Article

A Method for Electrochemical Detection of Brain Derived Neurotrophic Factor (BDNF) in plasma.

Marina Bockaj, Barnabas Fung, Michael Tsoulis, Lauren (Warren) Foster, and Leyla Soleymani

Anal. Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.analchem.8b01642 • Publication Date (Web): 22 Jun 2018

A Method for Electrochemical Detection of Brain Derived Neurotrophic Factor (BDNF) in Plasma

Bockaj M.[1,2], Fung B.[1], Tsoulis M.[1], Foster WG.[1,2,†], and Soleymani L[1,†].

Department of Engineering Physics
School of Biomedical Engineering
McMaster University

Department of Obstetrics & Gynaecology
Michael G. DeGroote School of Medicine
McMaster University

[†]**Address for correspondence:** Dr. Leyla Soleymani soleyml@mcmaster.ca or Dr. Warren Foster fosterw@mcmaster.ca

Key words: Endometriosis, point-of-care diagnosis, electrochemical biosensing, BDNF, plasma.

ABSTRACT

Currently, a blood test for the diagnosis of endometriosis, a common estrogen-dependent gynecological disease, does not exist. Recent studies suggest that circulating concentrations of brain derived neurotrophic factor (BDNF) have potential for the diagnosis of endometriosis. However, at present BDNF can only be measured by ELISA which requires a clinic visit, a routine blood sample, and laboratory testing. Therefore, we developed a point-of-care device (EndoChip) for use with small blood volumes that can be collected through a finger prick. Specifically, the presented device is a polymer-based chip with a nanoporous, wrinkled gold film acting as the electrode/sensing layer, allowing for the electrochemical detection of BDNF in plasma. Increasing concentrations of BDNF (0.1 – 2.0 ng/ml) induced significant differences in redox current. The biosensor produces a signal readout in a matter of seconds, and is ideal for realizing multiplexing. Blood samples were collected from women (n=20) with chronic pelvic pain undergoing a diagnostic laparoscopy. Plasma BDNF concentrations measured by commercial ELISA were positively correlated (r^2=0.8033; p<0.001) with results from the EndoChip. Our results demonstrate a quick and reliable method for point-of-care quantification of circulating concentrations of BDNF and a promising diagnostic tool for endometriosis.

INTRODUCTION

Endometriosis is a common estrogen-dependent disease of unknown etiology characterized by growth of endometrial stroma and glands in extra-uterine sites[1]. Women with endometriosis report significant pain during menstruation and intercourse, leading to health distress and interference with normal activities including work and leisure time activities. In addition to pain and infertility, endometriosis has a deleterious effect upon women's social functioning, emotional well-being, vitality, employment, and relationships with medical practitioners[2]. The prevalence of endometriosis ranges from 7% of women in the general population to 30% of women with chronic pelvic pain and 50% of infertile women[3-6]; affecting approximately 176 million women worldwide. Unfortunately, diagnosis remains a challenge and is achieved through the assessment of patient history, signs and symptoms, imaging studies and ultimately confirmed by an invasive operative procedure, typically laparoscopy.

A significant obstacle to the timely diagnosis and treatment of endometriosis is the absence of a reliable blood test. Challenges facing health care providers include: early age at onset of symptoms, normalization of pain by primary care providers, and suppression of symptoms through intermittent use of oral contraceptive pills (OCP)[7-8]. As a result, women with endometriosis experience lengthy delays in diagnosis[9-10], which prevent timely access to appropriate treatment[1], leading to significant avoidable morbidity and economic burden for both patients and the health care system[9, 11-12]. The total annual costs associated with surgically confirmed endometriosis in Canada alone are

approximately $1.8 billion dollars[13]. Thus, identification of a clinically useful marker of endometriosis remains an urgent unmet need[14].

Identification of a clinical marker of endometriosis remains elusive. A plethora of biochemical differences in the peripheral circulation, peritoneal fluid, and endometrial tissues of women with endometriosis compared to healthy controls have been documented[15-17] and explored as markers of endometriosis. However to date, low sensitivity limits the use of putative clinical markers as single stand-alone diagnostic markers of endometriosis[1]. A proteomic analysis revealed that brain derived neurotrophic factor (BDNF) expression was greater in the endometrium of women with endometriosis compared to disease-free controls[18]. In a subsequent study[19], mean circulating concentrations of BDNF were greater than two times higher in women with endometriosis relative to healthy asymptomatic controls whilst no differences were found at three months after surgical removal of lesions. We also recently demonstrated that plasma BDNF concentrations were significantly higher in women with surgically confirmed endometriosis (all cases combined) compared to symptomatic controls[20]. Moreover, plasma BDNF concentrations were greater in women with stage I & II endometriosis compared to controls suggesting that circulating concentrations of BDNF may have value in detecting early stage active disease. Using a cut off point of 1,000 pg plasma BDNF/ml, we found the sensitivity and specificity for early stage disease was 100 and 81 %, respectively. Taken together, we suggest that BDNF is a promising clinical marker of endometriosis, particularly for detection of early stage disease. However, BDNF can currently only be quantified in plasma by commercially available Enzyme

Linked Immunosorbent Assays (ELISA) and thus require blood collection in a clinic, processing, specialized equipment, and technical skill for analysis. Ideally, useful clinical markers of endometriosis will be obtained relatively non-invasively and be characterized by high sensitivity and specificity point-of-care analysis for the diagnosis of endometriosis. We therefore sought to develop a simple alternative method for the rapid measurement of BDNF in small volumes of peripheral blood that could be carried out without the need for expensive specialized equipment or expert technical skill. Herein, we describe a facile electrochemical detection technique for BDNF in peripheral blood that is promising for point-of-care diagnostics.

RESULTS

Development of Immunosensing Chips: Nanoporous and wrinkled electrodes were fabricated on prestrained polystyrene (PSPS) substrates as previously described[21-22]. Briefly, PSPS substrates were first modified with an aminosilane layer in order to link a gold seed layer to the substrate (Figure 1 (a)-(i), (ii)). A vinyl mask with the desired electrode pattern was placed on the substrate and a seed layer containing gold nanoparticles was added (Figure 1 (a)-(iii)). Electroless deposition was used to create a nanoporous gold film on the seed layer (Figure 1 (a)-(iv)). Finally, the PSPS substrate was heat shrunk to induce micro/nanoscale wrinkles within the gold film (Figure 1 (a)-(v)).

Antibodies are widely used in numerous applications to quantify target proteins in tissue and blood samples. Therefore, an anti-BDNF monoclonal antibody from a commercial

ELISA kit employed in our previous study[20] was used as the biorecognition element on the electrode surface. To functionalize the electrodes as immunosensors, a self-assembled monolayer of cystamine was immobilized on the gold surface by thiol bonds. An aqueous solution of gluteraldehyde and cystamine was used to bond the antibodies to the electrode surface. The surface of the immunosensing electrodes was further passivated with Bovine Serum Albumin (BSA) to reduce non-specific adsorption (Figure 1(b))[23]. These immunosensing chips were stable (with a less than 5% variation in peak current) on the laboratory bench for at least one week (see Methods for details).

Quantification of BDNF: In order to measure the concentration of BDNF protein captured by the developed immunosensor, we employed an electrochemical detection method based on steric hinderance of a redox reporter, $[Fe(CN)_6]^{3-/4}$ [23]. In this assay, capture of the BDNF protein limits the access of $[Fe(CN)_6]^{3-/4-}$ to the electrode surface, which decreases the faradaic current generated through interfacial electron transfer (Figure 1(b)). Differential pulse voltammetry (DPV) was used before and after incubating the immunosensor with the target to measure the decrease in the oxidation current generated by the redox reporter. This decrease in current (ΔI) was quantified to assess the amount of BDNF captured by the immunosensor (Figure 1(c)). In order to compare the signal changes generated using planar and nanoporous, wrinkled electrodes, we performed DPV scans before and after incubating the immunosensors with solutions containing identical concentrations of BDNF protein in phosphate buffered solution (PBS) (Figure 2(a)). The DPV scans recorded using these two classes of electrodes, and their corresponding ΔI values (Figure 2(a),(c)) demonstrated a significantly larger mean

signal change for the nanoporous, wrinkled electrodes compared to planar electrodes. Following this finding, we used nanoporous, wrinkled electrodes to analyse known concentrations of BDNF protein in PBS. The DPV scans (Figure 2(b)) and their corresponding ΔI values (Figure 2(c)) demonstrate a monotonic decrease in the peak current measured with increasing concentrations of BDNF in the 0.1 to 2.0 ng/mL range. The limit of detection of BDNF using this system is 0.2 ng/mL considering a signal to noise ratio of 3 and a signal to background ratio of 2. Prior studies in women with endometriosis have measured circulating concentrations between approximately 0.2 and 2.0 ng/mL[19-20], demonstrating the applicability of this immunosensor for the diagnosis of endometriosis.

Correlation between ELISA and Chip methods of detection: To compare the results obtained using our newly-developed EndoChip with results obtained using a commercial ELISA method, plasma samples collected from women with endometriosis (35.6 ± 6.2 years) who were either treated with ovarian suppressing medications or were untreated were tested using both methods. The percentage current change detected was significantly lower ($p < 0.05$) in women whose plasma BDNF concentrations were below the diagnostic cut off of 1,000 pg/ml (Figure 3(a)) compared to women above. Plasma BDNF concentrations measured by commercial ELISA were positively correlated (r=0.8033) with ΔI obtained by the EndoChip (Figure 3(b)). Given the large amount of biological background material present in plasma, including a variety of proteins, this demonstrates the selectivity of our assay to BDNF. Taken together, these results suggest that the chip-based assay can be used with a set cut-off point in a binary manner to

discriminate between high and low circulating concentrations of BDNF as a basic diagnostic test for endometriosis. Through this study, we analyzed more than 40 biological (Figure 2) and clinical samples (Figure 3) using disposable chips with nanoporous, wrinkled electrodes. The number of samples analyzed, the trends observed with biological samples (signal to noise ratio of at least three, above 0.2 ng/mL), and the correlation of clinical results with ELISA highlight the repeatability of the designed method.

DISCUSSION

Results of the present study demonstrate that BDNF in plasma can be detected using immunosensing chips. The electrochemical signal changes across the range of circulating BDNF concentrations detected in human plasma correlated well with results using a commercially available ELISA method. Taken together, our results using immunosensing chips and electrochemical detection provide the foundation for a novel point-of-care device to detect circulating concentrations of BDNF for the diagnosis of endometriosis.

Lengthy delays in the diagnosis of endometriosis arise in part from the lack of suitable diagnostic blood tests. In the present study, we combined the sensitivity and selectivity of an immunoassay with portability and ease-of-use of electrochemical detection devices. Specifically, we developed a new class of immunosensing chips by integrating nanoporous, wrinkled electrodes[22] with an electrochemical readout method designed for protein detection[23]. The nanoporous, wrinkled electrodes used in this study are facile and inexpensive to fabricate on polymer substrates, and have previously demonstrated an

enhanced sensivity in electrochemical biosensing compared to planar electrodes[21]. The DPV scans recorded using these two classes of electrodes, and their corresponding ΔI values demonstrated a significantly larger mean signal change for the nanoporous, wrinkled electrodes compared to planar electrodes, which is in line with previous reports that used three-dimensional, high surface area nanostructured electrodes in affinity-based biosensing[24-26]. The percentage change in detected current was substantially lower for study subjects whose circulating BDNF concentrations were below compared to those above the cut-off value established in our prior study[20]. Indeed, the percentage change in detected current was significantly correlated with the plasma BDNF concentration measured in the study participants using conventional ELISA methods. Taken together, we suggest that the electrochemical chip described in this report has value as a rapid screening method in combination with clinical judgment and imaging studies for the detection of endometriosis.

In summary, we have developed a polymer-based chip with nanoporous, wrinkled electrodes functionalized with monoclonal BDNF antibody as immunosensors for detecting BDNF protein. Our results reveal that our chip based method operates in the dynamic range needed for the diagnosis of endometriosis (200-2000 pg/mL), and results correlate well with those generated using a conventional ELISA based method. We propose that the described chip can be utilized as a point of care device in health care providers' offices to assist decisions around medical management of endometriosis.

METHODS

Materials: BDNF antigen and anti BDNF antibody, gold chloride (HAuCl) , 50% (w/w) glutaraldehyde, potassium ferricyanide (FiCN, 99.0%), and cystamine were purchased from Sigma-Aldrich (Mississauga, ON). Potassium chloride (KCl, ≥99.0%) and phosphate buffered solution (PBS, 1.0M, pH 7.4) were purchased from Anachemia (Rouses Point, NY). Sulfuric acid (H_2SO_4, 98%), 2-propanol (99.5%) were purchased from Caledon Laboratories (Georgetown, ON). Ethanol was purchased from Commercial Alcohols (Brampton, ON). Immobilized tris (2-carboxyethyl) phosphine (TCEP) was purchased from Thermo Scientific (Rockford, Il). Tris- (hydroxymethyl)aminomethane (tris, ≥99.9%) was purchased from BioShop Canada (Burlington, ON). The hydrogen peroxide (H_2O_2) was purchased from Caledon. Milli-Q grade ultrapure water (18.2 MΩ·cm) was used to prepare all solutions and for all washing steps.

Device Fabrication: The commercially-available, planar macroelectrodes (CH Instruments, Austin, TX) were cleaned by polishing followed by sonication in ethanol for two 5 minute intervals, DI water for 30 seconds, and air dried. The nanoporous and wrinkled electrodes were fabricated as follows. Commercially available polysterene (PS) substrate (Graphix Shrink Film Graphix, Maple Heights, Ohio) was plasma treated and incubated in a 10% APTES solution in double distilled water. The APTES acts as a self-assembled monolayer with amine affinity for gold (Au). The substrate was covered with a self-adhesive vinyl sheet. A Robo Pro CE5000-40-CRP vinyl cutter equipped with a CB09UA supersteel blade was used with Adobe Illustrator [v.16.0.3] CAD modeling software to create the desired electrode configuration on the vinly masks. The masked

surface is attached to the substrate and incubated in Au nanoparticle (NP) solution overnight to form a seed layer. Electroless deposition was performed on the seed layer by immersion into chloroauric acid ($HAuCl_4$) followed by the injection of hydrogen peroxide (H_2O_2) for 2 minutes and then the Au treated substrate was rinsed with water. Following the electroless Au deposition process, the vinyl mask was removed with tweezers and rinsed with DI water. The devices were heated for 3 minutes at 160 °C in order to be shrunk and rinsed again.

Scanning Electon Microscopy (SEM) Characterization: The wrinkled surfaces were characterized using SEM. SEM images of the gold films before and after shrinking were obtained using a JEOL JSM-7000S scanning electron microscope with an accelerating voltage of 2 kV, working distance of 6 mm, and low probe current.

Deposition of Immunosensor Probes for Protein Detection: For both planar and porous electrode experiments, a self-assembled monolayer (SAM) of antibodies was created using a 2 M cystamine solution at room temperature overnight with gluteraldehyde as the primary linker of the detection device[44]. Before applying the cystamine solution, a 1 hour reduction was performed with TCEP disulfide reduction solution to lyse the sulfide bond in the cystamine molecule. After being rinsed with DI water, 2.5% glutaraldehyde in water, was added to the chip for 1 hour and rinsed again[44].

To test the ability of the device to detect BDNF protein, 10 μg/mL anti BDNF-antibody in phosphate buffered saline (PBS) was incubated with the device at room temperature

for 1 hour. After being washed with PBS twice for 5 minutes, 5% (w/v) bovine serum albumin in PBS was applied to the sensor surface in order the block the unreacted aldehyde groups[44]. The sensors were washed 3 times with PBS. Increasing concentrations of BDNF (0.25, 1, and 2 ng/ml) in PBS were applied to the immunosensors for 40 minutes at 37 °C and washed with PBS and water before electrochemical measurements were assessed.

Electrochemical Measurements: All electrochemical experiments were performed using a CH1 660D potentiostat (CH Instruments, Austin, TX) connected to a 3-electrode arrangement consisting of a reference electrode Ag/AgCl (1.0 M KCl) and a counter electrode platinum wire. The nanoporous, wrinkled working electrodes were created as three 2 x 2 mm^2 electrodes per device connected with thin gold lines to contact pads. Differential pulse voltammograms (DPV) were obtained with a potential step of 5 mV, pulse amplitude of 50 mV, pulse width of 50 ms, and a pulse period of 100 ms[44] before and after incubation of the chip with solutions containing BDNF protein.

Protein Detection: Electrochemical signals were measured in an electrochemical solution containing 2.5 mM $K_3[Fe(CN)_6]$, 2.5 mM $K_2[Fe(CN)_6]$, 0.1 M KCl, and 10 mM phosphate buffer solution (PBS)[44]. Signal changes corresponding to target protein binding to the antibody were determined by calculating the difference between negative and positive current peaks for three aliquots from the same sample and then averaging them.The difference was calculated as follows: Δ I(%) = [(Negative Reduction Peak (uA))-(Positive Reduction Peak (uA))/(Negative Reduction Peak (uA))] x 100. Three

differential pulse voltammograms were performed per reduction peak to ensure accuracy of the reading.

Study participants: To demonstrate that ability of the device to detect plasma BDNF concentrations and discriminate between cases and controls, plasma samples (n=20) from our endometriosis tissue bank were used. Briefly, blood samples were collected immediately before induction of anesthesia from women undergoing a diagnostic laparoscopy at McMaster University Medical Centre. Women were between the ages of 18 and 45 years and had given informed consent. All procedures were conducted according to an approved institutional review board ethics protocol (T-06-064). Plasma samples were collected and stored at -80ºC until required for analysis. Twenty plasma samples from women with surgical confirmation of endometriosis were randomly selected from the tissue bank and coded before analysis. Ten plasma samples were from women who reported use of ovarian suppressing medications (low circulating BDNF concentrations) and ten samples were from women that reported no use of ovarian suppressing medications in the three months preceeding surgery (high circulating BDNF concentrations). Each sample was tested in triplicate by a study team member (MB) blinded to prior study results. Circulating concentrations of BDNF were also determined by a commercial ELISA (BDNF Emax immunoassay ELISA, Promega, Madison, WI) as previously described[20]. Following analysis the sample code was broken and results compared.

Statistical analyses: Three individual electrodes were deposited with the same sample of BDNF target analyte in PBS for each concentration. The average of the three separate electrodes yield the average percentage differences with corresponding standard deviation. In addition, each individual electrode has three separate differential pulse voltammograms that are averaged for the final reading of controls with corresponding standard error. Data are presented as the mean ± SD. Comparisons between groups were made by two-tailed Students t-test using SigmaStat Software (Systat Software Inc., Sigma-Aldrich Chemical Co. Mississauga, ON). Correlation between BDNF results obtained by chip and ELISA methods was carried out by linear regression (SigmaStat). A $p<0.05$ was considered statistically significant for all statistical procedures employed. Signal to noise ratio is defined as the ratio of ΔI at a particular concentration to the standard deviation at that concentration. Signal to background ratio is defined as the ratio of ΔI at a particular concentration to the ΔI obtained using a blank solution of PBS.

Stability testing: Six freshly prepared chips (with the antibody biorecognition layer) were created for stability testing. The DPV scans of the chips were measured right after preparation and following a seven day aging period. During the aging period, the chips were left in a covered petri dish on the laboratory bench. The DPV scans demonstrated an average current change of less than 5%.

REFERENCES

1. Kennedy, S.; Bergqvist, A.; Chapron, C.; D'Hooghe, T.; Dunselman, G.; Greb, R.; Hummelshoj, L.; Prentice, A.; Saridogan, E., ESHRE guideline for the diagnosis and treatment of endometriosis. *Hum.Reprod.* **2005,** *20* (10), 2698-2704.
2. Jones, G.; Jenkinson, C.; Kennedy, S., The impact of endometriosis upon quality of life: a qualitative analysis. *J.Psychosom.Obstet.Gynaecol.* **2004,** *25* (2), 123-133.
3. Eskenazi, B.; Warner, M. L., Epidemiology of endometriosis. *Obstet.Gynecol.Clin.North Am.* **1997,** *24* (2), 235-258.
4. Meuleman, C.; Vandenabeele, B.; Fieuws, S.; Spiessens, C.; Timmerman, D.; D'Hooghe, T., High prevalence of endometriosis in infertile women with normal ovulation and normospermic partners. *Fertil.Steril.* **2009,** *92* (1), 68-74.
5. Howard, F. M., The role of laparoscopy in chronic pelvic pain: promise and pitfalls. *Obstet.Gynecol.Surv.* **1993,** *48* (6), 357-387.
6. Barbieri, R. L., Etiology and epidemiology of endometriosis. *Am.J.Obstet.Gynecol.* **1990,** *162* (2), 565-567.
7. Ballard, K.; Lowton, K.; Wright, J., What's the delay? A qualitative study of women's experiences of reaching a diagnosis of endometriosis. *Fertil Steril.* **2006,** *86* (5), 1296-1301.
8. Nnoaham, K. E.; Hummelshoj, L.; Webster, P.; D'Hooghe, T.; de Cicco, N. F.; de Cicco, N. C.; Jenkinson, C.; Kennedy, S. H.; Zondervan, K. T., Impact of endometriosis on quality of life and work productivity: a multicenter study across ten countries. *Fertil Steril.* **2011,** *96* (2), 366-373.
9. Simoens, S.; Dunselman, G.; Dirksen, C.; Hummelshoj, L.; Bokor, A.; Brandes, I.; Brodszky, V.; Canis, M.; Colombo, G. L.; DeLeire, T.; Falcone, T.; Graham, B.; Halis, G.; Horne, A.; Kanj, O.; Kjer, J. J.; Kristensen, J.; Lebovic, D.; Mueller, M.; Vigano, P.; Wullschleger, M.; D'Hooghe, T., The burden of endometriosis: costs and quality of life of women with endometriosis and treated in referral centres. *Hum Reprod* **2012,** *27* (5), 1292-9.
10. Simoens, S.; Hummelshoj, L.; D'Hooghe, T., Endometriosis: cost estimates and methodological perspective. *Human reproduction update* **2007,** *13* (4), 395-404.
11. Klein, S.; D'Hooghe, T.; Meuleman, C.; Dirksen, C.; Dunselman, G.; Simoens, S., What is the societal burden of endometriosis-associated symptoms? a prospective Belgian study. *Reproductive biomedicine online* **2014,** *28* (1), 116-24.
12. Simoens, S.; Hummelshoj, L.; Dunselman, G.; Brandes, I.; Dirksen, C.; D'Hooghe, T.; EndoCost, C., Endometriosis cost assessment (the EndoCost study): a cost-of-illness study protocol. *Gynecol Obstet Invest* **2011,** *71* (3), 170-6.
13. Levy, A. R.; Osenenko, K. M.; Lozano-Ortega, G.; Sambrook, R.; Jeddi, M.; Belisle, S.; Reid, R. L., Economic burden of surgically confirmed endometriosis in Canada. *J.Obstet.Gynaecol.Can.* **2011,** *33* (8), 830-837.
14. Rogers, P. A.; D'Hooghe, T. M.; Fazleabas, A.; Giudice, L. C.; Montgomery, G. W.; Petraglia, F.; Taylor, R. N., Defining Future Directions for Endometriosis Research: Workshop Report From the 2011 World Congress of Endometriosis in Montpellier, France. *Reprod.Sci.* **2013**.

15. May, K. E.; Conduit-Hulbert, S. A.; Villar, J.; Kirtley, S.; Kennedy, S. H.; Becker, C. M., Peripheral biomarkers of endometriosis: a systematic review. *Hum.Reprod.Update.* **2010,** *16* (6), 651-674.
16. May, K. E.; Villar, J.; Kirtley, S.; Kennedy, S. H.; Becker, C. M., Endometrial alterations in endometriosis: a systematic review of putative biomarkers. *Hum.Reprod.Update.* **2011,** *17* (5), 637-653.
17. Fassbender, A.; Vodolazkaia, A.; Saunders, P.; Lebovic, D.; Waelkens, E.; De, M. B.; D'Hooghe, T., Biomarkers of endometriosis. *Fertil.Steril.* **2013,** *99* (4), 1135-1145.
18. Browne, A. S.; Yu, J.; Huang, R. P.; Francisco, A. M.; Sidell, N.; Taylor, R. N., Proteomic identification of neurotrophins in the eutopic endometrium of women with endometriosis. *Fertil.Steril.* **2012,** *98* (3), 713-719.
19. Giannini, A.; Bucci, F.; Luisi, S.; Cela, V.; Pluchino, N.; Merlini, S.; Casarosa, E.; Russo, M.; Cubeddu, A.; Daino, D.; Artini, P. G.; Genazzani, A. R., Brain-derived neurotrophic factor in plasma of women with endometriosis. *Journal of Endometriosis* **2010,** *2* (3), 144-150.
20. Wessels, J. M.; Kay, V. R.; Leyland, N. A.; Agarwal, S. K.; Foster, W. G., Assessing brain-derived neurotrophic factor as a novel clinical marker of endometriosis. *Fertil Steril* **2016,** *105* (1), 119-128 e5.
21. Adams-McGavin, R. C.; Chan, Y.; Gabardo, C. M.; Yang, J.; Skreta, M.; Fung, B. C.; Soleymani, L., Nanoporous and wrinkled electrodes enhance the sensitivity of glucose biosensors. *Electrochimica Acta* **2017,** *242*, 1-9.
22. Gabardo, C. M.; Adams-McGavin, R. C.; Fung, B. C.; Mahoney, E. J.; Fang, Q.; Soleymani, L., Rapid prototyping of all-solution-processed multi-lengthscale electrodes using polymer-induced thin film wrinkling. *Sci Rep* **2017,** *7*, 42543.
23. Das, J.; Kelley, S. O., Protein detection using arrayed microsensor chips: tuning sensor footprint to achieve ultrasensitive readout of CA-125 in serum and whole blood. *Anal Chem* **2011,** *83* (4), 1167-72.
24. Soleymani, L., Fang, L., Kelley, S.O., Sargent, E.H., Integrated nanostructures for direct detection of DNA at attomolar concentrations. *Appl. Phys. Lett.* **2009,** *95* (14).
25. Soleymani, L.; Fang, Z.; Sargent, E. H.; Kelley, S. O., Programming the detection limits of biosensors through controlled nanostructuring. *Nat Nanotechnol* **2009,** *4* (12), 844-8.
26. Soleymani, L.; Li, F., Mechanistic Challenges and Advantages of Biosensor Miniaturization into the Nanoscale. *ACS Sens* **2017,** *2* (4), 458-467.

Acknowledgements: We thank the study participants and staff of the Endo@Mac program for their assistance and selfless contributions to this project. The authors gratefully acknowledge the assistance of Annette Bullen with study participant recruitment, sample collection and data extraction. This study was supported by the Canadian Institutes of Health Research (CIHR; grant MOP142230; to WGF) and the Natural Sciences and Engineering Research Council (NSERC) of Canada. L.S. is the Canada Research Chair (Tier II) in Miniaturized Biomedical Devices.

Author contributions: WGF wrote the grant application and prepared the ethics applications for this project. Sample collection, processing of plasma samples and ELISA's were carried out by MT. Microchips were prepared and samples analyzed by MB and BF under the supervision of LS. The first draft fo the manuscript was prepared by MB, edited and submitted by LS and WGF.

Competing financial interests: Patent submissions have been made by WGF for the use of BDNF as a diagnostic tool in endometriosis and LS holds a patent for the electrochemical device.

Legend to Figures

Figure 1. a) Schematic diagram demonstrating the fabrication of nanoporous, wrinkled gold electrodes. b) Thiol bonding immobilizes the primary linker cystamine followed by the reactive secondary linker glutaraldehyde to create a self-assembled monolayer of aldehyde groups. Anti-BDNF (Brain Derived Neurotrophic Factor) monoclonal antibody (mAb) was immobilized via the secondary linker to assemble the electrochemical detection device. Unreacted aldehyde groups are blocked using 5 % (w/v) Bovine Serum Albumin (BSA). The target analyte, BDNF protein, will attach to its mAb. The redox reporter system of $[Fe(CN)_6]^{3-/4-}$ is used to electrochemically detect the presence of protein. c) Differential Pulse Voltammogram illustrating how the binding of BDNF to the antibody inhibits the interfacial electron transfer reaction therefore decreasing current signal.

Figure 2. (a) Differential pulse voltammetry of planar (top) and porous (bottom) electrodes in an electrochemical solution of 2.5 mM $[Fe(CN)_6]^{3-/4-}$ before (black) and after (red) adding an 1.0 ng/mL concentration of BDNF in PBS. The inset demonstrates the scanning electron micrographs of the surface of plannar and porous electrodes illustrating the increased surface area of the wrinkled surface induced by heat shrinkage. (b) Differential pulse voltammograms illustrating the difference in electrochemical signals before and after the addition of target analyte at increasing BDNF concentrations (0.25, 1.0, and 2.0 ng/mL). (c) In the first panel, comparison of signal changes of planar and nanoporous, rinkled immunosensors using a BDNF concentration of 1 ng/mL. In the

second panel (c) the mean ± SD percentage change in detected current using increasing BDNF concentrations (0.1, 0.2, 0.5, 0.6, 1.0, and 2.0 ng/mL).

Figure 3. (a) Percentage change in detected current in study participants with plasma BDNF concentrations below versus above the diagnostic cut off (1,000 pg/ml). (b) Linear regression line ($r^2=0.8033$) of plasma BDNF concentrations determined using a commercial ELISA method as previously described[20] and differences in current signal detected using the EndoChip device.

Fig. 1.

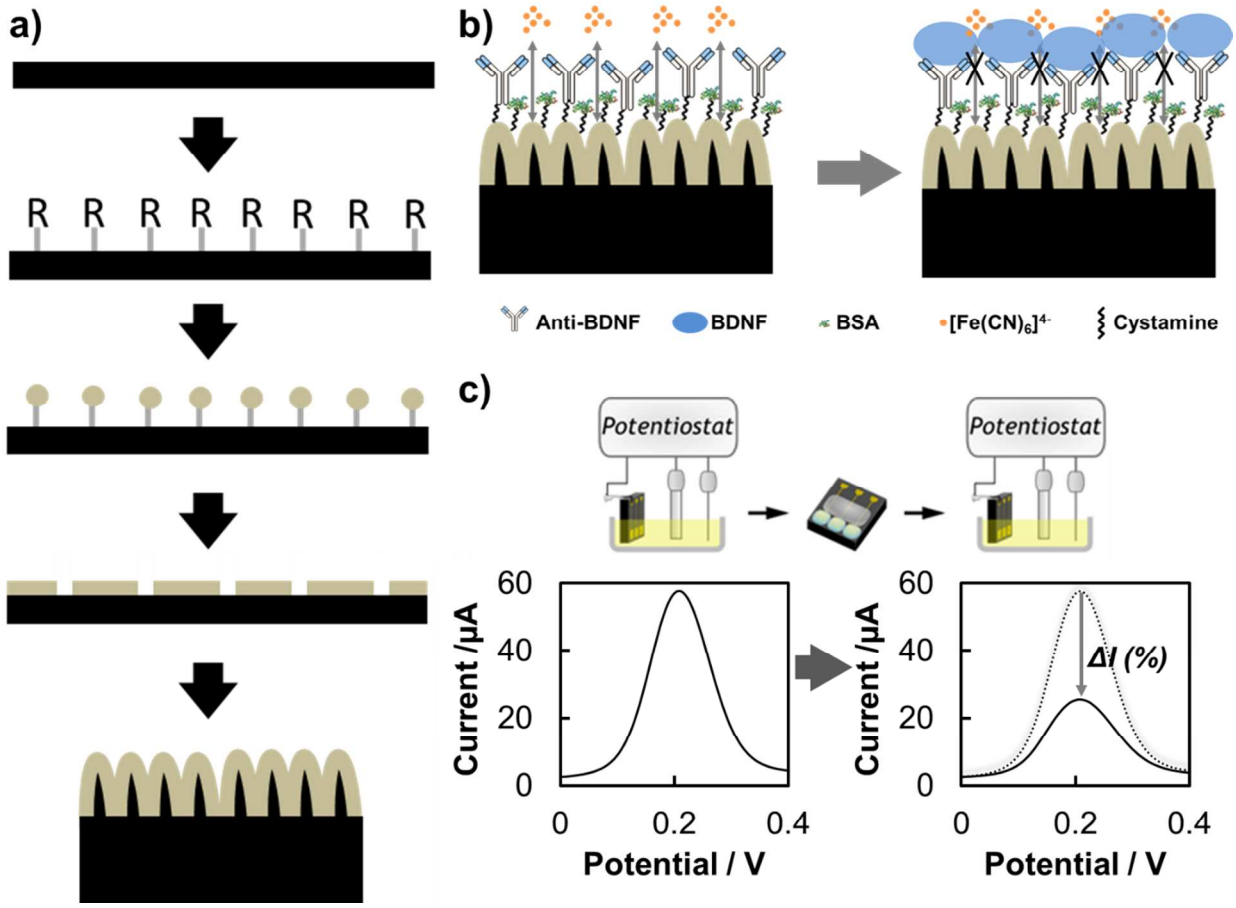

a)
R R R R R R R R
b)
Anti-BDNF
BDNF
BSA
[Fe(CN)6]4-
Cystamine
c)
Potentiostat
Potentiostat
Current /μA
60
40
20
0
0
0.2
0.4
Potential / V
Δl (%)

Fig. 2.

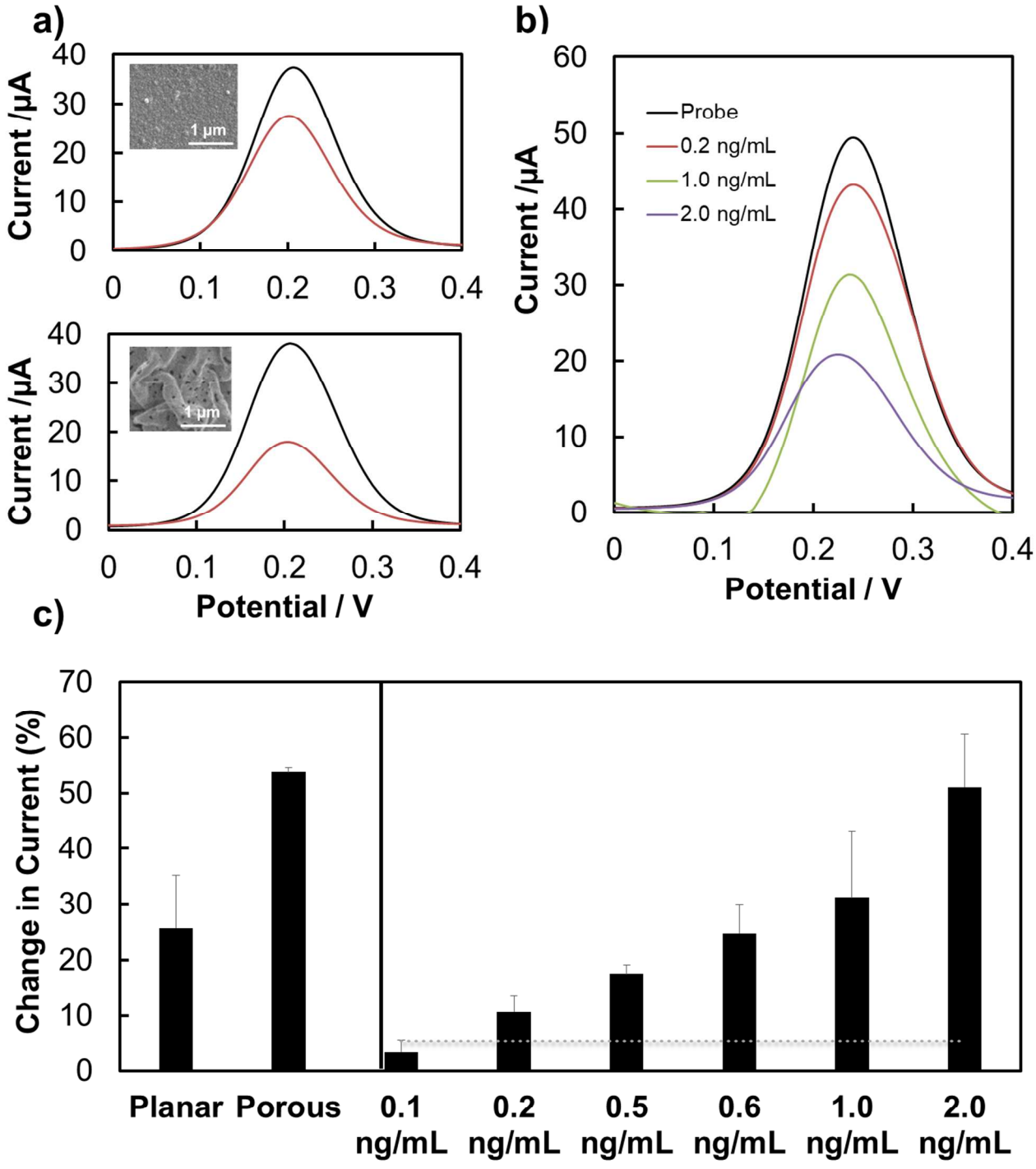
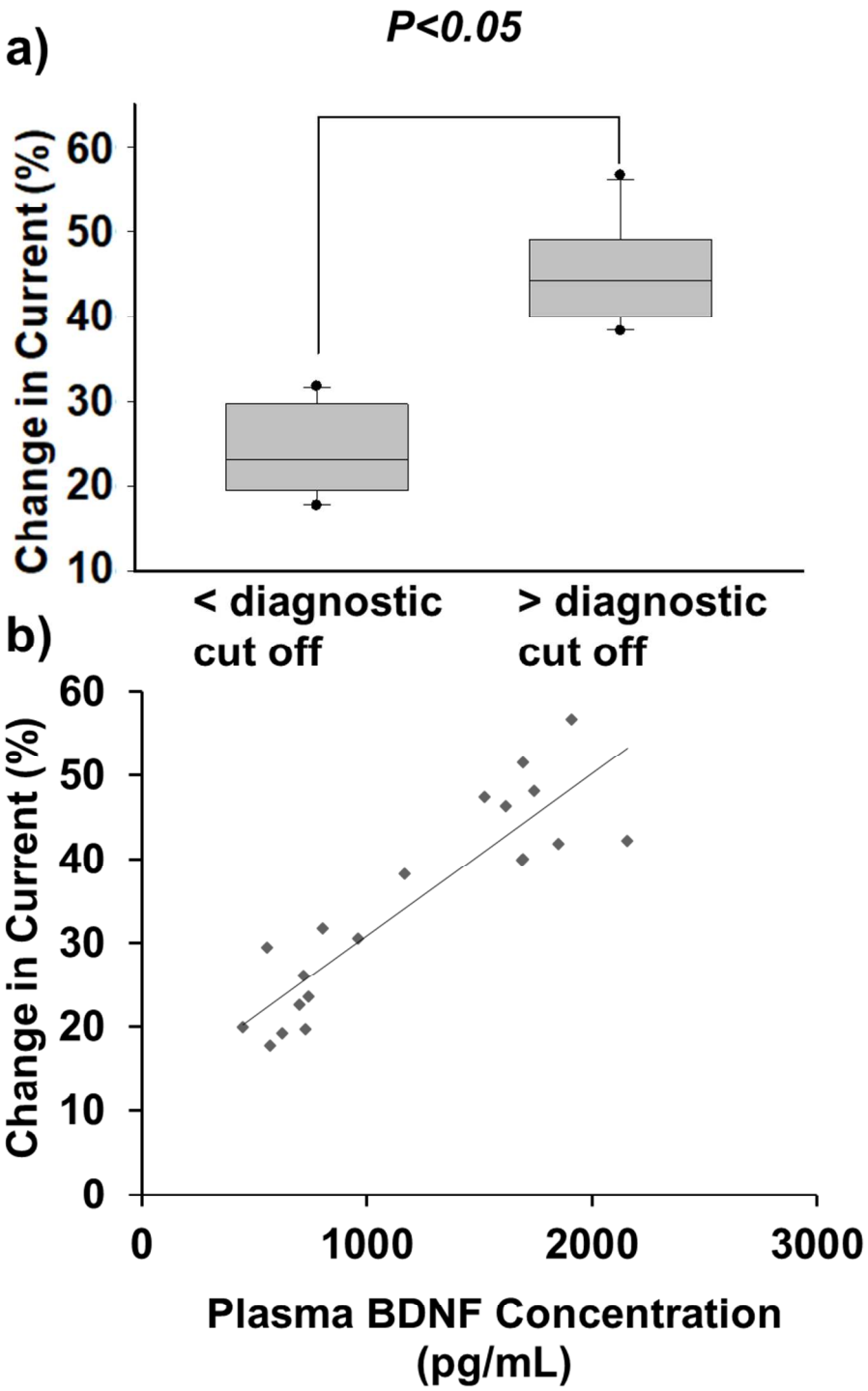

a)
b)
c)
Current /μA
Potential / V
1 μm
Probe
0.2 ng/mL
1.0 ng/mL
2.0 ng/mL
Change in Current (%)
Planar
Porous
0.1 ng/mL
0.2 ng/mL
0.5 ng/mL
0.6 ng/mL
1.0 ng/mL
2.0 ng/mL

Fig. 3


a)
P<0.05
Change in Current (%)
60
50
40
30
20
10
< diagnostic cut off
> diagnostic cut off
b)
Change in Current (%)
60
50
40
30
20
10
0
0
1000
2000
3000
Plasma BDNF Concentration (pg/mL)

TOC Figure