Supplementary information

Hierarchical Surface Restructuring of Ultra-Thin Electrodes and Microelectrode Arrays for Neural Interfacing with Peripheral and Central Nervous Systems

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Supplementary experimental details

Biocompatibility Studies

a) *In vitro* cytotoxicity

In vitro elution tests were performed according to ISO 10993-5:2009(E) for cytotoxicity assessment, evaluating the viability of mammalian cell cultures exposed to extracts prepared from restructured Pt10Ir electrodes. For this study, L929 murine fibroblast cell line (obtained from ATCC, NCTC clone 929) was chosen as the test system due to its sensitivity and reliability in assessing cytotoxic effects. To prepare the cells for testing, L929 cells were cultured in Complete Minimum Essential Medium (Eagles) with Earle's Balanced Salts, supplemented with 10% fetal bovine serum and an antibiotic-antimycotic solution. Confluent stock cultures were trypsinized and re-plated in 35 mm sterile, tissue culture grade petri plates and incubated for a minimum of 24 hours at 37 ± 1°C in a humidified atmosphere with 5 ± 1% CO_2 to achieve an approximately 80% confluent monolayer. Four electrodes with a surface area of 12 cm^2 were incubated in extraction medium at a ratio of 6 cm^2/ml. As part of the experiment, a positive control was established using a ratio of 0.2 gm/ml of latex rubber to extraction medium, while a negative control utilized a ratio of 3 cm^2/ml of the USP High-Density Polyethylene Reference Standard to extraction medium. Additionally, a blank sample containing only the extraction

medium was prepared. After the extraction period, the extracts were gently agitated and transferred into dry, sterile vessels without any abnormalities or particles observed. The test extract, negative control extract, and vehicle blank were clear and particle-free, while the positive control extract was cloudy, which is considered normal. Therefore, no further processing was needed, and the extracts were immediately used for testing. Following incubation, cultures with approximately 80% confluency and healthy morphology were selected for testing. The extraction medium was removed from each monolayer, and 2 ml of test extract, positive control, negative control, and blank were added to their respective 35 mm plates in triplicate. All plates were then placed back in incubation at 37 ± 1°C with 5 ± 1% CO_2 in air for 48 ± 2 hours. After incubation, qualitative morphological reactivity was assessed using the grading system outlined in Table S1.

Table S1: The grading system used to determine qualitative morphological cytotoxicity of the plates following incubation

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmic granules; no cell lysis, no reduction of cell growth.
1	Slight	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules or show changes in morphology; occasional lysed cells are present; only slight growth inhibition observable.
2	Mild	Not more than 50% of the cells are round, devoid of intracytoplasmic granules, no extensive cell lysis; not more than 50% growth inhibition observable.
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition observable.
4	Severe	Nearly complete or complete destruction of the cell layers

b) Intradermal Reactivity

An intracutaneous (intradermal) reactivity test was performed according to ISO 10993-10:2010(E) to assess the potential of the electrodes to produce irritation following a single intradermal injection of extracts prepared from restructured Pt10Ir electrodes. Healthy, young adult albino rabbits from a single strain (NZW/SPF), female, weighing not less than 2 kg, previously unused for skin irritation studies, and whose skin was free from mechanical irritation or trauma were used as the test system in this study. Caging and caring of the animals were done in compliance with the Animal Welfare Act, USDA (1995 and subsequent revisions) and the guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, 2011. Test article extracts were prepared following ISO 10993-12 guidelines. Each test article had a surface area of 12 cm^2, with a 6 cm^2/ml ± 10% ratio of test article to extraction medium applied. The intact test articles were placed in type I glass extraction vessels, with six articles per extract immersed in the required volume (72 cm2/13.2 ml) of USP saline (polar) and vegetable oil (non-polar) extraction medium. Controls consisting of

only extraction media only were also prepared. The extraction was performed by heating the vessels to 37 ± 1°C for 72 hours ± 2 hours. After the extraction period, the extracts were shaken or agitated and aseptically transferred into dry, sterile vessels. The saline and vegetable oil test and control extracts were clear and particle-free upon transfer. These extracts were stored at room temperature and utilized within 24 hours.

Four to eighteen hours prior to the test, the fur on each animal's back was closely clipped on both sides of the spinal column to expose a sufficient test area. The extracts were agitated, and each of three rabbits received intracutaneous injections of 0.2 ml of the polar (saline) test extract at five sites on the right side of the spinal column, and similarly, the non-polar (vegetable oil) test extract at five sites on the same side. This process was repeated on the opposite side using corresponding polar and non-polar blanks as reagent controls. The positive control (0.1% sodium dodecyl sulfate in 0.9% sodium chloride) was intracutaneously injected with 0.2 ml at five sites on the right side of the spinal column, while the negative control (saline diluent) received 0.2 ml intracutaneously at five sites on the left side. Immediate examination and follow-ups at 24 ± 2, 48 ± 2, and 72 ± 2 hours post-injection assessed gross evidence of tissue reaction (erythema and edema) at the injected sites. Observations were rated on a numerical scale, utilizing the scoring system in Table S2. The score for each test sample or blank on an individual animal was determined by dividing each total by 15 (3 scoring time points x 5 test or blank sample injection sites). Mean scores were calculated across the 3 animals, and the average scores from the blanks were subtracted from the treated scores for final evaluation.

Table S2: Scoring system for evaluation of skin reactions, as defined in ISO 10993-10:2010(E)

Erythema and Eschar Formation	**Score**
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate erythema	3
Severe erythema (beet-redness) to eschar formation preventing grading of erythema	4
Edema Formation	**Score**
No edema	0
Very slight edema (barely perceptible)	1
Well defined edema (edges of area well defined by definite raising)	2
Moderate edema (raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond exposure area)	4

c) Skin Sensitization

Skin sensitization tests were carried out to determine the potential of the electrodes to elicit contact dermal allergenicity. The Guinea Pig Maximization Test (GPMT) was used to assess sensitization, as defined in ISO 10993-10:2010(E). Healthy, young adult, albino guinea pigs from

a single outbred strain (Dunkin Hartley) weighing between 300-500g and previously unused for testing were used. Female animals were nulliparous and not pregnant. The animals were acclimated to the laboratory conditions for a minimum of five days before the start of the experiment. Caging and caring of the animals were in compliance with the Animal Welfare Act, USDA (1995 and subsequent revisions) and the Guide for the Care and Use of Laboratory Animals, National Research Council, 2011. Extracts of the electrode test articles were prepared in accordance with ISO 10993-Part 12: Biological Evaluation of Medical Devices - Sample Preparation and Reference Materials. Each electrode extract was prepared as above in the intradermal reactivity tests. The 1:1 mixture of vehicle/ being round and the test extract was subsequently homogenized by mixing until emulsified. Prior to the intradermal induction phase, the intrascapular region of each of the 45 guinea pigs was shaved and cleansed with isopropyl alcohol. Injections were done in a single row of three injections on each side of the midline, within a 4 x 6 cm area. Injection #1 was administered most cephalic, injection #3 was most caudal and injection #2 was between #1 and #3 but noticeably closer to #1. The negative and positive controls were injected in the same manner. Twenty-four hours before topical exposure during the induction phase, the injection sites were shaved and treated with 10% sodium dodecyl (lauryl) sulfate in petrolatum. The topical induction phase began 7 days after the intradermal induction phase during which a 2 x 4 cm gel blot paper soaked with the electrode test extract (0.5 mL) was placed over the injection sites and covered with an adhesive bandage. The challenge phase commenced 15 days after completing the topical induction phase. Immediately before the challenge phase, the flanks of all guinea pigs were clipped free of hair. On the right flank, electrode test extract (0.25 mL) was applied to both the test and negative control groups, while the left flank received a patch with the vehicle alone. For the positive control group, 0.1% dinitrochlorobenzene in propylene glycol was applied to the right flank, and propylene glycol alone was applied to the left flank. Patches remained on injection sites for 48 hours. Skin evaluations for adverse reactions were conducted 24 and 48 hours after bandage removal, following the criteria listed in Table S3.

Table S3:Magnusson and Kligman scale for evaluation of adverse reactions in the skin sensitization studies, as defined in ISO 10993-10:2010(E)

Reaction	Grading Scale
No visible change	0
Discrete or patchy erythema	1
Moderate and confluent erythema	2
Intense erythema and/or swelling	3

Supplementary figures

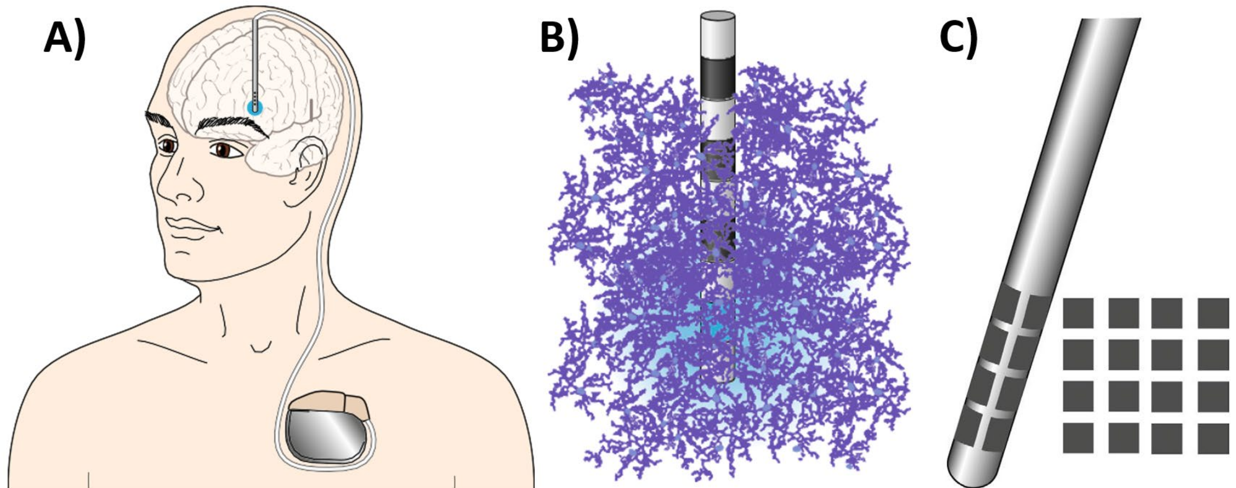

Figure S1: Schematic of, A) an implantable pulse generator and a four-contact cylindrical microelectrode array implanted near the subthalamic nucleus (Photo reproduced with permission from Cagnan, H [1]); B) four-contact cylindrical microelectrode array implanted near the subthalamic nucleus which contains ~ 250,000 neurons (illustrated in blue) that are much denser in reality than shown on the image (Photo reproduced with permission from Cagnan, H [1]); c) sixteen-contact flat microelectrode array developed for current steering or directional stimulation [1,2] (Photo reproduced with permission from Cagnan, H [1])

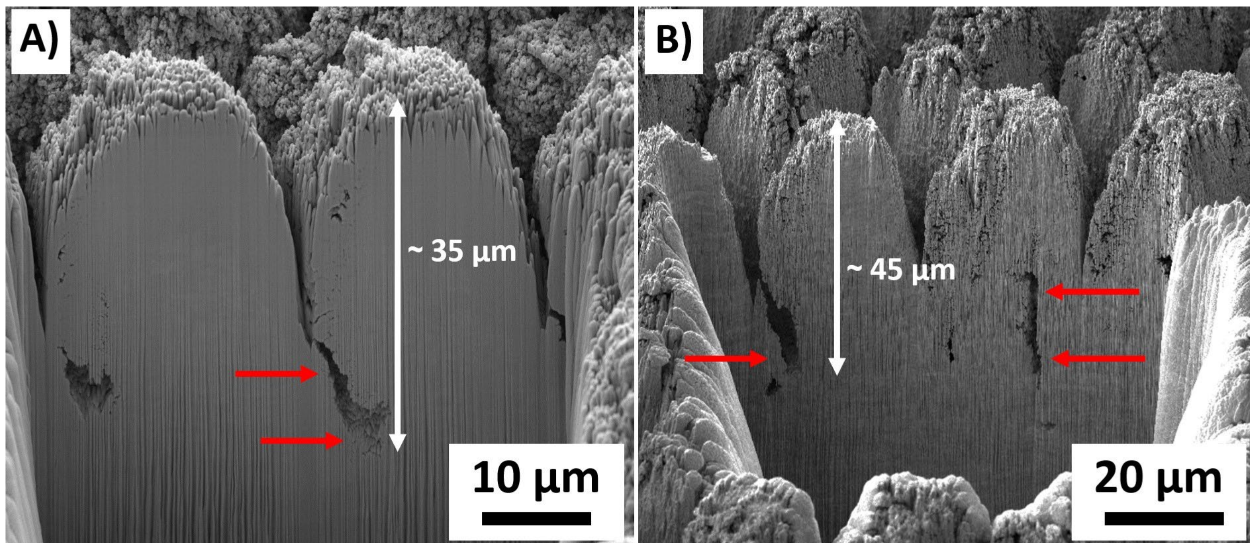

Figure S2: FIB cross-sections of a hierarchically restructured Pt10Ir electrode fabricated at, (A) 3.33 kJ/cm^2 fluence with an average restructuring depth of ~35 µm [3], and, (B) 3.39 kJ/cm^2 fluence with a depth of restructuring of ~ 45 µm [4]; Red arrows show subsurface features that are likely attributed to laser shock waves during femtosecond laser hierarchical surface restructuring.

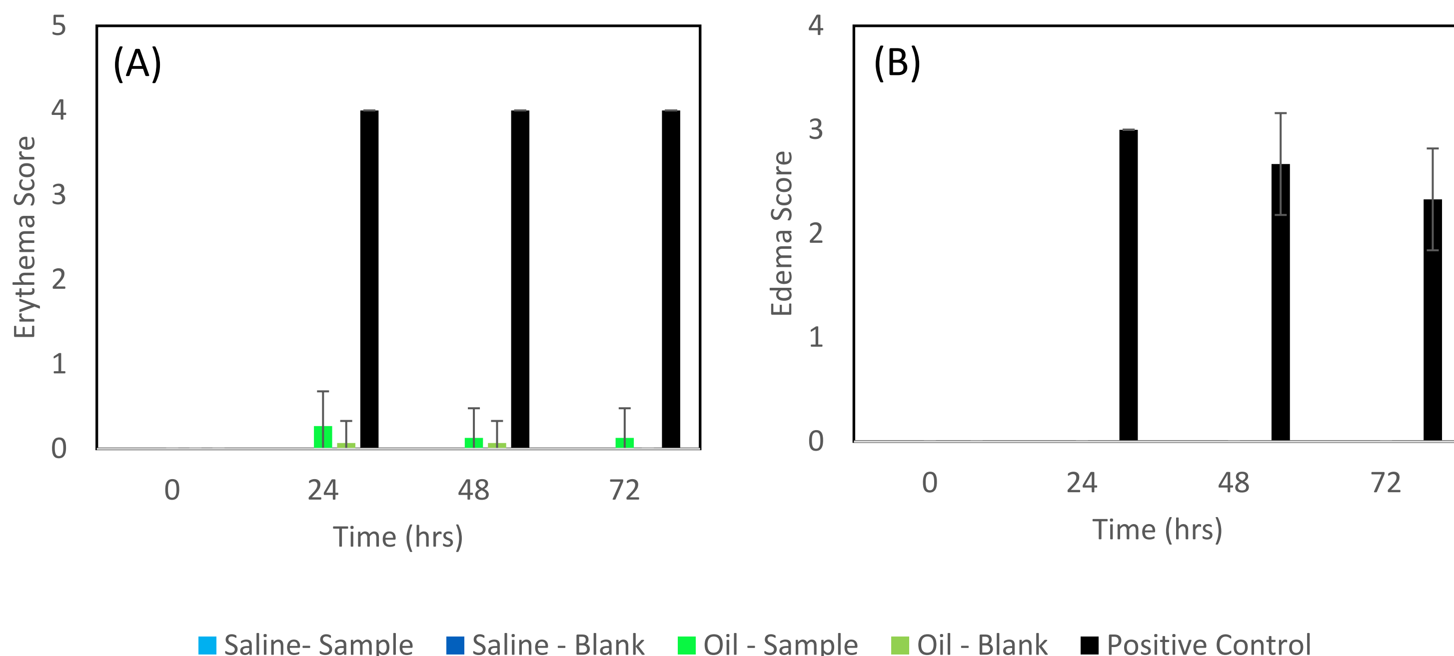

Figure S3: In vivo skin irritation assays. Intradermal irritation in a rabbit model system was evaluated for aqueous and oil-based extracts from restructured Pt10Ir electrodes according to ISO 10993-10(2010). Irritation was determined by evaluating, A) erythema (redness, top panel), and, B) edema (swelling, bottom panel) at the site of injection. Negative controls were vehicle only, while positive controls consisted of 0.1% sodium dodecyl sulfate injections. Data are an average of three injections of each experimental condition per animal across 6 total animals.

Supplementary references

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4 Amini, S., Seche, W., May, N., Choi, H., Tavousi, P. & Shahbazmohamadi, S. Femtosecond laser hierarchical surface restructuring for next generation neural interfacing electrodes and microelectrode arrays. *Sci Rep* **12**, 13966 (2022). https://doi.org:10.1038/s41598-022-18161-4