Incorporation of extra amino acids in peptide recognition probe to improve specificity and selectivity of an electrochemical peptide-based sensor

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Abstract

We investigated the effect of incorporating extra amino acids (AA) at the n-terminus of the thiolated and methylene blue-modified peptide probe on both specificity and selectivity of an electrochemical peptide-based (E-PB) HIV sensor. The addition of a flexible (SG)₃ hexapeptide is, in particular, useful in improving sensor selectivity, whereas the addition of a highly hydrophilic (EK)₃ hexapeptide has shown to be effective in enhancing sensor specificity. Overall, both E-PB sensors fabricated using peptide probes with the added AA (SG-EAA and EK-EAA) showed better specificity and selectivity, especially when compared to the sensor fabricated using a peptide probe without the extra AA (EAA). For example, the selectivity factor recorded in the 50% saliva was ~2.5 for EAA sensor, whereas the selectivity factor was 7.8 for both the SG-EAA and EK-EAA sensors. Other sensor properties such as the limit of detection and dynamic range were minimally affected by the addition of the six AA sequence. The limit of detection was 0.5 nM for the EAA sensor and 1 nM for both SG-EAA and EK-EAA sensors. The saturation target concentration was ~200 nM for all three sensors. Unlike previously reported E-PB HIV sensors, the peptide probe functions as both the recognition element and antifouling passivating agent; this modification eliminates the need to include an additional antifouling diluent, which simplifies the sensor design and fabrication protocol.

Keywords: Electrochemical peptide-based sensors, human immunodeficiency virus antibodies, methylene blue, antifouling agents, alternating current voltammetry, cyclic voltammetry

1. Introduction

Since the 1980s, HIV (Human Immunodeficiency Virus) has been an epidemic in the United States. According to the Centers for Disease Control, approximately 48,000 people were diagnosed with HIV and 28,000 people with AIDS (Acquired Immune Deficiency Syndrome) in 2012 [1]. The most commonly used method of detecting and monitoring HIV is through the use of polymerase chain reaction (PCR). While the PCR-based viral load test is extremely sensitive and accurate, it is considered invasive since it requires the use of blood. Furthermore, this test has to be performed by trained professionals in clinics or hospitals. The availability of a user-friendly HIV self-test that could be performed in the privacy of one's home will undoubtedly lead to higher rates of testing, reducing the number of undiagnosed infection and the risk of onward transmission.

There are several home testing kits for HIV diagnosis that are based on lateral flow immunoassays, however, none of them utilize an electrochemical signal transduction mechanism. Electrochemical-based sensors have demonstrated to be well-suited for commercialization, for example, glucose biosensors accounted for approximately 85% of the world market for biosensors. Its widespread commercial success is attributed to its superb analytical performance; it is sensitive, specific and works well under a wide range of experimental conditions. More importantly, it is selective enough to be employed directly in complex matrices such as whole blood. However, for diagnosis of highly contagious diseases such as HIV, a non-invasive sensor that is functional in less transmissible and invasive matrices, such as urine and saliva, is highly desirable [2-5]. Although HIV tests that are based on the detection of anti-HIV antibodies in saliva have recently been developed, [6-8] to our best knowledge, there is no equivalent electrochemical HIV sensor that is operational in saliva currently commercially available [9].

The electrochemical peptide-based (E-PB) sensor, with attributes similar to the well-characterized electrochemical DNA (E-DNA) and aptamer-based (E-AB) sensors, could potentially fill this unmet need [10-13].

While most E-DNA and E-AB sensors are functional in a wide range of realistically complex samples, this HIV E-PB sensor has only been tested in urine proxy [10]. The ability of these sensors to operate in various complex samples will likely broaden their applicability in disease diagnosis and monitoring. To date, a wide range of antifouling materials, including proteins, surfactants, and polymers, have been incorporated in the design and fabrication of amperometric and impedance sensors, as well as immunoassays [14-16]. Among all known antifouling or passivating proteins, bovine serum albumin (BSA) is the most common employed, presumably due to its effectiveness and low cost [15]. Covalently or electrostatically bound surfactants and polymers such as tween 20, polyethylene-glycol, carboxy-methyl dextran, polylysine, and cetyl trimethylammonium bromide, have also been shown to be capable of resisting non-specific adsorption of matrix contaminants [17-23]. Despite the fact that all these surface modification strategies have demonstrated to be effective, not all of them are compatible with E-PB sensors. Similar to the E-DNA and E-AB sensors, E-PB sensors are self-assembled monolayer (SAM)-based sensors; the sensor surface is composed of surface immobilized biorecognition probes and diluent alkanethiols [10,24-36]. Physisorption of large proteins such as BSA on top of the monolayer could affect the electron transfer between the redox-modified probe and the substrate electrode. The use of electrostatically bound antifouling molecules is one of the simplest ways to passivate a surface; however, since the molecules are not covalently attached to the sensor surface, stability of the passivating layer could be an issue, in particular, for sensors designed for use in flow-based microfluidic devices. Thus, antifouling molecules that

are more permanently bound to the surface are more suitable for this class of SAM-based biosensors. To this end, we have recently succeeded in enhancing both the specificity and selectivity of a different E-PB sensor by incorporating thiolated oligothymine into the monolayer [32]. The addition of negatively-charged diluents has shown to be effective in preventing surface fouling.

In this study, we explored an equally effective strategy for surface passivation, albeit without the use of an additional thiolated antifouling diluent. The peptide recognition probes used here were modified with extra amino acids (AA) that have been shown to have antifouling properties. We compared both the specificity and selectivity of the sensors fabricated using these peptides to the one fabricated using a peptide without this addition. Our results highlight the capability of these modified peptide probes in resisting non-specific adsorption of matrix contaminants. This strategy has one major advantage over previously employed methods, in which the peptide probe functions as both the target recognition element and the antifouling coating. It is also generalizable and can potentially be used with other detection platforms that utilize surface-immobilized biorecognition probes (e.g., surface plasmon resonance (SPR) spectroscopy) [37].

2 Materials & Methods

2.1 Materials and Reagents

All reagents and solvents were used as purchased. Human immunoglobulin G antibodies (IgG Ab), creatinine, trizma-base, tris-hydrochloride, sodium chloride, potassium chloride, magnesium chloride, calcium chloride, sulfuric acid, acetonitrile (ACN), and 8-mercapto-1-octanol (C8-OH) were purchased from Sigma-Aldrich (St. Louis, MO). The methylene blue

(MB)-modified peptides were purchased from Xaia Peptides (Göttenborg, Sweden) (Figure S1).

The three peptide sequences are shown below, the added six AA are underlined.

EAA peptide: (n) HS-(CH₂)₁₁- EAAEWDRVHP-K-MB (c)

SG-EAA peptide: (n) HS-(CH₂)₁₁- SGS GSG EAAEWDRVH P-K-MB (c)

EK-EAA peptide: (n) HS-(CH₂)₁₁- EKE KEK EAAEWDRVHP-K-MB (c)

The lyophilized peptides were reconstituted in 10% ACN (90% deionized (DI) water) to produce a 0.5 mM stock solution. The anti-p24 antibody (Anti-p24 Ab) was purchased from ProSpec-Tany Technogene Ltd. (East Brunswick, NJ). The sensors were interrogated in a Phys2 buffer (Phys2) containing 9.2 mM Trizma-base, 10.8 mM Tris-HCl, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1 mM CaCl₂ (pH 8.6). Synthetic urine solution (urine proxy) was obtained from Ricca Chemical Company (Arlington, TX); 11 mM creatinine was added to this solution. Synthetic human saliva was purchased from US Biocontract (San Diego, CA).

2.2 Instrumentation

A 1040B CH Instruments Workstation (Austin, TX) was used for all electrochemical measurements. Gold working electrodes of 2 mm diameter, Pt auxiliary electrode, and Ag/AgCl (1M KCl) reference electrode made up the electrochemical cell. All electrodes in the electrochemical cell were also purchased from CH Instruments.

Alternating current voltammetry (ACV) was the main technique for sensor characterization. AC voltammograms were recorded from -0.15 V to -0.48 V at different frequencies and with an AC amplitude of 25 mV. Cyclic voltammetry (CV) was used to determine the probe coverage, heterogeneous electron transfer rate constant (k), apparent diffusion coefficient (D), and double layer capacitance (C_{dl}). All electrochemical measurements were performed at room temperature (~21 °C).

2.3 Sensor Fabrication

Prior to sensor fabrication, the gold electrodes were mechanically polished using 0.1 μm diamond suspension (Buehler, Lake Bluff, IL). The electrodes were then sonicated in an ultrasonic bath for 5 minutes. They were electrochemically cleaned by cycling from -0.2 to 1.3 V at 4 V/s in 500 mM H_2SO_4 until minimal change in the current was observed. The electrodes were subsequently placed in 50 mM H_2SO_4 ; a CV scan was recorded at a scan rate of 100 mV/s. The charge under the cathodic peak at ~ 0.85 V in the CV scan was used to determine the actual area of the electrode. Electrodes with a ρ factor (actual area/geometric area) between 1.2 and 2 were deemed acceptable and were used to fabricate the sensors. The electrodes were rinsed with DI H_2O and dried with N_2 gas before SAM formation.

Sensor fabrication involved two steps; the electrodes were first placed in a Phys2 solution containing 1 μM peptide and 10 μM C8-OH for 6 hours. They were rinsed with DI H_2O and placed in 2 mM C8-OH in Phys2 for 14-16 hours. After the previous step, the electrodes were rinsed with DI H_2O , placed in 3 M KCl for 5 minutes, rinsed with DI H_2O , and placed in a new aliquot of Phys2. Prior to target interrogation, the electrodes were equilibrated in Phys2 until a stable MB peak current was obtained (~ 30 minutes).

2.4 Probe Coverage Determination and Sensor Characterization

CV was used to determine the number of peptide probes (Γ) using Equation 1, where Q is the charge associated with the reduction of MB at slow scan rates (10, 50, and 100 mV/s), F is the Faraday's constant, n is the number of electrons transferred in the electrochemical reaction ($n = 2$ for MB).

$$\Gamma = Q/nF \quad (1)$$

C_{dl} for each sensor was determined from CV scans recorded from -0.2 to 0.2 V at 51.2 V/s using Equation 2 [38,39].

$$C_{dl} = [|i_c| + |i_a|] / 2\nu \quad (2)$$

where i_c is the cathodic current and i_a is the anodic current at -0.05 V and ν is the scan rate.

Apparent diffusion coefficient (D) was calculated using Equation 3 [40].

$$(2Dt)^{1/2} \geq 10 L \quad (3)$$

where L is the length of the peptide ($L = \text{number of AA} \times 3.6 \text{ \AA}$), t is the time in the CV scan where the sensor system switches from diffusion independent (i.e., surface-confined) to diffusion limited regime, and D is the apparent diffusion coefficient.

Heterogeneous electron transfer (ET) rate constant (k) was calculated by Equation 4 [41].

$$\log k = \alpha \log(1 - \alpha) + (1 - \alpha) \log \alpha - \log \left(\frac{RT}{nF\nu} \right) - \frac{\alpha(1 - \alpha)nF\Delta E_p}{2.3RT} \quad (4)$$

where k is the rate of electron transfer, α is the transfer coefficient, R is the ideal gas constant, T is the temperature (in K), F is the Faraday's constant, n is the number of electrons transferred in the electrochemical reaction, ν is the scan rate, and ΔE_p is the difference between the cathodic ($E_{p,c}$) and anodic ($E_{p,a}$) peak potential (in V).

2.5 Target Interrogation

Sensor specificity was analyzed by first interrogating the sensor with 70 nM IgG Ab, followed by the addition of 70 nM Anti-p24 Ab. The change in the MB current was monitored until there was less than 0.5% signal change before the addition of the next target. In a separate experiment, the sensors were interrogated in either 50% synthetic human saliva with 50% 2X Phys2 or in 50% creatinine-supplemented urine proxy with 50% 2X Phys2. Similarly, the change in the MB current was monitored after target addition (70 nM Anti-p24 Ab) until there was less than 0.5%

signal change. In the calibration experiment, the sensors were allowed to equilibrate for 60 minutes after each addition of the target. AC voltammograms were recorded both before and 60 minutes after target addition. The limit of detection was identified as the lowest target concentration capable of generating a signal that is significantly different (i.e., $s/n = 3$) from the signal fluctuation in the absence of the target.

% signal suppression (%SS) was calculated using Equation 5 [10,32].

$$\%SS = [(I_0 - I)/I_0] * 100 \quad (5)$$

where I_0 is the baseline-subtracted MB current in the absence of the target and I is the baseline-subtracted MB current in the presence of the target. Unless stated otherwise, all data presented here are averages from three independent experiments ($n = 3$).

3. Results & Discussion

3.1 Sensor Design

There are several antigenic epitopes in the HIV-1 p24 protein, one of the most well-characterized epitopes is the core 10-AA peptide sequence used in this study ((n) EAAEWDRVHP (c)) [42,43]. As shown in Scheme 1, the E-PB sensors used in this study are "signal-off" sensors. In the absence of the target, the MB-labeled peptide probe is highly flexible; electron transfer between the redox label and the electrode is efficient. Binding of the target antibody leads to a significant reduction in probe flexibility, resulting in a large decrease in the MB current. Three different peptide probes were used in this study, with the goal of elucidating the effect of the added AA on sensor specificity and selectivity (Figure S1). The first peptide probe (EAA) contains the core 10-AA sequence, whereas the second peptide probe (SG-EAA) has six additional AA (SGSGSG) at the n-terminus. The addition of this (SG)₃ sequence at the proximal end of the probe (i.e.,

closest to the thiol linker) could provide the extra flexibility necessary for target binding. This sequence has been used in previous studies to improve peptide flexibility [44]. This neutral hydrophilic sequence could have protein-resistant and/or antifouling properties. Bolduc et al. studied the effect of peptides of differing lengths and compositions on their resistance to nonspecific adsorption. Their results show that a monolayer composed of oligoserine (e.g., S₅) is capable of minimizing nonspecific adsorption even when exposed to undiluted bovine serum [45]. The third peptide probe (EK-EAA) also has six additional AA at the n-terminus; while the “effective” charge of this added sequence is zero, both glutamic acid and lysine are charged under the current experimental condition. The added charged residues could have antifouling properties; modification of a sensor surface with highly charged species is often employed to prevent non-specific adsorption of matrix contaminants [46,47]. White et al. evaluated the extent of fibrinogen and lysozyme adsorption on various peptides-modified gold surfaces using SPR; their results show that the (EK)₇-modified surface is highly resistive to protein adsorption, its performance is comparable to the ultra-low protein fouling standard of <5 ng cm⁻² of fibrinogen [47]. In addition, our previous study on the use of negatively-charged oligothymines as antifouling diluents further supports the idea that charged and/or hydrophilic surfaces are less susceptible to protein adsorption and surface fouling [32]. Despite the differences in the peptide sequence, all three peptide probes have a similar pI and the net charge is ~-2 (Table S1).

3.2 Sensor Characterization Using ACV and CV

ACV is not only a good sensor interrogation technique, it is particularly effective in determining the conformation of the redox-modified probe on the electrode surface. In this case, we first recorded AC voltammograms at different frequencies and utilized the frequency-dependent current profile to deduce probe conformation. All three sensors displayed a rather similar

frequency-dependent profile, in which the normalized MB peak current increased drastically with increasing frequency, followed by a large reduction in the peak current at higher frequencies (Figure 1a). For this class of sensors, as the applied frequency approaches a critical value above which electron transfer can no longer keep up with the rapidly oscillating potential, the peak current diminishes relative to the background current [48-51]. The profiles shown in Figure 1a are often seen with the linear probe E-DNA sensor, suggesting that the peptide probes are likely in a flexible linear confirmation rather than a folded structure with limited surface diffusivity. Despite the similarity in the overall trend, there are differences between the three sensors. For example, the EK-EAA sensor showed a slightly different profile when compared to the other two sensors. The “threshold” frequency was lower (~ 30 Hz), the drop in the current beyond the “threshold” frequency was steeper as well. The difference in the “threshold” frequency can be clearly identified in the semi-log plot shown in Figure 1b. This difference could be, in part, attributed to the difference in the probe coverage and flexibility; these aspects will be addressed in the following section.

CV was also used to characterize these three sensors. To confirm probe attachment, we obtained CV scans for all three sensors over a wide range of scan rates. As shown in Figure 1c, the MB peak current increased linearly with scan rate between 0.01 and 100 V/s. The hysteresis (i.e., peak-to-peak separation) was minimal at slow scan rates, further confirming that the peptides are surface-confined. However, a deviation from linearity was evident at scan rates beyond ~ 100 V/s, suggesting that while the probes are attached to the gold surface via the thiol group, they retain surface diffusivity similar to other reported sensors of this class [12,40]. At higher scan rates, we observed a linear relationship between the MB peak current and the square root of scan rate (Figure 1d). This behavior confirms the similarity between these E-PB sensors

and the linear probe E-DNA sensor. It is worth noting that all three sensors showed a slightly different scan rate-dependent current profile, but the overall trend was the same.

Another crucial property of a monolayer is the C_{dl} ; a well-packed monolayer should have a high impedance barrier with charge-transfer resistance in the order of $k\Omega/cm^2$ and capacitance of the order of $1-5 \mu F cm^{-2}$ [52]. To determine C_{dl} for all three sensors, we employed a previously established method that relies on the analysis of CV scans recorded at 51.2 V/s [38,39]. The values obtained for the EAA ($4.4 \pm 0.2 \mu F cm^{-2}$), SG-EAA ($3.8 \pm 0.1 \mu F cm^{-2}$), and EK-EAA ($3.9 \pm 0.2 \mu F cm^{-2}$) sensors are all within expected values for a mixed C11/C8 monolayer, suggesting that all three monolayers have a similar packing density. The lower C_{dl} seen with both SG-EAA and EK-EAA sensors could be due to the longer peptide length and higher probe coverage [52-54]. The probe coverage for each sensor was calculated using CV. The values obtained from CV for the EAA, SG-EAA, and EK-EAA sensors were $1.9 (\pm 0.7) \times 10^{11}$, $3.5 (\pm 0.2) \times 10^{11}$, and $3.0 (\pm 0.4) \times 10^{11}$ molecules cm^{-2} . The slight difference in the probe coverage between the three sensors is likely due to the difference in the charge and size of the peptide probe (Table S1). These factors are known to influence probe immobilization and monolayer formation [55]. However, it is worth noting that these probe coverages, while lower than that used with E-DNA and E-AB sensors, were found to be ideal for capturing large targets such as the Anti-p24 Ab [10,27].

Although probe flexibility cannot be accurately measured and quantified, sensor properties such as apparent diffusion coefficient (D) and heterogeneous ET rate constant (k) can be used to infer probe structure, conformation, and flexibility (Figure 2). As described by Huang and White, a tightly packed monolayer free of defects with no motion of the redox label is required for accurate measurement of the heterogeneous ET rate [40]. This is clearly not the case here; the

surface-immobilized peptide probes are capable of short-range motion. Depending on the probe coverage and other experimental conditions such as the scan rate, ET in MB-modified peptide probes can be correlated with either a surface-confined or a diffusion limited ET reaction [56]. The calculated D value provides information on the surface diffusivity of the redox label on the electrode surface. The k value, on the other hand, describes ET kinetics of electrochemical reactions with adsorbed redox molecules in a diffusionless system [42]. Although the systems discussed here are not diffusionless, the calculated k values should still be useful in analyzing ET in sensing systems that are relatively similar (i.e., the three E-PB sensors used in this study). As shown in Figure 2, among the three sensors, the EAA sensor had the highest D ($14 (\pm 2) \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$) and k ($137 \pm 5 \text{ s}^{-1}$) values. These results are expected since the EAA peptide is the shortest; the redox label is closer to the electrode surface and the hemispherical volume is smaller [57]. This behavior could also be, in part, attributed to the slight lower probe coverage. The D value ($3.7 (\pm 0.3) \times 10^{-11} \text{ cm}^2 \text{ s}^{-1}$) was the lowest for the SG-EAA sensor, but its k value ($83 \pm 6 \text{ s}^{-1}$) was slightly higher than that of the EK-EAA sensor. As can be seen here, there is a clear correlation between surface diffusivity and electron transfer efficiency, a high D value corresponds to a high k value for systems that are similar and characterized under the same experimental condition.

3.3 Sensor Sensitivity

The goal of this study is to evaluate the effect of the added AA on sensor specificity and selectivity, but it is necessary to determine whether the addition will interfere with target binding. To achieve this, we first analyzed the sensors' response to the correct target and the binding kinetics. In the presence of 70 nM Anti-p24 Ab, we observed 50% of the total signal change (normalized for all three sensors) in ~20 minutes. The time required to reach 80% of the total

signal change, however, was different for the three sensors. The EAA, SG-EAA, and EK-EAA sensors required 70, 90, and 80 minutes, respectively, to reach this level of signal change (data not shown). The slower binding kinetics, especially when compared to the E-DNA and E-AB sensors, could be due to the large target size (~150 kDa) and that stirring was not used in these experiments. To determine the limit of detection (LOD) and dynamic range, we obtained a dose-response curve for each sensor. As shown in Figure S2, all three sensors responded to the target in a concentration-dependent manner; the saturation concentration was ~200 nM. The EK-EAA sensor consistently showed higher %SS at all target concentrations, the reason behind this is not well-understood and is currently under investigation in the lab. Despite this difference, the linear dynamic range was the same for all three sensors. The LOD was 0.5 nM for the EAA sensor and 1 nM for both SG-EAA and EK-EAA sensors. The slight difference in the LOD could be, in part, attributed to the differences in the probe coverage. The probe coverage was higher for the SG-EAA and EK-EAA sensors when compared to the EAA sensor. A high probe coverage is not ideal for these sensors because of the large target size. The dissociation constants (K_D) were 17 ± 1 , 24 ± 7 , and 15 ± 7 nM for the EAA, SG-EAA, and EK-EAA sensors, respectively. Our results indicate that the addition of the extra AA at the n-terminus of the peptide epitope does not have a major negative impact on probe-target interactions and sensor sensitivity.

3.4 Sensor Specificity

To determine sensor specificity, we interrogated all three sensors first with 70 nM IgG Ab, followed by 70 nM Anti-p24 Ab at a wide range of AC frequencies. As can be seen in Figure 3, the sensors responded differently to IgG Ab and Anti-p24 Ab at different AC frequencies. The overall SS in the presence of 70 nM IgG Ab were ~10, 8, and 5 % for the EAA, SG-EAA, and EK-EAA sensors, respectively. But the %SS was significantly higher for all three sensors in the

presence of Anti-p24 Ab. The best method to compare sensor specificity is to compare the specificity factor, which is obtained by simply dividing the %SS recorded in the presence of Anti-p24 Ab by the %SS recorded in the presence of IgG Ab. For example, at 20 Hz, the specificity factor was found to be 2.4 ± 0.4 for both the EAA and SG-EAA sensors, whereas a higher specificity factor (3.1 ± 0.3) was determined for the EK-EAA sensor. However, sensor specificity is known to be dependent on the applied frequency, given that the sensor's response to both IgG Ab and Anti-p24 Ab could be different at different frequencies. At 80 Hz, the specificity factors were 2.2 ± 0.3 , 3.0 ± 0.3 , and 3.6 ± 0.7 for the EAA, SG-EAA and EK-EAA sensors. These results suggest that the added AA clearly has an effect on sensor specificity; the (EK)₃ hexapeptide is slightly better than the (SG)₃ hexapeptide for this application. The addition of charged residues such as lysine and glutamic acid appears to be more effective in reducing non-specific adsorption and interactions of IgG Ab with the sensor surface.

3.5 Sensor Selectivity

For an E-PB sensor to be useful in a real world setting, sensor selectivity is as important as sensor specificity. To evaluate the sensors' selectivity, we analyzed their response in two different noninvasive samples demonstrated to be useful for HIV diagnosis. Shown in the Figure S3 are the AC voltammograms of the three sensors recorded in 50% synthetic human saliva. When interrogated at the optimal frequency, all three sensors showed good selectivity. For example, even for the EAA sensor, the SS observed after the sensor was placed in the saliva matrix (SS_{saliva}) was 10 ± 5 %; the SS recorded after the addition of 70 nM Anti-p24 Ab (SS_{p24}) was 25 ± 4 %. For the SG-EAA sensor, the SS_{saliva} was 4 ± 3 % and SS_{p24} was 31 ± 2 %. Last, for the EK-EAA sensor, the SS_{saliva} and SS_{p24} were 5 ± 2 % and 39 ± 3 %, respectively. The selectivity factor, which is determined by dividing the %SS recorded in the presence of Anti-p24 Ab by the

%SS recorded in the complex matrix, was ~ 2.5 for the EAA sensor, lower than that obtained for the SG-EAA and EK-EAA sensors (7.8 for both sensors). Sensor selectivity is clearly improved by addition of the extra AA in the peptide probe sequence.

The sensors were also found to be functional in 50% urine proxy; AC frequency-dependent plots for the sensors are shown in Figure 4. For the EAA sensor, we observed a slight reduction in the MB current after the sensor was placed in 50% urine proxy. This behavior was seen over a wide range of AC frequencies. Nevertheless, the sensor was functional, as verified by the %SS observed in the presence of 70 nM Anti-p24 Ab. It is worth noting that the selectivity factor (i.e., $\%SS_{p24}/\%SS_{urine}$) for the SG-EAA sensor, when interrogated at the optimal frequency (80 Hz), was ~ 51 , highest among all three sensors. For the EK-EAA sensor, the selectivity factor was ~ 7 at the optimal frequency. For both SG-EAA and EK-EAA sensors, the MB current was close to identical to that observed in a pure buffer at all applied frequencies, as evident by the overlapping AC frequency-dependent current profiles. This was not observed with the EAA sensor, further highlighting the advantages of incorporating the extra AA in the probe sequence, in this case for improving sensor selectivity. However, we are aware that there are limitations regarding the length of the added antifouling sequence; while the addition of a 6-AA sequence has demonstrated to be beneficial, incorporation of a longer sequence could have a negative effect on sensor sensitivity and/or binding kinetics. Thus, we are currently investigating the effect of both the length and location of the antifouling sequence on the overall sensor performance, these results will be disseminated in a publication in the near future.

4. Conclusion

We have successfully enhanced both the specificity and selectivity of an E-PB HIV sensor by incorporating AA that are known to improve probe flexibility and/or antifouling capability to the n-terminus of the peptide recognition probe. The sensors fabricated using peptide probes with an additional (SG)₃ or (EK)₃ sequence showed better specificity and selectivity when compared to the sensor fabricated with a peptide probe without the added sequence.). For the EAA, SG-EAA and EK-EAA sensors, the selectivity factors recorded in the 50% saliva were ~2.5, 7.8, and 7.8, respectively. It is worth noting that other sensor properties such as sensitivity and dynamic range were not severely affected by the addition of the extra AA. The LOD was 0.5 nM for the EAA sensor and 1 nM for both SG-EAA and EK-EAA sensors. The saturation target concentration was the same (~200 nM) for all three sensors. This design eliminates the need to include a thiolated antifouling diluent. The peptide probe, in this case, acts as both the target recognition element and antifouling passivating agent. With proper optimization, these sensors can potentially be used for detection of anti-HIV antibodies in real clinical samples such as urine and saliva. More importantly, this surface passivation strategy is versatile and compatible with other surface-based biosensing platforms that utilize peptide recognition probes.

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Scheme 1. Design and signaling mechanism of the E-PB sensors used in this study. The sensors are composed of a thiolated peptide (EAA, SG-EAA, or EK-EAA) and C8-OH.

Figure 1. Normalized AC frequency-dependent current for the three E-PB sensors (a). Also shown are the same data in a semi-log plot (b). Normalized DC current as a function of scan rate (c) and square root of scan rate (d). We normalized the current to facilitate comparisons between the three sensors. These data were collected in Phys2 at room temperature. Error bars represent one standard deviation of the means; $n = 3$.

Figure 2. Apparent diffusion coefficients (D) (black bars) and heterogeneous electron transfer rate constants (k) (grey bars) for the three sensors used in this study. Error bars represent one standard deviation of the means; $n = 3$.

Figure 3. %SS as a function of AC frequency in the presence of 70 nM IgG Ab and 70 nM Anti-p24 Ab for the EAA (a), SG-EAA (b), and EK-EAA (c) sensors. Error bars represent one standard deviation of the means; n = 3.

Figure 4. AC frequency-dependent current response for the EAA (a), SG-EAA (b), and EK-EAA (c) sensors in Phy2, 50% urine proxy, and in the presence of 70 nM Anti-p24 Ab.

Graphical Abstract: