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Detection of Norepinephrine in Whole Blood via Fast Scan Cyclic Voltammetry

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Abstract

Bioelectronic Medicines is an emerging field that capitalizes on minimally-invasive technology to stimulate the autonomic nervous system in order to evoke therapeutic biomolecular changes at the end-organ. The goal of Bioelectronic Medicines is to realize both 'precision and personalized' medicine. 'Precise' stimulation of neural circuitry creates biomolecular changes targeted exactly where needed to maximize therapeutic effects while minimizing off-target changes associated with side-effects. The therapy is then 'personalized' by utilizing implanted sensors to measure the biomolecular concentrations at, or near, the end-organ of interest and continually adjusting therapy to account for patient-specific biological changes throughout the day. To realize the promise of Bioelectronic Medicines, there is a need for minimally invasive, real-time measurement of biomarkers associated with the effects of autonomic nerve stimulation to be used for continuous titration of therapy. In this study we examine the feasibility of using fast scan cyclic voltammetry (FSCV) to measure norepinephrine levels, a neurochemical relevant to end-organ function, directly from blood. FSCV is a well-understood method for measuring electroactive neurochemicals in the central nervous system with high temporal and high spatial resolution that has yet to be adapted to the study of the autonomic nervous system. The results demonstrate that while detecting the electroactive neurochemical norepinephrine in blood is more challenging than obtaining the same FSCV measurements in a buffer solution due to biofouling of the electrode, it is feasible to utilize a minimally invasive FSCV electrode to obtain neurochemical measurements in blood.

Keywords

norepinephrine; bioelectronics medicines; minimally invasive; fast scan cyclic voltammetry (FSCV); blood; autonomic nervous system

I. Introduction

Implantable or non-invasive devices designed to interface with the autonomic nervous system (ANS) in order to elicit therapeutic biomolecular changes at end-organs, known as 'Bioelectronic Medicines', are an emerging field that has been the focus of a number of recently launched multi-million dollar programs within industry and the government, including the National Institutes of Health's (NIH) Stimulating Peripheral Activity to Relieve Conditions (SPARC) Program, the Defense Applied Research Projects Agency's (DARPA) ElectRx Program, and the GlaxoSmithKline (GSK) Bioelectronic Medicine's Initiative. In 2013 the NIH, DARPA, and GSK held a joint summit consisting of leading

researchers in academia and industry to create a research roadmap to realize 'the vision of a new class of medicines based on modulating the electrical signaling patterns of the peripheral (autonomic) nervous system'. A 'research imperative' identified through this planning effort was to develop '*in vivo* sensors for molecular and physiological markers' required to fundamentally understand and continuously titrate autonomic nervous system neuromodulation therapies in real-time. [1]

Fast-scan cyclic voltammetry (FSCV) is an existing technique to measure changes in electroactive neurochemical concentrations with high temporal and high spatial resolution that has been extensively utilized in the study of the central nervous system (CNS) [2,5,6], but has yet to be applied to the study of the autonomic nervous system *in vivo*. Devices capable of electrically stimulating CNS nerves and simultaneously measuring neurochemical concentrations via FSCV while minimizing stimulation induced artifact have already been developed [4]. Chronic FSCV neurochemical measurements in the CNS have already been demonstrated in the rodent model [5]. Moreover, FSCV has already been demonstrated to measure changes in norepinephrine (NE) in the CNS [6,7] - a neurochemical known to be directly relevant to the beneficial effects of ANS neuromodulation therapies for inflammation, hypertension, and heart failure [3,8,9] - utilizing a potentially minimally-invasive seven micron diameter carbon fiber electrode. These results suggest existing FSCV techniques may be utilized to develop a minimally-invasive sensor system to measure neurochemical biomarkers relevant to ANS neuromodulation therapies.

An attractive option for minimally-invasive deployment of an FSCV electrode would be direct placement into an easily accessible artery or vein near the surface of the skin. Two major difficulties are anticipated in obtaining neurochemical concentrations relevant to autonomic nervous system neuromodulation therapies from circulating blood, namely 1) biofouling of the electrode that occurs over time after placement in blood which diminishes the sensitivity of the electrode to neurochemicals of interest, and 2) distance from the neurochemical source in conjunction with known neurochemical reuptake rates lead to very low concentration changes in blood at a vein/artery distant from the end-organ. Recent imaging of the vasculature in the brain indicates that implantation of even very small microelectrodes penetrates small blood vessels [15]. Consequently *in vivo* CNS FSCV recording electrodes have always been exposed to blood, suggesting recordings from circulating blood may be possible. In order to better understand the impact exposure to blood may have on FSCV measurements taken by carbon fiber microelectrodes (CFMs), here FSCV measurements of known concentrations of NE added to heparinized blood drawn from a pig are directly compared to FSCV measurements taken at the same concentration of NE in a tris buffered saline (TBS) solution.

II. Methods and Materials

A. Chemicals and Solutions

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). TBS consisted of 150 mM sodium chloride and 12 mM trizma base dissolved in distilled deionized (DI) water. pH was adjusted to 7.4 using a 10 M hydrochloric acid solution. A 10 mM NE stock solution was prepared by dissolving NE hydrochloride in DI water, and a two-step dilution from the

stock to 100 μM to final desired concentrations was performed in TBS or blood. Blood was collected from the femoral vein in anesthetized domestic swine, and heparinized within the syringe using 5 mL of heparin for 30 mL of blood. Blood was kept at room temperature to allow it to equilibrate to the temperature of the TBS; blood measurements were made within one hour of blood being drawn.

B. Carbon Fiber Microelectrode Specifics and Construction

Carbon fiber microelectrodes (CFMs) were fabricated by sealing the junction of a carbon fiber and a silica tube with polyamic acid (polyamide) and using nitinol (alloy of nickel and titanium) as an extension wire. A single polyacrylonitrile-based carbon fiber (7 μm diameter; Cytec, Woodland Park, NJ) was aspirated into a silica tube (100 μm diameter; Polymicro Technologies, Phoenix, AZ) and a sealed tip was formed by curing polyamide. This assembly was connected to a nitinol extension wire using a mixture of 50% polyamide and 50% silver powder (Strem Chemicals, Newburyport, MA). The combined assembly was covered using polyamide tubing (300 μm diameter; Small Parts Inc., Logansport, IN), and sealed at the tip by curing polyamide. The exposed carbon fiber was cut to a final length of approximately 100 μm by cleaving with a scalpel blade.

C. Fast Scan Cylic Voltammetry Recordings

Experiments were performed in a beaker utilizing the Universal Electrochemical Instrument (University of North Carolina, Chapel Hill, NC) to produce FSCV waveforms and to collect the resulting cyclic voltammograms (CVs). CFMs were pre-conditioned using a holding potential of −0.4 V that was then ramped to 1.3 V and back to −0.4 V at 400 V/s vs silver chloride (Ag/AgCl) reference in the TBS solution for fifteen minutes at 30 Hz prior to beginning the experiments [14]. The experiments were performed using similar FSCV parameters, but at 10 Hz instead of 30 Hz. Separate beakers containing TBS with 0, 0.1, 0.5, and 1 μM NE, respectively, were prepared. Similarly, separate beakers containing blood with 0, 0.1, 0.5, 1, 5, 10, and 15 μM NE added were also prepared. The highest concentrations (5, 10, and 15 μM) of NE were used in anticipation of difficulties detecting NE in blood. FSCV measurements were performed for three minutes in each beaker starting with the lowest concentration of NE in TBS and ending with the highest concentration of NE in TBS, and then tested in the same order in blood using the same set of electrodes. Scanning electron microscope (SEM) images of a subset of electrodes were collected before experiments, after recordings in TBS, and after recordings in blood.

The current measured during an FSCV scan consists of a background component generated by capacitive current and faradaic current generated by electrochemical reactions unrelated to the analyte of interest, as well as faradaic current resulting from reduction or oxidation reactions involving the analyte of interest, NE. For simplicity, this manuscript refers to ‘background currents’ as the capacitive current plus any faradaic current generated by electrochemical reactions unrelated to NE oxidation/reduction, and current resulting from the oxidation/reduction of NE as faradaic current. To minimize the impact of capacitive current on the measurements, as per standard practice CVs were first collected in solutions without NE and subtracted from CVs collected in solutions containing NE in order to obtain the background subtracted cyclic voltammograms (BSCV). The BSCV therefore provides an

estimate of the faradaic current resulting from reduction and oxidation reactions involving NE. Utilizing the BSCV, the characteristic oxidation and reduction currents for NE became visibly apparent.

Two methods of quantifying the oxidation/reduction currents from the BSCV were used. First, the peak current amplitude for the characteristic oxidation and reduction humps in the BSCV were noted (i.e. the faradaic current), and the voltage location of the peak current amplitude for the oxidation hump was identified. Second, the area under the CV, and the areas under the oxidation and reduction peaks in the BSCV were calculated via trapezoidal integration (See Table 1). For the BSCV in blood, the oxidation and reduction humps were often superimposed on an underlying low-frequency drift apparently caused by progressive biofouling of the electrodes. To minimize this drift, additional ad hoc manipulation was employed. Visual inspection was used to identify the beginning and the end of the oxidation and reduction potential “humps”, and a line was drawn from the beginning of the hump to the end of the hump to correct for drift. Only the area under the hump above this correction line was measured to isolate the signal from drift. These isolated humps were also used to collect peak amplitudes corrected for drift.

III. Results

A. Changes in the Voltammogram

Immediately upon the start of recording, CVs collected in heparinized blood were noticeably different in overall shape as compared to recordings in TBS (Figure 1A). The voltage location of the oxidation and reduction potentials measured in blood shifted approximately +100 mV and −60 mV, respectively, from their locations in TBS (Figure 1B). Finally, there was an approximate 12% overall decrease in capacitive, non-faradaic current as calculated by the area under the full CV (Table 1). Over the course of recording for three minutes, the overall amplitude of the CV in blood shifted approximately ten nA (Figure 2C).

B. Calibration to Norepinephrine

A typical representation of a BSCV for a NE signal, measured in TBS, can be found in Figure 1. The oxidation and reduction peak signals, found at 0.67 V and −0.2V respectively, are visually evident. For the same concentration in blood using the same CFMs, the oxidation peak (now at 0.77V) and the reduction peak (now at −0.26V) are less obvious, but still visible. Visually obvious oxidation and reduction peaks were obtained at concentrations 5 μM NE and above in blood (Figure 1B). A summary of the CV characteristics, peak currents and integrals, can be found in Table 1. Finally, a clear linear correlation between concentration and obtained current for both the oxidation and reduction potentials of NE was evident in blood after employing our simple ad-hoc signal processing method (Figure 3).

C. Scanning Electron Microscopy

Comparison of the SEM images before recordings and after use in TBS showed no apparent difference. Comparison after use in TBS and after use in blood showed a coating that preferentially covered the surface of the CFM near the interface between the insulation, polyamide, and the electroactive surface area, carbon fiber (Figure 4B).

IV. Discussion

A. Biofouling at the Surface of the Carbon Fiber Microelectrode

The immediate and long-term changes to the CV, sensitivity to NE, and surface of the electrode as visualized by SEM all support the conclusion that blood produces biofouling at the surface of CFMs used for FSCV. The effect of biofouling could be explained in two ways that are not necessarily mutually exclusive. First, the layer of biofouling may increase the impedance that must be overcome to result in application of the expected voltage at the working electrode. For example, if the peak voltage is set to 1.3V, the actual voltage exhausted across the working electrode may only be 1.2V, as additional voltage is lost across the impedance of the layer of biofouling. This could in turn change the shape of the CV, since the shape of the CV is known to change when using different potential ranges [2,10]. This could also explain the shift in location of the oxidation and reduction peaks in blood compared to those in TBS; in effect the FSCV system would record that the peak oxidation current was obtained at 0.77V, when the voltage drop across the biofouling limited the effective voltage at the working carbon fiber electrode to 0.67V. The observed 13 percent decrease in measured capacitive current in the CV when placed in blood is consistent with this hypothesis. Second, biofouling may block sites at the electrode surface for adsorption of NE and inhibit diffusion to the surface of the electrode. Blocking sites for adsorption would decrease faradaic currents [11]. Loss due to blocked sites for neurochemical adsorption may explain why the capacitive current decreased by only 13% in blood compared to TBS, whereas the oxidation faradaic current decreased by 92% in blood compared to TBS.

B. Rejuvenation and Signal Processing

Although the results here demonstrate that it is possible to measure 0.1 micromolar NE in blood, previous studies have shown that concentrations relevant to ANS neuromodulation therapies are in the 1 nanomolar range. For example, the approximate mean concentration of NE in the blood is normally 500 pM, and in patients with heart failure is 2 nM [8,9]. These data suggest that a resolution of at least 0.5–1 nM would be required to differentiate between successfully treated and untreated patients. However, now that FSCV measurement of NE in blood has been shown to be feasible, several methods can be explored in future work to increase measurement sensitivity to the ranges relevant to ANS neuromodulation therapies.

One potential method is optimization through advanced signal processing. Note in Table 1 that measurement of the oxidation peak in blood directly from the BSCV gives a negative signal, whereas correcting for drift and isolating the oxidation hump in ad-hoc fashion gives a positive signal correlated with concentration down to 0.1 micromolar. Also worth noting is that the standard deviation drops an order of magnitude for blood. The effect of this drift on multiple concentrations of NE in blood can be found in Figure 2D. We are actively exploring more advanced signal processing techniques such as principle components regression and wavelet analyses to use the entire waveform to quantify faradaic changes [12,16], as well as algorithms to automatically correct for drift, minimize noise, and isolate faradaic components. Secondly, applying a higher peak voltage at a faster frequency for a short period of time has been shown to “rejuvenate” the surface of CFMs and temporarily remove biofouling [2]. We hypothesize that the rejuvenation protocol could be applied to CFMs in

blood to obtain improved sensitivity recordings in an intermittent fashion. Third, different waveforms could be used to optimize recorded FSCV signal. Greater sensitivity to electroactive neurochemicals has previously been demonstrated by scanning to higher anodic potentials [13], as well as using faster scan rates [10]. Finally, locating the electrode closer to the source of the NE should minimize the impact of dilution in circulating blood, which could increase the concentration of NE that we are trying to measure, thus reducing the sensitivity requirement. A relevant source of NE in the viscera is the kidneys, so one might use a catheter system to deliver CFMs to the renal vein or artery to increase the concentration of NE measured.

V. Conclusions

Although biofouling in blood was found to dramatically reduce the sensitivity of the FSCV electrode to NE both immediately and over time, oxidation/reduction peaks characteristic of NE were still observable at concentrations 1–2 orders of magnitude more than concentration changes anticipated to be relevant to ANS neuromodulation therapies when measured in circulating blood. In conjunction with anticipated sensitivity gains from future work to optimize electrode design, FSCV protocol, signal processing, and catheter delivery close to the organ of interest, these findings suggest that it may be feasible to develop a minimally invasive FSCV sensor system for obtaining neurochemical measurements from circulating blood at concentrations relevant to the development of Bioelectronic Medicines.

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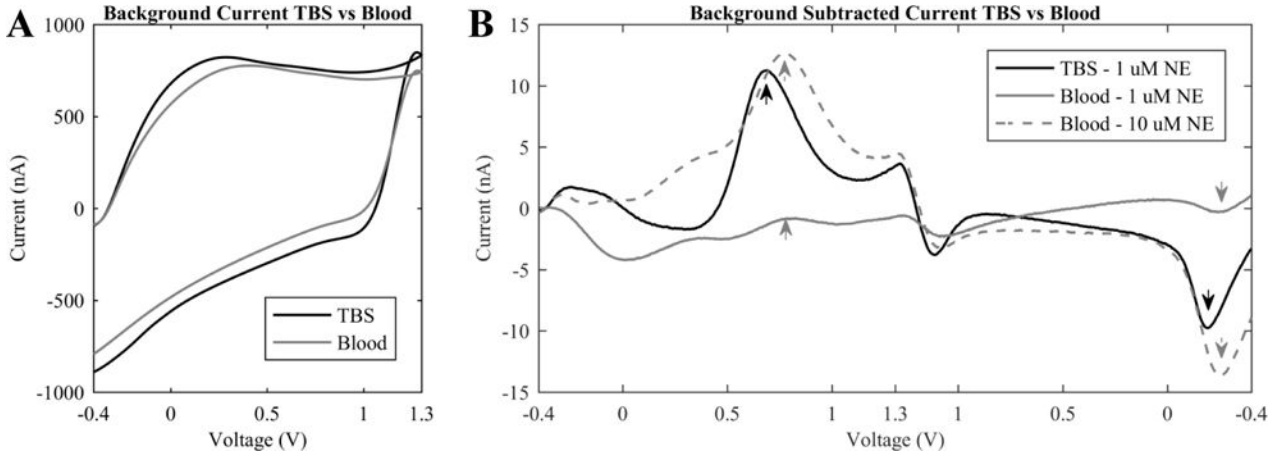

Figure 1.

Differences in static cyclic voltammorgrams (CV) between TBS and blood. A) background CVs for TBS and blood after approximately fifteen minutes of continuous application of FSCV in each solution. B) background subtracted CVs for 1 uM NE in both TBS and blood, as well as 10 uM NE in blood for clarification of oxidation and reduction peaks.

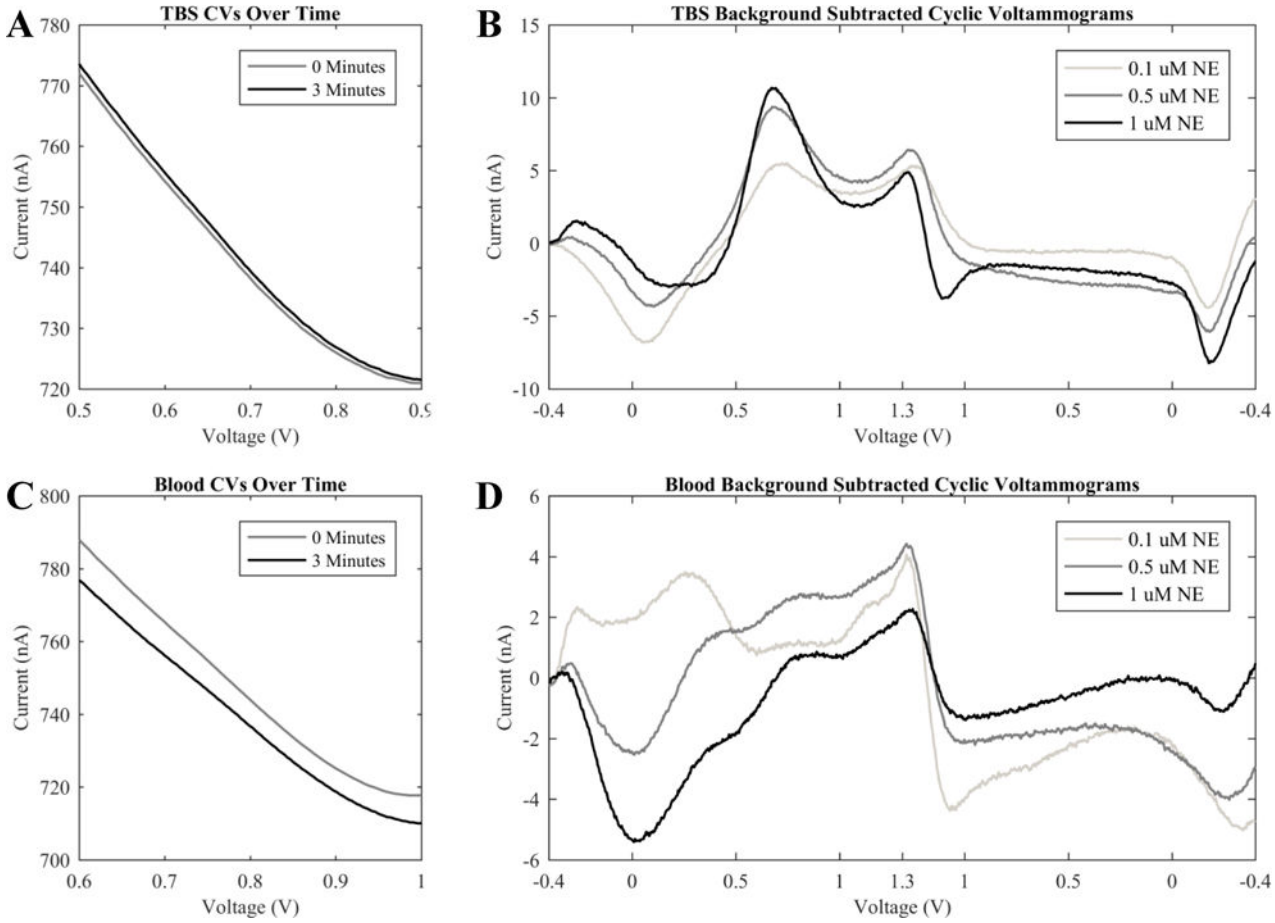

Figure 2.

TBS and blood cyclic voltammograms (CVs) over time. Left panels are background CVs zoomed into the voltage range surrounding the oxidation potential for each solution recorded at the beginning and the end of a 3 minute recording, and right panels are background subtracted cyclic voltammograms (BSCVs) collected for 0.1, 0.5 and 1 uM of NE. A–B) Datacollected in TBS. C–D) Data collected in blood.

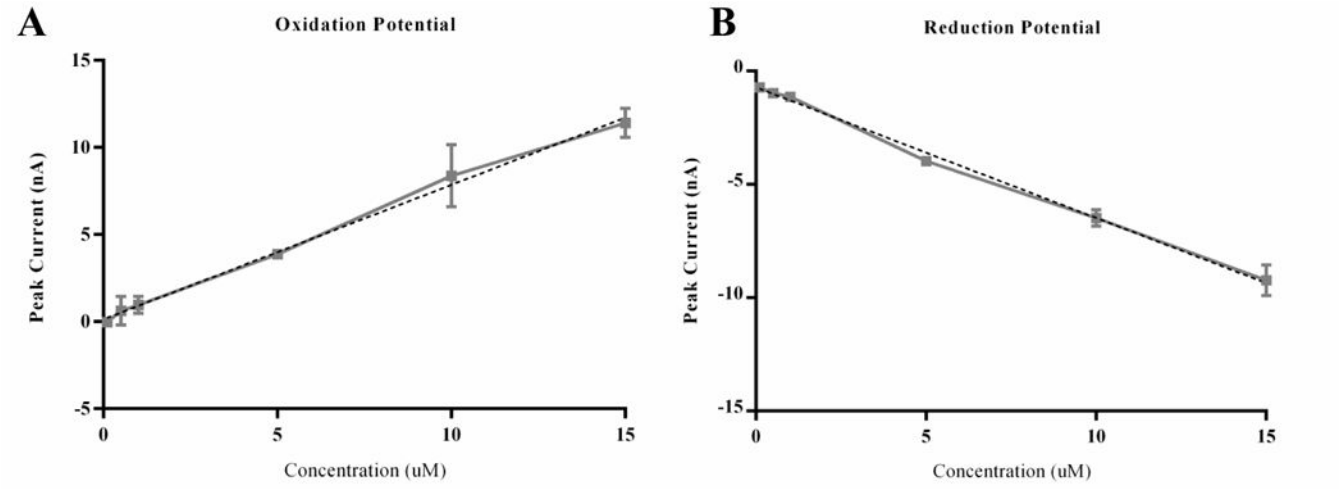

Figure 3.

Calibration curves to NE at CFMs in blood. A) oxidation potential peak currents collected from background subtracted cyclic voltammograms (BSCVs) at 0.77V. B) reduction potential peak currents collected from BSCVs at −0.26V. Grey squares are the mean values of peak currents collected at 4 CFMs for 6 concentrations: 0.1, 0.5, 1, 5, 10, and 15 μM. Grey error bars are the standard error of the mean. Black dotted line is a linear fit with R squared values of 0.96 and 0.99 for theoxidation and reduction, respectively.

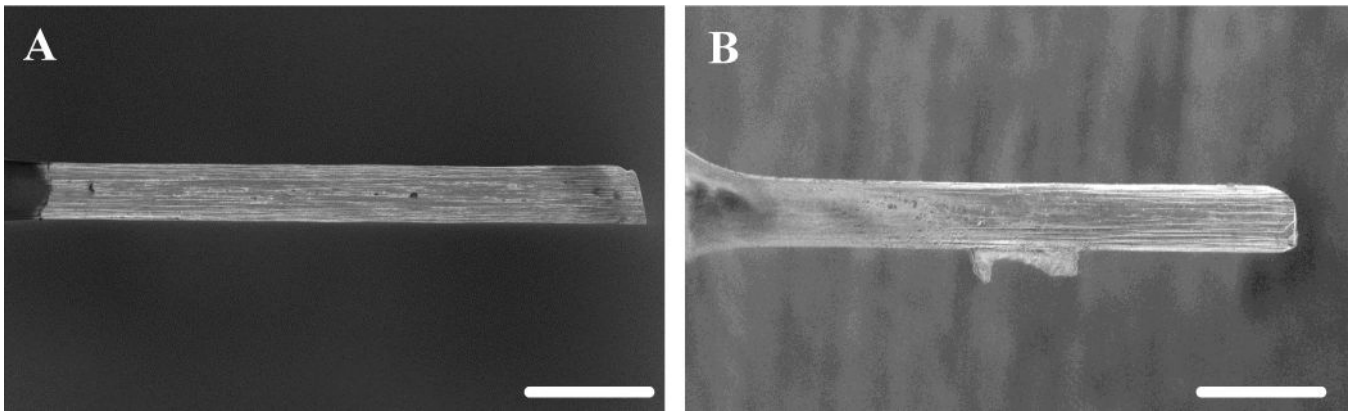

Figure 4.

Scanning electron micrographs of CFMs before and after FSCV in whole heparinized blood. A) Representative CFM before FSCV use in any solution. B) Representative CFM after FSCV use in blood for 15 minutes. Scale bars are 20 μm.

TABLE I

Cyclic Voltammogram Characteristic Comparison TBS vs Blood[a]

Characteristic	TBS Peak (nA)	Blood Peak (nA)	TBS Integral (nA*V)	Blood Integral (nA*V)
Background Current	763.1 ± 55.9	721.7 ± 31.6	473,800 ± 37,525	412,310 ± 24,644
Oxidation Current[b]	10.7 ± 1.5	−0.7 ± 2.3	989 ± 38.5	84.7 ± 9.7
	10.9 ± 0.6	1.2 ± 0.1		
Reduction Current[b]	−8 ± 4.6	−1 ± 2.3	−329.5 ± 138.4	−84.4 ± 30
	−6.1 ± 2.2	−1.4 ± 0.3		

[a]Data collected from the 1 uM norepinephrine CVs shown in Figure 1. All values are the mean and standard deviation of data for 4 electrodes.

[b]Data above is without ad-hoc correction for drift, and below is with ad-hoc correction.