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Aptamer-Based Nano-Slit Platforms for Characterizing Human Performance Biomarkers

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"Aptamer-based nano-slit platforms for characterizing human performance biomarkers"

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Abstract: The need for methods to assess for human performance biomarkers that indicate fatigue, vigilance, and stress among DoD personnel during key missions has been identified as a major priority by the AFOSR. We seek to develop aptamer-based sensing platforms within or immobilized to nanoscale structures to enrich biomarkers of interest, for downstream coupling to multiplexed detection strategies from biofluids that contain physiological levels of interfering molecules. The chief scientific advancements include the development of electric field-based biomarker enrichment platforms, nanoconfined strategies for determining aptamer binding kinetics to target biomarkers and nanoparticle strategies for surface immobilization-free detection of biomarkers. The work was performed in collaboration with AFRL's 711th Human Performance Wing.

Introduction: Monitoring the expression profile of stress-related biomarkers can enable an improved understanding of the underlying signaling pathways that cause stress and lead to the development of therapies to mitigate stress. In this project, we seek to apply electrokinetic force fields for selective enrichment of stress-related neurological biomarkers within nano-slit devices, so that they could be coupled to aptamer-based detection strategies to assess human performance. The overall aims include:
(1) Enhance detection sensitivity by electrokinetic enrichment of biomarkers within nano-slit device platforms
(2) Enhance detection selectivity using aptamers and frequency selective AC fields
(3) Quantify biomarker binding interactions of aptamer libraries with target biomarkers

Experiment: The enabling device used in this work is a nanoslit device with nanoscale confinement in depth. Per Fig. 1, a nanoslit to nanoscale depth (50-200 nm) is microfabricated by etching into quartz or imprinting into COC (cyclic olefin copolymer). This device was immobilized with aptamers of interest using gold-thiol chemistry and room temperature bonding under pressure to ensure biofunctionality. In some situations, the sensing was performed without surface immobilization.

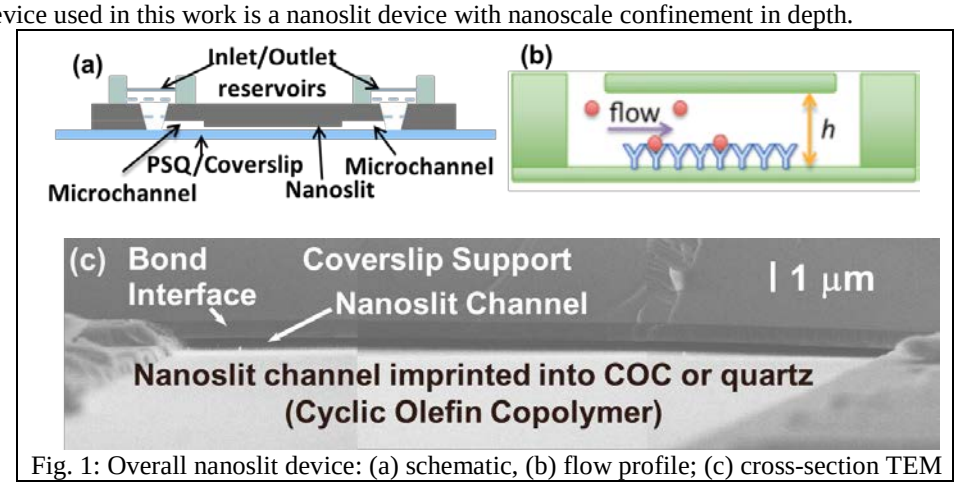


Fig. 1: Overall nanoslit device: (a) schematic, (b) flow profile; (c) cross-section TEM

Results and Discussion: In the following sections, we describe the highlights of results in each significant publication from this work.

1. “Aptamer-Functionalized Nanoparticles for Surface Immobilization-Free Electrochemical Detection of Cortisol in a Microfluidic Device”, B. J. Sanghavi; J. H Moore; J. L Chavez; J. Hagen; N. Kelley-Loughnane; C. –F. Chou, N. S. Swami*. *Biosens. Bioelectron.* (2016), 78, pp. 244-252; DOI: 10.1016/j.bios.2015.11.044. Journal Impact Factor =7.8; Times Cited = 80.

Monitoring the periodic diurnal variations in cortisol from small volume samples of serum or saliva is of great interest, due to the regulatory role of cortisol within various physiological functions and stress symptoms. Current detection assays are immunologically based and require cumbersome antibody immobilization chemistries, thereby limiting the assay versatility, kinetics, and reproducibility. We present a quantitative aptamer-based detection methodology for cortisol that does not require target labeling, capture probe immobilization on the detection surface or wash steps prior to readout. Using a recognition system of aptamer functionalized gold nanoparticles pre-bound with electro-active triamcinolone, the cortisol level is detected based on its competitive binding to the aptamer by following signal from the displaced triamcinolone using square wave voltammetry at patterned graphene-modified electrodes in a microfluidic or nanoslit device. Due to the 3D analyte diffusion profile at the aptamer interface and the ability to enhance the surface area for cortisol capture, this assay shows signal linearity over a five-log analyte concentration range (10 µg/mL – 30 pg/mL) and exhibits rapid binding kinetics with cortisol versus other glucocorticoids, as apparent from the absence of interferences from estradiol, testosterone and progesterone. The assay is carried out within the biologically relevant range for glucocorticoids in serum and saliva matrices, and benchmarked versus ELISA and radioimmunoassays. Based on absence of cumbersome surface immobilization and wash steps for carrying out this assay, its quantitative signal characteristics and its ability to resist interferences from other glucocorticoids, we envision its application towards routine monitoring of cortisol within bio-fluids.

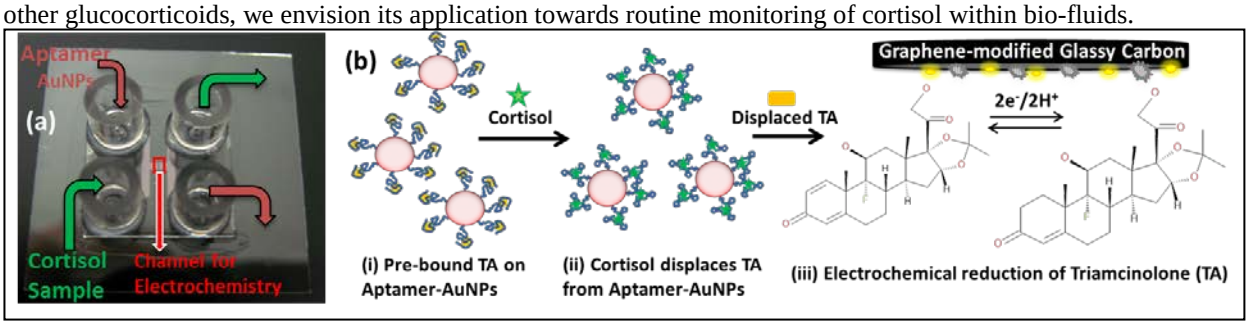


Fig. 2: (a) Top view of device on glass coverslip within an insulator layer for fluidic and electrical isolation; (b) Displacement assay for aptamer-based cortisol detection, due to signal from electrochemical reduction of displace triamcinolone (TA) on graphene-modified electrodes.

The electrochemical aptamer assay for cortisol is based on close similarity in its size and structure to that of electro-active triamcinolone (TA). While this similarity causes the aptamer to bind with triamcinolone (Fig. 1b(i)), it is displaced from the aptamer in the presence of cortisol (Fog. 1b(ii)), since the aptamer was specifically selected for preferential binding to cortisol. The displaced triamcinolone can be electrochemically reduced (Fig. 1b(iii)) for detection at graphene-modified electrodes. The chief analytical figures of merit are summarized in Fig. 3. This aptamer-functionalized nanoparticle platform has the following features:

(i) the high capture-probe level enables a wide dynamic range (10 µg/mL – 10 pg/mL);
(ii) the 3D diffusion profile of cortisol towards aptamers for rapid binding;
(iii) wash-free detection – allows for real-time monitoring

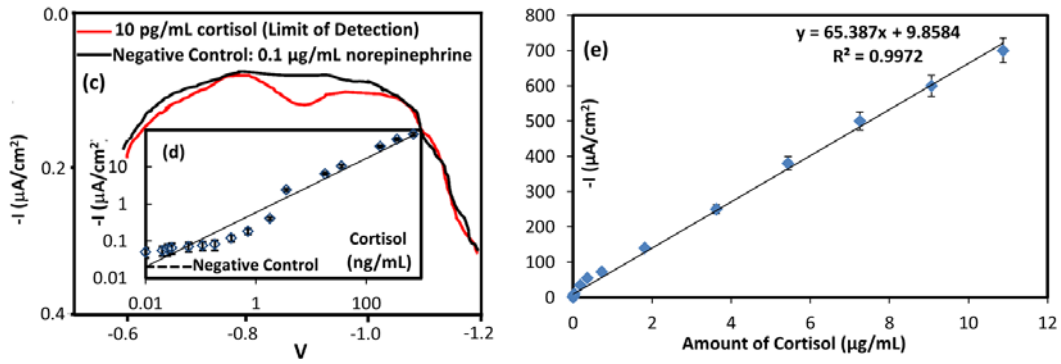
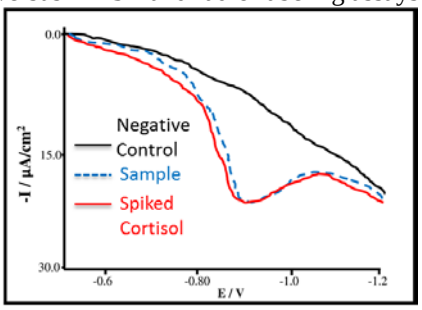


Fig. 3: (c) Cortisol signal at its limit of detection (LOD) at 10 pg/mL versus negative control (0.1 µg/mL of Neuropeptide Y); (d) Dilution plot close to LOD; and (e) Dilution plot over five-log signal linearity range (30 pg/mL to 10 µg/mL). Error bars are mean ± standard deviation.

Figure 4 shows the quantification of samples using the voltammetry assay (left) and validation of voltammetry versus ELISA and radio-labeling assays for three samples from AFRL.



Serum Sample	Voltammetry	ELISA	Radio-labeling
1 (Sample 17)	0.09 ± 2.3	0.08 ± 3.9	0.11
2 (Sample 20)	0.15 ± 2.1	0.13 ± 3.4	0.21
3 (Sample 54)	0.11 ± 2.5	0.09 ± 3.6	0.16

Fig. 4 (left) Sample quantification using voltammetry; (right) validation of voltammetry versus ELISA & radio-labeling assays for three samples from AFRL (±%).

2."Nanoslit design for ion conductivity gradient enhanced dielectrophoresis for ultrafast biomarker enrichment in physiological media", A. Rohani, W. Varhue, K. -T. Liao, C. –F. Chou, N. Swami*. Biomicrofluidics (2016), 10 (3) 033109:1-14. Journal Impact Factor =3.1. Times Cited = 8.

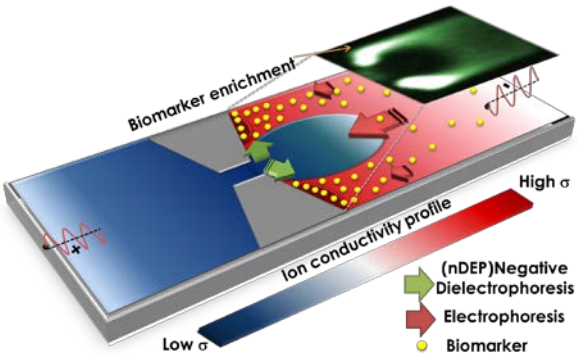


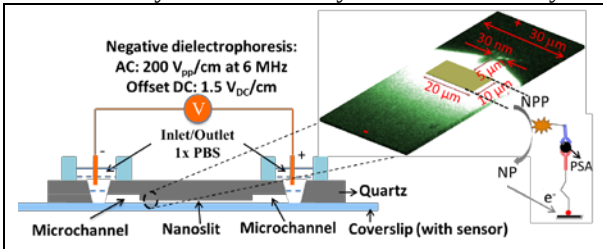
Fig. 5: Overview of method for AC electrokinetic enrichment of biomarkers in nanoslits

Selective and rapid enrichment of biomolecules is of great interest for biomarker discovery, protein crystallization and in biosensing for speeding assay kinetics and reducing signal interferences. The current state of the art is based on DC electrokinetics, wherein localized ion depletion at the microchannel to nanochannel interface is used to enhance electric fields and the resulting biomarker electromigration is balanced against electro-osmosis in the microchannel to cause high degrees of biomarker enrichment. However, biomarker enrichment is not selective and the levels fall off within physiological media of high conductivity, due to a reduction in ion concentration polarization and electro-osmosis effects. Herein, we present a methodology for coupling AC electrokinetics with ion concentration polarization effects in nanoslits under DC fields, for enabling ultrafast biomarker enrichment in physiological media. Per Fig. 5, using AC fields at the critical frequency necessary for negative dielectrophoresis of the biomarker of interest, along with a critical offset DC field to create proximal ion accumulation and depletion regions along the perm-selective region inside a nanoslit, we enhance the localized field and field gradient to enable biomarker enrichment over a wide spatial extent along the nanoslit length. While enrichment under DC electrokinetics relies solely on ion depletion to enhance fields, this AC electrokinetic mechanism utilizes ion depletion as well as ion accumulation regions to enhance the field and its gradient. Hence, biomarker enrichment continues to be substantial in spite of the steady drop in nanostructure perm-selectivity within physiological media.

3. "Ultrafast immunoassays by coupling dielectrophoretic biomarker enrichment on nanoslit molecular dam with electrochemical detection on graphene", B.J. Sanghavi+, W. Varhue, A. Rohani, K.T. Liao, L. Bazydlo, C.F. Chou, N.S. Swami*; *Lab Chip* (2015), 15, 4563 – 4570. Journal Impact Factor =6.1; Times Cited = 64.

Heterogeneous immunoassays usually require long incubation times to promote specific target binding and several wash steps to eliminate non-specific binding. Hence, signal saturation is rarely achieved at detection limit levels of analyte, leading to significant errors in analyte quantification due to extreme sensitivity of the signals to incubation time and methodology. The poor binding kinetics of immunoassays at detection limit levels can be alleviated through creating an enriched analyte plug in the vicinity of immobilized capture probes to enable signal saturation at higher levels and at earlier times, due to higher analyte association and its faster replenishment at the binding surface. Herein, we achieve this by coupling frequency-selective dielectrophoretic molecular dam enrichment of the target biomarker in physiological media to capture probes immobilized on graphene-modified surfaces in a nanoslit to enable ultrafast immunoassays with near-instantaneous (< 2 minutes) signal saturation at dilute biomarker levels (picomolar) within ultra-low sample volumes (picoliters). This methodology is applied to the detection of Prostate Specific Antigen (PSA) diluted in serum samples, followed by validation against a standard two-step immunoassay using three de-identified patient samples. Based on the ability of dielectrophoretic molecular dam analyte enrichment methods to enable the detection of PSA at 1-5 pg/mL levels within a minute, and the relative

insensitivity of the signals to incubation time after the first two minutes, we envision its application for improving the sensitivity of immunoassays and their accuracy at detection limit levels.



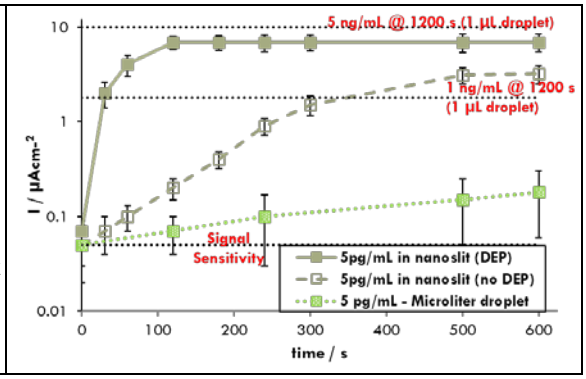


Fig. 6: (Top) Electrokinetic enrichment in nanoslit dielectrophoresis coupled to detection;
(right) enhanced detection kinetics comparing:
(i) droplet; (ii) nanoslit only; (iii) nanoslit + DEP

4. "Aptamer-functionalized graphene-gold nanocomposites for label-free detection of dielectrophoretic-enriched neuropeptide Y"; R. E. Fernandez, B. J. Sanghavi, V. Farmehini, J. L. Chavez, J. A. Hagen, N. Kelley-Loughnane, C. F. Chou, N. S. Swami*. *Electrochemistry Communications* (2016), 72, 144-147. Journal Impact Factor =4.6. Times Cited = 19.

We present an electrokinetically enhanced aptamer sensing platform on a disposable plastic chip for label-free detection of neuropeptide Y (NPY), which is a key neurological biomarker. The sensor consists of aptamer-functionalized graphene-gold nanocomposites (Gr-AuNs) patterned inside a nanoslit that is embossed on cyclic olefin copolymer via nanoimprint lithography. Analyte molecules are dielectrophoretically focused through the nanoslit onto aptamer-immobilized Gr-AuNs for rapid and selective electrochemical detection of NPY at picomolar levels. Per Fig. 8, picomolar level detection limits can be achieved under electric fields to enrich NPY, which was then confirmed for selectivity for serum samples with cortisol spiked in at µg/mL levels.

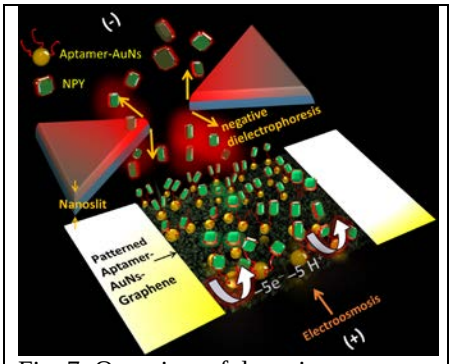
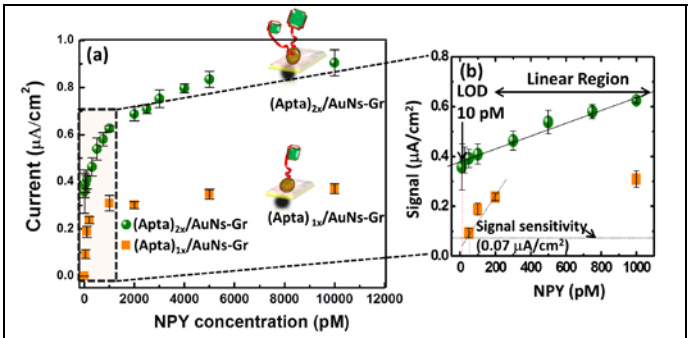


Fig. 7: Overview of detection strategy



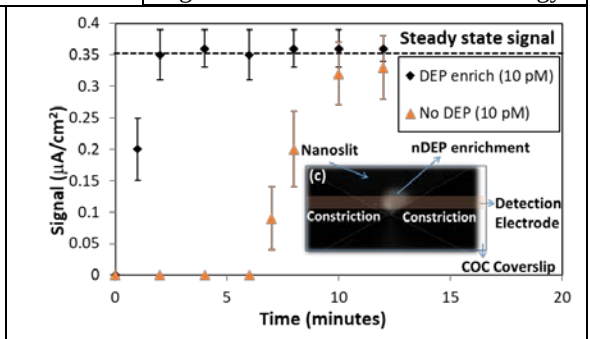


Fig. 8: (a) Dynamic range and (b) linear detection regions for NPY; and (c) detection kinetics.

5. "Frequency-selective electrokinetic enrichment of biomolecules in physiological media"

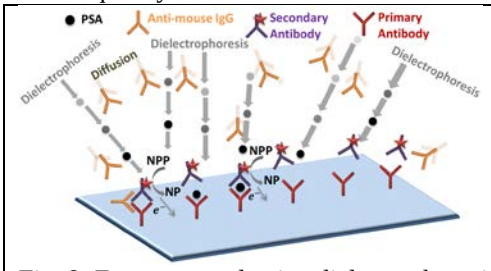


Fig. 9: Frequency-selective dielectrophoresis is used for selective enrichment of prostate specific antigen versus anti-mouse immunoglobulin to red immunoassay signal interference

A. Rohani, B. J. Sanghavi+, A. Salahi, K. –T. Liao, C.-F. Chou, N.S. Swami*, Nanoscale (2017), 9, 12124 – 12131. Impact Factor =7.4; Cited = 15.

Proteomic biomarkers of interest to the early diagnosis of diseases and infections are present at trace levels versus interfering species. Hence, their selective enrichment is needed within bio-assays for speeding binding kinetics with receptors and for reducing signal interferences. While DC fields can separate biomolecules based on their electrokinetic mobilities, they are unable to selectively enrich biomarkers versus interfering species, which may possess like-charges. We present the utilization of AC electrokinetics to enable

frequency-selective enrichment of nanocolloidal biomolecules, based on the characteristic time constant for polarization of their electrical double-layer, since surface conduction in their ion cloud depends on colloidal size, shape and surface charge. In this manner, using DC-offset AC fields, differences in frequency dispersion for negative dielectrophoresis are balanced against electrophoresis in a nanoslit channel to enable the selective enrichment of prostate specific antigen (PSA) versus anti-mouse immunoglobulin antibodies that cause signal interferences to immunoassays. Through coupling enrichment to capture by receptors on graphene-modified surfaces, we demonstrate the elimination of false positives caused by anti-mouse immunoglobulin antibodies to the PSA immunoassay.

List of Publications and Significant Collaborations that resulted from your AOARD supported project:

1. A. Rohani, B. J. Sanghavi, A. Salahi, K. –T. Liao, C.-F. Chou, N.S. Swami*, "Frequency-selective electrokinetic enrichment of biomolecules in physiological media based on electrical double-layer polarization"; *Nanoscale* (2017), 9, 12124 – 12131. Impact Factor =7.4; Cited = 22.
2. R. E. Fernandez, B. J. Sanghavi, V. Farmehini, J. L. Chávez, J. Hagen, N. Kelley-Loughnane, C.-F. Chou, N. S. Swami; "Aptamer-functionalized graphene-gold nanocomposites for label-free detection of dielectrophoretic-enriched neuropeptide Y", *Electrochem. Comm.* (2016), 72, 144-147. Times Cited = 19.
3. B.J. Sanghavi, W. Varhue, A. Rohani, K.T. Liao, L. Bazydlo, C.F. Chou, N.S. Swami*; "Ultrafast immunoassays by coupling dielectrophoretic biomarker enrichment on nanoslit molecular dam with electrochemical detection on graphene", Lab Chip (2015), 15, 4563 – 4570. Times Cited = 64.
4. B. J. Sanghavi; J. A Moore; J. L Chavez; J. Hagen; N. Kelley-Loughnane; C. –F. Chou, N. S. Swami*. ""Aptamer-Functionalized Nanoparticles for Surface Immobilization-Free Electrochemical Detection of Cortisol in a Microfluidic Device", *Biosens. Bioelectron.* (2016), 78, pp. 244-252; Times Cited = 80
5. A. Rohani, W. Varhue, K.-T. Liao, C.-F. Chou, N.S. Swami; "Nanoslit design for ion conductivity gradient enhanced dielectrophoresis; *Biomicrofluidics* (2016) 10, 033109; Times Cited = 8.
6. P Teerapanich, M Pugnière, C Henriquet, YL Lin, CF Chou, T Leïchlé, Nanofluidic Fluorescence Microscopy (NFM) for real-time monitoring of protein binding kinetics and affinity studies, *Biosensors & Bioelectronics*, 2017, 88, 25–33. Times Cited = 6.
7. Pattamon Teerapanich, Martine Pugniere, Corinne Henriquet, Yii-Lih Lin, Antoine Naillon, Pierre Joseph, Chia-Fu Chou, Thierry Leichle, Nanofluidic fluorescence microscopy with integrated concentration gradient generation for one-shot parallel kinetic assays, *Sensors and Actuators B: Chemical* (2018), 274, 338-342.
8. KK Sriram, S Nayak, S Pengel, CF Chou, A Erbe, 10 nm deep, sub-nanoliter fluidic nanochannels on germanium for attenuated total reflection infrared (ATR-IR) spectroscopy, *Analyst* (2017) 142 (2), 273-278

Collaborative interactions with industry or with Air Force Research Laboratory scientists:

- Joint papers with the following scientists from AFRL's 711-th Human Performance Wing: Jorge Chavez, Nancy Kelley-Loughnane, and Joshua Hagen
- Collaboration with UES, Inc. on multi-target sensor development

Attachments: Publications a), b) and c) listed above if possible.

DD882: As a separate document, please complete and sign the inventions disclosure form.