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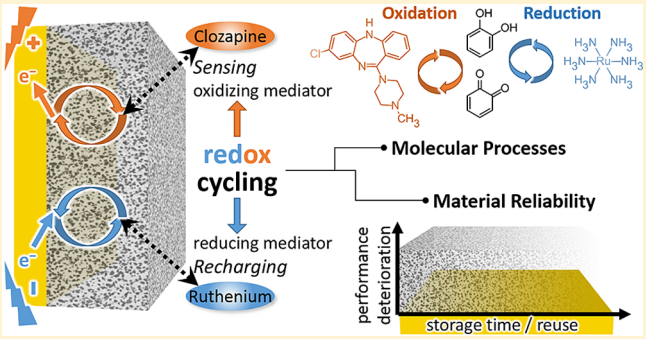
Electrochemical Study of the Catechol-Modified Chitosan System for Clozapine Treatment Monitoring

Thomas E. Winkler,[⊥,†,‡] Hadar Ben-Yoav,[⊥,†] Sheryl E. Chocron,[†,‡] Eunkyoung Kim,[§] Deanna L. Kelly,[∥] Gregory F. Payne,[‡,§] and Reza Ghodssi*[,†,‡]

[†]MEMS Sensors and Actuators Laboratory (MSAL), Institute for Systems Research, Department of Electrical and Computer Engineering, [‡]Fischell Department of Bioengineering, and [§]Institute for Bioscience and Biotechnology Research, University of Maryland, College Park, Maryland 20742, United States

[∥]Maryland Psychiatric Research Center, University of Maryland School of Medicine, Baltimore, Maryland 21228, United States

ABSTRACT: This work presents a thorough electrochemical and reliability analysis of a sensing scheme for the antipsychotic clozapine. We have previously demonstrated a novel detection approach for this redox-active drug, highly effective in schizophrenia treatment, based on a catechol-modified chitosan film. The biomaterial film enables amplification of the oxidative current generated by clozapine through redox cycling. Here, we study critical electrochemical and material aspects of the redox cycling system to overcome barriers in point-of-care monitoring in complex biological samples. Specifically, we explore the electrochemical parameter space, showing that enhanced sensing performance depends on the presence of a reducing mediator as well as the electrochemical technique applied. These factors account for up to 1.75-fold and 2.47-fold signal enhancement, respectively. Looking at potential interferents, we illustrate that the redox cycling system allows for differentiation between selected redox-active species, clozapine's structurally largely analogous metabolite norclozapine as well as the representative catecholamine dopamine. Furthermore, we investigate material stability and fouling with reuse as well as storage. We find no evidence of film fouling due to clozapine; slow overall biomaterial degradation with successive use accounts for a 2.2% absolute signal loss and can be controlled for. Storage of the redox cycling system appears feasible over weeks when kept in solution with only 0.26%/day clozapine signal degradation, while ambient air exposure of three or more days reduces performance by 58%. This study not only advances our understanding of the catechol-modified chitosan system, but also further establishes the viability of applying it toward sensing clozapine in a clinical setting. Such point-of-care monitoring will allow for broader use of clozapine by increasing convenience to patients as well as medical professionals, thus improving the lives of people affected by schizophrenia through personalized medicine.



1. INTRODUCTION

Schizophrenia is a challenging and complex disorder with 30–50% of patients not responding to first line antipsychotic treatment.[1,2] Clozapine is the only antipsychotic approved by the FDA for treatment-resistant schizophrenia, and is the most effective antipsychotic medication currently available.[3,4] Yet, clozapine remains underutilized because of the burden associated with the requirement for performing frequent and invasive white blood cell counts due to risk of agranulocytosis, a rare but potentially fatal side effect of clozapine.[5−7] Additional monitoring for clozapine blood levels to facilitate accurate dosage control is proven to improve treatment outcomes (> 1 μM) and lower the risk of drug toxicity (< 3 μM).[8−11] However, these supplementary blood tests are rarely implemented due to the associated extra burden to patients. Rapid point-of-care clozapine testing using small sample volumes could significantly reduce the inconvenience and allow for more rapid adjustment of dosage to reach a safe and effective blood levels.

Direct electrochemical detection of the redox-active clozapine is appealing, especially since no specific biorecognition elements (antibodies, aptamers, and so forth) are available.[12,13] This established sensing modality is furthermore well suited for miniaturization, required for small-volume point-of-care solutions.[14,15] We have recently presented the application of the catechol-modified chitosan redox cycling system to amplify and detect the electrochemical clozapine signal in undiluted human serum.[16]

In this study, we expand on our previous work to gain a more complete understanding of the material and electrochemical aspects of the sensor. In the first part, we investigate material

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reliability as well as fouling of our redox cycling system in terms of both long-term storage and reusability. In the second part, we explore the electrochemical parameter space—including the presence of various redox species—to facilitate targeted optimization of the system for accurate clozapine monitoring in complex biological samples.

The approach taken here is in contrast to our previous work, and to that of most sensor characterization work in the literature, which focused on figures of merit such as limit of detection and sensitivity. While those parameters are significant, other aspects must not be neglected when developing a new detection scheme, and assessing its feasibility for transitioning from a research setting to a point-of-care one. Detection in complex biological samples such as blood plasma is associated with a number of electrochemical and material challenges. The thousands of species in blood plasma—ranging from large proteins to elemental ions—can interact with the sensor in numerous ways.[17] Any of these species—including clozapine itself—may adsorb to the electrode or become embedded in our biomaterial matrix, causing fouling and leading to degradation of signal magnitudes. Species may also bind or react with clozapine, altering its characteristic redox response. Furthermore, the subset of redox-active species may interfere more directly by causing nonspecific electrochemical signals. In miniaturization, we expect these challenges to be exacerbated due to the generally lower signal-to-noise ratio, combined with the higher surface-to-volume ratio allowing for relatively more nonspecific interactions.[14] A point-of-care device places additional requirements on the sensor and materials in that performance needs to remain conserved over time in storage prior to use; unlike in a research setting, on-site electrode modification is impractical as it would increase required user interaction and likely reduce repeatability.

These challenges cannot be tackled without a well-founded understanding of the molecular mechanisms involved. The comprehensive exploration of the available, as-yet unexplored, electrochemical parameter space yields just such valuable insights. The ability to differentiate various redox species using the same technology illustrates potential for applying it toward other analytes. The light shed on clozapine kinetics, in particular, opens up new avenues for signal enhancement in most detection approaches for this analyte. Knowledge on material degradation, and methods to adjust for its impact on the electrochemical signal, may translate well to a variety of other redox-based sensors. Altogether, our study encompasses important steps toward application as well as understanding of the redox cycling system for clozapine detection.

1.1. Catechol-Modified Chitosan Redox Cycling System. Our sensor relies on a combination of biomaterials to provide electrochemical signal amplification through redox cycling. Chitosan is a versatile polymer that can be selectively electrodeposited as a thin film, and further modified with redox-active catechol (E_0 = +0.132 V vs Ag/AgCl 1 M KCl)[18] by electro-grafting, illustrated in Figure 1a.[19,20] As previously described, this yields a redox capacitor.[21] Clozapine ($E_0 \approx$ +0.4 V vs Ag/AgCl 1 M KCl) can freely diffuse through the film and be oxidized at the electrode, yielding a current (Figure 1b,c).[16,22] Oxidized clozapine can subsequently be reduced by the grafted catechol moieties in close proximity to the electrode. This allows for continuous redox cycling wherein clozapine acts as an oxidizing mediator in electron transfer from the redox capacitor to the electrode. While the measured current is still dependent on the amount of clozapine present, it is no longer limited by that concentration (rather by the redox capacity of the film), resulting in significant amplification of the signal. To recharge the redox capacitor, negative potential is applied in the presence of a reducing mediator such as hexaammineruthenium(III) ($Ru(NH_3)_6$; E_0 = −0.135 V vs Ag/AgCl 1 M KCl).[23]

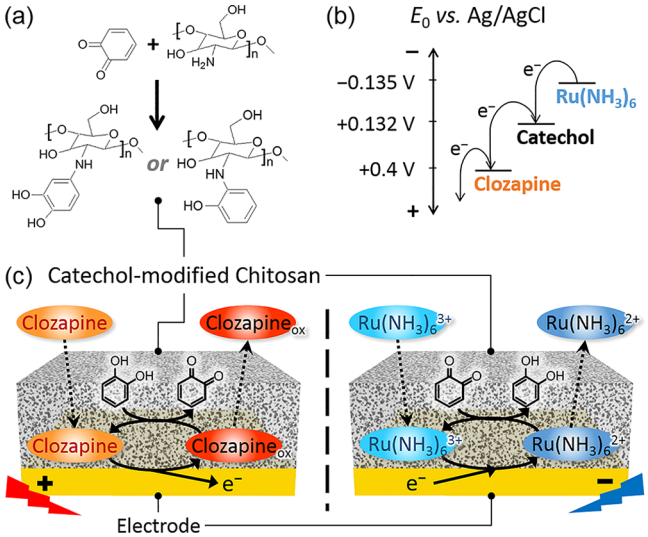

Figure 1. (a) Putative chemistry of catechol grafting to chitosan (adapted from ref 20). (b) Standard reduction potentials and overall electron transfer scheme for the major redox species discussed. (c) Schematic of the catechol-modified chitosan redox cycling system. The diffusing clozapine acts as an oxidizing mediator; the redox capacitor allows for a repeating cycle of clozapine reduction in the presence of reduced catechol followed by clozapine reoxidation at the anode (left; E > 0.4 V). Conversely, under negative potentials, $Ru(NH_3)_6$ in solution acts as a reducing mediator regenerating the oxidized catechol (right).

2. MATERIAL AND METHODS

2.1. Experimental Setup. We fabricated planar gold working electrodes (7.5 × 7.5 mm^2, 200 nm Au on 20 nm Cr adhesion layer) using standard photolithography processes on Si/SiO_2 wafers. The three-electrode cells were completed with a platinum foil counter electrode and an Ag/AgCl reference electrode (1 M KCl electrolyte; CH Instruments; Austin, TX). For the experiments investigating the impact of negative potential application, commercial 2 mm diameter gold disk working electrodes and platinum wire counter electrodes (CH Instruments; Austin, TX) were utilized to achieve an oxygen-free environment where needed. In this case, nitrogen was bubbled into the test solution through tubing for 10 min, and gas flow was cut off immediately prior to the measurement. In contrast to the custom electrodes mentioned above, the commercial ones enable hermetic sealing of the electrochemical cell to retain the nitrogen-rich atmosphere throughout the experiment. Electrochemistry was controlled through a CHI660D potentiostat (CH Instruments), or a VSP-300 potentiostat (Biologic; Claix, France) in the case of oxygen-free environment. All voltages are denoted with reference to the Ag/AgCl 1 M KCl electrode.

2.2. Redox Cycling System Biofabrication. We followed published protocols on chitosan electrodeposition and catechol electrografting.[21] We deposited chitosan from solution (1% in dilute HCl, pH 5–6) onto the working electrodes at constant cathodic current (6 A/m^2) for 45 s. The chitosan forms a film as it turns insoluble near the electrode due to the local pH increase deprotonating its amine groups. We graft catechol onto the chitosan amine groups by applying constant anodic potential (+0.6 V) for 180 s in solution (5 mM in 0.1 M pH 7 phosphate buffer (PB)). All

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chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and solutions prepared with deionized (DI) water (R > 18 Ω cm).

2.3. Experimental Protocols. To carry out measurements, the three electrodes were immersed in test solutions based on PB. The solutions were spiked with $Ru(NH_3)_6$ and an oxidizing mediator—clozapine, its metabolite norclozapine, or dopamine. Unless otherwise noted, cyclic voltammetry was recorded between −0.4 V and +0.7 V at a scan rate of 0.02 V/s. Each electrode was used for two experiments: a baseline measurement in solution without oxidizing mediator, and a sample measurement in the actual test solution. The exception was reusability testing, where this alternating mode of measurements was carried on for a total of 18 experiments. Between experiments, the electrodes were rinsed in DI water. Phosphate buffered saline (PBS) for storage purposes was prepared as 0.01 M PB and 0.1 M NaCl.

2.4. Data Analysis. The baseline data allow for two modes of data processing. First, background subtraction refers to the subtraction of the baseline signal I_B from the sample signal I, such that $\hat{I} = I - I_B$. This allows for better visualization of the signal due to the analyte (or interferent) of interest. Second, normalization refers to dividing both baseline and sample signal by the total charge transfer per voltammetry cycle $Q = \int_{cycle} dI_B \times 50$ s/V measured in the baseline, such that normalized signal $S = I / Q$. As laid out in Section 3.1, this procedure is useful in situations where biomaterial degradation plays a role. All processing was carried out in OriginPro (OriginLab; Northampton, MA). To characterize goodness of fit, we utilize R^2 as well as the norm of residuals NR.

3. RESULTS AND DISCUSSION

3.1. Catechol-Modified Chitosan Reliability. We seek to understand the material-related performance degradation of our redox cycling system with reuse as well as with storage. This will enable us to better assess fouling mechanisms in transitioning to complex biological samples, as well as to gauge applicability of our system as a packaged point-of-care sensor. While biofabrication enables our unique sensor functionality, the biomaterial films are inherently more fragile than solid-state components.

To test reusability, we perform 9 experiments with fresh 25 μM clozapine samples in sequence using the same modified electrode, interleaved with baseline measurements. The cyclic voltammograms in Figure 2a reveal a linear decrease in peak current of 1.04 ± 0.03 μA (2.2% of the absolute signal) with each consecutive clozapine sample (R^2 = 0.992). At the same time, the potential where the peak is observed increases by 5.47 ± 0.22 mV (1.2%) each time (R^2 = 0.988). We find that the signal degradation can be accounted for when normalizing each experiment with respect to the immediately preceding baseline measurement. Specifically, we evaluate the total charge transfer Q in the baseline voltammetry. Thus, we derive a normalized signal $S = I / Q$ for the clozapine samples from the measured current I. In Figure 2b, the nearly constant level of S with progressing experiments becomes immediately apparent. Indeed, linear regression analysis of peak signals fails to yield $R^2 > 0$, indicating an S independent of the number of successive experiments; for a horizontal line with S = 24.8 ks^{-1}, we determine a norm of residuals of only NR = 0.7 ks^{-1} (2.9%). The concurrent decrease of both baseline and sample signals observed indicates that the dominant mechanism affecting reusability is film degradation from repeated electrochemical measurements (and/or associated rinses), rather than film fouling due to clozapine. In the latter case, the baseline and sample signal would be expected to increase with each exposure to clozapine, as more becomes entrained in the film. The observed shift in peak potential implies a slowing down of the redox cycling kinetics.[23] This is consistent with the hypothesis

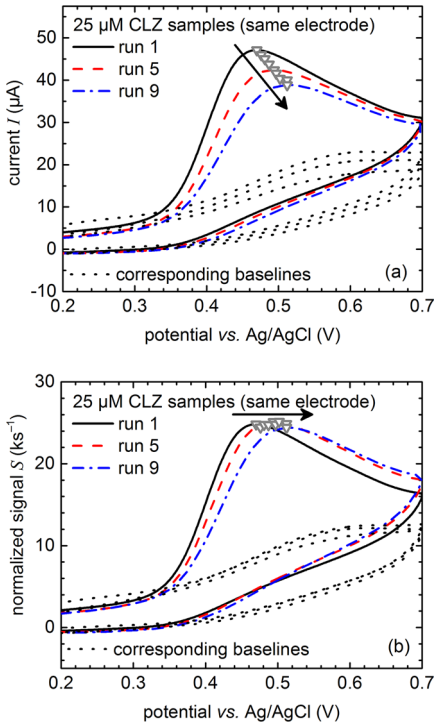

Figure 2. Selected cyclic voltammograms (lines; oxidative region shown) and clozapine signal peaks (gray triangles) for solutions containing 25 μM clozapine measured with the same catechol-modified chitosan electrode. Corresponding baseline measurements are included (black dots). The arrows indicate the progression of measurement runs. (a) The measured signal decreases by 2.4% with each electrode use, accompanied by a 1.2% increase in peak potential. (b) Normalization with respect to immediately preceding baseline measurements reveals constant sensor performance.

of catechol-modified chitosan film degradation, where grafted catechol may be lost over time, leading to longer mean diffusion time of clozapine between the electrode and electron-donating catechol.

Overall, while limited film degradation is observed, the intrinsic performance of our sensor appears to be well conserved, potentially allowing for feasibility of eventual continuous monitoring systems. When transitioning to blood plasma samples, we expect signal degradation to be more pronounced due to electrode fouling with other compounds, especially proteins. However, the normalization is clearly promising, and can at the very least simplify experimental procedures in a research setting. It may further be of use for other redox-based sensors, especially those based on biomaterial constructs.

Another important factor is the stability of the biomaterials over prolonged times of storage, especially toward real-world application. To evaluate catechol-modified chitosan degradation, we biofabricated multiple catechol-modified chitosan films (day 0). Half of these were immersed in PBS for storage, half were kept in air, both under ambient light conditions. We removed individual electrodes at either day 3, 10, 17, or 24 and proceeded with clozapine measurements. For PBS-stored films, another round of tests was conducted on days 56, 63, 70, and 77. Analogous to the reusability study, we visualize baseline charge-normalized data for select time points. Figure 3a illustrates a consistent 57.5 ± 3.0% loss in peak signal for air storage independent of storage time (days 3 through 24; S =

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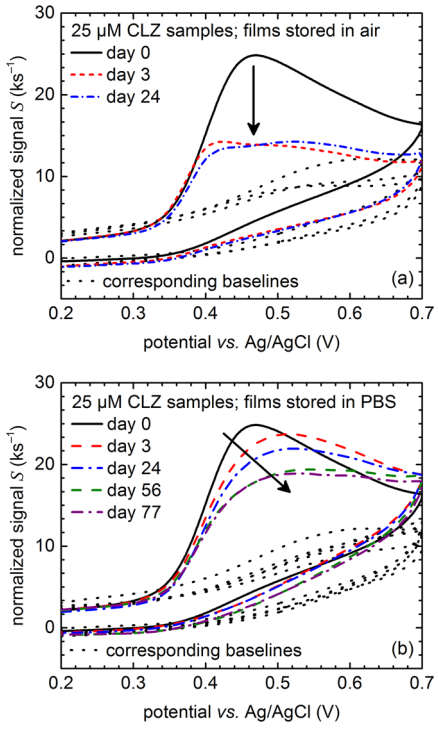

Figure 3. Normalized cyclic voltammograms (oxidative region shown) for solutions containing 25 μM clozapine measured with catechol-modified chitosan electrodes immediately after biofabrication (black solid) and for various storage conditions. Corresponding baseline measurements are included (black dots). The arrows highlight the impact of storage on the clozapine signal. (a) The signal decreases sharply by 58% with storage in air for 3 days (red short dash), with no significant changes through 24 days (blue short dash dot). An average baseline signal is shown for reference (black dots). (b) When stored in PBS, with days 3 (red dash), 24 (blue dash dot), 56 (green dash), and 77 (purple dash dot) shown, the signal linearly degrades by 0.26% per day. An average baseline signal is shown for reference (black dots).

14.3 ks^{-1}; NR = 1.3 ks^{-1} (9%)) compared to day 0 (S = 24.8 ks^{-1}). Electrodes in solution, conversely, display very gradual degradation in the recorded peak signals in Figure 3b throughout the time series, at 0.255 ± 0.072% per day (R^2 = 0.64; NR = 4.1 ks^{-1} (16%)). We attribute the observed behavior to an irreversible collapse of the chitosan hydrogel matrix upon prolonged exposure to air. Although quantification of the chitosan thickness in its hydrated state was not possible, this is in line with qualitative visual observations of the films. Such a collapse may restrict diffusion in the chitosan matrix, yielding low peak signals. In solution, the mechanism appears to be distinct, the steady decrease in performance accompanied by a peak potential shift reminiscent of what was observed in the reusability study. This suggests a similar degradation of the catechol-modified chitosan film, combined with a gradual degrafting of catechol into the storage solution. As oxygen was present both in air (free) and in solution (dissolved), we do not believe it to be the determining factor in either mechanism; the same applies for natural variations in temperature and light.

Both storage approaches have distinct advantages with respect to eventual point-of-care application. At a performance loss of less than 10% over one month of storage in solution, the redox cycling system retains high sensitivity, but will suffer from increased storage time- and condition-dependent variability. Conversely, with storage in air, the signals show 3-fold lower variability compared to solution, at the cost of lower sensitivity. Without the option of storage, biofabrication would have to occur on-site outside of a controlled environment, likely decreasing accuracy as well as ease of use. If signal degradation with reuse cannot be corrected for when measuring blood plasma samples, then this concern is compounded, as every sample would require a single-use catechol-modified chitosan film. Investigation of different storage environments (argon, vacuum, different buffers, and so forth) may further enhance shelf life in the future.

3.2. Impact of Reducing and Oxidizing Mediators. Of similar importance to understanding material system properties is that of the redox reactions involved. These have significant impact on the signal-to-noise ratio of the sensor. For our redox cycling system, we identify two basic categories: (1) the reducing mediator to restore catechol to its reduced state, ensuring continued amplification and (2) the oxidizing mediator, i.e., the analyte of interest (clozapine) as well as possible interferents with similar properties. These factors become especially important in transitioning to complex biological samples, where thousands of species—some of them redox-active[24]—can interact with the redox cycling in numerous ways to depress the signal-to-noise ratio.

3.2.1. Reducing Mediator. First, we consider the role of the reducing mediator, which is expected to play a significant role in ensuring consistent clozapine signal amplification. In Figure 4a, we plot the cyclic voltammetry signal for 25 μM clozapine test solutions with varying concentrations of $Ru(NH_3)_6$, subtracted by the respective baseline without clozapine. The

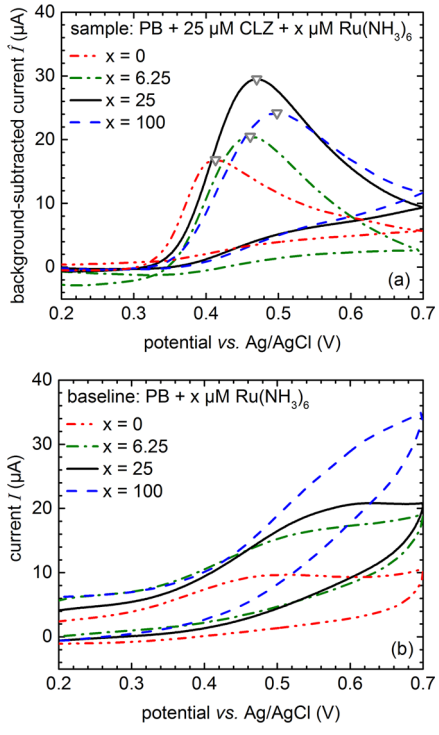

Figure 4. (a) Cyclic voltammograms (oxidative region shown; baseline subtracted) measured with catechol-modified chitosan electrodes for solutions containing 25 μM clozapine and $Ru(NH_3)_6$ at 0 μM (red dash dot dot), 6.25 μM (green dash dot), 25 μM (black solid) or 100 μM (blue dash). The reducing mediator enhances clozapine signal amplification up to 1.75 times. (b) Corresponding baseline measurements (i.e., without clozapine) illustrating the increase in noise with the addition of $Ru(NH_3)_6$.

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utility of the reducing mediator becomes immediately apparent—the clozapine peak current is up to 1.75 times higher in the presence of $Ru(NH_3)_6$ compared to its absence. The peak potential increases by 46 ± 1 mV in the presence of $Ru(NH_3)_6$, and an additional positive shift of 391 ± 15 μV per μM of $Ru(NH_3)_6$ is indicated (R^2 = 0.998). The mechanism here is distinct from that underlying the shifts observed in 3.1, where the chitosan matrix or the grafted catechol were compromised. Instead, with the kinetics likely unaffected, the $Ru(NH_3)_6$ enables longer sustenance of the redox cycle, resulting in a mainly temporal delay of the peak potential. Furthermore, we observe a nonlinear dependence of clozapine signal amplification on $Ru(NH_3)_6$ concentration. At 6.25 μM, $Ru(NH_3)_6$ is significantly less abundant than the clozapine. In this case, the oxidizing mediator is more efficient at discharging the catechol than the reducing mediator is at recovering it, resulting in suboptimal amplification of the clozapine signal. At 25 μM, we record maximum amplification, the clozapine oxidation now no longer limited by insufficient recharging of the catechol. While intuitively, an even higher concentration of 100 μM $Ru(NH_3)_6$ should not adversely affect redox cycling performance, we note a drop in amplification performance. We hypothesize this is due to the large concentration of $Ru(NH_3)_6$ causing a very high intrinsic background signal, yielding smaller relative changes when introducing clozapine. The supporting data is displayed in Figure 4b, showing the successive increase in recorded current from baseline measurements with increasing $Ru(NH_3)_6$. Thus, while the reducing mediator is essential for sustained clozapine signal amplification, its concentration needs to be chosen with the dynamic range of the application in mind. When considering blood plasma samples, this implies that the signal-to-noise ratio of our approach may be impacted by the concentration of endogenous strong reversible reducers with standard reduction potentials similar to that of $Ru(NH_3)_6$, such as ubiquinone.[25] Such species will require further investigation on their ability to interact with the film—if they are active as reducing mediators, a strategy for ensuring consistent levels between samples will have to be researched.

In the absence of $Ru(NH_3)_6$, omission of the negative potential region in CV cycles would not be expected to impact clozapine amplification positively or negatively. The negative potentials facilitate recharging of the catechol in the presence of $Ru(NH_3)_6$. Without a reducing mediator, we would expect a decrease in clozapine peak current with increasing number of cycles as the catechol is discharged, independent of the negative potential. However, in Figure 5a,b, we illustrate that the omission of the negative potential in cyclic voltammetry (i.e., recording between 0 and +0.7 V) yields a 2.47-fold decrease in the clozapine signal compared to measurements over the full potential range. This decrease can be recovered by application of a 30 s, −0.35 V pulse. We hypothesize this is related to electrophoretic transport of clozapine ($pK_{a,1}$ = 3.92 and $pK_{a,2}$ = 7.75; predominantly positive charge state at pH 7)[26] enhancing the locally available concentration at the electrode.[27] We note that we are operating at an ionic strength of 223 mM, and thus charge screening effects need to be considered. The Hückel equation allows for first-order approximation of electrophoretic mobility $\mu^{0,\infty}$, which can further be expressed as a function of ionic strength μ^0(IS) using the extended Onsager model.[28,29] For clozapine, the decrease in electrophoretic mobility in PB can thus be estimated to be μ^0(223 mM) / $\mu^{0,\infty} \approx 0.5$ (allowing for some uncertainty in its hydrodynamic radius), supporting

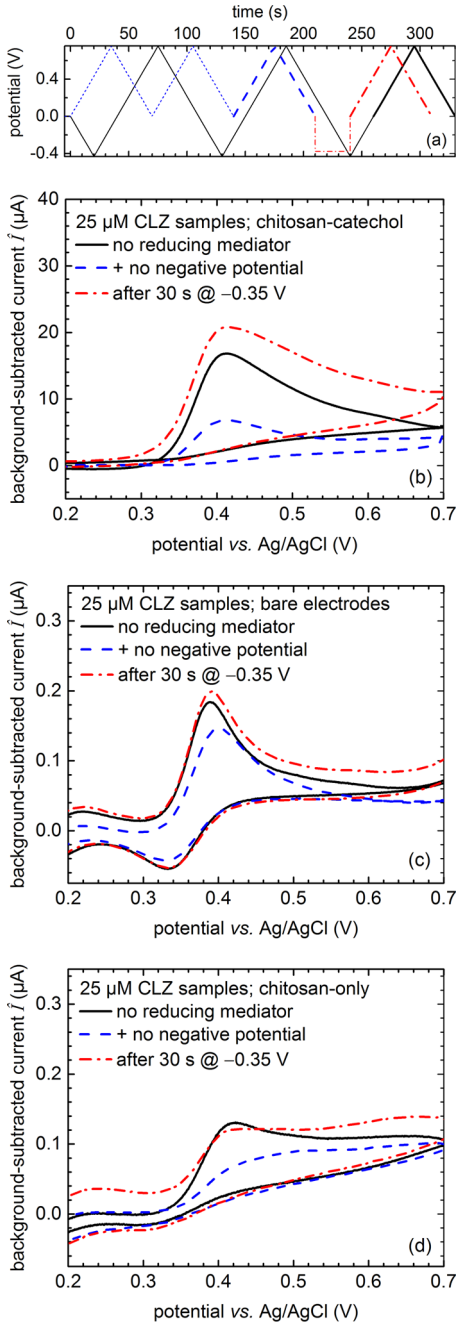

Figure 5. (a) Schematic of potential sweeps utilized in these experiments: Standard full voltammetry cycle (black solid); omission of negative potential range (blue dash); Application of negative potential pulse (red dash dot). Bold sections are plotted below. (b–d) Impact of applied negative potential on amplification, showing electrostatically driven transport of clozapine for catechol-chitosan-modified, bare, and chitosan-only-modified electrodes.

that electrophoretic effects cannot be neglected. Moreover, similar conditions in terms of pH and ionic strength have previously been utilized in capillary electrophoresis studies of clozapine.[30,31]

To further test our hypothesis, we conduct identical experiments using electrodes coated only with chitosan, or entirely unmodified. These are carried out in nitrogen-rich environment to further rule out the possibility oxygen-related interference—oxygen reduction is prominent at −0.2 V. Due to the different nature of the electrochemical cell necessitated by

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the environmental requirements, the absolute current values cannot be directly compared to previous results. Relatively, we indeed observe similar behavior on bare electrodes in Figure 5c, a 1.26-fold decrease is apparent which is subsequently recovered. On chitosan-only electrodes in Figure 5d, the effect becomes more pronounced with a factor of 1.87. Therefore, we believe that in a diffusion-limited environment (the chitosan) electrophoretic transport plays a significant role, while diffusion dominates for bare electrodes. This electrophoretic effect may explain preconcentration effects observed and utilized, but not further investigated, by other authors.[32−35] The significant signal gain observed in these studies on bare electrodes is likely due to the different nature of the electrodes utilized (carbon- or mercury-based rather than gold), which may serve to adsorb the transported clozapine until the measurement is initiated. Moving toward complex biological samples, fully utilizing this electrophoretic effect may prove critical in achieving a clinically relevant detection limit. The approach is clearly not limited to the catechol-modified chitosan system, and understanding its nature will allow a wide range of clozapine detection schemes to better take advantage of it.

3.2.2. Oxidizing Mediator. Human blood plasma is a highly complex fluid, containing a wide variety of chemical species, some of which are known to be electrochemically active.[17] We have previously demonstrated dose-dependent response in human serum.[16] However, for our redox cycling system to serve as an effective clozapine sensor, we need to understand the impact of other redox species on the recorded signals. We selected two prime candidates for investigation: norclozapine, the pharmacologically active metabolite of clozapine, featuring nearly identical structure;[36] and dopamine (E_0 = +0.182 V vs Ag/AgCl),[37] a well-studied representative catecholamine present in blood, which may be expected to interact with the catechol-based detection mechanism. The relevant chemical structures and similarities are illustrated in Figure 6a. In Figure 6b, we record signals from solutions spiked with either clozapine or one of two potential interferents. From the graph, we find that norclozapine displays similar amplification behavior to clozapine with a dose-dependent response. This shows that norclozapine, like clozapine, is able to act as an oxidizing mediator in the catechol-modified chitosan system. However, the peak potential is +0.11 V higher compared to clozapine. This limits the potential for interference, and may even enable simultaneous monitoring of both compounds in mixed samples using peak deconvolution algorithms. While norclozapine blood concentration does not have a proven correlation with treatment efficacy, it is still routinely reported in current practice, and data on its impact on treatment safety is insufficient.[11] Hence, a dual sensor for both the clozapine and its metabolite offers added utility both for the point-of-care as well as clinical research. Dopamine shows a concentration-dependent redox signal around 0.2 V, in line with expectations and far removed from the signal observed with either clozapine or norclozapine.[37] The higher peak current from dopamine is likely due to faster electron transfer kinetics, and hence higher redox cycling efficiency, over clozapine and norclozapine, which is also reflected in the sharper definition of the dopamine peak. We also note that dopamine did not effect changes in baseline recordings conducted after dopamine sample measurements, indicating no permanent changes in our redox cycling system in spite of its catechol-like nature. It appears the catechol occupies a near-equilibrium fraction of exposed chitosan amine groups, and thus combined with the short residence time of dopamine

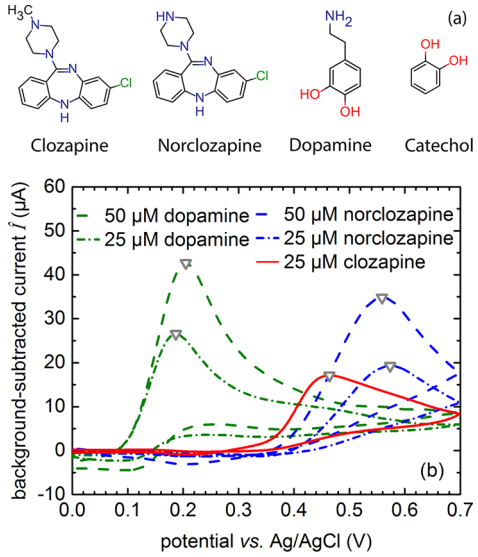

Figure 6. (a) Chemical structures of (from left to right) clozapine, its metabolite norclozapine, the catecholamine dopamine, and catechol. (b) Cyclic voltammograms (oxidative region shown; baseline subtracted) measured with the catechol-modified chitosan electrodes for solutions containing various oxidizing mediators: 25 μM clozapine (red solid); 25 μM (blue dash dot) or 50 μM (blue dash) norclozapine; and 25 μM (green dash dot) or 50 μM (green dash) dopamine. This illustrates the capability of the redox cycling system to differentiate certain redox-active species.

in its oxidized form due to redox cycling only negligible dopamine grafting occurs. Not observing the detrimental impact of dopamine on the catechol-modified chitosan system at the high concentrations investigated, its presence—and that of other catecholamines—in blood samples is clearly not a concern at the typical concentrations around 1 nM.[38]

Overall, these experiments demonstrate that selectivity based on standard reduction potential is conserved in the redox cycling system. This is in addition to multiple inherent properties of the redox capacitor contributing to clozapine selectivity. First, because the amplification relies on redox cycling, redox species that show irreversible reduction or oxidation will contribute significantly less to the electrochemical signal. Only the signal from reversible or quasi-reversible species, such as clozapine, will be amplified. Second, the catechol has a standard reduction potential of E_0 = +0.132 V vs Ag/AgCl, limiting signal amplification to species with a higher E_0. Those with lower E_0 cannot participate in discharging the redox capacitor. In combination with our previous results in human serum, we believe this is encouraging for applying the redox cycling system toward sensing in real-world biological samples with mixtures of analyte and interferents. However, further study of cross-reactions and interference is clearly required.

4. CONCLUSIONS

Our in-depth study of the material and electrochemical parameter space furthers the understanding of the catechol-modified chitosan films as sensors and of the electrochemical behavior of clozapine, as well as their interplay. We illustrate the critical function of the reducing mediator in this redox cycling approach, where careful attention has to be paid to the choice of concentration, contributing up to 1.75-fold to signal amplification. The study of electrical potential application

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illustrates electrophoretic transport of clozapine playing a similarly significant role in signal enhancement, especially in diffusion-limited environments, accounting for a factor of up to 2.47. This fact may be exploited in the future to enhance sensitivity and selectivity of not just our redox cycling approach, but also other electrochemical clozapine sensors. We further demonstrate the ability to differentiate three distinct redox-active species—clozapine, its structurally largely analogous metabolite norclozapine, and the representative catecholamine dopamine—with the catechol-modified chitosan system, showcasing aspects of selectivity. This also opens up the possibility of applying the redox cycling approach to detect other reversible redox species such as dopamine, where the knowledge gained in our study will provide guidelines for optimization. We moreover demonstrate the feasibility of film storage over weeks, critical when considering packaged point-of-care sensors, with performance degradation limited to 0.26%/day, which may be further optimized by investigating a range of storage solutions. The signal degradation trends with reuse, where absolute signal loss of 2.2% can be accounted for with signal processing, demonstrate the overall robustness of our biomaterial system and show that clozapine itself does not foul the sensor. This will be useful in comparison to similar studies in plasma to elucidate fouling mechanisms. In the future, our understanding may further benefit from comparative studies between our biomaterial-based redox cycling system and solid-state ones.[39−41]

Transitioning to complex samples such as blood plasma and miniaturizing the system will both put stringent requirements on sensor performance. As mentioned before, plasma contains many potential interferents, and film fouling is expected to become more substantial. A microenvironment is dominated by different physicochemical effects than macro-scale systems, with e.g. diffusion being of much greater importance. We expect the insights gained through the experiments presented here will guide the necessary optimization toward a lab-on-a-chip system to monitor clozapine treatment. Such a system will allow for broader use of clozapine by increasing convenience to patients as well as medical professionals, thus improving the lives of people affected by schizophrenia through personalized medicine.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +1 (301) 405-8158. Fax: +1 (301) 314-9920. E-mail: ghodssi@umd.edu.

Author Contributions

⊥T.E.W. and H.B.-Y. contributed equally to this paper.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Carlstedt, R. A. *Handbook of Integrative Clinical Psychology, Psychiatry, and Behavioral Medicine: Perspectives, Practices, and Research*; Springer Publishing Company: New York, 2010.

(2) Sadock, B. J.; Kaplan, H. I.; Sadock, V. A. *Kaplan & Sadock's Synopsis of Psychiatry: Behavioral Sciences/Clinical Psychiatry*, 10th ed.; Lippincott Williams & Wilkins: Philadelphia, 2007.

(3) Conley, R. R.; Tamminga, C. A.; Kelly, D. L.; Richardson, C. M. Treatment-Resistant Schizophrenic Patients Respond to Clozapine after Olanzapine Non-Response. *Biol. Psychiatry* **1999**, *46*, 73−77.

(4) Breier, A.; Buchanan, R. W.; Kirkpatrick, B.; Davis, O. R.; Irish, D.; Summerfelt, A.; Carpenter, W. T., Jr. Effects of Clozapine on Positive and Negative Symptoms in Outpatients with Schizophrenia. *Am. J. Psychiatry* **1994**, *151*, 20−26.

(5) Kelly, D.; Kreyenbuhl, J.; Buchanan, R.; Malhotra, A. Why Not Clozapine? *Clin. Schizophr. Relat. Psychoses* **2007**, *1*, 92−95.

(6) Cohen, D.; Bogers, J. P. A. M.; van Dijk, D.; Bakker, B.; Schulte, P. F. J. Beyond White Blood Cell Monitoring. *J. Clin. Psychiatry* **2012**, *73*, 1307−1312.

(7) Novartis. CLOZARIL Prescribing Information, 2013.

(8) Buchanan, R. W.; Kreyenbuhl, J.; Kelly, D. L.; Noel, J. M.; Boggs, D. L.; Fischer, B. A.; Himelhoch, S.; Fang, B.; Peterson, E.; Aquino, P. R.; et al. The 2009 Schizophrenia PORT Psychopharmacological Treatment Recommendations and Summary Statements. *Schizophr. Bull.* **2010**, *36*, 71−93.

(9) Stark, A.; Scott, J. A Review of the Use of Clozapine Levels to Guide Treatment and Determine Cause of Death. *Aust. N. Z. J. Psychiatry* **2012**, *46*, 816−825.

(10) Freeman, D. D. J.; Oyewumi, L. K. Will Routine Therapeutic Drug Monitoring Have a Place in Clozapine Therapy? *Clin. Pharmacokinet.* **1997**, *32*, 93−100.

(11) Spina, E.; Avenoso, A.; Facciolà, G.; Scordo, M. G.; Ancione, M.; Madia, A. G.; Ventimiglia, A.; Perucca, E. Relationship between Plasma Concentrations of Clozapine and Norclozapine and Therapeutic Response in Patients with Schizophrenia Resistant to Conventional Neuroleptics. *Psychopharmacology (Berl.)* **2000**, *148*, 83−89.

(12) Kauffmann, J.-M.; Patriarche, G. J.; Christian, G. D. Electrochemical Oxidation of Derivatives of Dibenzodiazepin. Dibenzothiazepin and Dibenzoxazepin. *Anal. Lett.* **1979**, *12*, 1217−1234.

(13) Leeuwen, S. M. van; Blankert, B.; Kauffmann, J.-M.; Karst, U. Prediction of Clozapine Metabolism by on-Line Electrochemistry/liquid Chromatography/mass Spectrometry. *Anal. Bioanal. Chem.* **2005**, *382*, 742−750.

(14) Bashir, R. BioMEMS: State-of-the-Art in Detection, Opportunities and Prospects. *Adv. Drug Delivery Rev.* **2004**, *56*, 1565−1586.

(15) Yu, D.; Blankert, B.; Viré, J.; Kauffmann, J. Biosensors in Drug Discovery and Drug Analysis. *Anal. Lett.* **2005**, *38*, 1687−1701.

(16) Ben-Yoav, H.; Winkler, T. E.; Kim, E.; Chocron, S. E.; Kelly, D. L.; Payne, G. F.; Ghodssi, R. Redox Cycling-Based Amplifying Electrochemical Sensor for in Situ Clozapine Antipsychotic Treatment Monitoring. *Electrochim. Acta* **2014**, *130*, 497−503.

(17) Psychogios, N.; Hau, D. D.; Peng, J.; Guo, A. C.; Mandal, R.; Bouatra, S.; Sinelnikov, I.; Krishnamurthy, R.; Eisner, R.; Gautam, B.; et al. The Human Serum Metabolome. *PLoS One* **2011**, *6*, e16957.

(18) Clark, W. M. *Oxidation Reduction Potentials of Organic Systems*; Williams & Wilkins: Baltimore, 1960.

(19) Koev, S. T.; Dykstra, P. H.; Luo, X.; Rubloff, G. W.; Bentley, W. E.; Payne, G. F.; Ghodssi, R. Chitosan: An Integrative Biomaterial for Lab-on-a-Chip Devices. *Lab. Chip* **2010**, *10*, 3026.

(20) Kim, E.; Liu, Y.; Shi, X.-W.; Yang, X.; Bentley, W. E.; Payne, G. F. Biomimetic Approach to Confer Redox Activity to Thin Chitosan Films. *Adv. Funct. Mater.* **2010**, *20*, 2683−2694.

(21) Kim, E.; Gordonov, T.; Bentley, W. E.; Payne, G. F. Amplified and in Situ Detection of Redox-Active Metabolite Using a Biobased Redox Capacitor. *Anal. Chem.* **2013**, *85*, 2102−2108.

(22) Ben-Yoav, H.; Winkler, T. E.; Kim, E.; Kelly, D. L.; Payne, G. F.; Ghodssi, R. *Catechol-Modified Chitosan System as a Bio-Amplifier for Schizophrenia Treatment Analysis*; MRS Online Proceedings Library: San Francisco, California, 2013; Vol. *1572*.

(23) Bard, A. J.; Faulkner, L. R. *Electrochemical Methods: Fundamentals and Applications*; Wiley: New York, 2000.

(24) Kohen, R.; Nyska, A. Invited Review: Oxidation of Biological Systems: Oxidative Stress Phenomena, Antioxidants, Redox Reactions, and Methods for Their Quantification. *Toxicol. Pathol.* **2002**, *30*, 620−650.
(25) Tinoco, I.; Sauer, K.; Wang, J. C.; Puglisi, J. D.; Harbinson, G.; Rovnyak, D. *Physical Chemistry: Principles and Applications in Biological Sciences*, 5th ed.; Prentice Hall: New Jersey, 2013.
(26) http://www.chemicalize.org/. Clozapine Properties (accessed Sep 1, 2014).
(27) Kalvoda, R. Adsorptive Stripping Voltammetry in Trace Analysis. In *Contemporary Electroanalytical Chemistry*; Ivaska, A., Lewenstam, A., Sara, R., Eds.; Plenum Press: New York, 1990; pp 403−405.
(28) Li, D.; Fu, S.; Lucy, C. A. Prediction of Electrophoretic Mobilities. 3. Effect of Ionic Strength in Capillary Zone Electrophoresis. *Anal. Chem.* **1999**, *71*, 687−699.
(29) Plasson, R.; Cottet, H. Determination of Homopolypeptide Conformational Changes by the Modeling of Electrophoretic Mobilities. *Anal. Chem.* **2005**, *77*, 6047−6054.
(30) Pucci, V.; Raggi, M.; Kenndler, E. Separation of Antipsychotic Drugs (clozapine, Loxapine) and Their Metabolites by Capillary Zone Electrophoresis. *J. Chromatogr. A* **1999**, *853*, 461−468.
(31) Hillaert, S.; Snoeck, L.; Van den Bossche, W. Optimization and Validation of a Capillary Zone Electrophoretic Method for the Simultaneous Analysis of Four Atypical Antipsychotics. *J. Chromatogr. A* **2004**, *1033*, 357−362.
(32) Hernández, L.; González, E.; Hernández, P. Determination of Clozapine by Adsorptive Anodic Voltammetry Using Glassy Carbon and Modified Carbon Paste Electrodes. *Analyst* **1988**, *113*, 1715−1718.
(33) Hammam, E.; Tawfik, A.; Ghoneim, M. M. Adsorptive Stripping Voltammetric Quantification of the Antipsychotic Drug Clozapine in Bulk Form, Pharmaceutical Formulation and Human Serum at a Mercury Electrode. *J. Pharm. Biomed. Anal.* **2004**, *36*, 149−156.
(34) Farhadi, K.; Karimpour, A. Electrochemical Behavior and Determination of Clozapine on a Glassy Carbon Electrode Modified by Electrochemical Oxidation. *Anal. Sci.* **2007**, *23*, 479−483.
(35) Mashhadizadeh, M. H.; Afshar, E. Electrochemical Investigation of Clozapine at TiO2 Nanoparticles Modified Carbon Paste Electrode and Simultaneous Adsorptive Voltammetric Determination of Two Antipsychotic Drugs. *Electrochim. Acta* **2013**, *87*, 816−823.
(36) Bondesson, U.; Lindström, L. H. Determination of Clozapine and Its N-Demethylated Metabolite in Plasma by Use of Gas Chromatography-Mass Spectrometry with Single Ion Detection. *Psychopharmacology (Berl.)* **1988**, *95*, 472−475.
(37) Mohammad-Shiri, H.; Ghaemi, M.; Riahi, S.; Akbari-Sehat, A. Computational and Electrochemical Studies on the Redox Reaction of Dopamine in Aqueous Solution. *Int. J. Electrochem. Sci.* **2011**, *6*, 317−336.
(38) Buhler, H. U.; Prada, M. D.; Haefely, W.; Picotti, G. B. Plasma Adrenaline, Noradrenaline and Dopamine in Man and Different Animal Species. *J. Physiol.* **1978**, *276*, 311−320.
(39) Neugebauer, S.; Müller, U.; Lohmüller, T.; Spatz, J. P.; Stelzle, M.; Schuhmann, W. Characterization of Nanopore Electrode Structures as Basis for Amplified Electrochemical Assays. *Electroanalysis* **2006**, *18*, 1929−1936.
(40) Kätelhön, E.; Wolfrum, B. On-Chip Redox Cycling Techniques for Electrochemical Detection. *Rev. Anal. Chem.* **2012**, *31*, 7−14.
(41) Ma, C.; Contento, N. M.; Gibson, L. R.; Bohn, P. W. Redox Cycling in Nanoscale-Recessed Ring-Disk Electrode Arrays for Enhanced Electrochemical Sensitivity. *ACS Nano* **2013**, *7*, 5483−5490.