An in-vitro system for closed loop neuromodulation of peripheral nerves

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***Abstract*— Current neuromodulation research relies heavily on in-vivo animal experiments for developing novel devices and paradigms, which can be costly, time-consuming, and ethically contentious. As an alternative to this, in-vitro systems are being developed for examining explanted tissue in a controlled environment. However, these systems are typically tailored for cellular studies. Thus, this paper describes the development of an in-vitro system for electrically recording and stimulating large animal nerves. This is demonstrated experimentally using explanted pig ulnar nerves, which show evoked compound action potentials (eCAPs) when stimulated. These eCAPs were examined both in the time and velocity domain at a baseline temperature of 20°C, and at temperatures increasing up to those seen in-vivo (37°C). The results highlight that as the temperature is increased within the in-vitro system, faster conduction velocities (CVs) similar to those present in-vivo can be observed. To our knowledge, this is the first time an in-vitro peripheral nerve system has been validated against in-vivo data, which is crucial for promoting more widespread adoption of such systems for the optimisation of neural interfaces.**

I. INTRODUCTION

Neural stimulation is used extensively in medical and therapeutic contexts, including in the treatment of drug-resistant epilepsy via vagus nerve stimulation [1]. However, a crucial limitation in the majority of these systems is that stimulation is typically conducted without measuring the resultant neural response, meaning that if the efficacy decreases over time, it is difficult to investigate the underlying problem. A novel approach to address this issue is closed loop neuromodulation, which aims to record the elicited neural response and thus enable adaptive stimulation.

In electrical neuromodulation for the peripheral nervous system, different electrode configurations and geometries are used, such as intrafascicular electrodes and multi-electrode cuffs. Depending on the invasiveness of the device, recordings often contain a combination of activity from individual fibres, particularly when using extraneural electrodes. This compound activity is referred to as the electrically-evoked compound action potential (eCAP) when elicited by stimulation, or simply CAP when naturally occurring.

The most common method to validate and test novel neural interfaces and configurations is via in-vivo experiments on animals. As an alternative, in-vitro systems have been proposed, which aim to replicate the in-vivo environment at a miniaturised scale in order to grow and maintain cells or tissues [2]. These have been investigated using cell cultures, but not as much from the perspective of explanted whole nerves and tissues [3], [4], [5]. Whole nerve in-vitro models would be highly beneficial in reducing the overall number of animals required for optimising neural interfaces and maximising the overall volume of nerves to be examined, particularly for non-recovery experiments where typically only a single nerve is analysed in-vivo. In all, there has yet to be an in-vitro platform proposed for investigating closed loop neuromodulation using cuff electrodes on large nerves, and that considers nerve viability over time.

More importantly, an in-vitro large nerve platform should be validated against in-vivo recordings to verify how closely the nerve response matches that in its natural environment, and hence whether it is a suitable complement for in-vivo experiments. There are several stages in the development and optimisation of neural interfaces, such as the development of electrode geometries or sizes, and choice of amplifiers, which would benefit from being trialled initially in an in-vitro preparation prior to further in-vivo studies. To our knowledge, an in-vitro system to address this challenge has not been explored yet.

Thus, this paper proposes an approach for developing and testing an in-vitro system capable of sustaining explanted peripheral nerves for up to five hours. This approach was validated using an explanted pig ulnar nerve, for which multiple sets of experiments were run. Initially, the nerve response at a baseline temperature of 20°C was investigated, following prior work on a similar large nerve in-vitro model [3]. Experiments were also conducted at temperatures increasing up to those seen in-vivo (37°C). Furthermore, these recordings were compared to in-vivo recordings using the same stimulation and recording configuration, to assess the similarity between both experimental setups. This was achieved by examining the data both in the time and velocity domain. With increasing temperature, the results highlight an increase in eCAP conduction velocity (CV) approaching that seen in-vivo.

II. METHODS

A. In-vitro system

An in-vitro system was devised to hold explanted animal nerves and conduct both electrical stimulation and recording using cuff electrodes, as shown in Fig. 1.

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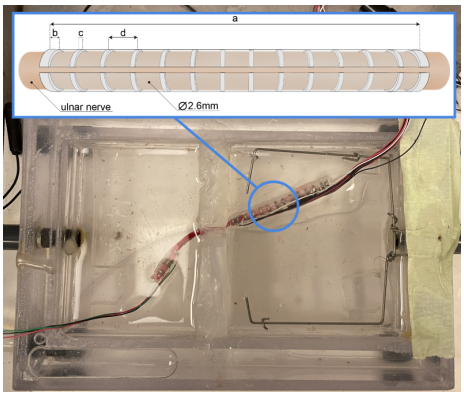

Fig. 1. In-vitro system with explanted pig ulnar nerve in a stimulation and recording cuff. The container was surrounded by a water jacket warmed to a range of temperatures for different experiments, ranging from 20 to 37°C, and the two chambers contained the nerve in Ringer's solution. *Inset:* Recording cuff electrode geometry and dimensions. The diameter of the cuff was 2.6 mm, the length of the whole cuff (a) was 50 mm, the guard electrode width (b) was 1 mm, the recording electrode width (c) was 0.5 mm, and the inter-electrode spacing (d) was 3.5 mm. Diagram was adapted with permission from Felipe Rettore Andreis et al., Sensors; published by MDPI in 2022 [6].

This system consisted of an acrylic container split into two chambers, with a small channel in the middle connecting the two, a water jacket for maintaining the culture medium at a set temperature, a water bath, and a separate container for the culture medium. The culture medium was a modified Ringer's solution (NaCl: $146\,\mathrm{mM\,L^{-1}}$, KCl: $5\,\mathrm{mM\,L^{-1}}$, MgCl2: $1\,\mathrm{mM\,L^{-1}}$, CaCl2: $2\,\mathrm{mM\,L^{-1}}$, HEPES: $10\,\mathrm{mM\,L^{-1}}$, Glucose: $11\,\mathrm{mM\,L^{-1}}$, PH adjusted to 7.4 using NaOH) as an isotonic solution for promoting the viability of the explanted nerve [3]. Both chambers were filled with culture medium warmed to approximately 20°C, and the nerve was threaded through the narrow channel and submersed in culture medium. Stimulation and recording cuffs were placed in separate chambers to minimise the magnitude of stimulation artefacts on the recordings. Over the course of the experiments, the water jacket temperature was initially kept constant at 20°C, and later increased up to 37°C.

B. Surgical methods

All animal procedures were performed in accordance with the *Danish Animal Experiments Inspectorate* (ethics approval number 2017-15-0201-01317), as well as the care and use of laboratory animals as described by the *U.S. National Institutes of Health*. An adult Danish Landrace pig with a weight of approximately $34.5\,\mathrm{kg}$ was anaesthetised using Zoletil and kept sedated using sevoflurane (1.5 to 2.5% minimum alveolar concentration), propofol ($2\,\mathrm{mg\,h^{-1}\,kg^{-1}}$), and fentanyl ($10\,\mathrm{\mu g\,h^{-1}\,kg^{-1}}$). The animal was mechanically ventilated at 15 cycles per minute.

The ulnar nerve was exposed via a 20 cm incision and dissected as far distally as permissible. A section of approximately 15 cm was freed. The stimulation and recording cuffs were implanted on the exposed nerve section to conduct in-vivo recordings and ascertain nerve health over the course of five hours. These recordings are not included in this paper. A silicone sheet was applied around the cuffs to minimise current leakage, and sutures were used at each end and in the centre of each cuff to ensure these were closed. Once in-vivo recordings were complete, the nerve was explanted and stored in a closed flask with Ringer's solution at 4°C, prior to being transported to the in-vitro system after one hour. After the nerve was dissected, the animal was euthanised by an overdose of pentobarbitone.

C. Cuff design

Stimulation and recording cuffs were manufactured and implanted on the ulnar nerve, first in-vivo and subsequently in-vitro. Both cuffs were manufactured following the technique described by Haugland [7]. The stimulation cuff was approximately 10 mm long, with an inner diameter of 1.8 mm, and was configured in a tripolar manner, with three platinum-iridium ring electrodes with 3 mm centre-to-centre distance. The recording cuff was approximately 50 mm long and consisted of 12 centre electrodes and an additional two guard electrodes on either end of the cuff. The two guard electrodes were short-circuited and used as a reference. The inter-electrode distance was 3.5 mm, and the widths of the electrode rings were 0.5 mm for the recording electrodes, and 1 mm for the guard electrodes.

D. Electronic apparatus

A programmable current stimulator (STG4008, Multichannel Systems) was configured with a stimulation sequence of charge-balanced pulses, with the primary cathodic phase increasing from an amplitude of 0 to $16\,\mathrm{mA}$ (beyond supramaximal level). The cathodic phase was $100\,\mathrm{\mu s}$ in duration, followed by a $32\,\mathrm{ms}$ delay, and subsequently the anodic phase lasted $1\,\mathrm{ms}$ at an amplitude of 10% of the cathodic amplitude. The maximum cathodic stimulation current corresponds to a charge density of $39.2\,\mathrm{\mu C\,cm^{-2}}$. There was a $1\,\mathrm{s}$ break between each biphasic pulse, and the entire stimulation protocol lasted approximately 5 min. This protocol was repeated seven times. The stimulation profile increased amplitude in smaller steps of $50\,\mathrm{\mu A}$ at the lower end, and in larger steps of $1\,\mathrm{mA}$ past the $7\,\mathrm{mA}$ mark, given that most fibres should be recruited at this stage. In this paper, only recordings at supramaximal stimulation levels ($5\,\mathrm{mA}$) were considered.

The recording cuff was connected to SmartProbes (AI402 SmartProbes, Axon Instruments, Inc.) and an amplifier bank (CyberAmp 380, Axon Instruments, Inc.) in a bipolar configuration. The total amplifier gain was 25,000 and the data was digitised using a PCIe-6363 card (National Intsruments) at 90 kS/s.

E. Data analysis

Once digitised, the data were bandpass filtered between 100 and 10,000 Hz using FIR lowpass and highpass filters, with filter orders of 100 and 20 respectively. The data were then analysed both in the time domain and velocity domain. For the time domain analysis, recordings were extracted both at a constant temperature of 20°C (n=3) and at temperatures

increasing up to 37°C. For the velocity domain analysis, *delay-and-add* was used to produce a representation of the data in the velocity domain, in the form of an intrinsic velocity spectrum (IVS) [8]. This is based on the principle that the eCAPs recorded at adjacent electrodes appear with a delay corresponding to both the electrode spacing and CV of the eCAP. Thus, an artificial delay can be swept over a range of values and applied to each channel data, which after being summed, produces a maximum value at a point when the artificial delay cancels out the previously mentioned naturally occurring one. This technique has the added benefits of increasing the effective SNR by $\sqrt{C}$, where C is the number of channels, and minimising the effect of common-mode interference. Similarly to the time-domain analysis, IVSs were generated both for multiple channels at a constant temperature 20°C (n=3) and for a single channel with temperatures increasing up to 37°C.

III. RESULTS

A. Time-domain eCAP recordings

The nerve remained viable for up to five hours after being explanted. Continuous time recordings of eCAPs were obtained following the stimulation protocol and recording method described in Section II-D. Fig. 2 shows successive bipolar recordings from seven channels of the recording cuff, with each coloured line corresponding to the average waveform from each channel (n=3), and the grey lines under each plot represent three repeats from the same channel. A single CAP can be observed which propagates along the cuff, and as it does, the amplitude decreases and duration increases. A stimulation artefact is also visible at the start of each recording.

Additional recordings were also conducted with increasing temperature beyond 30°C. Fig. 3 highlights the change in eCAP morphology when changing from an initial temperature of 20°C up to 37°C. A bipolar waveform can be observed at all temperatures for each eCAP, with the peak amplitude being $\sim 0.2\,\mathrm{mVpp}$ at 32°C, and the smallest being $\sim 0.05\,\mathrm{mVpp}$ at 37°C.

B. Velocity-domain analysis

Bipolar recordings were analysed in the velocity domain using the *delay-and-add* process described in Section II-E and in [8]. This process was applied to $15\,\mathrm{ms}$ windows from each channel containing the eCAP observed in the time-domain data at a stimulation current of $5\,\mathrm{mA}$ and temperature of 20°C. The range of velocities considered in this analysis was between 5 and $100\,\mathrm{m\,s^{-1}}$ with a step size of $0.5\,\mathrm{m\,s^{-1}}$. At the end of the process, the maximum values obtained were used to produce the IVS shown in Fig. 4(a) (black line). At this temperature, a peak of $0.87\,\mathrm{mV}$ can be clearly seen at $17\,\mathrm{m\,s^{-1}}$ in the IVS plot, which suggests this was the primary CV of the eCAP.

The same analysis was repeated for eCAPs at increasing temperatures up to 37°C. These results have also been included in Fig. 4(a). When increasing the temperature to 32°C, 33°C, and finally 37°C, the peak velocities in the IVS plots change to 24, 27, and $43.5\,\mathrm{m\,s^{-1}}$, respectively. Additionally, the corresponding amplitudes first increase to $1\,\mathrm{mV}$, and subsequently decrease to $0.9\,\mathrm{mV}$ and $0.62\,\mathrm{mV}$. Lastly, the IVS for eCAPs at temperatures of 32°C and 33°C exhibit a second, less prominent peak, at 35 and $40\,\mathrm{m\,s^{-1}}$, respectively. An IVS for eCAPs recorded in-vivo for a pig ulnar nerve have also been included in Fig. 4(b), highlighting a peak at $58.5\,\mathrm{m\,s^{-1}}$. This IVS was also compared against in-vitro results at the same temperature.

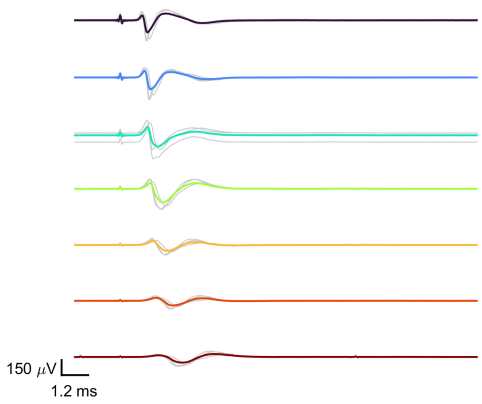

Fig. 2. Time-domain recording of eCAPs at a stimulation level of $5\mathrm{mA}$ and a constant temperature of 20°C. Each coloured trace corresponds to an average waveform from 3 repeats across seven channels, and the thin grey lines highlight any variation from each repeat.

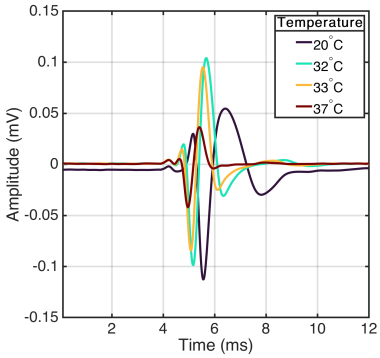

Fig. 3. Variation in eCAP shape from a single channel with increasing temperature, covering a temperature range from 20 to 37°C.

IV. DISCUSSION

eCAP recordings were successfully obtained up to five hours after the nerve had been explanted from the animal. When conducting the recordings at an initial temperature of 20°C, an eCAP can be observed with a peak amplitude of $0.14\,\mathrm{mVpp}$. As this eCAP propagates along the nerve, the amplitude decreases and time duration increases. This has been discussed previously and arises because an eCAP is comprised of individual action potentials travelling at different speeds, hence dispersing in time as they propagate along the recording array [9]. When examining the eCAPs in the time domain at temperatures reaching up to 37°C, it can be seen that the amplitude of the eCAP peaks at

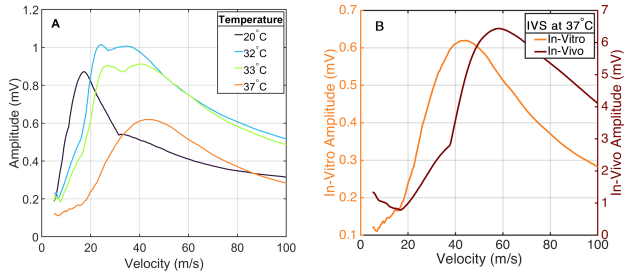

Fig. 4. IVS for recorded in-vitro and in-vivo eCAPs at stimulation level of 5 mA. **(A)** IVS for recorded eCAPs in vitro with increasing temperature from 20 to 37°C. The predominant CV of the eCAP shifts from $17\,\mathrm{m\,s^{-1}}$ to $43.5\,\mathrm{m\,s^{-1}}$, and the amplitude increases to a maximum of 1 mV and subsequently decreases to 0.62 mV. **(B)** Comparison of IVS for recorded eCAPs in-vivo and in-vitro at a temperature of 37°C. Compared to the in vitro data, the peak in the IVS is now at a velocity of $58\,\mathrm{m\,s^{-1}}$ and a considerably larger amplitude of 6.4 mV

32°C, but decreases as temperature increases further. Given that the nerve was explanted, kept at a significantly colder temperature compared to that in-vivo, and subsequently reheated, it is possible that these environmental changes and lack of supplemental oxygen caused some deterioration in the nerve response. A decrease in nerve diameter over time, and hence increased distance to the recording electrode, would also affect the electrical coupling between the nerve and electrode. This microscopic change is sufficient to attenuate the recorded amplitude by an order of magnitude, and hence is also a possible explanation for the decrease in amplitude seen [10], [11].

When examining the eCAPs at supramaximal stimulation at 20°C in the velocity domain, a predominant velocity $\sim 17\,\mathrm{m\,s^{-1}}$ was seen. Prior in-vivo cuff recordings of the ulnar nerve have shown peak velocities reaching up to $58\,\mathrm{m\,s^{-1}}$ [6]. This indicates that maintaining an explanted nerve at a temperature of 20°C results in a substantial decrease in the modal CV of the eCAP. This decrease in CV has been previously reported in studies in cat peripheral nerves and has been attributed predominantly to sodium channel function, as well as potassium channels [12], [13]. These studies reported that at lower temperatures, there is a slower onset of sodium channel depolarisation, producing slower CVs.

At 32°C and beyond, there is a shift in the IVS towards faster CVs, highlighting that as temperature approaches that seen in-vivo, as do the CVs. The temperature dependency of nerve CV has been previously quantified to be approximately 5% per degree centigrade [14]. In this paper, the change in CV per degree centigrade varied significantly between $0.5\text{-}3\,\mathrm{m\,s^{-1}\,°C^{-1}}$, or 3-12.5% per degree centigrade. It is worth noting that the IVS amplitudes also decrease with increasing temperatures, which may be due to nerve deterioration, as previously discussed.

Finally, at temperatures of 32 and 33°C, two peaks were seen in the IVS which were not previously present at 20°C, and are also not present at a higher temperature of 37°C. We hypothesise that this indicates the presence of multiple velocity modes contributing to the nerve response at these temperatures.

V. CONCLUSIONS

This paper introduces an in-vitro system for the electrical stimulation and recording of explanted peripheral nerves using multi-electrode cuffs. This was demonstrated with an explanted pig ulnar nerve, which was maintained for five hours post-explantation, stimulated using a tripolar cuff, and the neural response captured using bipolar, multi-channel recordings. Recordings were performed both at a constant temperature of 20°C and increasing temperatures up to 37°C, and compared against in-vivo data. These showed a change in eCAP morphology in the time domain, and a shift in IVS in the velocity domain, indicating higher temperatures similar to those seen in-vivo are required for generating faster CVs. In future, this setup can be used with different nerves and culture media to aid with optimising novel devices, electrode geometries, and experimental setups. Overall, such an in-vitro platform for peripheral nerve studies would be highly beneficial for reducing the amount of whole animal experiments required in the development and optimisation of closed-loop neural interfaces.

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