

Toward Subcutaneous Electrochemical Aptasensors for Neuropeptide Y

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Abstract — Subcutaneous sensors, similar to the continuous glucose monitor, are advantageous for identifying healthy and pathological patterns of circulating biomarkers. A biosensor for the detection of neuropeptide Y (NPY), a marker of stress, has been designed and tested for operation in a flexible microneedle form factor. The biosensing principle used is affinity binding of NPY to a DNA aptamer-functionalized electrode. A gold microelectrode was functionalized by formation of a self-assembled monolayer (SAM) of a thiol-modified NPY-binding aptamer and poly(ethylene glycol) methyl ether thiol (PEG). The sensors were evaluated by cyclic voltammetry and electrochemical impedance spectroscopy, resulting in a response to NPY over 400 pM to 200 nM when tested in KCl and K₃[Fe(CN)₆]/K₄[Fe(CN)₆], and PBS.

Keywords—aptamer; biosensor; neuropeptide Y; flexible electronics, cyclic voltammetry, electrical impedance spectroscopy

I. INTRODUCTION

With the commercialization and popularity of continuous glucose monitors, *i.e.*, Dexcom and FreeStyle Libre, there is significant motivation to develop medical devices toward longitudinal biomarker monitoring. Measuring instantaneous concentrations of biomarkers can indicate when it is necessary to administer treatment or medication. Chronic medical conditions require longitudinal patient monitoring and biomarker pattern recognition. Conditions like anxiety, depression, addiction, and PTSD often rely on self-recognition of symptoms and subjective clinical consultation to diagnose. A novel wearable biosensor that could continuously quantify stress-related modulation of biomarkers, *e.g.*, cortisol and adrenaline, can fill in the gaps in our fundamental understanding of stressor impact on health and improve our current patient care paradigms. Significant efforts are ongoing to develop continuous sensors for cortisol [1], which is positively associated with acute stress response. Development of sensors for concomitant biomarkers, such as neuropeptide Y (NPY), are necessary to better identify the physiological responses to stressors and manifestation as anxiety or performance reduction.

NPY is a 36-amino acid anxiolytic hormone formed in the nervous system that regulates body functions like appetite, sleep patterns, and blood pressure. In alarming or threatening situations, NPY levels become slightly elevated to mollify stress responses in the body [2-4]. Furthermore, individuals that have stress-related disorders or have undergone an intense period of extreme distress (*e.g.*, post-traumatic stress disorder, PTSD) can have elevated basal NPY levels, when compared to those without disorders. NPY plasma-levels

range from 250 to 950 nM [5]. Furthermore, NPY levels have been quantified in sweat (0.19 to 17.21 pM) [6], offering multiple avenues for wearable sensor development. Continuous NPY monitoring has a clear position as a quantifiable metric to improve diagnoses and management of stress disorders.

Electrochemical aptamer-based biosensors, *i.e.*, aptasensors, are ideal low-power point-of care systems with features like high sensitivity, low sample consumption, and miniaturization. Aptamer sequences can be identified for myriad targets with binding constants (K_D) ranging from μ M to pM affinity [7]. Aptamer-based biorecognition can be coupled with different transduction principles too. Common electrochemical aptasensors employ potentiometry [8] and impedance spectroscopy [9, 10].

Recently, aptasensors for NPY have been demonstrated. Lopez et al. measured a concentration range from 10 to 1000 ng/mL NPY on platinum and carbon electrodes [9]. They compared the peptide adsorption on electrodes functionalized with aptamer and electrodes without by comparing the change in $-\omega^*Z_{\text{imag}}$ at different frequencies and potentials via electrochemical impedance spectroscopy (EIS). Also using EIS, Churcher et al. determined their NPY detection by calculating the ratio change in impedance through Z_{mod} [10]. Although the capture probe used was an antibody, the characterization methodology was relevant to this work. Herein, we combine an electrochemical aptasensor with a flexible microelectrode cell. This system provides a preliminary demonstration of an NPY aptasensor for use in a subcutaneous system for longitudinal assay of dermal interstitial fluid (ISF). Fig. 1 shows the flexible microneedle cell and the sensor functionalization. Aptamer

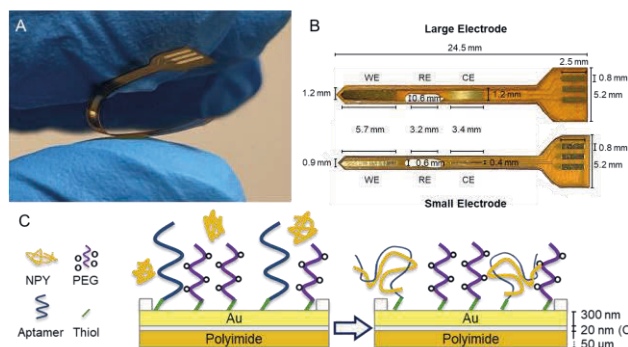


Fig. 1. (A) Mechanical deformation of microneedle to demonstrate flexibility. (B) Schematics of microneedle and its working (WE), reference (RE), and counter electrodes (CE). (C) Symbolic representation of NPY-aptamer binding on electrode surface.

immobilization was validated using cyclic voltammetry (CV) to compare curve morphology. The electrochemical modulations present on the electrode surface due to the target molecule binding were detected using EIS.

II. EXPERIMENTAL PROCEDURE

A. Materials and Sensor Functionalization

All chemicals were purchased from Sigma Aldrich [St. Louis, MO] unless noted and used as received.

The aptamer sequence 5'-Thiol-MC6-S-S-AGCAGC-ACAGAGGTCAGATGCAAACCACAGCCTGAGTGGTTAGCGTATGTCATTTACGGACCTATGCGTGCTACCGTGAA-3' was synthesized by Integrated DNA Technologies (IDT) Inc. [Coralville, IA]. To resuspend the aptamers, a pH 8.0 buffer with 10 mM tris(hydroxymethyl)aminomethane (Tris) and 0.1 mM ethylenediaminetetraacetic acid (EDTA) was also purchased from IDT. To reduce the disulfide bond formation, tris[2-carboxyethyl] phosphine (TCEP) was added to the aptamer solution to reach 100x excess, *i.e.*, 100 mM TCEP to 100 μ M aptamer stock solution, and allowed to equilibrate at room temperature for three hours.

The self-assembled monolayer (SAM) was formed via gold-sulfur interaction at ambient conditions [11]. Electrodes were cleaned with ethanol then incubated with 100 μ L of 1 μ M aptamer solution for 12 hours. Then, the electrodes were rinsed with DI water and exposed to 1 mM of poly(ethylene glycol) methyl ether thiol (PEG) in resuspension buffer for three hours. Electrodes were characterized immediately following the functionalization process.

NPY (YPSKPDNPGEDAPAEDMARYYSALRHYIN-LITRQRY-NH₂) was purchased from GenScript Biotech Corp. [Piscataway, NJ]. The aptamer sequence and NPY have been validated for specific binding with a reported K_D constant of 300 nM [12]. NPY was dissolved in pH 7.4 phosphate buffer saline (PBS) (7.5 mM Na₂HPO₄, 2.5 mM NaH₂PO₄, 2.7 mM KCl and 137 mM NaCl) from Cytiva [Marlborough, MA]. For electrochemical analysis, a 1 M KCl solution with 10 mM K₃[Fe(CN)₆]/K₄[Fe(CN)₆] from VWR [Radnor, PA] was created.

B. Flexible Microneedle Fabrication

Fabrication of the flexible microneedle cells was completed by standard lithography and metallization processes. Briefly, a carrier layer of 10:1 base:crosslinker polydimethylsiloxane (PDMS) was deposited onto a clean soda lime glass 100 mm wafer. A 50 μ m thick layer of

polyimide film was then placed onto the PDMS layer. Cr adhesion and Au layers were deposited by DC Sputtering for a 20 nm and 300 nm thick layer, respectively. Traces, pads, and electrodes were patterned by photolithography. After being cleaned, a 4 μ M layer of parylene was coated onto the wafer by thermal deposition. The parylene was patterned and etched by DRIE to expose the desired contact pads and electrodes. As shown in Fig. 1, two electrode sizes were considered. A laser cutter was utilized to cut the desired shape of the electrodes out of the polyimide film. The RE was drop cast with Ag/AgCl ink from Sun Chemical Ltd [Midsomer Norton, UK] and cured at 80°C for 10 minutes. Ink was refrigerated at 4°C when not in use.

A printed circuit board was custom designed and composed of a dielectric support, milled copper substrate, and gold traces. This board was used in both CV and EIS measurements and suspended above the solution while fully immersing the three electrodes of the microneedle.

C. Sensor Characterization

A Reference 600+ Gamry potentiostat/Galvanostat/ZRA was used for all CV and EIS measurements. For CV, the cell voltage was swept from -0.5 V to 0.5 V at 10, 100, and 250 mV/s and 1 mV step size. Three cycles were measured for each scan rate. Parameters for EIS measurements were derived from previously reported methods by Lopez et al. [9]. EIS was performed with an initial and final frequency of 5 MHz and 10 Hz, respectively. AC voltage was set to 10 mV rms. The DC offset was chosen based on the CV peaks: -0.4 V for electrodes in PBS and +0.26 V in KCl solution.

When conducting measurements in a solution containing NPY, the appropriate volume from a stock concentration of 2 μ M was added to the electrochemical cell. Once added, solution was stirred for three minutes at 200 rpm and then allowed to settle for one minute before measurement. Subsequent concentrations were calculated based on total volume as it changed with increasing NPY additions.

III. RESULTS

A. Cyclic Voltammetry

An initial CV study was done to establish the baseline behavior of the functionalized electrodes and the anodic and cathodic peak currents of the cell in an electrolyte. Fig. 2a-c display the cyclic voltammograms of the bare gold and functionalized microneedle in KCl solution and in PBS. The peak shape of the cyclical voltammogram for the bare gold microneedles illustrates the expected response of the reversible redox couple. For the functionalized

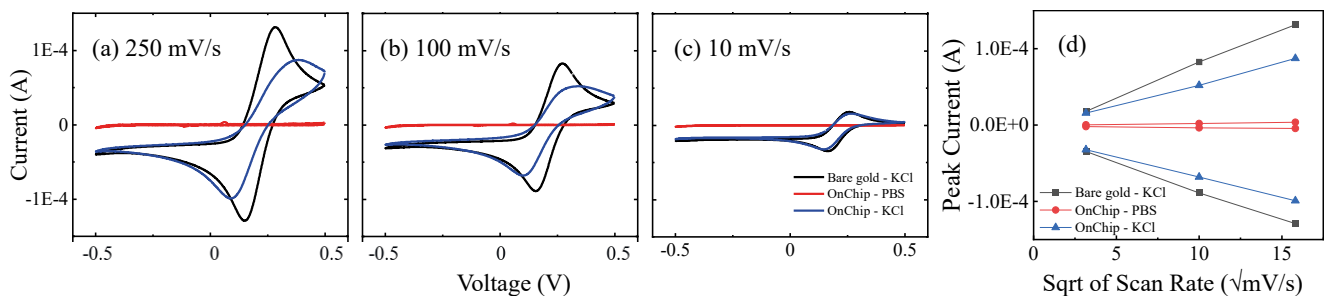


Fig. 2. Cyclic voltammograms of a bare gold microneedle in KCl (black), a functionalized microneedle with NPY-binding aptamer and PEG in KCl (blue) and a functionalized microneedle in PBS (red). The three different scan rates of (a) 250 mV/s, (b) 100 mV/s, and (c) 10 mV/s exhibited slightly different peak shapes. (d) Plot of peak current vs. square root of scan rate suggests reversible redox reactions.

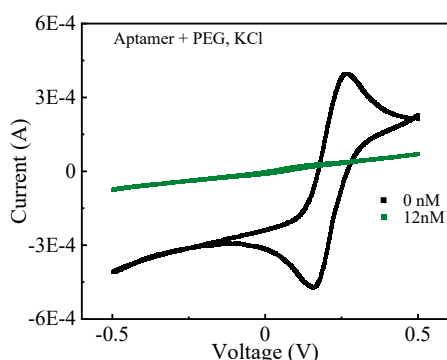


Fig. 3. Cyclic voltammogram of 0 nM and 12 nM NPY on a functionalized microneedle in KCl solution. The oxidation peak measured at 0 nM was not present at increasing NPY concentration.

microelectrodes, the cyclic voltammogram shows a reduced current and broadening of the redox peaks, indicative of a surface-limiting effect on the charge transfer between the redox couple and electrode. The peaks are not visible when measured in PBS, as expected. CV plots were all generated with large microelectrodes (Fig. 1). These results point to the successful functionalization of the NPY-binding aptamer and PEG, and are further corroborated by analysis of the peak redox currents versus the square root of scan rate for the different microelectrodes (Fig. 2d). A linear response ($R^2 > 0.97$) is observed, and the change is dependent on the surface, supporting the interpretation that surface functionalization impacts the electrochemical reaction rate.

The NPY concentration-dependent effect on the cyclic voltammetry behavior was investigated. Fig. 3 shows a representative comparison of cyclic voltammograms for the aptasensor before and after NPY exposure. Immediately following the addition of NPY into the potassium ferrocyanide solution, CV measurements were taken with parameters identical to those run in an NPY-free cell. As is depicted in Fig. 3, redox sites initially available in the 0 nM NPY solution are eliminated by the addition of NPY into the cell, indicative of NPY binding to the aptasensor.

B. Electrochemical Impedance Spectroscopy

In addition to CV, EIS was evaluated as a transduction method for quantification of NPY. NPY concentrations of 12 nM to 200 nM were individually measured on small electrodes. Frequencies from 5 kHz to 50 kHz were selected to show the parallel concentration dependence. Initial EIS

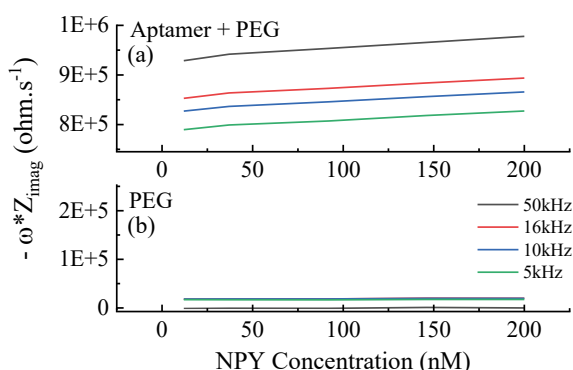


Fig. 4. $-\omega * Z_{\text{imag}}$ (ohm/s) vs NPY at 12 nM to 200 nM using electrodes with (a) aptamer+PEG and (b) PEG-only functionalization. Both microneedles were tested in KCl solution.

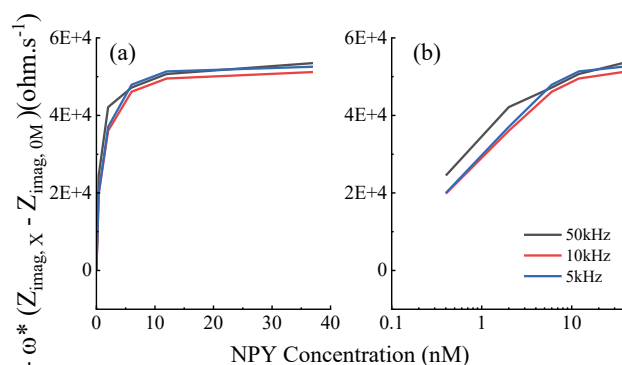


Fig. 5. Differential $-\omega * Z_{\text{imag}}$ (ohm/s) vs. NPY for a small microelectrode functionalized with aptamer and PEG in PBS plotted on with a (a) linear and (b) logarithmic scale x-axis. Larger increases in response occur at low concentrations (400 pM–37 nM).

with the redox couple was conducted on microneedles with and without an aptamer monolayer. Microneedles without aptamer were introduced only to PEG to form an appropriate reference surface for result comparison. Results in Fig. 4 show that the aptamer-functionalized electrode has a clear concentration dependence across the entire frequency range; the reference sensor shows a lower magnitude of response, confirming a small amount of non-specific adhesion to the aptamer-less surface.

Fig. 5 shows EIS performed in PBS (no redox couple), which represents a realistic deployment of this sensor, in anticipation of future subcutaneous usage. The differential $-\omega * Z_{\text{imag}}$ is plotted following subtraction of the initial reference measurement conducted in buffer. At higher concentrations, a saturation of the surface by NPY is observed. A more significant response was captured at concentrations below 12 nM down to 400 pM. This range more closely mimics that naturally occurring in human serum and ISF, and thus provides a clear next step toward realizing the end use case for the aptasensor proposed herein.

IV. CONCLUSION

Flexible microelectrodes for future subcutaneous and continuous biosensing were designed, fabricated, and tested with an affinity-based binding to NPY, an important marker for stress. CV was used to confirm functionalization of the working electrode with an NPY-binding DNA aptamer. EIS measurements were conducted to characterize the electrode behavior following exposure to different concentrations of NPY. Successful detection of NPY in PBS – without the use of a redox couple – with a minimum detectable signal of 400 pM was demonstrated. Through these techniques we confirmed that the proposed aptasensor specifically detected NPY in solution and shows potential for use in subcutaneous sensors. In the future, the NPY concentration will be reduced to explore the limit of detection and dynamic range of the sensors. Tests will also be conducted in artificial ISF to demonstrate clinical relevance.

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REFERENCES

- [1] D. Kinnamon, et al. "Portable biosensor for monitoring cortisol in low-volume perspired human sweat," *Sci Rep.*, vol. 7, pp. 13312, October 2017.
- [2] U. Tural, and D. V. Iosifescu. "Neuropeptide Y in PTSD, MDD, and chronic stress: A systematic review and meta-analysis," *J. Neuro Res.*, vol. 98, pp. 950–963, February 2020.
- [3] F. Reichmann, and P. Holzer. "Neuropeptide Y: A stressful review," *Neuropeptides*, vol. 55, pp. 99–109, February 2016.
- [4] K. Tatemoto, et. al. "Neuropeptide Y: history and overview," *handbook of experimental pharmacology*, vol. 162. pp. 2–15, 2004.
- [5] M. E. Beckner, et al. "Impact of simulated military operational stress on executive function relative to trait resilience, aerobic fitness, and neuroendocrine biomarkers," *Physiology & Behavior*, vol. 236, pp. 113413, July 2021.
- [6] G. Cizza, et al. "Elevated neuroimmune biomarkers in sweat patches and plasma of premenopausal women with major depressive disorder in remission: the POWER study," *Bio. Psychiatry*, vol. 64, pp. 907-911, November 2008.
- [7] S. Cai, J. Yan, H. Xiong, Y. Liu, D. Peng and Z. Liu. "Investigations on the interface of nucleic acid aptamers and binding targets," *Analyst*, vol. 143, pp. 5317-5338, October 2018.
- [8] Ahmad, et. al. "Graphene-based electrolyte-gated field-effect transistors for potentiometrically sensing neuropeptide Y in physiologically relevant environments," *ACS Applied Nano Materials*, vol. 3, pp. 5088-5097, April 2020.
- [9] L. López et al. "Measurement of neuropeptide Y using aptamer-modified microelectrodes by electrochemical impedance spectroscopy," *Anal. Chemistry*, vol. 93, pp. 973-980, January 2021.
- [10] N. K. M. Churcher, S. Upasham, P. Rice, C. F. Greyling, and S. Prasad. "Sweat based-multiplexed detection of NPY-cortisol for disease diagnostics and stress management," *Electroanalysis*, in press, May 2021).
- [11] C. D. Bain and G. M. Whitesides. "Molecular-level control over surface order in self-assembled monolayer films of thiols on gold," *Science*, vol. 240, pp. 62-63, April 1988.
- [12] S. Mendonsa, M. Bowser. "In vitro selection of aptamers with affinity for neuropeptide Y using capillary electrophoresis," *J Am Chem Soc.* vol. 127, pp. 9382-9383, July 2005.