat cancer (Hall et al., 2007). Recently, much work has focused on screening the routes of cellular uptake and sites of action (Perez et al., 2001) as well as cytotoxicity for new Pt-based anticancer compounds (Senerovic et al., 2015). However, there is a lack of understanding in the neural stimulation community of how the Pt species released from platinum-containing stimulating electrodes (as well as other potential damage mechanisms) may affect the health of the surrounding tissue and cells (Merrill et al., 2005). Indeed, it has been suggested that charge injection in conjunction with electrode chemistry, surface area, configuration, and the *in vivo* environment are all important factors to consider interdependently (Green et al., 2014). An easily envisioned *in vitro* setup to address this question involves the stimulation of a platinum electrode in direct proximity to cultured cells. However, there are a number of competing hypotheses explaining observed *in vivo* tissue damage that would also be present as confounding factors in this seemingly simple setup (Agnew et al., 1977; Robblee et al., 1983; Agnew et al., 1990; Morton et al., 1994; Elwassif et al., 2006; Butterwick et al., 2007). It is for this reason that a 'cleaner' assay is of interest: isolating Pt as a mechanism of damage. By developing an assay platform that is capable of isolating causes of stimulation-induced cytotoxicity, future studies can combine factors in order to uncover potential synergistic mechanisms and better define the root causes of stimulation-induced tissue damage.

In order to simplify and expedite the evaluation of the effects of released Pt species from stimulating electrodes, the current manuscript aims to develop an *in vitro* assay of Pt toxicity using glial and neural cell types. In addition to being a platform for stimulation toxicity studies, it is envisioned that this model may be used to help predict *in vivo* tissue damage given a stimulation protocol and electrode geometry by first observing the effects of soluble Pt on relevant cell populations. Here, we quantify the release of platinum species from a stimulated commercial electrode and investigate the dose- and time-dependent cytotoxicity of a model platinum compound on multiple neural cell lines.

2. Materials and methods

2.1. Materials

For cell culture and staining, phosphate-buffered saline (PBS, cat. #14040-133), Dulbecco's modified eagle's medium (DMEM, 11965-092), DMEM/F-12 (11039-021), fetal bovine serum (FBS, 16000-044), penicillin/streptomycin (pen/strep, 15070-063), MEM non-essential amino acids (NEAA, 11140-050), 0.25% trypsin-EDTA (25300-054), Live/Dead Viability/Cytotoxicity Kit (L3224), and Hoechst 33342 trihydrochloride, trihydrate (H1399) were obtained from Life Technologies (Carlsbad, CA). An Apoptotic/Necrotic/Healthy Cells Detection Kit (PK-CA707-30018) was purchased from PromoKine (Heidelberg, Germany). Retinoic acid (R2625), sodium hydroxide (221465), and sulfuric acid (258105) were purchased from Sigma-Aldrich (St. Louis, MO) while ethanol (S25310A) was from Fisher Scientific (Pittsburgh, PA). Platinum ICP standard, 1000 ppm in HCl (RPPT1KH0) was obtained from Ricca Chemical (Arlington, TX). NSC-34 mouse motor neuron-like hybrid cells (CLU140) were purchased from CELLutions Biosystems (Burlington, NC), and BV-2 mouse microglia were generously donated by Dr. Stephen Selkirk of the Louis Stokes Cleveland Department of Veterans Affairs Medical Center.

2.2. Electrode platinum release

A microcentrifuge tube was filled with 200 μL of supplemented DMEM (10% and 1% v/v FBS and pen/strep) and a Deep Brain Stimulation (DBS) electrode (working electrode) was gently lowered into the tube along with a graphite sheet counter electrode immediately after removal from sterile packaging. This supplemented media is identical to the media in which cells are cultured in subsequent experiments. Prior to the experiment, the DBS electrode was washed with soap and rinsed several times with ultrapure deionized water. Both the DBS and graphite sheet electrode were then sterilized via ethylene oxide gas. A commercial DBS lead with Pt electrodes was used, and each electrode contact had a geometric surface area of 6.1 mm^2. Of the four Pt contacts on the electrode, three were submerged in media during the experiment, but only the bottom-most one was stimulated. Electric pulse generation was carried out using an in-house designed and fabricated instrument. The device produces biphasic, cathodic-first, charge-balanced pulse trains at a 50-Hz pulse frequency. Often known as capacitor-coupled stimulation, the device outputs an initial constant cathodic current phase of 100 μs, followed by an open-circuit inter-phase delay of 100 μs, followed by an anodic phase produced by a discharge capacitor (1 μF), which provides the balancing charge. Two stimulation levels were tested: the 100-μs cathodic phases of each had amplitudes of 4.7 mA ($k = 0.57$, charge density of 7.83 $\mu C/cm^2$) and 35 mA ($k = 2.3$, charge density of 57.7 $\mu C/cm^2$). A current-potential (i-E) curve (a.k.a. cyclic voltammogram) was acquired with an Autolab Potentiostat (PGSTAT128N, Metrohm Autolab, The Netherlands), equipped with the linear scan generator module (SCAN250) at 100 mV/s until the i-E curve became stable, starting and stopping at the open circuit potential. The i-E curve was acquired in the same well in which the cells were present. In order to confirm that the potential across the graphite counter electrode did not change during stimulation from its open circuit value, we used the graphite sheet as a working electrode, applied 35 mA current pulses and verified that the interface potential did not change from its open circuit value. We used k from the Shannon plot (Shannon, 1992) to describe the level of charge injection; $k = \log Q + \log D$, where Q is charge (in μC) and D is charge density (in $\mu C/cm^2$). It is also important to note the charge densities given above for each k value in order to fully understand our specific stimulation system. With respect to the pre-clinical data originally used