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Ruthenium oxide based microelectrode arrays for *in vitro* and *in vivo* neural recording and stimulation

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Abstract

We have characterized the *in vitro* and *in vivo* extracellular neural recording and stimulation properties of ruthenium oxide (RuOx) based microelectrodes. Cytotoxicity and functional neurotoxicity assays were carried out to confirm the *in vitro* biocompatibility of RuOx. Material extract assays, in accordance to ISO protocol “10993-5: Biological evaluation of medical devices”, revealed no significant effect on neuronal cell viability or the functional activity of cortical networks. *In vitro* microelectrode arrays (MEAs), with indium tin oxide (ITO) sites modified with sputtered iridium oxide (IrOx) and RuOx in a single array, were developed for a direct comparison of electrochemical and recording performance of RuOx to ITO and IrOx deposited microelectrode sites. The impedance of the RuOx-coated electrodes measured by electrochemical impedance spectroscopy was notably lower than that of ITO electrodes, resulting in robust extracellular recordings from cortical networks *in vitro*. We found comparable signal-to-noise ratios (SNRs) for RuOx and IrOx, both significantly higher than the SNR for ITO. RuOx-based MEAs were also fabricated and implanted in the rat motor cortex to demonstrate manufacturability of the RuOx processing and acute recording capabilities *in vivo*. We observed single-unit extracellular action potentials with a SNR >22, representing a first step for neurophysiological recordings *in vivo* with RuOx based microelectrodes.

Graphical abstract

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Data Availability

The authors declare that all processed data supporting the findings of the present study are available on request.

Conflicts of interest

The authors have no conflicts of interest to declare.

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In vitro microelectrode array recordings

Acute *in vivo* single unit recordings

Keywords

Ruthenium oxide; iridium oxide; multi-electrode arrays; cortical networks; neuronal recording

1. Introduction

Clinical neuroprosthetics rely primarily on metal-based electrode sites which can deliver current or voltage pulses and record neuronal signals with high selectivity for chronic time periods [1-3]. Microelectrode arrays (MEAs) have potential applications as prostheses for sensory and motor deficits in conditions such as vision and auditory loss as well as paralysis [4-6] [7]. Regardless of the application, the microelectrode sites must exhibit relatively low impedance, biocompatibility, and electrochemical stability when exposed to the biological microenvironment. Additionally, for electrical stimulation, microelectrode materials must demonstrate effective charge injection capacities while remaining within a potential range that does not give rise to irreversible faradic reactions that generate neuro- or cytotoxic chemical species at the electrode-electrolyte interface [8].

Previously, metal-based electrodes, which have been used for *in vitro* or *in vivo* neural recording and stimulation studies, have included platinum, tungsten, titanium nitride, indium tin oxide (ITO), and gold. The widespread use of platinum microelectrodes has been limited due to relatively high-impedance, low charge injection capacity, and possible cytotoxicity caused by dissolution of free ions during stimulation [9-11]. Moreover, the high-impedance of candidate materials such as platinum or gold, requires surface modification such as platinization [12], nano-structuring [13-17], or coating with conductive polymers to increase the surface area and improve neural recording and stimulation [18,19]. Metal oxides such as iridium oxide (IrOx) are often used as electrode coatings and have been widely used in neuroprosthetics due to their high charge injection capacity and low impedance compared to other electrode materials. IrOx has been prepared via activation of iridium metal in

electrolyte solutions to form activated iridium oxide films (AIROF) or by reactive sputtering to form sputtered iridium oxide films (SIROF) [20]. Irrespective of the deposition method, the electrochemical properties of the microelectrode are improved after coating.

Another transition metal oxide which is promising for neural applications is ruthenium oxide (RuOx) as it shares similar electrochemical characteristics to IrOx, undergoing reversible faradaic reactions between several oxidation states. RuOx has been previously investigated in supercapacitor [21,22] and biosensing [23] applications, making use of its enhanced electrochemical properties. Prior electrochemical characterization of RuOx microelectrodes demonstrated enhanced charge storage capacity, lower impedance, and a high electrochemically active surface area [24,25]. However, prior studies have failed to provide a detailed evaluation of RuOx as a candidate material for neural interfaces, namely, for neural recording and stimulation.

In the present study, we report a characterization of RuOx as a neural interface. Thin film RuOx microelectrodes were tested for cytotoxicity and functional neurotoxicity to elucidate any acute cytotoxic effects of the material associated with neural tissue contact *in vivo* or in neural cell cultures. We then developed and fabricated planar MEAs consisting of ITO electrode sites coated with RuOx and IrOx for a direct comparison of extracellular recording and stimulation of cortical networks *in vitro*. Lastly, we developed RuOx modified electrode sites on neural probes and demonstrated acute *in vivo* recordings in the rat motor cortex.

2. Methods

2.1 Cytotoxicity and functional neurotoxicity of ruthenium oxide films

2.1.1. Embryonic cortical neuron dissociation and culture—Murine derived cortical neurons were obtained from embryonic age (E14-E16) mice in accordance with The University of Texas at Dallas Institutional Care and Use Committee. Individual embryos were surgically removed via caesarean section from anesthetized time pregnant female mice (ICR-CD1, Envigo RMS Inc, Indianapolis) after euthanasia via cervical dislocation. Embryos were immediately transferred and washed once in ice cold Hanks Balanced Salt Solution (HBSS). Individual embryos were removed from amniotic sacs, decapitated, and stored in ice cold HBSS. Primary cortical neurons were derived from cortices that were dissociated and cultured as described previously. Briefly, surgically isolated frontal cortices sections were pooled in an enzyme dissociation buffer composed of 200 U/ml DNAase and 1X papain reconstituted in HBSS and incubated for 30 minutes at 37°C. Following incubation in enzyme buffer, whole tissue sections were dissociated via mechanical trituration using a 9"-fire polished glass Pasteur pipette until all tissue was visibly homogenously distributed and no aggregates were apparent. Enzyme buffer solution was quenched with 2 ml Dulbecco's Modified Eagle's Medium supplemented with 5% horse serum, 5% fetal bovine serum, and 1% penicillin-streptomycin (SDMEM 5/5). Cells were centrifuged at 300 g for 7 minutes and resulting pellet was resuspended in fresh SDMEM 5/5. Cell count and viability was determined using a hemocytometer and further dilution in cell medium was performed to concentrate 90,000 viable cells in a 5 µL cell seeding solution. 90,000 cells were plated per array by placing 5 µL of the seeding solution at the center of pre-treated electrode arrays and incubated in the cell culture incubator for

approximately 30–45 minutes at 37°C. After cell adhesion, MEA wells were carefully flooded with 600 µL of pre-warmed SDMEM 5/5 and maintained for 48 hours after which serum concentrations were reduced to prevent the over proliferation of non-neuronal cells. Cells were maintained thereafter by 50% medium exchanges every 48 hours for at least a period of 4 weeks.

2.1.2 In vitro cytotoxicity—Cytotoxicity assays using murine-derived primary cortical neurons were carried out in a manner consistent with ISO protocol “10993-5: Biological evaluation of medical devices” and as previously described Charkhkar et al. 2014 [26]. Briefly, the cytotoxicity of RuOx material extract was tested against copper (positive control) and silicon (negative control) material extract. Normalized percent cell viability above 70% deemed the material non-cytotoxic as stated by ISO protocol. 24 well polystyrene plate (Greiner Bio-One, Austria) was pretreated prior to cell seeding with 20 µg/ml laminin and 50 µg/ml poly-d-lysine (PDL) (Sigma-Aldrich, USA). Cortices were surgically dissected from E-18 mouse embryos as described in section 2.1.1 and cells were seeded at a density of 90,000 cells per well. Material extracts were made by soaking 3 cm²/ml films of RuOx on a silicon wafer, positive control, and negative control materials in SDMEM for 24 hours at 10% CO₂, 37°C, and 95% humidity. On DIV 15, cortical cultures were exposed to material extracts for 24 hours at 10% CO₂, 37°C, and 95% humidity. On DIV 16, cells were stained with a LIVE/DEAD cytotoxicity kit (Thermo Fisher, L3324) according to manufacturer protocol and as previously described (Rihani et al. 2018). Epifluorescent 10x images were taken with an inverted microscope (Nikon Ti eclipse, Nikon, Japan). Cells were counted using ImageJ (NIH). A 2.0 Gaussian blur was applied to images and cells were counted based on local intensity maxima. Cells stained with both live and dead markers were identified using a custom MATLAB code. These cells were regarded as apoptotic, and thus transferred from the live count to the dead count during analysis. Cell viability percentages were normalized to the negative control.

2.1.3 In vitro functional neurotoxicity—To determine the effects of RuOx material extracts on spontaneous neuronal network activity, functional *in vitro* neurotoxicity assays were carried out as described previously [26–28]. Previously prepared material extracts of RuOx, silicon (negative control), and copper (positive control) as described in section 2.1.2 were added to DIV 19 cortical networks cultured on commercially available multi-well MEA plates (Axion Biosystems). Briefly, a 10 minute baseline was acquired on DIV 19 followed by a 100% medium exchange and exposure to the prescribed ruthenium oxide extract and positive/negative controls. Cells were incubated with extracts for 48 hours at 10% CO₂, 37°C, and 95% humidity. Following incubation, spontaneous extracellular recordings were carried out at DIV 21. The quantification of functional neurotoxicity relied on metrics of neural activity such as spiking rate and bursts. Only active electrodes were considered for analysis and were defined as a microelectrode having a spiking rate of at least 5 spikes/min. A burst was defined as microelectrode having 5 consecutive spikes with an inter spike interval of 100 ms.

2.2 Fabrication of RuOx microelectrode arrays

2.2.1. Thin film-sputtering technique for Ruthenium oxide and Iridium oxide

—RuOx films were deposited in a custom-modified DC magnetron sputtering system (AJA International, MA). Initially the chamber was pumped down to a pressure of $\sim 5 \times 10^{-7}$ Torr, using a turbomolecular pump. During the deposition, a chamber pressure of 30 mTorr was maintained by throttling a gate valve between the chamber and the system turbopump using the output of a capacitance manometer as feedback for active pressure control. The sputtering gases included argon and a mixture of oxygen and water vapor. For sputtered iridium oxide film (SIROF) deposition, the argon (Ar) gas flow rate into the chamber was maintained at 18 sccm and oxygen (O₂) and water vapor (H₂O) were added at 10 sccm and 30 sccm, respectively. The gas flow rates for RuOx deposition were 20 sccm of Ar, 7.5 sccm of O₂ and 22.5 sccm of H₂O. The gas composition in the plasma was monitored by an MKS e-Vision 2 residual gas analyzer. The film thickness was maintained around 300 nm for both films and was monitored using a profilometer.

2.2.2. Fabrication of planar MEAs and modification of electrode sites with RuOx and IrOx

—MEA fabrication began with commercially available ITO-coated glass (Luminescence Technology Corp., Taiwan) as shown in Fig1. A-C, with side length of 4.9 cm (Fig 1A-C). The ITO film had a resistivity of 15-19 Ω m, and a thickness of 120-160 nm. The ITO was patterned using S1813 photolithography processes and etched with concentrated HCl, to yield 59 circular MEA electrodes, one ground electrode, bond pads, and connective traces. The S1813 resist was removed with acetone, isopropyl alcohol, and water. A negative photoresist, nLOF 2020, was spin-cast on the MEA surface, and patterned using photolithography, to selectively open the resist over approximately 1/3 of the electrode areas. An IrOx film was sputtered on the resist-coated sample. The bulk metal oxide film and resist were removed by soaking in acetone for 4 hours, leaving 1/3 of the MEA electrodes coated with IrOx. A fresh nLOF 2020 film was deposited on MEA and patterned with photolithography processes using a different photomask that selectively opened the resist on a different subset of electrodes. A RuOx film was sputtered on the MEA surface. After liftoff of metal and resist, this resulted in an MEA with 1/3 electrodes coated with RuOx, 1/3 electrodes coated with IrOx, and 1/3 bare ITO electrodes. The electrodes were insulated with a patterned polystyrene film. The MEA substrates were prepared for polystyrene adhesion by dip coating the substrates in a 1% solution of γ -methacryloxypropyltrimethoxysilane (A-174) (Specialty Coating Systems, USA). A 0.04 g/mL solution of polystyrene, average molecular weight $\sim 280,000$ (Sigma Aldrich, USA), dissolved in toluene was spin-cast on the surface of the MEA to yield a polystyrene film that was approximately 500 nm thick. After curing the film on a hotplate for an hour at 90° C, a 100 nm hardmask SiO₂ film was deposited on the MEA by PECVD at 100 °C. The hardmask film protected the polystyrene from solvent exposure in the subsequent steps. The SiO₂ film was patterned with S1813 photolithography and etched with a BOE 7:1 etch to selectively expose the polystyrene film. The polystyrene film was etched using O₂ reactive ion etch (Sirius T2, Trion Technology, USA) at 200 W and 200 mT pressure. Etching the polystyrene selectively removed the insulation to form openings in the insulation over the MEA electrode areas and the bond pads. Remaining photoresist was removed with a blanket UV exposure and resist develop, and the SiO₂ hardmask was removed with BOE 7:1. A

polycarbonate ring with a height of 0.6 cm, 2.0 cm inner diameter, and 2.2 cm outer diameter, was attached to the MEA using a biocompatible silicone adhesive (MED-4213, Nu-Sil, USA). The attached ring contained plated cells and liquid media as it was added to the MEA over the course of cell culture. Just prior to cell culture, the MEA substrate was treated with UV/ozone exposure (UV-1, Samco Inc., USA), with O₂ flow of 0.7 L/min at atmospheric pressure in order to render the polystyrene surface hydrophilic for cell culture.

2.2.3. Electrochemical Characterization—Electrochemical impedance spectroscopy (EIS) measurements were made in a three-electrode cell comprising the ITO, SIROF and RuOx on the MEAs as working electrodes, using a Gamry Reference 600 potentiostat. Ag|AgCl (3M KCl) and a large-area platinum electrode were used as the reference and counter electrodes respectively. EIS measurements were obtained over a frequency range of 1 to 10⁵ Hz using a 10 mV RMS sinusoidal voltage applied about the open circuit potential. Phosphate buffer saline (PBS) solution having a composition of 126 mM NaCl, 22 mM NaH₂PO₄·7H₂O, and 81 mM Na₂HPO₄·H₂O and pH = 7.2, was used as the electrolyte.

2.3 In vitro neuronal recording and stimulation

2.3.1. Microelectrode array preparation—Prior to cell culture, fully fabricated arrays were sterilized under ethylene oxide for a 12-hour cycle. Post sterilization, MEAs were coated with 50 µg/ml poly-D-lysine (PDL) by application of a 5 µL volume solution of PDL to the center of each device and incubated overnight in 10% CO₂, 37°C, and 95% humidity. Subsequently, devices were washed three times with deionized water (DI) and allowed to dry under laminar flow in a cell culture hood. Following treatment with PDL, devices were coated similarly with 20 µg/ml laminin for 2 hours at 37°C. Laminin was removed from all devices prior to cell seeding as described in section 2.1.1., but the surface was not allowed to dry completely.

2.3.2. Spontaneous extracellular recordings and stimulation of cortical networks in vitro—Spontaneous extracellular recordings from murine-derived cortical cultures were carried out starting on DIV 21. For all recording sessions, MEAs were housed in a stage-top environmental control chamber maintained at 37°C, 10% CO₂, and 95% humidity (OKOLAB USA Inc., Burlingame, CA). Neuronal activity was acquired in the form of extracellular voltage recordings simultaneously from all electrode sites over a frequency range of (0.1 Hz–7000 Hz with a sampling rate of 40 kHz using an Omniplex data acquisition system (Plexon Inc., Dallas, TX, USA). Wide band data were filtered using a 2-pole Butterworth bandpass filter (250 Hz – 7000 Hz) and individual spikes were detected in the form of threshold crossings by setting a $\pm 5.5\sigma$ from the RMS noise associated with each recording channel. Only active electrodes were considered for further analysis and were defined as an electrode having a mean firing rate of at least 5 spikes/min. Spike data were resolved into extracellular waveforms or single units based on the 2D principal component (PC) analysis of extracellular waveforms by scanning K-means and identifying distinct clusters in PC space. Analysis of mean firing rates, peak-to-peak amplitude, and signal to noise ratio (SNR) were performed in NeuroExplorer (Version 5, NEX technologies, USA) or via custom matlab scripts. The RMS noise of recording electrodes were acquired by subtracting extracellular action potentials from the original signal based on a $\pm 5.5\sigma$. The

SNR was calculated as the ratio of the peak to peak amplitude of the extracellular waveform and the associated mean RMS noise. The peak to peak amplitude was measured as the difference between the minimum and maximum point of the mean extracellular wave form recorded on each individual channel. For electrical stimulation protocols, PlexStim (Plexon Inc., USA) was used to deliver a biphasic current pulse (cathodal phase first), with a pulse width of 200 μ s, interphase delay of 100 μ s, at 1 Hz frequency. Stimulation was applied to RuOx electrode sites and recorded from other available recording sites on the MEA.

2.4 In vivo neuronal recording

2.4.1 Devices and Surgical Implantation—All procedures performed were approved by the University of Texas at Dallas Institutional Animal Care and Use Committee. An adult Long Evans male rat was implanted with a 4 shank, 16 channel MEA made of amorphous silicon carbide (a-SiC). Shanks were 1.3 mm in length, 20 μ m wide at the base, and 6 μ m thick. Shank pitch was 400 μ m and distance between electrodes was 150 μ m. Electrode sites composed of sputtered RuOx were 200 μ m². The array was connected directly to an Omnetics connector. Surgical procedures were similar to those outlined previously[29]. Briefly, the rat was anesthetized using 2-3% isoflurane and administered an intraperitoneal injection of a 65 mg/ml ketamine, 13.3 mg/ml xylazine, 1.5 mg/ml acepromazine cocktail. The scalp was shaved and cleaned, and the rat was placed into a stereotaxic frame (Kopf 940, Kopf Instruments, USA) and the animal was supplied with a constant flow of 2% isoflurane mixed with 100% oxygen during the surgical procedure. Following a midline incision and cleaning of the skull, 2 bone screws were placed to provide reference and ground contacts for the array. A craniotomy was performed above the left motor cortex and the meninges was resected. Afterwards, the reference and ground wires were wrapped around the 2 bone screws, and the array was implanted at 100 μ m/sec using a pneumatic inserter (Kopf 940, Kopf Instruments, USA). After the neural recording was taken, the rat was sacrificed using a 200 mg/kg intraperitoneal injection of sodium pentobarbital.

2.4.2 Electrophysiological recordings and single unit analysis—Wideband data (0.1-7000 Hz) were collected at a sampling rate of 40 kHz and recorded simultaneously from all 16 microelectrodes for 10 minutes (Omniplex, Plexon, USA). Data were then processed in PlexControl software using a 4-pole, high-pass Butterworth filter with a 250 Hz cutoff frequency. Single unit activity was distinguished using a -4σ threshold below the RMS noise for each individual channel. Separation in principal component space was used to manually sort single units. The SNR was calculated as previously described [17] and additional analysis was performed using NeuroExplorer (Nex Technologies) software and custom MATLAB scripts.

2.5 Statistical Analysis

All statistical analysis was performed in OriginPro software (Origin Lab Corporation, Northampton, MA, United States). For normally distributed data, comparisons between groups were made using a two-sample t-test. For non-normally distributed sets, comparisons between two groups were made using a Mann-Whitney U test and between group effects were measured using a Kruskal-Wallis ANOVA test. Unless otherwise stated, all data are

represented as $\pm$ standard error of the mean (SEM). In all cases, $p < 0.05$ was considered as statistically significant.

3. Results

3.1 In vitro cytotoxicity assay of RuOx using embryonic cortical neurons

To evaluate the presence of any acute adverse biological effects from RuOx material extracts, standard cytotoxicity assays were performed in accordance to ISO 10993-5 using primary embryonic (E15-E18) cortical neurons. Material extracts were prepared by soaking processed and sterilized samples of RuOx films in cell medium for 24 hours and compared to silicon and copper extracts as negative and positive controls, respectively. Following incubation, material extracts were applied to DIV15 primary cortical cultures for 24 hours and a live/dead assay was performed to generate normalized cell viability percentages, normalized to the negative control (silicon). According to the standard cytotoxicity method, in the elution test, a material is deemed non-cytotoxic if the normalized percent cell viability exceeds 70%. RuOx material extract treatment had a normalized percent viability of 104.5 ± 1.6 % (mean $\pm$ SEM, $n=5$ wells) (Fig. 2A, B). In comparison, the negative control (silicon) had a percent viability of 100 ± 2.37 % (mean $\pm$ SEM, $n=5$ wells). The copper exposed medium produced a significantly lower normalized percent cell viability of 0 ± 0 % (mean $\pm$ SEM, $n=5$) (Fig. 2B). Since treatment with material extracts eluted from RuOx passed the 70% threshold of normalized percent cell viability, the data suggest that the RuOx is non-cytotoxic to neuronal tissue supporting its biocompatibility as a neural interface material.

3.2 MEA-based in vitro functional neurotoxicity assay of ruthenium oxide using embryonic cortical networks

Embryonic cortical neurons cultured on MEAs exhibit spontaneous network formation *in vitro*. Over time in culture, extracellular action potentials can be detected non-invasively from these networks to serve as a function-based assay. Over 21 *days in vitro*, such patterns of activity emerge in the form of tonically firing and/or bursting that is indicative of synaptic formation and neurotransmission (Fig. 3A). Therefore, we used cortical networks to test the effects of RuOx material extracts on intrinsic and bursting activity of neuronal networks to evaluate the presence of neurotoxicity. A 10 min baseline recording of spontaneous activity at DIV19 was acquired using a multi-well recording system (Axion Maestro, Axion Biosystems). Material extracts of RuOx, silicon (negative control), and copper (positive control) were prepared as previously described, applied to respective treatment groups, and allowed to incubate for 48 hours. Post-incubation, a second recording was acquired and activity metrics such as mean firing rate (MFR) and burst frequency (BF) were computed and compared to baseline levels. The MFR of RuOx, silicon, and copper after treatment resulted in normalized MFRs of 94.2 ± 14.7 , 104.2 ± 11.4 , and 0.0219 ± 0.0105 (mean $\pm$ SEM, $n = 3$ wells), and BF of 87.9 ± 16.8 , 112.2 ± 17.7 , 0 ± 0 (mean $\pm$ SEM, $n = 3$ wells), respectively. In comparison to the negative control, RuOx failed to affect both MFR and BF of cortical cultures based on a Mann-Whitney U test ($Z(700) = -0.51$, $p = 0.61$ for MFR and $Z(883) = 1.30$, $p = 0.19$ for BF). In contrast, treatment with copper material significantly inhibited both spontaneous MFR and BF after a 48 hour period ($p < 0.001$) (Fig. 3A, B).

3.3 Electrochemical characterization of RuOx microelectrodes

Post-fabrication, *in vitro* MEAs (see methods section and Fig. 1A-C) composed of ITO, IrOx, and RuOx microelectrode sites were assessed in terms of their electrochemical performance using electrochemical impedance spectroscopy and cyclic voltammetry. Representative cyclic voltammograms for the IrOx, RuOx and ITO electrodes are compared in figure 4A at a near equilibrium scan rate of 50 mV/s. The current response for ITO is considerably lower in comparison to both IrOx and RuOx, reflecting the limited charge available from the capacitive double-layer charging of the ITO compared to the faradaic redox capacity of the oxide films[30]. The cathodal charge storage capacity (CSCc) calculated from the CVs for these electrodes demonstrate the higher CSCc of IrOx and RuOx compared to ITO. The CSCc at 50 mV/s for IrOx and RuOx were $59 \pm 38 \text{ mC/cm}^2$ (mean $\pm$ STD, $n = 19$ microelectrodes) and $49 \pm 20 \text{ mC cm}^2$ (mean $\pm$ STD, $n = 17$ microelectrodes), respectively. No significant difference was found in the CSCc between RuOx and IrOx ($p = 0.47$, Two sample t-test) microelectrodes. In comparison, the CSCc of ITO was found to be $1 \pm 2 \text{ mC/cm}^2$ (mean $\pm$ STD, $n = 19$ microelectrodes). Figure 4B illustrates the Bode representation of the average impedance spectra for the three microelectrode material types. The average impedance, as measured in PBS, at 1 KHz was found to be $1.2 \pm 0.5 \text{ M}\Omega$ (mean $\pm$ STD, $n = 29$ microelectrodes), $166.1 \pm 103.1 \text{ k}\Omega$ (mean $\pm$ STD, $n = 30$ microelectrodes), and $73.83 \pm 59.91 \text{ k}\Omega$ (mean $\pm$ STD, $n = 22$ microelectrodes) for ITO, IrOx, and RuOx respectively measured from two arrays. A Kruskal-Wallis ANOVA test revealed statistically significant differences in the impedance values associated with the three different microelectrode types ($\chi^2(2) = 54.85$, $p < 0.001$). Additional post-hoc tests revealed a significant reduction in impedance of both RuOx and IrOx compared to ITO ($Z(5) = -5.96$, $p < 0.001$ for RuOx and $Z(32) = -6.10$, $p < 0.001$ for IrOx, Mann-Whitney U test). In total, our data suggest that the electrochemical properties of RuOx were comparable to that of IrOx in terms of increased cathodal charge storage capacity and decreased impedance compared to native ITO sites.

3.4 In vitro neuronal recording and stimulation

To evaluate the performance of RuOx-modified electrodes to record and evoke neuronal activity, murine-derived cortical networks were cultured on $n=4$ substrate integrated MEAs and spontaneous extracellular recordings were performed at 21 days *in vitro*. For a direct comparison of performance of RuOx to IrOx and ITO, 59 available ITO microelectrode sites were “split” between 20 electrode sites modified by sputtering IrOx on ITO sites, 19 electrode sites modified by sputtering RuOx, and 20 electrodes maintained as ITO on a single array. Figure 5(A) illustrates DIV21 cortical neurons cultured on an array consisting of distinct electrode sites of RuOx, IrOx, and ITO. For all cultures, we observed excellent cellular adhesion and physical extension of neurites from individual neurons across all three electrode sites. Spontaneous extracellular recording sessions on DIV21 taken simultaneously from all electrode sites revealed the ability to discriminate well-resolved, all-or-nothing, extracellular action potentials recorded from all three electrode types (Fig. 5B). On further discrimination of single action potentials (unit sorting), extracellular waveforms consistent with the time course, amplitude, and shape of extracellular action potentials were readily observed on RuOx and IrOx-modified microelectrode sites (Fig. 5C). Similar results were observed on ITO sites. Additionally, the signal-to-noise ratio (SNR) acquired from both

RuOx and IrOx-modified sites (SNR = 9) was modestly higher when compared to ITO sites (SNR = 7) ($Z(11) = 2.28$, $p = 0.0225$, Mann-Whitney Test). However, the SNR of RuOx and IrOx sites were comparable and no significant differences were observed between these coatings ($Z(12) = 0.240$, $p = 0.810$, Mann-Whitney Test).

We further evaluated the capability of delivering a current stimulus through RuOx microelectrode sites to evoke neuronal activity. When biphasic current pulses (cathodic leading phase) at a frequency of 1 Hz, interphase delay of 100 μ s, and pulse width of 200 μ s with 20 repetitions was delivered through a RuOx site, evoked neuronal responses were recorded from adjacent electrode sites. In response to a 20 μ A current stimulus, bursting activity was readily observed with action potential amplitudes exceeding 30 μ V (Fig. 5D). We found the latency of the evoked action potentials to be 5.6 ± 0.8 ms (mean $\pm$ STD, $n = 10$ stimuli), which was found to be consistent with the onset of evoked action potentials following electrical stimulation of *in vitro* preparations [31]. Additionally, we investigated the voltage transients produced by RuOx and ITO microelectrodes in response to cathodal-leading biphasic current pulses (20 μ A, 200 μ s/phase, 100 μ s interpulse interval) which was previously observed to elicit neural activity (Fig. 5E). The benefit of RuOx over ITO electrodes for stimulation capabilities is readily apparent, as the driving voltage required to inject a stimulus pulse was considerably lower for RuOx. For example, the average driving voltage for bare ITO was reduced from 1.63 ± 0.5 V (mean $\pm$ STD, $n = 13$ microelectrodes) to 0.95 ± 0.3 V (mean $\pm$ STD, $n = 8$ microelectrodes). In total, these data suggest that RuOx-modified electrodes sites demonstrate a significant improvement in neuronal recording and stimulation capability compared to ITO, and are comparable to IrOx.

3.5 Acute in vivo single unit recordings

We also investigated the ability of RuOx-microelectrodes to record neural signals in an acute *in vivo* study of spontaneous neural activity in rat motor cortex. A 4 shank, 16 channel MEA composed of 200 μ m² sputtered RuOx microelectrode sites was implanted unilaterally in the motor cortex of an adult male Long Evans rat (Fig. 6A). Figure 6(B) shows filtered continuous data from three representative channels demonstrating individual bursting patterns of firing as well as apparent network level activity with a robust SNR from 9.9-52.5 with an average of 22.1 ± 3.9 (mean $\pm$ SEM). The active electrode yield was 56%, with 10 units recorded across 9 channels demonstrating the ability to record from multiple distinct neurons. On further analysis of the single action potentials, spikes were discriminated (sorted) via clusters in principle component space (Fig. 6C) illustrating distinct neurons with extracellular waveforms consistent with the time course, shape, and amplitude of extracellular action potentials. Furthermore, the RMS noise was relatively low and similar across channels (4.9 ± 0.1 μ V, mean $\pm$ SEM). Overall, these data suggest that RuOx microelectrodes can stably record localized spontaneous multi-unit neural activity *in vivo* under acute preparations with relatively low noise and excellent SNR.

Discussion

In the present study we explored the electrochemical performance, neural recording and stimulation capability of RuOx microelectrodes. A significant concern for the use of neural

interfaces *in vitro* or *in vivo* is the interaction of the electrode material with the neural tissue. If the material is unstable and produces chemical species in the surrounding tissue, possible cytotoxic and neurotoxic outcomes may impede chronic recording or stimulation. To evaluate RuOx as a potential biocompatible electrode, cytotoxicity and neurotoxicity assays were conducted with murine-derived cortical networks to directly assess the potential for acute toxicity. Material extract treatment of RuOx, carried out in accordance with ISO protocol 109935, caused no significant change in cell viability nor modulated the spontaneous neural activity of cortical networks. Consistent with these results, RuOx was deemed non-cytotoxic and may cause no potential local toxicity via corrosion and dissolution in the surrounding tissue, at least in acute studies.

To determine the feasibility of RuOx as a neural interface for recording and stimulation, *in vitro* substrate integrated MEAs were initially fabricated and consisted of ITO sites which were modified via reactive sputtering of IrOx and RuOx. Examination of the electrochemical performance, as compared to bare ITO electrode sites, revealed a significant reduction in impedance in RuOx and IrOx-modified electrodes. Prior studies have demonstrated via scanning electron microscopy and atomic force microscopy that functional SIROF sites exhibit reduced impedance due to the rough and porous structure of the site that increases the effective electrochemically active surface area of the electrode [31]. Similar impedance reductions were observed for RuOx that appeared to be significantly lower and may be attributable to a similar roughness and porosity (Fig. 1C) [8,20,30]. To our knowledge, only one prior study has reported impedances for RuOx films deposited via electrochemical deposition and atomic layer deposition. Albeit our method was reactive sputtering, the study reported impedances consistent with the present study (approximately 100 k Ω) [25].

The low impedance of RuOx microelectrodes was associated with an apparent improved recording capability compared with the uncoated ITO electrodes. The SNR of the top 25% of recorded RuOx sites exceeded 11. This was comparable to the IrOx electrode sites and, in our present study, the SNR was approximately 12 for the top 25% of the electrodes. Preliminary current stimulation of cortical networks using RuOx microelectrodes demonstrated typical evoked bursting behavior of neurons recorded from adjacent electrodes. We additionally conducted acute neuronal recordings using a multi-shank device consisting of 16 RuOx electrode sites implanted in the rat motor cortex. We observed individual and bursting activity on >9 channels with a mean peak-to-peak amplitude of 106.7 ± 19.1 μ V (mean $\pm$ SEM), relatively low noise, with an excellent SNR > 20. To further extend the utility of RuOx as a neural interface, future studies will be needed to investigate the chronic reliability of RuOx *in vivo* and to elucidate the long-term recording and stimulation capabilities of RuOx-coated electrodes.

We have investigated the cytotoxicity and electrochemical properties of RuO_x relevant to its use as a coating for neural stimulation and recording electrodes. Our findings suggest that the overall suitability, electrochemical performance, and recording capability are similar to those of IrOx. One potential advantage of ruthenium over iridium is the reduced cost of the metal sputter target. Additionally, ruthenium oxide is of interest because of the multiple redox states of the oxide that are accessible within the electrochemical potential limits for water electrolysis[30,32]. These redox states result in a high charge-storage capacity that

may result in improved stimulation charge-injection properties compared with iridium oxide. More importantly, the present study supports the further use and investigation of alternate transition metal oxides which exhibit enhanced electrochemical performance and may be used in acute and chronic neural interface technology.

Although the present study highlights the capability and functionality of RuOx as an electrode coating, its chronic reliability is yet to be determined. Ullah et al. (2015) reported on the long term stability of a bi-metal composite of iridium/ruthenium oxide (Ir/Ru-oxide) for neural electrode applications. The composite was found to have a 56% greater charge storage capacity, compared to IrOx alone, and displayed stability under intense electrochemical cycling with a 5V potential window. This suggests that the addition of ruthenium oxide may prevent degradation and that RuOx electrode coatings may be suitable for chronic neural interfaces (Ullah and Omanovic, 2015). Additionally, prior studies of the electrochemical performance of ruthenium oxide electrodes have reported high charge storage capacities, high electrical conductivity, and a large electrochemically active surface areas [22,25,33-37]. While these and our studies report promising results, future studies are necessary to determine the chronic reliability of implantable RuOx electrodes for recording and stimulation applications.

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Statement of Significance

A critical challenge in neural interface technology is the development of microelectrodes that have recording and electrical stimulation capabilities suitable for bidirectional communication between the external electronic device and the nervous system. The present study explores the feasibility and functional capabilities of ruthenium oxide microelectrodes as a neural interface. Significant improvement in electrochemical properties and neuronal recordings are reported when compared to commercially available indium tin oxide and was similar to that of iridium oxide electrodes. The data demonstrate the potential for future development of chronic neural interfaces using ruthenium oxide based microelectrodes for recording and stimulation.

Fig 1.

Fabrication of RuOx based microelectrode arrays for in vitro neuronal recording and stimulation. (A) Assembly and fabrication steps of MEAs. Approximately 1/3 of the sites were modified with sputtered IrOx, 1/3 of the sites were modified with sputtered RuOx, and 1/3 were left as bare ITO. (B) Complete distribution of three electrode materials on the single array. Red sites correspond to ITO, orange sites correspond to IrOx, and blue sites correspond to RuOx sites. (C) Scanning electron microscopy image of RuOx deposited on and cleaved from a silicon wafer. Horizontal scale bar indicates 200 nm. (D) Scanning electron microscopy image of fully fabricated IrOx microelectrode. (E) Scanning electron microscopy image of fully fabricated RuOx microelectrode. Horizontal scale bar indicated 15 μm .

Fig 2.

Cytotoxicity assay of RuOx material extract using primary cortical neurons. (A) Representative Epifluorescent image of DIV15 primary cortical neurons following incubation with calcein-AM (green) and ethidium homodimer (red) indicating live and dead cells, respectively, after prior incubation with RuOx material extracts for 24 hours *in vitro*. Scale bar represents 150 μm . (B) Normalized mean percentage cell viability quantified from the live/dead assay in the presence of negative control (silicon), positive control (copper), and RuOx material extracts. Error bars denote SEM for $n= 5$ replicates. The blue dashed line indicates the 70% threshold which denotes the “passing” criteria and qualifies a material as non-cytotoxic and biocompatible based on ISO 10993-5.

Fig 3.

Functional neurotoxicity assay of primary cortical networks following RuOx material extract treatment. (A) Representative raster plots of spontaneous activity from a single well (n=16 electrodes) before exposure (baseline) and after exposure (treatment) for 48 hours with material extract from RuOx, silicon (negative control), and copper (positive control). Wavelets of activity represented by collection of blue dashes is indicative of single channel bursts. Red dashed line indicates the 48 hour treatment period with respective material extracts. (B) Mean firing rates (mean $\pm$ SEM) expressed as percentage of baseline after 48 hour incubation with material extracts from RuOx and positive/negative controls. (C) Burst frequency (mean $\pm$ SEM) expressed as percentage of baseline after 48 hour incubation with material extracts from RuOx and positive/negative controls. Statistical tests performed via Mann-Whitney U test and RuOx treatment were compared to positive and negative controls. (***) indicates $p < 0.001$, N.S. indicates no significant difference $p > 0.05$).

Fig 4. Electrochemical characterization of 30 μm diameter ITO, IrO_x, and RuO_x microelectrode sites. (A) CV at 50 mV/s scan rate. (B) Typical average impedance magnitude spectra of ITO (n=29), IrO_x (n=30), and RuO_x (n=22). Data are reported as mean $\pm$ STD.

Fig 5.

In vitro neuronal recording and stimulation of cortical networks using RuOx microelectrodes. (a) DIV21 murine-derived cortical networks cultured on MEA substrates with RuOx (top), IrOx (middle), and ITO (bottom) electrode sites on a single array. (b) Representative raw traces of filtered continuous data on DIV21 from RuOx (top), IrOx (middle), and ITO (bottom) electrodes demonstrate detection of well-resolved extracellular action potentials from neuronal cells. (c) Mean extracellular waveforms consistent with time and shape of extracellular action potentials recorded from single representative RuOx (top, blue and green waveforms represent two distinct units as identified in PC space) and IrOx (bottom, blue and red waveforms represent two distinct units as identified in PC space) electrode sites. (D) Representative raw trace of evoked bursting activity in response to a biphasic current stimulus (cathodic leading edge, 200 μ s pulse width, 20 stimuli at 1 Hz frequency) applied through a RuOx microelectrode. Red arrow indicates the beginning of the current stimulus and inset displays single evoked action potentials > 30 μ V in response to a single 20 μ A current pulse. (E) Representative voltage transients recorded on RuOx and ITO microelectrode sites in response to a 20 μ A current with pulse width of 200 μ s.

Fig 6.

Acute in vivo implantation and recording from rat motor cortex using a 4-shank, 16-channel array with RuOx coated sites. (A) Surgical set up and implantation of a 4 shank, 16 channel array made of amorphous silicon carbide, consisting of 16 RuOx microelectrode sites. (B) Representative filtered continuous data from 3 channels indicating both individual and network level activity. (C) Representative single-unit sorting based on amplitude and separation in 2D principal component space. (D) Resultant waveforms from single-unit sorting demonstrating multi-unit activity from a single electrode. Blue and green color codes represent distinct extracellular waveforms, in terms of amplitude and time course, as identified in PC space.