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Aptasensor for Detection of Influenza-A in Human Saliva*

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***Abstract*— Access to low-cost, rapid, individualized diagnostics at point-of-care and point-of-need is vital to minimize the impact of highly infectious viruses, such as influenza. Herein, a biosensor for detecting hemagglutinin (HA), an abundant capsid protein in H1N1 viruses, is demonstrated. A gold working electrode was functionalized with a thiol-modified, HA-binding aptamer derivatized with a methylene blue modification for redox reporting. The aptamer was characterized by surface plasmon resonance to confirm its biorecognition activity for HA. The aptasensor was characterized by square wave voltammetry to quantify the sensor's response to varying concentrations of HA. The sensor exhibited a lower limit of detection of 1.5 pM with linear detection of up to 1.2 nM in both Tris buffer and simulated human saliva, thus encompassing the clinically relevant HA range in saliva. Average sensitivity was measured at 21.083 nA·nM^{-1} in Tris and 14.5 nA·nM^{-1} in artificial saliva across clinically relevant HA titers. Sensor stability across time was also investigated, providing a preliminary understanding of the translational viability of the aptasensors for mobile and remote diagnostic applications.**

I. Introduction

As the world continues to adapt in response to the evolving COVID-19 pandemic, a significant societal need for accessible, accurate, and portable viral testing has clearly emerged. Access to low-cost, individualized diagnostics provides the necessary information to inform medical intervention on individuals and communities. Before the COVID-19 pandemic, clinical testing for influenza and other transmissible respiratory disease was considered the gold standard, despite requiring in-person clinic visits by patients to obtain nasopharyngeal, nasal, or oropharyngeal tissue swabs for processing, exacerbating the risk of viral spread [1]. As significant efforts towards point-of-need testing continue [2-4], there is great potential for the parallel development of at-home testing for other highly infectious viruses, such as influenza.

H1N1 is a type of influenza A virus, airborne and highly transmissible between humans. This strain of influenza was responsible for the "swine flu" pandemic, which spanned over a year between June 2009 and August 2010. Individuals infected with H1N1 present viral loads in saliva of 10^{-10} viral particles·mL^{-1} [5]. Hemagglutinin, or HA, is a small (Stokes' radius of 8.5 nm) yet abundant membrane protein displayed on the surface of H1N1 and responsible for the virus' ability to infect [6]. Vaccinations against H1N1 rely on targeting of HA [7]; analogously, the HA titer in saliva can be directly correlated to viral load in the sample, with the relevant pathological range falling between 0 and 10,000 pg·mL^{-1} [5].

Significant progress has been made in the development of electrochemical biosensors, which operate by transducing a bio-recognition phenomenon - namely, the formation of an affinity complex between the target analyte and a ligand (e.g., an anti-body, aptamer, peptide, enzyme, etc.) immobilized on the sensor - into an electrical signal. Electrochemical sensors have been shown to be ideal wearable and point-of-care biosensors for various conditions [8, 9]. Chief among the ligands employed for target recognition, aptamers are short, single strands of RNA or DNA, often chosen through the SELEX process, known for their highly selective target binding [10]. Recent studies demonstrated the use of aptamers for rapid influenza detection via SERS (Surface-Enhanced Raman Spectroscopy); these sensors, however, do not support point-of-care deployment [11]. The combination of aptamer ligands with electrochemical sensors, *i.e., an aptasensor*, show promise as highly specific, stable, and versatile devices. Aptasensors have been developed in a variety of electrochemical formats, including but not limited to electrochemical impedance spectroscopy and voltammetry [12-16].

In this study, we present the demonstration of a novel aptasensor for the detection of HA in human saliva. The operational principle of this sensor relies on the formation of a self-assembled monolayer of electrochemically active aptamers, RHA0006, reported in literature to have a K_D value between 15.3 nM and 24.7 nM for HA [17]. **Fig. 1** shows the architecture of the proposed aptasensor. The aptamer was derivatized with thiol modification at the 5'-terminus and methylene blue modification at the 3'-terminus to enable conjugation to the electrode and electrochemical transduction of conformational changes triggered due to HA binding, respectively. The sensor was interrogated via square wave voltammetry, which is preferred for its ability to produce highly sensitive results by eliminating contributions from background and non-faradaic currents [18], since increasing HA concentrations cause a dampening of peak current response. The viability of this sensor was demonstrated in both non-competitive conditions (aqueous buffer) and artificial

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human saliva, with focus on aptamer viability, biological relevance, and sensor stability.

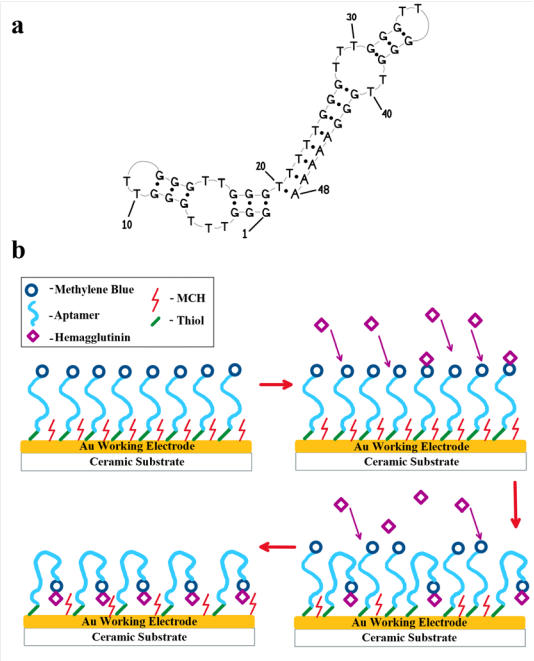

Fig. 1. Sensor Principle. (a) RHA0006 aptamer structure ValFold prediction as determined by Shiratori *et al.* [17], (b) Sensing mechanism with (clockwise) functionalized sensor, introduction of HA, aptamer conformational change, and sensor saturation.

II. Materials & Methods

A. Aptamer Preparation

The aptamer sequence RHA0006 (5'-GGG TTT GGG TTG GGT TGG GTT TTT GGG TTT GGG TTG GGT TGG GAA AAA-3'), derivatized with a thiol (SH-C_6-) modification on the 5' terminus and a methylene blue modification on the 3' terminus was synthesized by IDT Technologies (Coralville, IA). A solution of aptamer in MilliQ water at 100 µM was mixed with 20 mM tris[2-carboxyethyl] phosphine (TCEP) (MilliporeSigma, St. Louis, MO) in the dark for one hour at room temperature, or until the solution became clear. To screen the negative charge on the aptamer backbone and ensure the formation of a proper monolayer, a solution of 1 mM $MgCl_2$ in phosphate buffer saline (PBS) at pH 7.4 was and added to the reduced aptamer in a 50:1 volumetric ratio.

B. Electrode Functionalization Specifications

DropSens electrodes (DRP-220BT-U75; Metrohm, Riverview, FL) with a gold working, gold auxiliary, and silver reference were prepared for functionalization via cyclic voltammetry (CV) cleaning procedures based on that described by Fischer et al. [19]. CV was conducted for 15 cycles in 0.5 M H_2SO_4 at 300 mV·s^{-1} across a voltage window from –0.4 V to +1.4 V, with both an initial and final voltage of 0 V. DropSens electrodes were introduced to the aptamer solution only atop the working electrode to ensure reproducibility and minimize additional impedance on the auxiliary and reference electrodes. Uncoated gold Surface Plasmon Resonance (SPR) chips (BioNavis, Tampere, Finland) were initially cleaned with ethanol, acetone, and MilliQ water. An aliquot of 300 µL of aptamer solution at 1µM, prepared as detailed above, was placed on the chip surface, and incubated overnight, in a moist, dark environment to allow the formation of a self-assembled monolayer of aptamer, as depicted in **Fig. 2a**. After incubation, the sensors were rinsed with MilliQ water, dried gently with N_2 gas, and contacted with a 2 mM solution of 6-mercapto-1-hexanol (MCH) (MilliporeSigma, St. Louis, MO) in PBS to backfill ("blocking") the gold surface. The electrodes were rinsed with MilliQ water, dried with N2 gas, and immediately tested. **Fig. 2b**. shows the impact of functionalization through square wave voltammetry (SWV), producing the expected current peak in the redox potential window of methylene blue.

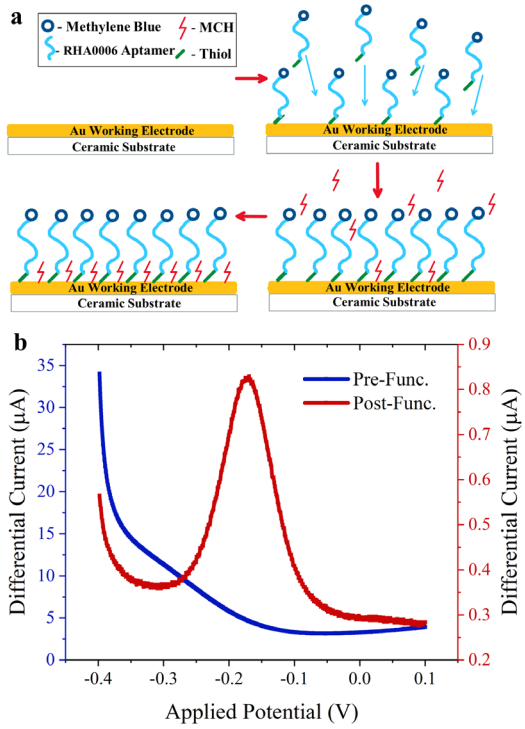

Fig. 2. Sensor Functionalization. (a) Symbolic demonstration of functionalization with (clockwise) cleaned bare gold, SAM formation, MCH backfilling, and final sensor, (b) SWV peak before and after functionalization, measured in Tris-HCl.

C. Surface Plasmon Resonance

Hemagglutinin (HA) from recombinant H1N1 (A/California/04/2009) obtained from Sino Biological (Wayne, PA) was reconstituted in PBS. The SPR buffer was prepared as described by Shiratori et al. [17] (50 mM Tris HCl, pH 7.4, 100 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 0.01% v/v Tween 20) and degassed. A KSV SPR 200 (BioNavis, Tampere, Finland) was used for all SPR measurements. Following chip insertion, baseline data were collected by flowing the Tris buffer at 30 µL·min^{-1} for 10 minutes. Aliquots of 400 µL of HA solution at 10nM, 25nM, 50nM, 65nM, 85nM, 100nM, 125nM, and 150nM were injected into the flow channels at 30 µL·min^{-1} until saturation of the signal, at which time reflow of Tris buffer was reinitiated. SPR data was analyzed in Sigma Plot (Systat Software, Inc., Berkshire, UK). A K_D value of 1.79x10^{-8} M was derived from the SPR sensorgrams.

D. Square Wave Voltammetry

Square Wave Voltammetry was conducted using an AutoLab Potentiostat (PGSTAT and the DropSens electrode interface; Metrohm, Riverview, FL). HA was initially dissolved in either Tris HCl buffer or Artificial Saliva (Pickering Test Solutions, Mountain View, CA); the values of HA titer in solution – from 0 to 1200 pM – encompassed the clinically relevant range [5]. Unless otherwise specified, SWV was conducted from –0.4 V to +0.1 V, with a voltage step of 1 mV, and modulation amplitude of 25 mV at 50 Hz. The test solutions were placed atop the electrode and allowed to equilibrate for 1 min before SWV was performed.

III. Results & Discussion

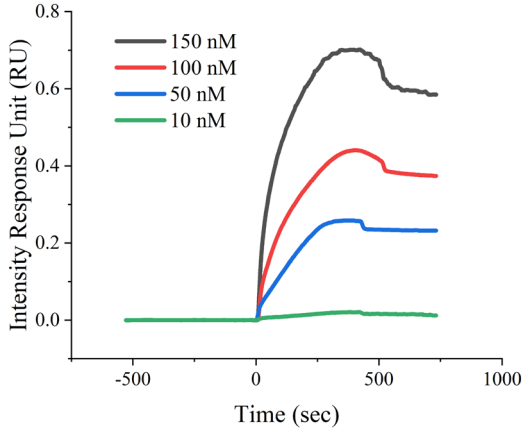

Fig. 3. Surface Plasmon Resonance. SPR binding curves for RHA0006 and HA in Tris HCl buffer with initial baseline normalized to 0. Separation between initial and final baselines is used to determine K_D for an aptamer/analyte biorecognition pair, with higher baseline separation indicating increased aptamer activity.

A. Surface Plasmon Resonance

To validate the applicability of aptamer RHA0006 for HA sensing, a SPR study was initially conducted using 8 solutions of HA in Tris buffer at titers ranging from 10 nM to 150 nM. **Fig. 3** shows the normalized results for 10 nM, 50 nM, 100 nM, and 150 nM (the Response Units were determined by normalizing the final baseline to the initial baseline). Fitting to single-site binding regression model returned a binding constant of 17.9 nM, on par with reported values (K_D ~ 15.3 - 24.7 nM [17]). As anticipated, the baseline shift at 10 nM HA was still significant and detectable, although lower in comparison to those obtained at higher titers. With a 10 nM HA sample, SPR results showed a shift of ~20 RUs, which validated the applicability of RHA0006 at lower, clinically relevant titers.

B. Square Wave Voltammetry

Square wave voltammetry (SWV) was used to quantify the correlation between the aptamer's conformational change and the variation in current flow, simulating the vertical displacement of the methylene blue redox probe that occurs upon analyte binding. As shown in the literature, increasing the titer of the analyte is associated with a reduction in peak current [20, 21]. Initial testing was conducted using samples of HA in Tris HCl with titers ranging from 1.5 pM to 1.2 nM. **Fig. 4a** shows the sensor's response to increasing HA titers. **Fig. 4b** shows the relationship between the peak differential current and HA titer on a logarithmic scale; these data indicate that the sensor operates in a linear range of detection across the clinically relevant titers of HA.

Additional SWV analysis was conducted on HA samples prepared in artificial saliva to simulate the application in relevant point-of-care testing environments. Artificial saliva simulates the mineral composition, pH, and mucin content of human saliva and provides a better understanding of the sensor's capability as an actual diagnostic. Accordingly, the SWV test protocols were repeated using HA in artificial saliva, with results seen in **Fig. 4, c-d**. Indicative of HA sensing, **Fig. 4c** shows dampening of the peak differential current magnitude as the HA titer increases, although with a different baseline and slightly reduced sensitivity when compared to results obtained in aqueous buffer. **Fig. 4d** supports previous data in **Fig. 4b**, suggesting that the sensor operates in a linear detection range across all tested titers.

To understand the potential for long-term deployment at point-of-care, the temporal stability of the aptamer monolayer was evaluated. Functionalized sensors were stored dry at 4°C before testing at 24 h or 72 h. Stability tests conducted in both Tris buffer and artificial saliva showed that the peak differential current response vs. HA titer increased with sensor age, as shown in **Fig. 5**. Although sensor baseline shifts occurred across all electrodes (see "Conclusions & Future Work") independently of time, a significant linear response was observed in the µA range across all electrodes, including those not tested until 72 hours after preparation. All electrodes tested in aqueous buffer exhibited a sensitivity based on $y \propto 0.2 \ln(x)$, while the majority of those tested in artificial saliva

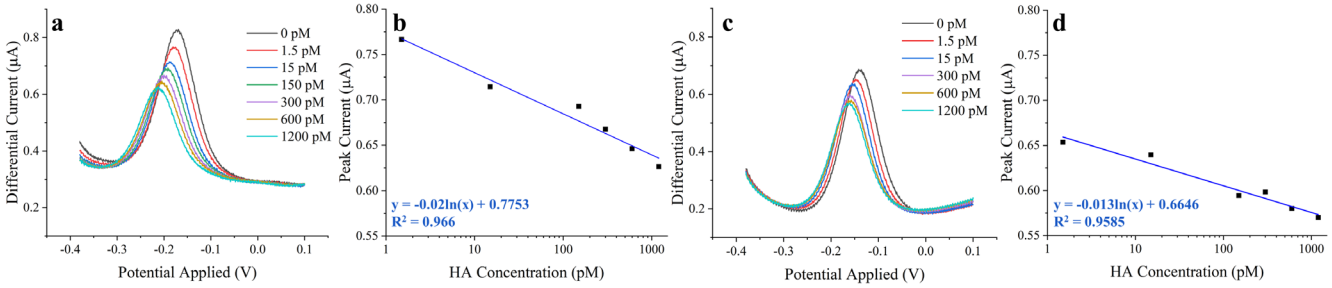

Fig. 4. SWV in Tris HCl Buffer and Artificial Saliva. (a-b) SWV on a functionalized electrode showing (a) differential current (µA) vs. potential (V), with decreased peak magnitude indicative of increased analyte presence and (b) peak current magnitude (µA) vs. logarithmic concentration (pM) for 1.5pM to 1.2nM HA, exhibiting a linear detection range across samples. (c-d) show experimental replication of (a-b) in artificial saliva with mucin.

featured a slightly lower slope. These results recommend further exploration into the viability of the proposed sensor in point-of-care conditions, due to the wide accessibility of the storage environment necessary to maintain sensor stability.

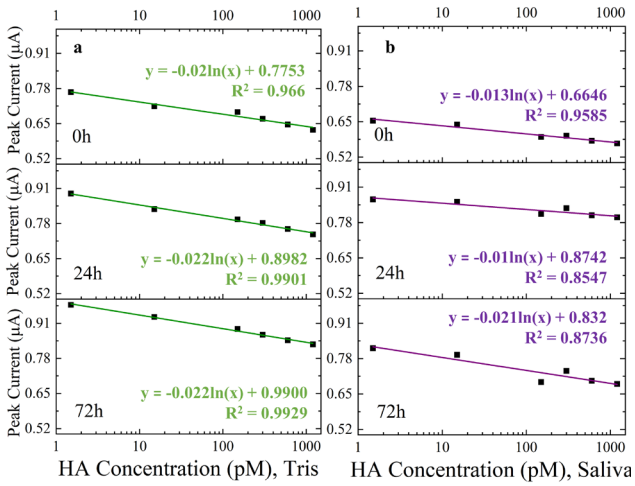

Fig. 5. Sensor Stability. SWV results at 0h, 24h, and 72h from functionalization time showing differential current (µA) vs. concentration (pM) for 1.5pM to 1.2nM HA in (a) Tris HCl buffer and (b) artificial saliva to evaluate sensor stability over time.

IV. Conclusions & Future Work

This study presents the design and characterization of a novel aptasensor for point-of-care or point-of-need influenza diagnosis in human saliva. SPR was initially used to confirm binding affinity in line with literature values. SWV was then utilized to transduce the sensor response across the relevant analyte range in human saliva. SWV results in aqueous buffer and simulated human saliva showed a linear, concentration-dependent response, with a lower limit of detection of 1.2 pM and average sensitivity 21.083 $nA \cdot nM^{-1}$ (aqueous buffer) and 14.5$nA \cdot nM^{-1}$ (artificial saliva). Sensor stability was investigated over 72 hours, with sensors maintaining linear response in both test media across all electrode ages, pointing to the translational potential of the sensor. The results demonstrated herein show promise for the use of RHA0006 aptamer for specific, non-invasive influenza sensing at the point-of-care. Future research will look to minimize baseline drift and investigate the biological viability and selectivity against interferents in human saliva. Carboxyl polystyrene nanoparticles with diameter comparable to H1N1 and coated with HA will be used to simulate a viral load and evaluate the sensor's response to virus-like particles in saliva.

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