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Versatile potentiometric metabolite sensing without dioxygen interference

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ABSTRACT

The field of electrochemical biosensors has been dominated by amperometric and voltammetric sensors; however, these are limited greatly in their signal dependence on electrode size. Open circuit potentiometric sensors are emerging as an alternative due to their signal insensitivity to electrode size. Here, we present a second-generation biosensor that uses a modified chitosan hydrogel to entrap a dehydrogenase or other oxidoreductase enzyme of interest. The chitosan is modified with a desired electron mediator such that in the presence of the analyte, the enzyme will oxidize or reduce the mediator, thus altering the measured interfacial potential. Using the above design, we demonstrate a swift screening method for appropriate enzyme-mediator pairs based on open circuit potentiometry, as well as the efficacy of the biosensor design using two dehydrogenase enzymes (FADGDH and ADH) and peroxidase. Using 1,2-naphthoquinone as the mediator for FADGDH, dynamic ranges from 0.1 to 50 mM glucose are achieved. We additionally demonstrate the ease of fabrication and modification, a lifetime of $\geq$28 days, insensitivity to interferents, miniaturization to the microscale, and sensor efficacy in the presence of the enzyme's natural cofactor. These results forge a foundation for the generalized use of potentiometric biosensors for a wide variety of analytes within biologically-relevant systems where oxygen can be an interferent.

1. Introduction

Electrochemical biosensors are advantageous in that they do not require expensive instrumentation, are portable, rapid, inexpensive, and highly sensitive and selective (Parrilla et al., 2016; Guadarrama-Fernandez et al., 2018; Ding and Qin, 2020). As a result, there are countless electrochemical biosensors that exist with applications in food science, environmental monitoring, and medical diagnostics (Chen et al., 2020). The vast majority of biosensors are based on amperometric or voltammetric techniques (Pishko et al., 1991; Luo et al., 2004; Bollella et al., 2017; Rassas et al., 2019). However, the use of potentiometry is increasingly attractive as it passes negligible current when making a measurement (Lee et al., 2019; Smith et al., 2020). Advantages of potentiometric measurements then include: reduced interferent effects, slower fouling of the sensor, insensitivity to electrode size, ease of miniaturization, and longer lifetimes (Park et al., 2013; Smith et al., 2020; Walker and Dick, 2021). Thus, the use of potentiometric techniques, such as open circuit potentiometry, allow for the creation of more reliable, long-lasting and easily miniaturizable biosensors.

When the biorecognition element is an enzyme, biosensors can be sorted into three generations (Bollella et al., 2017; Lee et al., 2019). First-generation biosensors contain oxidase enzymes and monitor either the consumption of oxygen or the production of hydrogen peroxide to quantify the analyte. Second-generation enzymatic biosensors often use artificial electron mediators with either oxidases or dehydrogenases in order to avoid the sensitivity to oxygen that plagues first-generation biosensors, while third-generation biosensors utilize enzymes capable of direct electron transfer (DET) with the electrode surface. In our previous work (Walker and Dick, 2021), we entrapped oxidase enzymes on the surface of platinum electrodes with chitosan to make biosensors for detection of glucose and galactose. In this versatile design, simply changing out the enzyme for another oxidase enzyme formed a biosensor specific for a new analyte. Mechanistically, the reaction of the enzyme with oxygen and the generation of hydrogen peroxide changed the interfacial potential, allowing construction of calibration curves on electrodes ranging in size from millimeters to micrometers.

While promising for sensing applications, a drawback of using oxidase enzymes is that dioxygen is a dominant interferent. Furthermore, the signal was dependent on a mixed potential and resulted in unstable baselines and differing response between macro- and microbiosensors.

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In order to overcome these disadvantages, a second or third-generation version is needed. Though there are many advantages to third-generation biosensors (Lee et al., 2019), there are relatively few enzymes capable of direct electron transfer (Ghindilis et al., 1997; Gorton et al., 1999; Ma et al., 2019; Okuda-Shimazaki et al., 2020) currently available. Thus, a second-generation biosensor would allow for a more versatile platform capable of detecting a wide variety of analytes by changing the enzyme used and would not suffer from dioxygen interferents.

There are a large number of second-generation enzymatic biosensors present in the literature already. However, the majority of them put the artificial electron mediator in the test solution (Lu et al., 2012; Dai et al., 2015). This not only would require sample preparation steps and the use of larger amounts of sometimes expensive mediators, but would greatly restrict use as many of these mediators are often toxic (Bollella et al., 2017). Others adsorb the mediators and enzymes to metal nanoparticles or carbon nanotubes (Navaee and Salimi, 2018; Boussema et al., 2018), but this has the risk of leakage of the mediator as adsorption is not the strongest method of binding. Other biosensors bind the usual enzymatic cofactor NAD(H) to the surface or a membrane (Karra et al., 2013; Hughes et al., 2016), but NAD(H) is not the most stable mediator and it requires a high overpotential to detect. Some utilize redox-active polymers to entrap the enzymes (Hickey et al., 2016; Pinyou et al., 2016a, 2016b), but most of these still use oxidase enzymes and thus can be affected by environmental oxygen levels. Similarly, some groups chemically conjugate artificial electron mediators or NAD(H) to polymers (Karra and Gorski, 2013; Milton et al., 2015) to mediate the enzymatic reaction, but these require chemical modification of the mediators prior to conjugation and thus more complicated synthesis processes. One thing all of these previous biosensors also have in common is they almost exclusively use amperometric or voltammetric methods of detection.

In this work, we address limitations of first-generation closed-circuit biosensors by creating a second-generation open circuit biosensor. The development of a second-generation biosensor necessitates enzymes capable of communicating with the electrode when trapped in the hydrogel. This communication is achieved by functionalizing the backbone of the hydrogel with an appropriate mediator to facilitate the enzymatic reaction and change the interfacial potential. This design is not only simple and quick to make, but also easy to modify to functionalize with other enzyme-mediator combinations. We detail a method for quickly screening the most effective mediator-enzyme combination, then describe the efficacy of this design utilizing the enzyme FAD-dependent glucose dehydrogenase (FADGDH) with three different mediators on both macro- and micro-electrodes. We also demonstrate versatility with the enzymes peroxidase and alcohol dehydrogenase. To the best of our knowledge, this is the first example of a potentiometric second-generation biosensor wherein the hydrogel entrapping the dehydrogenase enzyme is capable of mediating the enzymatic reaction.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical grade unless noted otherwise and were used as received. FAD-dependent glucose dehydrogenase (FADGDH-AB) from recombinant *Aspergillus sojae* was obtained from Kikkoman Biochemifa (Vannoy et al., 2021). Peroxidase from Horseradish and Alcohol Dehydrogenase (ADH) from *Saccharomyces cerevisiae* were obtained from Sigma-Aldrich. Dextrose (D-Glucose) (ACS certified), Sucrose (ACS certified), Toluene (ACS certified), and Chloroform (HPLC-grade) were obtained from Fisher Scientific. Chitosan (from shrimp shells, ≥75% (deacetylated)), 5% Nafion, L-Ascorbic Acid (99%), Acetaminophen (≥99%), Methylene Blue (≥82%), Tris (2-2′-bipyridyl)dichloro ruthenium (II) hexahydrate ($Ru(bpy)_3Cl_2$) (99.95%), Menadione (K3) (Analytical Grade), 1,2-Naphthoquinone (1,2-NQ) (97%), Phenosafranin (≥80%), Gallocyanine (GCy) (≥90%), Phenazine methosulfate (PMS) (≥90%), Phenol Red sodium salt, O-Phenylenediamine (≥98%), P-Phenylenediamine (≥99%), Ferrocenemethanol (97%), D-(+)-Galactose (≥99%), Potassium hexacyanoferrate (III) (≥99%), and 25% glutaraldehyde in water (grade II) were obtained from Sigma-Aldrich. 1-Methoxy-5-methyl phenazine methyl sulfate (mPMS) (≥82%) was obtained from Dojindo Laboratories. Bromocresol Purple (Indicator Grade) was from Mallinckrodt Chemicals. 1,4-Benzoquinone (1,4-BQ) (99%) and Alpha-D-Lactose monohydrate (ACS reagent) was obtained from Acros Organics. NAD^+ (free acid) was purchased from Cayman Chemical Company. Dulbeccos' Phosphate Buffered Saline (DPBS) (1X) with calcium and magnesium (pH = 6.8) was purchased from Corning.

2.2. Instrumentation

All open circuit potentiometry experiments were performed on a CHI model 601E potentiostat (CH Instruments, Austin, TX, Input Impedance = 1 TΩ). Deposition of the hydrogel film occurred using the amperometry technique on a CHI model 601E potentiostat. All stock solutions were prepared in DPBS (1X) and vortexed using a vortex-genie (Scientific Industries, New York, Bohemia). Glassy carbon macroelectrodes (r = 1.5 mm), gold macroelectrodes (r = 1 mm), platinum macroelectrodes (r = 1 mm), and gold microelectrodes (r = 6.25 μm) were used as working electrodes. A silver/silver chloride reference electrode (stored in 1M KCl) and a glassy carbon rod as a counter electrode were used in every experiment. All electrodes were purchased from CHI (CH Instruments, Austin, TX).

2.3. Electron mediator screening

Analysis of which electron mediators worked best with the FADGDH enzyme was done using a method developed in our lab to determine the turnover rate of an enzyme-mediator pair (Smith et al., 2018). In brief, solutions of 0.5 mM mediator and 0.15 M glucose were made in DPBS. A bare glassy carbon macroelectrode and a Ag/AgCl reference electrode were used to make an open circuit potential measurement in the stirred solution for 10 min. After this blanking period was over, 50 μL of a solution of 1 mg of FADGDH in 1.5 mL PBS was spiked into the solution. The potential then changed as the enzymatic reaction reduced the mediator present in solution, and this slope could be mathematically correlated to the turnover rate of that enzyme-mediator pair. Example open circuit potential traces and a description of the calculation are provided in the Supplementary Information (Fig. S1).

This screening was done for FADGDH and nine mediators (methylene blue, 1,2-NQ, 1,4-BQ, menadione, PMS, mPMS, phenosafranin, GCy, and $Ru(bpy)_3Cl_2$) 4–5 times each. The average turnover rate (k_{turn}) of each of these enzyme-mediator pairs is reported in the Supplementary Information (Table S1). The same was done for horseradish peroxidase and eight mediators, and the average turnover rate of each of these enzyme-mediator pairs is reported in the Supplementary Information (Table S2), as well as for alcohol dehydrogenase and nine mediators (Table S3). Example open circuit potential traces and a description of the calculation are provided in the Supplementary Information (Figs. S2 and S3). Using this information, three mediators with high but differing k_{turn} values and functional groups that can be easily conjugated to chitosan were chosen for further use: 1,2-NQ, 1,4-BQ, and GCy.

2.4. Chitosan-mediator solution preparation

Chitosan was added to a solution of 1% (v/v) acetic acid in ultrapure water while stirring and gently heating until fully dissolved to make a 1% (w/v) chitosan solution. Chitosan-mediator solutions were made in ratios of 10:5:2.5, 10:5:1, 10:2.5:2.5, 10:2.5:1, and 10:1:1 chitosan: mediator: glutaraldehyde by molarity for 1,2-NQ and 1,4-BQ and in ratios of 10:1:4, 10:1:8, 10:1.5:6, 10:2:8, and 10:2.5:10 for GCy. To do

so, the appropriate mass of either 1,2-NQ, 1,4-BQ, or GCy was added to 10 mL of 1% chitosan and stirred vigorously until visibly homogeneous. Then, the corresponding amount of 25% aqueous glutaraldehyde solution was added to the solution, covered, and vigorously stirred for at least 4 h. Afterwards, the excess glutaraldehyde and mediator were extracted through three successive washes with toluene (4:1 toluene: chitosan-mediator solution), followed by at least three successive washes with chloroform (4:1 chloroform: chitosan-mediator solution), and three successive washes with diethyl ether (4:1 chloroform: chitosan-mediator solution), until no more colored mediator was removed during the wash steps. Finally, the solutions were allowed to dry under a hood for at least 1 h to ensure that all leftover organic solvents had evaporated off of the chitosan-mediator solutions.

2.5. Chitosan-mediator film optimization

Chitosan-mediator films were prepared on macroelectrodes initially without enzyme present to establish the optimal deposition conditions for each of the chitosan-mediator solutions. Otherwise, films were made as described in section 2.6 below. Biosensor electrodeposition time, electrodeposition potential, and chitosan: mediator: glutaraldehyde ratios were optimized in this way for each of the three chitosan-mediator solutions. Details of these optimizations can be found in the Supplementary Information (Figs. S4–7).

To determine the optimal mediator regeneration time and potential for each of the three chitosan-mediator solutions, biosensors were made as optimized above with 2.5 mg/mL FADGDH added to the deposition solution. Details of these optimizations can be found in the Supplementary Information (Fig. S8). Biosensors were also made as above with varying concentrations of FADGDH to determine the optimal enzyme concentration. Details of these optimizations can be found in the Supplementary Information (Fig. S9).

Finally, leakage tests were done to ensure the mediator was not leeching from the hydrogel over time. Hydrogels without enzyme were made using the above optimized conditions on glassy carbon macroelectrodes and left in solutions of DPBS over 60,000s (or 16.67 h). Analysis of the results demonstrated that no appreciable mediator leakage occurred over that time. The details can be found in the Supplementary Information (Fig. S10).

2.6. Glucose sensor preparation

To make the deposition solution, 2.5 mg of FADGDH was suspended in 0.5 mL of chitosan-mediator solution and vortexed until homogeneous for a final concentration of 5 mg/mL FADGDH. Deposition of the hydrogel occurred by applying the previously determined optimal conditions (see Table 1). The electrodes were then gently rinsed with ultrapure water and dried at room temperature. A protective layer of 10 μL of 1% Nafion (dissolved in ethanol) was dropcast onto the surface of the electrodes and allowed to dry at room temperature for at elast 10 min. This layer of Nafion is added on top of the hydrogel to prevent enzyme leakage or detachment of the hydrogel, and to lessen the effects of interferents (Luo et al., 2004; Parrilla et al., 2016; Lee et al., 2019). The electrodes were then thoroughly washed with ultrapure water to remove any unbound mediator. The electrodes were stored in a refrigerator at 4 °C when not in use.

For the sensor on a microelectrode, the same procedure was done except that 3 μL 1% Nafion in ethanol was dropcast on top of the dried hydrogel using a micropipet.

2.7. Hydrogen peroxide and NAD $^{+}$ sensor preparation

To make the hydrogen peroxide biosensor, the procedure from section 2.6 was used. The only difference was 2.5 mg HRP was used in place of 2.5 mg FADGDH. Similarly, to make the NAD^{+} sensor, 2.5 mg ADH was used in place of 2.5 mg FADGDH.

2.8. Open circuit potentiometry procedure

A standard three-electrode set-up was used (Elgrishi et al., 2018). Biosensors were rinsed gently with ultrapure water before and after every measurement. Open circuit potentiometry measurements were taken in a stirred solution of 10 mL of DPBS. An initial potential was applied to discharge any current that had built up on the surface of the electrode before the open circuit potentiometry measurement was taken (Lee et al., 2019; Reinmuth and Wilson, 1962) and to ensure the mediator at the electrode surface was in its oxidized form. For each of the chitosan-mediator solutions, this regeneration potential and the duration are listed above in Table 1. Following the end of the regeneration potential pulse, the potentiometric measurement began immediately. The open circuit potential was measured for 5 min, then the analyte was spiked in every 100 s. The recorded potential for each spike of analyte was the potential measured 100s after the spike occurred, regardless of if the potential had stabilized yet.

3. Results and discussion

3.1. Development of the chitosan-mediator-FADGDH biosensor

The enzyme FAD-dependent glucose dehydrogenase (FADGDH) converts D-glucose to D-glucono-1,5-lactone through a 2-electron and 2-proton mechanism, reducing another species in the process (Okuda--Shimazaki et al., 2020; Vannoy et al., 2021). Normally, this is either NAD^{+} or $NADP^{+}$, but artificial electron mediators can be used instead to mediate this reaction. In our earlier work (Walker and Dick, 2021), an oxidase enzyme was entrapped in chitosan to make a potentiometric glucose biosensor whose potential was controlled by mixed potential theory due to the lack of a well-defined redox couple in solution (Park et al., 2013; Percival and Bard, 2017; Baez et al., 2020). Here, however, there is a well-defined redox couple due to the covalent attachment of the mediator to the backbone of the hydrogel through crosslinking with glutaraldehyde (GA), so the potential of the system should be strongly controlled by the ratio of the surface concentrations of the oxidized and reduced forms of the mediator, as described by the Nernst equation. Additionally, by using a redox mediator with fast electron transfer kinetics instead of relying on the slower reactions that set the potential in a mixed system, a faster response should be obtained. Over the course of the enzymatic reaction, the oxidized form of the mediator initially present within the hydrogel will be reduced and this change will alter the electrode's measured potential (Fig. 1). The more glucose is present, the more of the mediator is reduced and the more the measured potential is altered.

We can predict what is expected to occur over the course of the enzymatic reaction using the Nernst equation: $E_{OCP} = E^{0'} + (RT/nF)\ln$

Table 1
Optimized hydrogel deposition and regeneration conditions on glassy carbon macroelectrodes.

Mediator	Deposition Potential (V)	Deposition Time (min)	Chitosan: Mediator: Glutaraldehyde Mole Ratio	Enzyme Concentraiton (mg/mL)	Regeneration Potential (V)	Regeneration Time (min)
1,2-NQ	−2.5	7.5	10:2.5:1	5.0	0.3	1
1,4-BQ	−3	7.5	10:2.5:1	5.0	0.6	1
GCy	−2.5	5	10:2:8	5.0	0.1	1

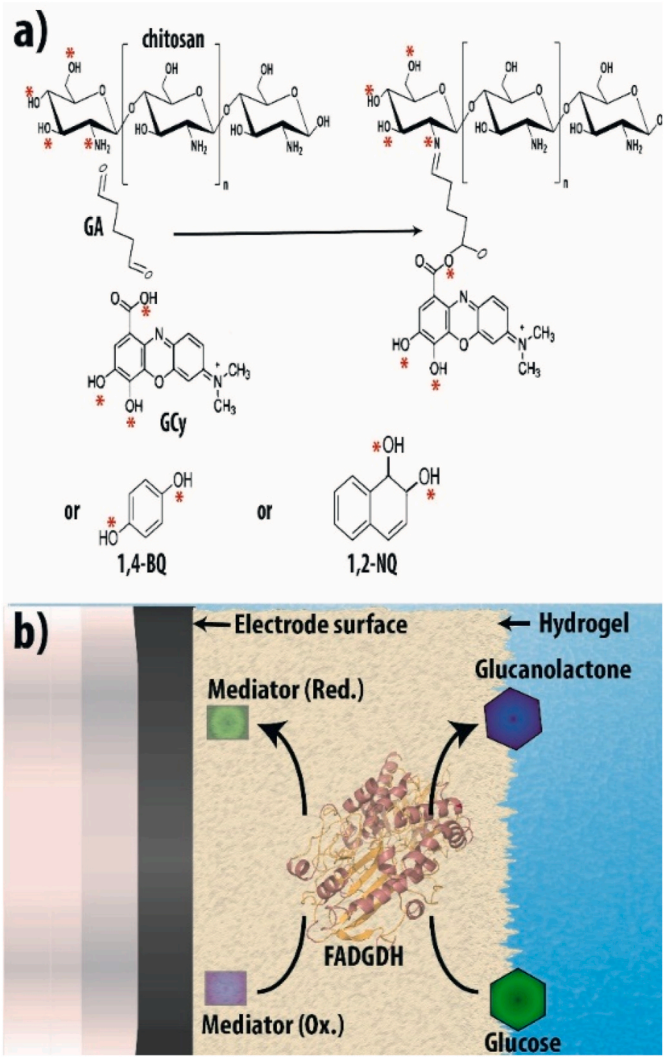

Fig. 1. a) Possible crosslinked structures of the three mediators to chitosan using glutaraldehyde in acidic solution, where the possible sites for reaction with glutaraldehyde are shown by red asterisks. **b)** The reaction that occurs on the surface of the chitosan-mediator-FADGDH carbon electrode to alter the surface potential. Glucose is oxidized to gluconolactone in the presence of the oxidant, which gets reduced to the reductant as a byproduct. The changed $[C_O]/[C_R]$ ratio in the hydrogel then alters the measured open circuit potential. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

$(C_{O(x=0)}/C_{R(x=0)})$. Here, E_{OCP} is the measured potential, $E^{0'}$ is the formal potential of the electron mediator in solution, R is the gas constant, T is the temperature in Kelvin, n is the moles of electrons involved in the redox reaction, F is Faraday's constant, $C_{O(x\,=\,0)}$ is the surface concentration of the oxidant, and $C_{R(x\,=\,0)}$ is surface the concentration of the reductant. Since more of the reduced form (reductant) of the mediator is present at the electrode surface and less of the oxidized form (oxidant) is present as the enzymatic reaction progresses, the value of E_{OCP} is expected to decrease as a result of the enzymatic reaction.

Example traces of the chitosan-1,2-NQ-FADGDH and chitosan-GCy-FADGDH biosensors on glassy carbon macroelectrodes are shown in Fig. 2a–b. As expected, additions of glucose cause the measured potential of the electrode to decrease. Example traces without mediator (Fig. S11) and without enzyme (Fig. S12) demonstrate that it is the enzymatic reaction with the mediator in the hydrogel causing the change in potential. Without either of the components needed for this enzymatic reaction to occur, there is no observed change in electrode potential. Further experiments demonstrate that the mediator in the hydrogel is setting the potential of the system, regardless of the electrolyte solution used (Fig. S13) or the electrode material (*i.e.,* platinum, gold, or glassy carbon macroelectrodes) (Fig. S14) down to a concentration of 625 μM mediator in the hydrogel (Fig. S15). Below this concentration, the potential appears to be controlled at least partially by the mixed potential of the system, rather than solely the mediator in the hdyrogel. This follows with what has been observed before by Percival and Bard. In their experiments, they diluted a 50:50 mixture of hexacyanoferrate (II/III) to probe at what concentration the redox pair fails to poise the electrode. They found that the concentration necessary to sufficiently poise the electrode is $>$ μM, indicating surface concentrations of mediator in our system must be greater than μM to observe appreciable potentiometric changes. (Percival and Bard, 2017).

Of note, the measured k_{turn} value of 1,2-NQ was higher than that of GCy in solution, thus the 1,2-NQ biosensor was expected to be faster and more sensitive than the GCy biosensor. However, the GCy biosensor is significantly more sensitive than the 1,2-NQ one. We hypothesize that this is because the crosslinker glutaraldehyde is capable of forming bonds with multiple groups on the GCy molecule, not just its redox active groups. On the other hand, the only locations for glutaraldehyde to bind to 1,2-NQ are its redox centers (see Table S1 for structures). This would dampen the ability of 1,2-NQ to react with the enzyme, thus effectively lowering its k_{turn} value and resulting in a less sensitive biosensor. A similar trend occurs with the 1,4-BQ hydrogel; however, the measured k_{turn} of 1,4-BQ in solution was an order of magnitude lower than that of 1,2-NQ to begin with. The combination of the lower k_{turn} value and glutaraldehyde crosslinking the mediator *via* its redox active groups results in a powerful dampening of the enzyme's ability to react with 1,4-BQ. Thus, the chitosan-1,4-BQ-FADGDH hydrogels reacted to glucose very poorly. Additions of very high concentrations of glucose (5 mM) caused only a few mV decrease in the measured open circuit potential. As a result of this poor performance, no calibration curves were able to be made.

The open circuit potentiometric response of this chitosan-mediator-FADGDH electrode to glucose (Fig. 2c–d) has a similar shape to a binding isotherm. In our previous work, we developed a semi-quantitative empirical model based off of mixed potential theory and Michaelis-Menten kinetics in order to describe the biosensor's observed behavior (Walker and Dick, 2021). This model was modified for this system because the derivation in the original was based upon a system set by a mixed potential. Here, we have a system whose potential is set by a well-defined redox couple, so it can be much more simply described by the Nernst equation. The specifics of the new derivation can be found in the Supplementary Information, as can be seen in Fig. S16, which demonstrates how closely the experimental and modelled data match for both the chitosan-1,2-NQ-FADGDH and the chitosan-GCy-FADGDH biosensors.

Of note here is that we use semi-log plots (Fig. 2e–f) to establish the three log-linear ranges of each of the chitosan-mediator-FADGDH biosensors (Table 2). Their use has been rightly called into question recently (Clark and Dick, 2020; Urban, 2020), but we have decided to report both plots both because of mathematical reasons and because use of semi-log plots is common in the literature. The mathematical reasoning is based upon the fact that the behavior of this biosensor is based upon a model function that utilizes a logarithm and Michaelis-Menten enzyme kinetics (see discussion of semiempirical model above). Taking the log of this function should give a log-linear range with a higher slope when the concentration of glucose is plus or minus an order of magnitude from K_M. On either side should then be a log-linear region of a lower slope, which is what we observe experimentally. As a result of this behavior, we determined that the use of semi-log plots is an appropriate way to represent this data.

These semi-log plots illustrate the three log-linear ranges of the two biosensors. Notably, the chitosan-1,2-NQ-FADGDH biosensor's middle region is linear from 1 to 10 mM, which contains the biologically

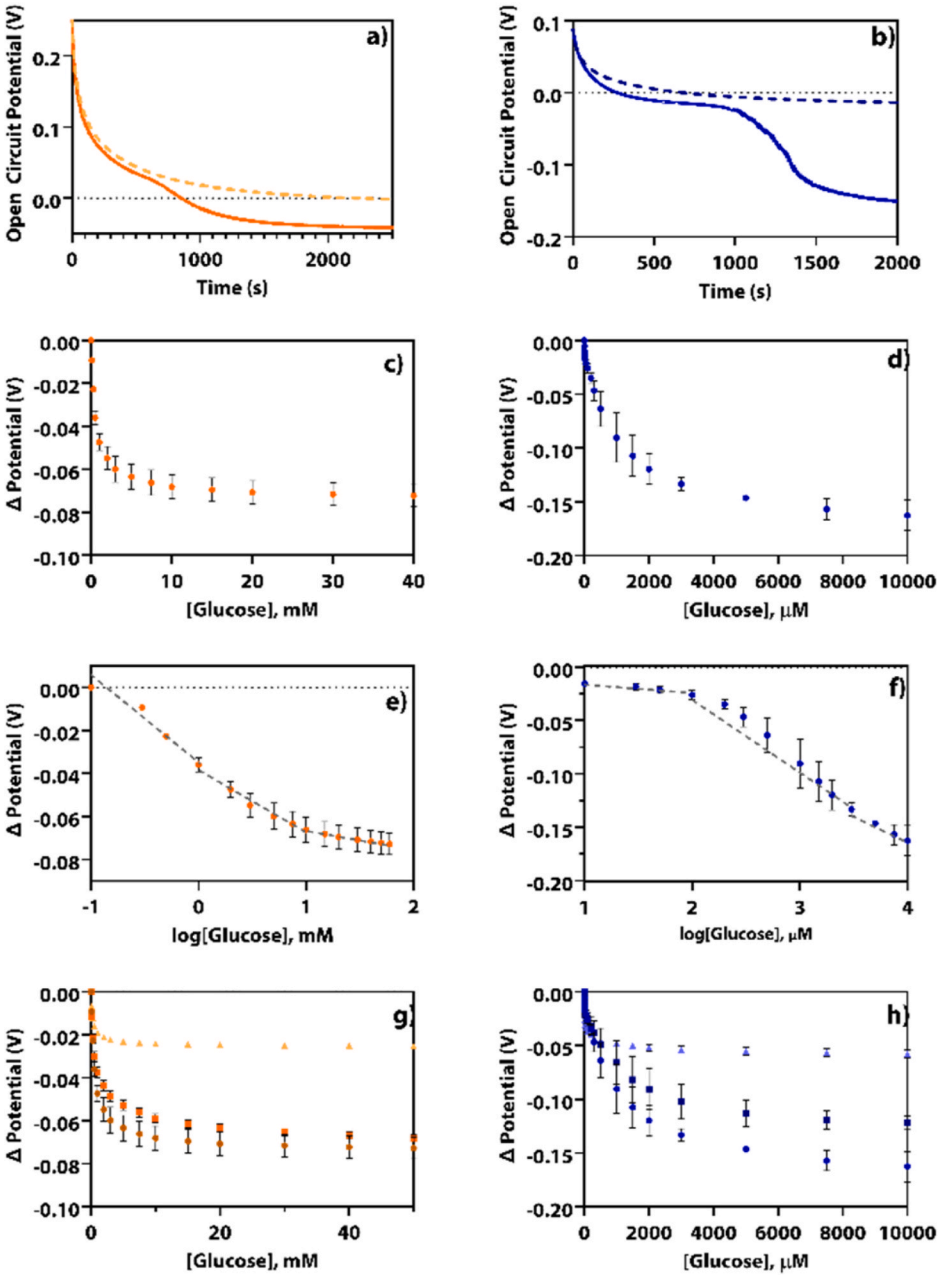

Fig. 2. The open circuit potential of a chitosan-mediator-FADGDH glassy carbon macroelectrode in (**dashed**) a blank solution of DPBS and (**solid**) as glucose is spiked into a solution of DPBS when the mediator is **a)** 1,2-NQ or **b)** GCy. Change in the open circuit potential from the initial blank potential as glucose is added to a solution of DPBS when the mediator is **c)** 1,2-NQ (n = 3) or **d)** GCy (n = 3). The semi-log plots of parts d-f when the mediator is **e)** 1,2-NQ (n = 3) or **f)** GCy (n = 3). The change in open circuit potential of three different chitosan-mediator-FADGDH glassy carbon macroelectrodes as glucose is spiked into a solution of DPBS when the mediator is **g)** 1,2-NQ or **h)** GCy.

Table 2
Figures of merit for the chitosan-mediator-FADGDH biosensors on glassy carbon macroelectrodes and gold ultramicroelectrodes.

Mediator in Biosensor	Linear Range 1 (mM)	Linear Range 2 (mM)	Linear Range 3 (mM)	Repeatability (%RSD, n = 3)	Reproducibility (%RSD, n = 3)	% Recovery
1,2-NQ	0.1–1	1–10	10–50	2.0–8.1%	42%	13% (97%, 250s)
GCy	0.01–0.1	0.1–3	3–10	5.2–20%	27%	117%
1,2-NQ (UME)	0.025–0.3	0.3–1	1–5	N/A	41%	N/A
GCy (UME)	0.001–0.03	0.03–0.3	N/A	N/A	77%	N/A

relevant range of blood glucose levels (Lee et al., 2019).

Repeatability was analyzed through repeated calibration of the same biosensor. As can be seen in Fig. 2c–d and Table 2, the repeatability of the GCy biosensor is poorer than that of the 1,2-NQ one. This is due to the poorer stability of the GCy hydrogel as discussed *vida infra*.

To evaluate the reproducibility, three glassy carbon macroelectrodes were prepared in batch for each chitosan-mediator-FADGDH biosensor and tested by spiking glucose into solutions of DPBS (Fig. 2g–h). As can be seen in this figure and Table 2, the reproducibility from sensor to sensor is low. There are several factors that could be responsible for irreproducibility from sensor to sensor, and we hypothesize that the main reason for this is the heterogeneous nature of the hydrogel. Surface coverage varies after electrodeposition, and there is likely quite a bit of heterogeneity within the chitosan-mediator solution itself due to the

nonspecific nature of glutaraldehyde crosslinking. As a result, there is a varied amount of mediator near the surface of the electrode in each biosensor and a varied amount of mediator near enough to react to the enzymes. In order to reduce these effects and further optimize these biosensors, the well-established heterogeneous nature of chitosan electrodeposition (Donahue et al., 2018) would need to be overcome through a more reproducible film formation process. Additionally, separation methods could be used to isolate the desired chitosan-mediator molecules from the chitosan-chitosan and the mediator-mediator molecules to ensure the deposition solution is as homogeneous as possible.

A double-blind study was done to test the accuracy of the two biosensors. A member of the lab made a solution of glucose in DPBS so another member could determine the concentration of glucose. Using the same procedure as when making the calibration curves, 10 μL of the solution of unknown concentration was spiked into the DPBS after the blanking period (n = 3 for each measurement). As seen in Table 1, there was a percent recovery of 13% and 117% for the 1,2-NQ and GCy biosensors, respectively. However, the 1,2-NQ biosensor responds much slower to spikes of glucose than the GCy biosensor does, due to the effectively lower k_{turn} value between the enzyme and the bound mediator. When the time period for reaction is extended to 250 s, the percent recovery of the 1,2-NQ biosensor is 97%. This observation brings up an important point about potentiometric biosensors that are being developed. When mediators are used that have slow transfer kinetics, a resistive component is introduced into the overall circuitry of the experimental setup. Thus, the time constant decay, which is the product of the total system resistance and total system capacitance, will get longer when using mediators that have slower kinetics (effectively increasing the resistance to charge transfer).

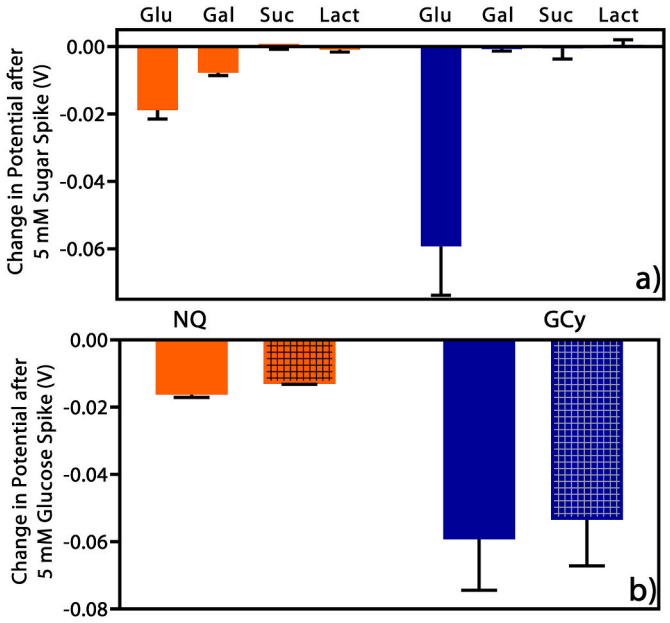

Fig. 3. **a)** The change in open circuit potential of a chitosan-mediator-FADGDH glassy carbon macroelectrode in as 5 mM of various sugars are spiked into a solution of DPBS when the mediator is **(orange)** 1,2-NQ or **(blue)** GCy (n = 3, each). **b)** The change open circuit potential of a chitosan-mediator-FADGDH glassy carbon macroelectrode in as 5 mM glucose is spiked into a solution of **(solid)** DPBS or **(checkered)** DPBS with 1.3 mM acetaminophen and 170 μM ascorbic acid when the mediator is **(orange)** 1,2-NQ or **(blue)** GCy (n = 3, each). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Analysis of biosensor selectivity

Selectivity was evaluated by spiking in 5 mM sucrose, lactose, or galactose in a solution of DPBS in place of glucose. As can be seen in Fig. 3a, the other sugars show a much lower binding affinity for the enzyme. Galactose has the highest response, being structurally almost identical to glucose, but it is still only a fraction of the response to glucose. Both the 1,2-NQ and the GCy biosensors have nearly no response to lactose or sucrose. Thus, the biosensors are both quite selective to our target metabolite.

To evaluate whether or not solution components have an effect on the measured open circuit potential of the biosensor, the potential of bare, pure chitosan hydrogels, and each of the three chitosan-mediator hydrogels was measured in solutions of DPBS, 100 mM KCl, and 100 mM KNO_3. As can be seen in Fig. S13, when only chitosan or the bare electrode are used, changing the electrolyte species or concentration (DPBS has a total salt concentration of ~150 mM), the measured open circuit potential varies greatly and is not stable. However, when any of the mediators are present within the hydrogel, the potential is much more reproducible and stable regardless of electrolyte species or concentration. This demonstrates that not only is the biosensor less affected by solution characteristics than the original chitosan-GOx biosensor was (Walker and Dick, 2021), but it provides significant evidence for the fact that the potential of the biosensors is being set by the mediators present and not by a mixed potential mechanism.

Further, to determine if electrochemically active interferents affect the measured open circuit potential of the biosensors, biologically relevant levels of ascorbic acid (170 μM) and acetaminophen (1.3 mM) (Bollella et al., 2017; Lee et al., 2019) were added to the DPBS solution before glucose was spiked in. As shown in Fig. 3b there is only a slight dampening on the 1,2-NQ biosensor's response to glucose in the presence of the interferents and no difference in the GCy biosensors response. Additionally, for both biosensors, the measured background potential was no different when the interferents were present than when they were not. This is different from the previous GOx-chitosan version of this biosensor, where electroactive interferents did change the measured background potential since they altered the mixed potential of the system. Here, as the mediator is setting the potential of the system, the interferents do not affect the measured potential, further demonstrating the power of the proposed method.

To evaluate the ability of the biosensors to operate in the presence of the natural cofactor, NAD^+, the open circuit potentiometric response of the chitosan-NQ-FADGDH and the chitosan-GCy-FADGDH biosensors to 5 mM glucose was determined in both blank DPBS and in DPBS containing biologically relevant concentrations of NAD^+. The amount of NAD^+ used was 100 μM to simulate the amount found in the cytosol of a cell (Cambronne et al., 2016). As can be seen in Fig. S17 in the Supplementary Information, the response to 5 mM glucose is unchanged when NAD^+ is added to the DPBS solution. Both the magnitude of response to glucose and the background potentials are the same in solutions of DPBS and in 100 μM NAD^+ in DPBS. These data, as well as the interferent studies above, indicate that the biosensors will be just as effective in buffer as they would be in a complex environment containing interferents, even if that interferent is the natural cofactor of the enzyme.

3.3. Analysis of biosensor stability

One chitosan-mediator-FADGDH macroelectrode was tested by repeated calibration over 28 days (Fig. 4) to analyze biosensor stability. After 28 days, the 1,2-NQ biosensor showed an improvement in its open circuit potentiometric response to glucose, maintaining 167% of the original signal on day 28. The GCy biosensor, on the other hand, lost signal quite rapidly. By day 7, the biosensor only has 60% of its original signal, and by day 28 it has 5% of its original signal. Repeated use of the biosensor led to the film visibly degrading and coming off the surface of

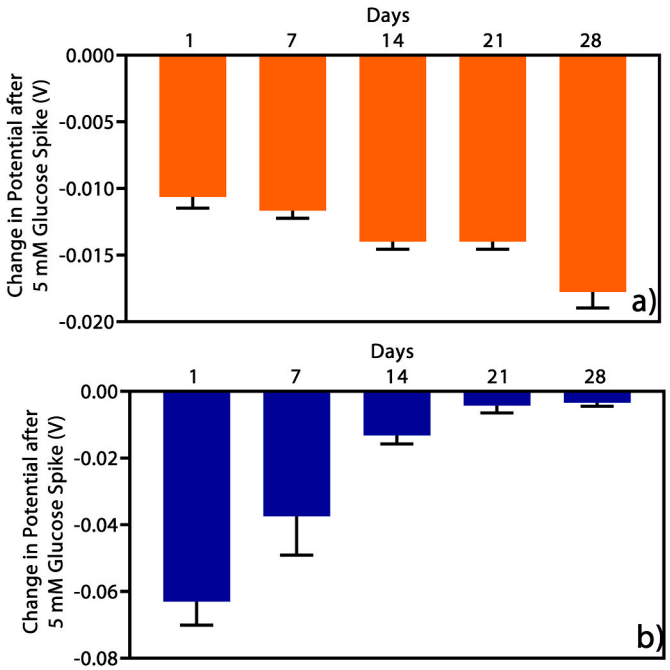

Fig. 4. The change in open circuit potential over 28 days of a chitosan-mediator-FADGDH glassy carbon macroelectrode in DPBS and as 5 mM glucose is spiked into a solution of DPBS when the mediator is **a)** 1,2-NQ (n = 5) or **b)** GCy (n = 5).

the electrode. We hypothesize this is because the mediator is sufficiently soluble in aqueous solution, thus it begins to pull the electrodeposited chitosan off the surface of the electrode over time. The 1,2-NQ hydrogel, on the other hand, has much lower solubility in aqueous solution so it does not experience this issue.

3.4. Miniaturization of the glucose biosensor to a microelectrode

Biosensor miniaturization is necessary for patient comfort and use with continuous monitoring devices. To demonstrate the ease with which an open circuit potentiometric biosensor can be miniaturized, the above hydrogels were deposited onto gold microelectrodes (r = 6.25 μm). As can be seen in Fig. 5, the chitosan-mediator-FADGDH microelectrode biosensors respond to glucose similarly to the macroelectrode versions. Like the biosensor on the macroelectrode, there are three linear regions present when the log of the glucose concentration is taken (Table 2). Of note, the GCy biosensor only has two log-linear regions and is lacking in the usual third, much less sensitive region. This is believed to be because of the instability of the film. As discussed above, the GCy hydrogels are unstable on macroelectrodes; however, on the microelectrode scale the films come off the electrode surface even quicker as they are quite small to begin with. Thus, they are not present enough to elicit a detectable response by the time additions of over 300 μM are added to the DPBS solution. However, if 5 mM glucose is added to the solution initially, there is a large response to glucose (Fig. S18), indicating that the GCy microbiosensors are functional at this concentration despite the inability to obtain a calibration curve to that concentration.

These microelectrode biosensors are more sensitive than their macroelectrode counterparts, with lower limits of detection and shorter linear ranges than their macroscale counterparts. This is hypothesized to be due to the increased diffusion of the analyte to a microelectrode surface, as well as that there are less mediator molecules on the electrode surface due to its smaller size. Thus, it takes less analyte for the enzymatic reactions to fully reduce the mediator at the surface of the microelectrode.

Notably, the earlier version of these biosensors (Walker and Dick, 2021) had both different directionality and magnitude responses to glucose at the microscale as compared to their macroscale counterparts. This is likely because the earlier version utilized glucose oxidase and its potential was controlled by a mixed potential that was altered by the enzymatic production of H_2O_2. We demonstrated then that a system whose open circuit potential is set by a mixed potential is affected by the size of the electrode, while a system whose open circuit potential is set by a redox couple is not. Since this new biosensor has an open circuit potential set by the mediator present within the hydrogel, its behavior should be independent of electrode size. We see here, that unlike the earlier version of this biosensor, the microelectrode biosensors have very similar responses to their macroelectrode counterparts in terms of both directionality and response magnitude.

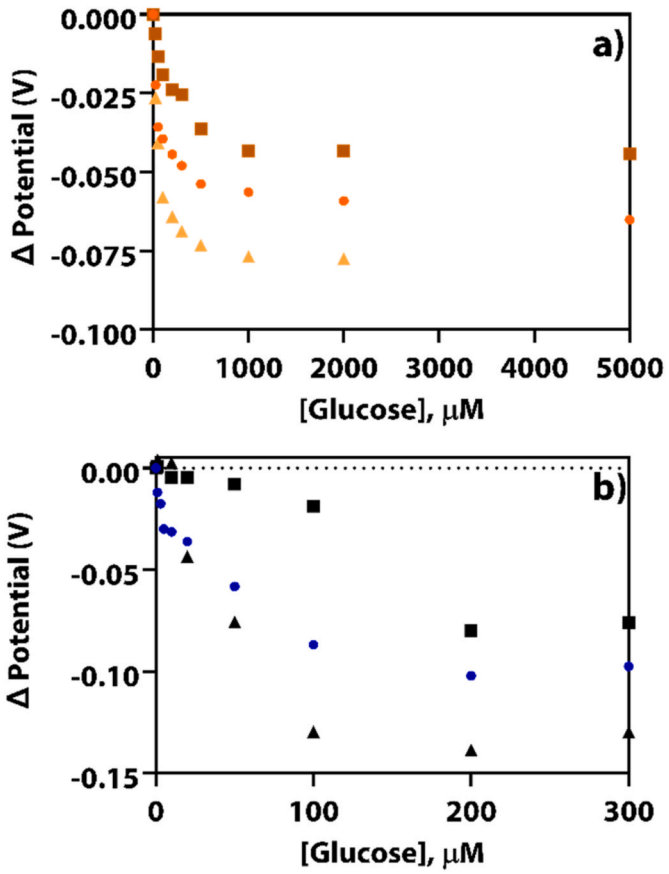

Fig. 5. The change in open circuit potential for three different chitosan-mediator-FADGDH gold microelectrodes as glucose is spiked into a solution of DPBS when the mediator is **a)** 1,2-NQ or **b)** GCy.

3.5. Demonstration of versatility using horseradish peroxidase and alcohol dehydrogenase

A biosensor for hydrogen peroxide was made by switching the FADGDH enzyme for horseradish peroxidase (HRP). This enzyme reduces H_2O_2 to H_2O, oxidizing an electron mediator in the process. While the FADGDH biosensor reduces the mediator present and thus sees a decrease in measured potential when the analyte is present, the HRP biosensor is expected to do the opposite since it is oxidizing the mediator present in the hydrogel. As the Nernst equation predicts, the presence of increasing amounts of the analyte cause more of the reduced form of the mediator initially present at the electrode surface to be converted to the oxidized form. Thus, the value of E_{OCP} is expected to increase as a result of the enzymatic reaction.

As demonstrated by the k_{turn} values in Table S2, HRP can oxidize GCy in the presence of H_2O_2. Thus, a hydrogel utilizing the same chitosan-

GCy deposition solution and conditions can be used for both the FADGDH enzyme and HRP. When deposited on glassy carbon macroelectrodes, the chitosan-GCy-HRP hydrogel measures an increased potential as H_2O_2 is added to the DPBS solution (Fig. 6a), as predicted by the Nernst equation. Without HRP, there is no response from the chitosan-GCy hydrogel as H_2O_2 is added to the DPBS (Fig. S19), indicating that it is the enzymatic reaction with H_2O_2 causing the change in potential. An example OCP trace using this biosensor and a comparison to a blank are shown in Fig. S20. Like the FADGDH biosensors, there are 3 linear regions: 30–300 μM, 300 μM to 15 mM and 15–50 mM (Fig. 6b), with an overall %RSD of 5.1%.

As demonstrated by the k_{turn} values in Table S3, ADH can reduce GCy in the presence of ethanol and NAD^+. Thus, a hydrogel utilizing the same chitosan-GCy deposition solution and conditions can be used for a variety of dehydrogenase enzymes, including ADH and FADGDH. When deposited on glassy carbon macroelectrodes, the chitosan-GCy-ADH hydrogel measures a decreased potential as NAD^+ is added to the DPBS solution containing 0.15M ethanol (Fig. 6c), as predicted by the Nernst equation. Without ADH, there is no response from the chitosan-GCy hydrogel as NAD^+ is added to the 0.15M ethanol in DPBS (Fig. S21), indicating that it is the enzymatic reaction with NAD^+ causing the decrease in potential. An example OCP trace using this biosensor and a comparison to a blank are shown in Fig. S22. As with the chitosan-GCy-FADGDH biosensors, there is poor reproducibility, with a %RSD of 24% (n = 3). There is one log-linear region from 0.25 to 10 mM NAD^+. (Fig. 6d). The response time was increased to 200 s for this biosensor, because NAD^+ is a negatively charged molecule, so its diffusion through the negatively charged Nafion membrane is slower compared to that of neutral molecules like glucose or ethanol (Karyakin et al., 1994).

The results from this study are compared to other, similar potentiometric biosensors for glucose detection in Table S4. This shows that the main advantage of this biosensor design over others is the quick and simple fabrication procedure that can be tailored to a particular enzyme and a particular log-linear range by altering the mediator bound to the chitosan. By covalently attaching the mediator to the biosensor, we minimize the potential for contaminating and altering the natural state of the system in which the measurement is being made (McCormick and Dick, 2021). Furthermore, using open circuit potentiometry minimizes perturbation of the system, while also allowing for the creation of a biosensor that has a ≥28 day lifetime and can be miniaturized easily. The main disadvantages are that there is low reproducibility between similarly-made biosensors.

4. Conclusion

In this study, we designed a simple and rapid method for screening enzyme-mediator pairs for use in biosensors, then apply those findings to design a platform for open circuit potentiometric biosensors that can be easily modified to detect various analytes at varied concentrations. The platform functions with dehydrogenase enzymes, as well as with peroxidase enzymes. When utilized with FADGDH to detect glucose, the biosensors are stable over 28 days and interferents do not affect the signal. This work continues to build a foundation for versatile metabolite sensing using oxygen-independent enzymes and open circuit potentiometry, a powerful technique that is insensitive to electrode size. This type of sensor will be necessary for the future development of implantable sensors in complex environments.

CRediT authorship contribution statement

Nicole L. Walker: Visualization, Investigation, Formal analysis, Writing – original draft. **Jeffrey E. Dick:** Conceptualization, Writing – review & editing, Funding acquisition.

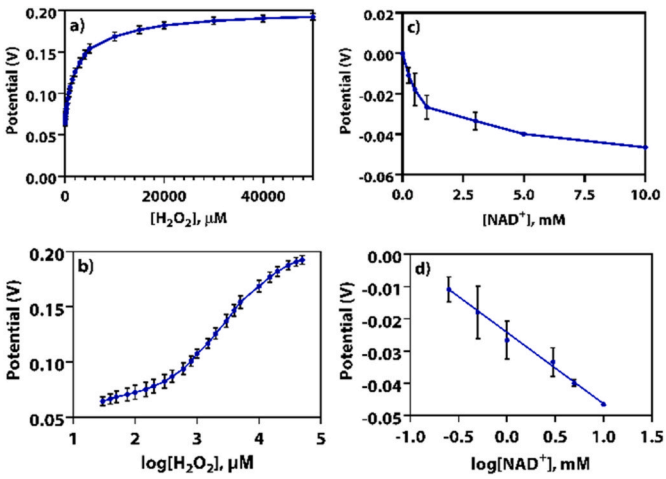

Fig. 6. a) The open circuit potential of a chitosan-GCy-HRP glassy carbon macroelectrode as 30 μM to 50 mM H_2O_2 is spiked into a solution of DPBS (n = 3). **b)** log scale of part a (n = 3). **c)** The open circuit potential of a chitosan-GCy-ADH glassy carbon macroelectrode as 0.25 mM–10 mM NAD^+ is spiked into a solution of DPBS with 0.15M ethanol (n = 3). **d)** log scale of part c (n = 3).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2021.113888.

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