Identifying Key Properties that Drive Redox Mediator Activity in *Lactiplantibacillus plantarum*

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ABSTRACT: *Lactiplantibacillus plantarum* is known to utilize exogenous small molecule quinone mediators to perform extracellular electron transfer (EET), which allows it to produce a detectable current in a bioelectrochemical system (BES). Utilization of quinone mediators by *L. plantarum* requires a type-II NADH dehydrogenase (Ndh2), however small structural variations in the core of 1,4-naphthoquinone EET mediators yields significantly different current outputs. Herein, we assembled a library of 30 naphthoquinone-based EET mediators in order to probe the important physicochemical properties and biochemical interactions that are responsible for Ndh2-dependent EET in *L. plantarum*. The library was designed with inspiration from naturally-occurring metabolites, and was assembled focused on structural modifications that diversified polarity (cLogP), reduction potential (E^o), and Ndh2 binding affinity as these properties are hypothesized to be driving EET activity. In general, Ndh2-dependent EET activity in an iron(III) oxide nanoparticle assay significantly correlates to the mediator's polarity and binding affinity. Five mediators were analyzed in BESs with *L. plantarum* and each generated Ndh2-dependent current with low background signal. Importantly, the amine containing mediators yielded incredibly stable current output over the course of the experiment (up to 5 days). These findings increase our understanding of structure-activity relationships for quinone-mediated EET and provide mediators for bioelectronic sensing.

KEYWORDS: extracellular electron transfer, 1,4-naphthoquinone, biosensing, mediator

INTRODUCTION:

All domains of life depend on redox chemistry to generate energy through respiration or fermentation.[1] Electrons flow from electron donors to various terminal electron acceptors (TEAs) through redox active biomolecules. Oxygen is the most common TEA and is used by all aerobic organisms; however, many microorganisms have evolved to utilize insoluble TEAs, such as metal oxides.[2,3] These molecules include proteins (cytochromes, oxidoreductases) and small molecules (e.g., quinones, flavins, phenazines) and all have the potential to change oxidation states under biological conditions.[4] Utilization of insoluble TEAs requires passing electrons through cellular membranes and is commonly referred to as extracellular electron transfer (EET).[3,5] There are three main mechanisms that are used to accomplish EET: 1) direct contact of redox proteins located in the cell envelope with the TEA, 2) secretion of cytochrome containing nanowires or outer membrane vesicles, or 3) use of small molecule redox mediators, also known as extracellular electron shuttles.[6] While many small molecule mediators have been identified, very little is known about the physicochemical properties driving their EET capabilities.

In addition to utilization of natural TEAs, many organisms that perform EET can also use an anode within a bioelectrical system (BES) as a TEA, thereby generating a current. The detectable current is an electrical feature that has been leveraged for biosensing applications as it can report on a microorganism's local environment.[7–10] For example, engineered bioelectronic signaling systems have successfully been designed to sense biologically-relevant small molecules, including pyocyanin[11] and riboflavin.[12] Also, an engineered two microbe system was designed to detect and degrade organophosphate pesticides, where a modified *Shewanella oneidensis* detects degradation products of the pesticides that are produced by *Escherichia coli*.[13] However, the readout is slow (requires translation), insensitive, and lacks the necessary selectivity.[14] Alternatively, *E. coli* has been engineered so that an analyte triggers a conformational change in one of the proteins in an EET pathway, allowing an endocrine

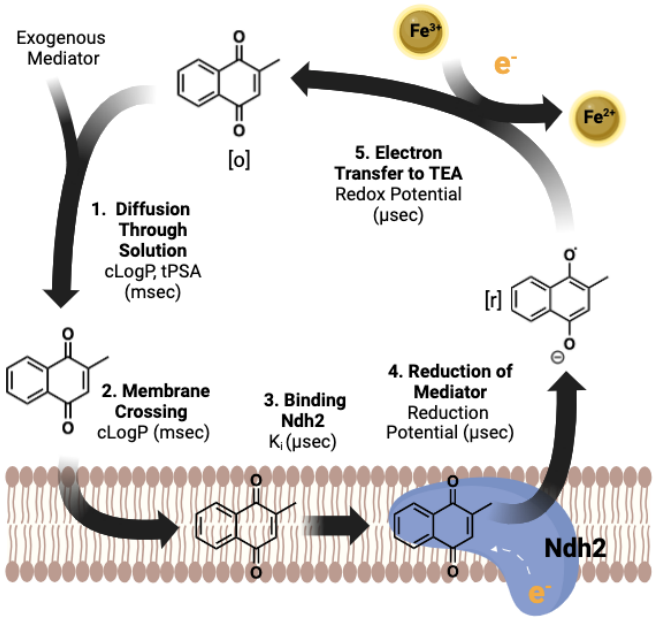


Figure 1. A diagram of the process an EET mediator must successfully complete. **(Step 1)** Diffusion through aqueous environment (msec), **(step 2**) enter the cellular membrane (msec), **(step 3)** bind to Ndh2 (µsec), **(steps 4 and 5**) have compatible reduction potentials to accept electrons from Ndh2 (µsec - msec) and pass them to a TEA (µsec). Created with Biorender.com (Agreement number: YB26T2V617)

disruptor to be detected in minutes.[15] However, these sensors rely on bespoke engineered electron transfer pathways and proteins that must be re-engineered for each analyte. Advancing our understanding of mediated EET has the potential to overcome some of these challenges.

Lactiplantibacillus plantarum, a commensal lactic acid bacterium, is capable of performing mediated EET using flavins and quinones as small molecule mediators, together with a highly conserved FLEET gene locus that encodes a type-II NADH dehydrogenase (Ndh2), flavin transport proteins, membrane demethylmenaquinone (DMK) synthesis proteins (DmkA, EetA, and EetB), flavin mononucleotide (FMN) cofactor (PlpA).[16–18] Recent studies by our group showed that Ndh2 is required for utilization of quinone mediators.[19] Although *L. plantarum* has defined pathways that utilize quinones, it is incapable of producing quinones and must depend on acquiring them exogenously from its environment.[20,21] Quinones are known to be abundant in many environmental niches, with high concentrations present in human gut.[22,23] However, *L. plantarum* can convert exogenously acquired DHNA (1,4-dihydroxy-2-naphthoic acid), an intermediate in the biosynthesis of menaquinone (vitamin K), to demethylmenaquinone through prenylation by DmkA.[16] *L. plantarum* uses a variety of exogenous quinone mediators, though very little is known about the specificity for particular mediators. A small study that evaluated the Ndh2-dependent EET of six commercially available mediators indicated that reduction potential and binding affinity were the primary drivers of EET activity; however, that study was limited in scope and only evaluated structurally related 1,4-naphthoquinones as mediators.[17]

For a mediator to be EET viable, it must possess certain physicochemical properties and favorable biological interactions. In addition to binding favorably to Ndh2, mediators must have a favorable reduction potential to be reduced by Ndh2 and oxidized by a given TEA, be lipophilic enough to diffuse through a lipid bilayer, yet have a diffusion coefficient that supports diffusion through aqueous environments to an insoluble TEA (**Fig. 1**).[17] However, a mediator's EET activity is unlikely to be equally dependent on each of these parameters as the timescale of each varies. Of the steps that a mediator must accomplish, the electron transfer between the mediator and Ndh2 or TEA is known to occur on the micro- to milli-second timescale and just requires an acceptable reduction potential to facilitate the transfer of electrons.[24] Meaning, the mediator must simply have a reduction potential between that of Ndh2 and the TEA. Flavin adenine dinucleotide is a cofactor within Ndh2 and its reduction potential is -0.22 eV[24], while the reduction potential of common insoluble TEAs is significantly higher (0.3 - 1 eV).[25,26] We hypothesize that other physicochemical properties of the mediator, such as diffusion coefficient (D_{water}), lipophilicity (cLogP), and total polarization area (tPSA), have a larger impact on EET activity as they drive slower, potentially rate-limiting steps. Passive transport through a membrane and diffusion through an aqueous environment occur on the timescale of milliseconds for compounds with favorable properties and even slower for compounds with more unfavorable properties.[27–29] This is up to an order of magnitude slower than the transferring of electrons and thus we hypothesize these properties are likely very important for EET activity.

Herein, we aim to better understand the chemical properties most important to mediating Ndh2-dependent EET in *L. plantarum*. A library of 1,4-naphthoquinones was designed to probe diverse chemical properties, with a particular focus on polarity and lipophilicity. The mediator library was inspired by natural products that have previously exhibited shuttling properties. The known electron shuttles includes those that are known to mediate Ndh2-dependent EET in *L. plantarum* [e.g., DHNA (**1**), menadione (**2**), naphthoquinone (**3**), juglone (**4**), and

lawsone (**5**)],[16,17] promote EET in *S. oneidensis* [ACNQ (**6**)],[30] and herbal plant natural products that were shown to bypass Complex I defects in humans though shuttling electrons [e.g., chimaphilins (**7** and **8**) and lapachones (**9** - **11**)].[31] Our library of 1,4-naphthoquinones was assembled using semi-synthesis to derivatize three scaffolds (e.g., **2**, **3**, and **7**), isolated from natural sources, or acquired commercially. Examining the relationship between Ndh2-dependent EET activity with physicochemical and biochemical properties of the mediators revealed that binding affinity and polarity significantly correlate to increased EET activity. Analysis of a subset of the best performing mediators in a BES confirmed that our mediators are capable of performing Ndh2-dependent EET using a carbon felt anode as the TEA, thereby producing a stable current for duration of the experiment (up to 5 days).

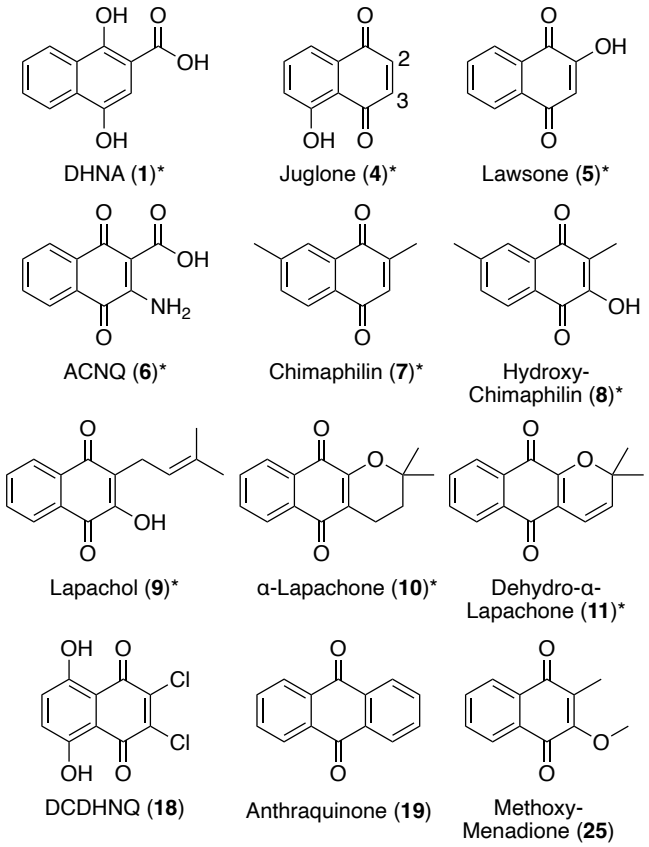


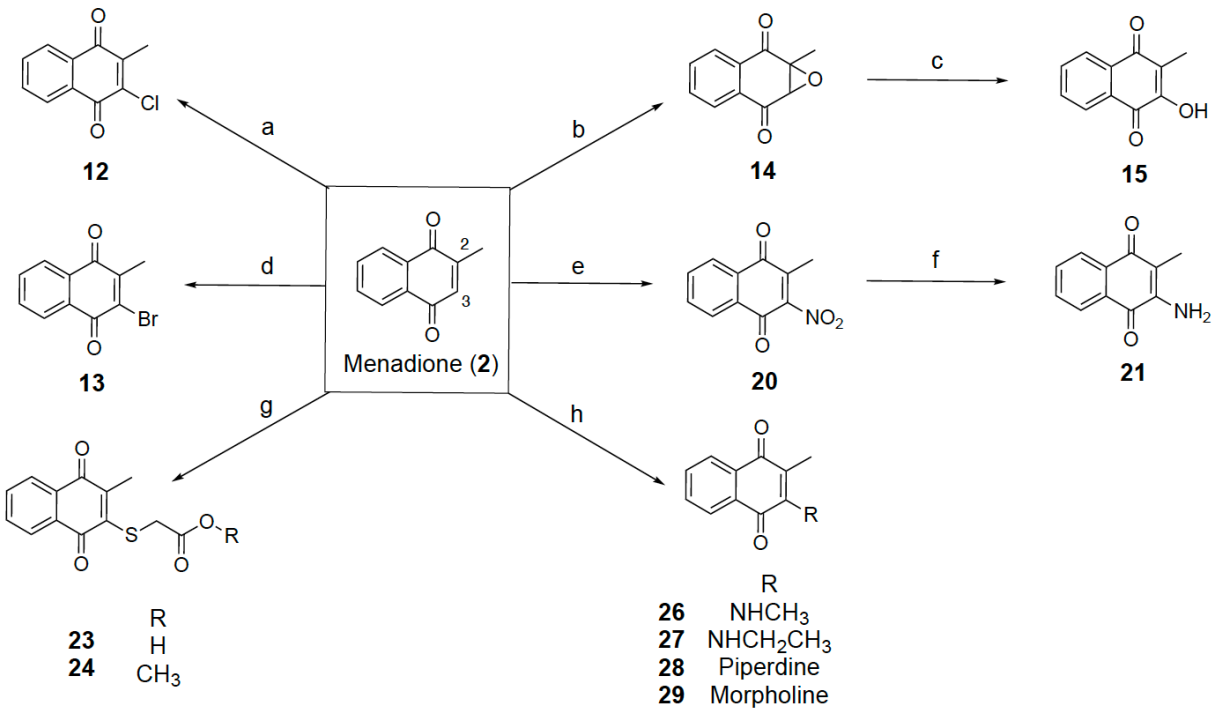
Figure 2. 1,4-Naphthoquinone mediators. Naturally-occurring mediators are indicated with an asterisk.

RESULTS AND DISCUSSION

To begin to probe the importance of physicochemical properties and biochemical interactions on Ndh2-dependent EET in *L. plantarum*, a library of 1,4-naphthoquinones (**1** - **13** and **15** - **31**) was designed by drawing inspiration from natural small molecules with known electron shuttling properties, including those that are known to mediate Ndh2-dependent EET in *L. plantarum*,[16,17] promote EET in *S. oneidensis*,[30] and bypass Complex I defects in humans though shuttling electrons.[31] Several quinones were obtained through commercial or natural sources (**Fig. 2**). Compounds **7** and **8** were isolated from lyophilized *Chimaphila umbellata* bark (**Fig. S1** - **S4**). Lapachol (**9**), α-lapachone (**10**), and dehydro-α-lapachone (**11**) are produced by the plant, Pau D'Arco, but the purification was challenging so they were purchased for this study. Finally, five commercially available naphthoquinones were purchased (**1**, **4**, **5**, **18**, and **19**). The remaining quinones were obtained through semi-synthesis (**Fig. S5** - **S40**), where we focused efforts on adding functionality to the unoccupied 3-position of **2** and **7** or the unoccupied 2- and 3-positions of the **3**, as these positions are in conjugation with the quinone functionality and are therefore hypothesized to have the largest impact on EET activity. Compounds **2** and **7** are commercially available and large quantities of **7** were obtained by isolation from freeze-dried plant bark (see experimental details).

The first phase of the mediator library was focused on diversifying the polarity of the mediators (cLogP) by installing modifications that are commonly found in nature, i.e, halogenations and hydroxylation. Using **2** and **3** as initial scaffolds, a variety of halogenated and hydroxylated analogs were synthesized using one or two step reactions (**Schemes 1** and **2**). First, the unoccupied 3-position was chlorinated by treatment with *N*-chlorosuccinimide and copper(II) chloride to yield chloro-menadione (**12**) in decent yields (53%).[32] Bromination of the same position involved treatment of **3** with bromine, sodium acetate, and acetic acid to yield bromo-menadione (**13**) in moderate yields (71%).[33] Hydroxylation of **2** was accomplished in two steps. First treatment of **2** with hydrogen peroxide (30%) in sodium carbonate generated 2,3-epoxide-menadione (**14**), which was subsequently treated with sulfuric acid to yield hydroxy-menadione (**15**) in an overall yield of 62%.[34] Dichlorination of **5** was accomplished through treatment with thionyl chloride and pyridine to yield dichloro-naphthoquinone (**16**) in relatively poor yields (21%) and dibromination of **3** involved using similar conditions as the bromination of **2** but needed to be heated to produce dibromo-naphthoquinone (**17**) in decent yields (45%).[35] Overall, the majority of the halogenation reactions proceeded very slowly and starting material was still observed when the reactions were quenched after 3 days.

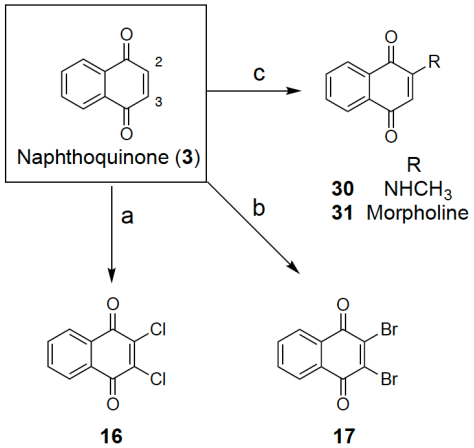
Many chemical properties of the mediators are easily obtained using available software or online tools,



Scheme 1. a) Copper (II) chloride, *N*-chlorosuccinimide, ACN, 80 °C, 72 h, 53% b) H_2O_2, Na_2CO_3, 0 °C, 0.5 h, 82%; **c)** H_2SO_4/H_2O, 5 min, rt, 62% d) Br_2, NaOAc, HOAc, rt, 72 h, 71%. e) H_2SO_4/HNO_3, H_2O, 60 °C, 40 min, 80 °C, 5 min, 24% f) 10% w/v Pd/C, H_2 (g), EtOAc, 30 °C, 6 h, 99% **c)** thioglycolic acid/methyl thioglycolate, MeOH, 25 °C, 24 h 18-21% h) $HClO_4/SiO_2$, amine, rt, sonicated, 20 min, 20 - 44%

such as ChemDraw and MolGpka,[36] which were used to determine cLogP, tPSA, and pKa (**Table 1** and **S2**). However, calculating a compound's redox potential is considerably more challenging. Previous literature established that there is a linear relationship between the energy difference of the ground state quinone and radical anion, and experimentally determined one electron ($1e^-$) reduction potentials (E°).[37] Therefore, we used Density Functional Theory (DFT) to calculate this energy on all mediators and the $1e^-$ reduction potential was experimentally measured for a subset (18 mediators) of the library (**Table S3**). Graphing these values against one another yielded a line of best fit ($R^2 = 0.82$) that was subsequently used to calculate the reduction potential for the remaining mediators (**Fig. S41 - S50**). All reduction potentials were calculated to fall between -1.45 - (-0.74) eV, thereby indicating they are capable of promoting Ndh2-dependent EET (**Table 1**). This method allowed us to calculate the reduction potential of **1**, which posed a challenge experimentally due to the inability to obtain the molecule in the quinone form. In addition, we were also able to ensure reduction potentials of the mediators were in the acceptable range prior to synthesis in future phases.

Ndh2-dependent EET activity in *L. plantarum* was quantified using an established iron(III) oxide nanoparticle reduction assay, which uses ferrozine to quantify the amount of Fe^{3+} that is converted to Fe^{2+} by *L. plantarum*.[17–19] Absent of any exogenous mediator, no Fe^{2+} is observed. Although *L. plantarum* is incapable of producing mediator, the bacterium does have the ability to metabolize these compounds, specifically DmkA has the ability to prenylate open 2- or 3-positions of naphthoquinone analogs to produce dimethyl-



Scheme 2. a) Pyridine, benzene, 80 °C, 4 h, 21% **b)** Br_2, glacial acetic acid, 110 °C, 3 h, 45% **c)** perchloric acid/SiO_2, amine, rt, sonicated, 20 min, 38 - 57%.

Table 1. Ndh2-dependent EET activity, physicochemical properties, and biochemical interactions for mediator library

	Compound	Ndh2 Dependent EET (Norm. to **15**)	cLogP (phys. pH)[a]	E^o (eV)	K_i (kcal/mol)
Phase 1	DHNA (**1**)	-1.9 ± 1.2	1.24 (-1.55)	-0.74	-5.4
	Menadione (**2**)	-0.078 ± 1.2	2.17	-1.10	-5.2
	Naphthoquinone (**3**)	-1.09 ± 0.28	1.65	-1.01	-5.1
	Juglone (**4**)	-6.8 ± 6.0	1.45 (-1.93)	-0.90	-5.0
	Lawsone (**5**)	-4.90 ± 11.1	1.41 (-2.16)	-1.12	-5.6
	Chimaphilin (**7**)	0.39 ± 1.64	2.67	-1.15	-4.7
	Hydroxy-chimaphilin (**8**)	32.0 ± 6.1	2.42 (-1.60)	-1.18	-4.9
	Chloro-menadione (**12**)	3.47 ± 0.60	2.94	-1.01	-3.6
	Bromo-menadione (**13**)	19.0 ± 2.2	3.03	-0.99	-3.4
	Hydroxy-menadione (**15**)	32.0 ± 6.1	1.92 (-2.10)	-1.45	-5.0
	Dichloro-naphthoquinone (**16**)	26.0 ± 1.8	3.20	-0.79	-5.1
	Dibromo-naphthoquinone (**17**)	14.1 ± 1.4	3.38	-0.78	-2.6
	DCDHNQ (**18**)	-6.56 ± 6.3	2.78 (-0.59)	-0.66	-3.1
	Anthraquinone (**19**)	2.90 ± 0.79	3.18	-1.35	-4.1
Phase 2	ACNQ (**6**)	65.3 ± 6.9	0.89 (-3.16)	-1.13	-4.2
	Lapachol (**9**)	37.4 ± 4.3	3.43 (-0.59)	-1.24	-5.2
	α-Lapachone (**10**)	5.87 ± 3.1	2.87	-1.31	-2.2
	Dehydro-α-lapachone (**11**)	-7.16 ± 7.9	2.65	-1.17	-2.5
	Nitro-menadione (**20**)	23.4 ± 3.9	0.39	-0.58	-2.5
	Amine-menadione (**21**)	21.0 ± 1.3	2.22 (1.22)	-1.37	-4.8
	Amine-chimaphilin (**22**)	17.4 ± 2.2	2.72 (1.72)	-1.41	-4.6
	Thioglycolic acid-menadione (**23**)	-3.4 ± 2.3	1.65 (-1.26)	-1.06	-3.8
	Methyl thioglycolate-menadione (**24**)	0.091 ± 0.79	1.96	-1.07	-3.8
	Methoxy-menadione (**25**)	0.023 ± 1.2	2.02	-1.19	-3.8
Phase 3	Methylamine-menadione (**26**)	32.3 ± 6.2	2.26 (1.98)	-1.38	-4.0
	Ethylamine-menadione (**27**)	18.9 ± 0.69	2.79 (2.51)	-1.38	-4.1
	Piperidine-menadione (**28**)	14.0 ± 0.55	3.70 (4.02)	-1.35	-3.8
	Morpholine-menadione (**29**)	9.44 ± 0.43	2.20 (1.97)	-1.29	-3.6
	Methylamine-naphthoquinone (**30**)	41.3 ± 7.4	1.74 (1.46)	-1.39	-5.2
	Morpholine-naphthoquinone (**31**)	9.30 ± 1.2	1.68 (1.45)	-1.27	-4.7

menaquinone analogs.[16] So, to ensure the observed EET is mediated by Ndh2, we compared the EET activity between two previously developed mutant strains of *L. plantarum* (*L. plantarum* Δ*dmkA*Δ*ndh1* and *L. plantarum* Δ*dmkA*Δ*ndh1/2*).[17] Removal of the genes *dmkA* and *ndh1* eliminates EET side-reactions that may consume the mediator, ensuring EET depends primarily on the interaction between Ndh2 and the mediator. All mediators were evaluated for EET activity at 5 μM and counter screened for toxicity at 10-times this concentration (**Fig. 3A**, **S51** and **S52)**. In addition, their stability under assay conditions was evaluated (**Fig. S53**). Overall, just under half of the mediators from the first phase were able to mediate Ndh2-dependent EET but to varying levels. Top performers were mediators with hydroxyls in the 3-position (**8** and **15**), while those with an open 2- or 3-position (**1** - **5**) exhibited little to no Ndh2-dependent EET. Previous literature has suggested that quinones with open 2- or 3-positions are more unstable, in particular, it is known that **1** quickly degrades in complex media, and this may be attributing to the lack of observed activity.[30]

A second phase of the library was designed to continue to probe the impact of polarity on EET and this included synthesizing naphthoquinones with nitro [nitro-menadione (**20**)], amines [amine-menadione (**21**) and amine-chimaphilin (**22**)], thioether [thioglycolic acid-menadione (**23**), and thioglycolic methyl ester-menadione (**24**)], and carboxylic acid functionality, while also eliminating the hydrogen bond donor in **15** through methylation [methoxy-menadione (**25**)]. Many of these compounds are significantly more polar than the previous mediators, and although these modifications also increase the range of $1e^-$ reduction

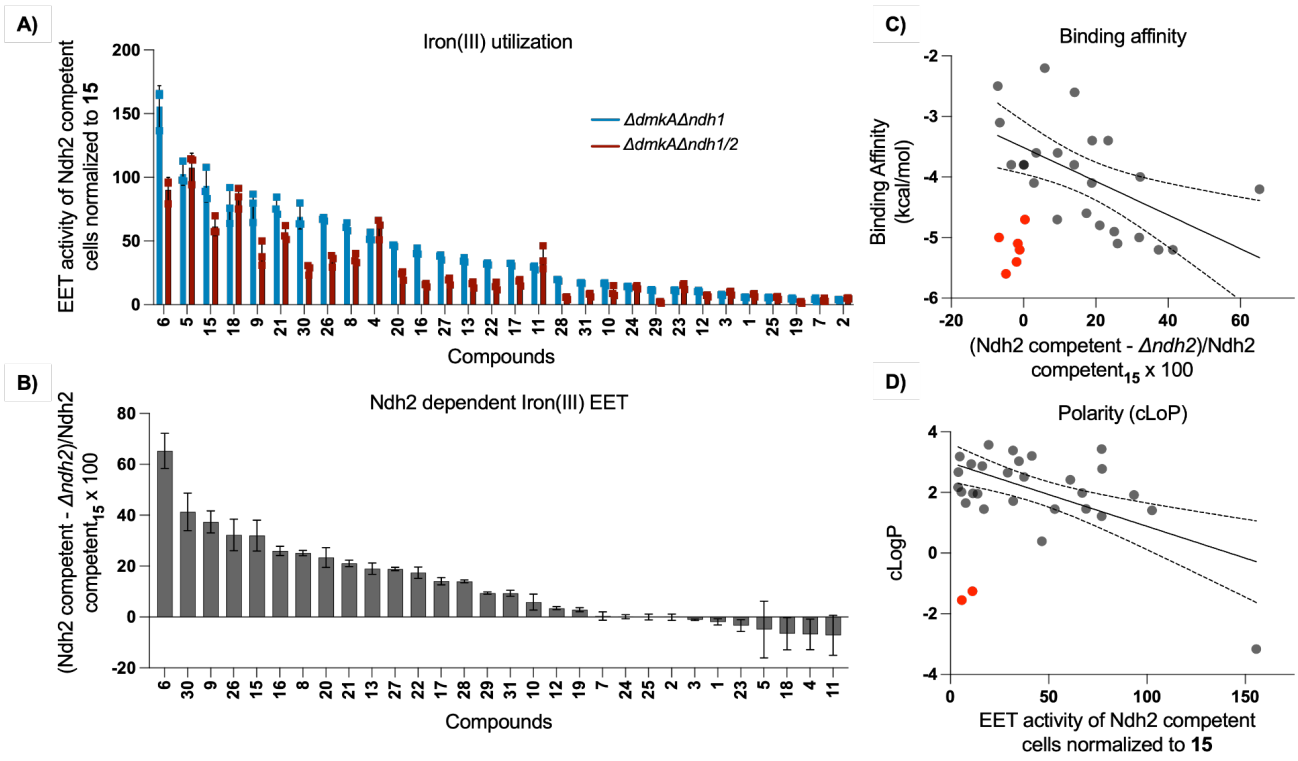


Figure 3. Ndh2-dependent EET activity of the library of mediators and the correlation to physicochemical properties and biochemical interactions. **A**) EET activity in iron(III) nanoparticle assay using Ndh2-competent (black bars) and Ndh2 deficient (gray) *L. plantarum* strains. Absorbance measurements were normalized to the activity of **15** with Ndh2 competent cells, **B**) Ndh2-dependent EET for each mediator, which was calculated by subtracting the activity in the Ndh2-competent by Ndh2-deficient cells in **A**, **C**) Correlation of Ndh2-dependent EET (data in **B**) to Ndh2 binding affinity for each mediator, excluding outliers (**1** - **5**, and **7**) ($R^2 = 0.31$, $p = 0.0047$), and **D**) Correlation of EET activity by the Ndh2-component strain compared to cLogP for each mediator, excluding outliers (**1** and **23**) ($R^2 = 0.36$, $p = 0.0008$). Error bars in A and B represent the standard deviation of the biological triplicates. The linear best fit lines in **C** and **D** were calculated using a simple linear regression model in GraphPad. Dotted lines in both represent the 95% confidence bands. Both showed significant non-zero slopes.

potentials (e.g. **20**: –0.58 eV, and **21**: -1.37 eV), all should still possess favorable properties. First, introduction of the nitro functionality involved treatment of **2** with nitric acid (*aq*) in concentrated sulfuric acid.[38] The orange crude product was recrystallized in ethanol to yield **20** in low yields (24%). Uncrystallized product was observed in the filtrate but was discarded rather than conducting biphasic extractions on concentrated nitric acid. Pure **20** was subsequently reduced with 10% Pd/C and H_2 (g) in quantitative yields to produce compound **21**.[39] The same two reactions were carried out on **7**, however the reaction was done on a smaller scale and necessitated a biphasic extraction to obtain the nitro-chimaphilin. The crude reaction mixture was then treated with 10% Pd/C and H_2 (g) to form **22** in low overall yields (12%). The thioglycolic-containing compounds were synthesized by treating **2** with either thioglycolic acid or thioglycolic methyl ester in methanol resulting in **23** and **24**, respectively, in low overall yields (18 - 21%).[39,40] In addition, compound **6** was obtained through derivatizing **2** with ammonium chloride following previously reported methods.[30] Finally, several attempts were made to synthesize **25** from **15** using methyl iodide and silver (I) oxide, however it yielded a mixture of inseparable products - **25** and 4-methoxynaphthalene-1,2-dione.[41] Therefore, **26** was obtained from a commercial source and no other attempts were made to derivatize the hydroxyl position in **15**.

Screening the new mediators for EET activity confirmed that polarity of the mediator appears to improve overall EET activity (**Fig. 3A** and **Table 1**). Specifically, having a free hydrogen bond donor in either the 2- or 3-position appears to improve overall activity, where **9** and **15** were more active than their

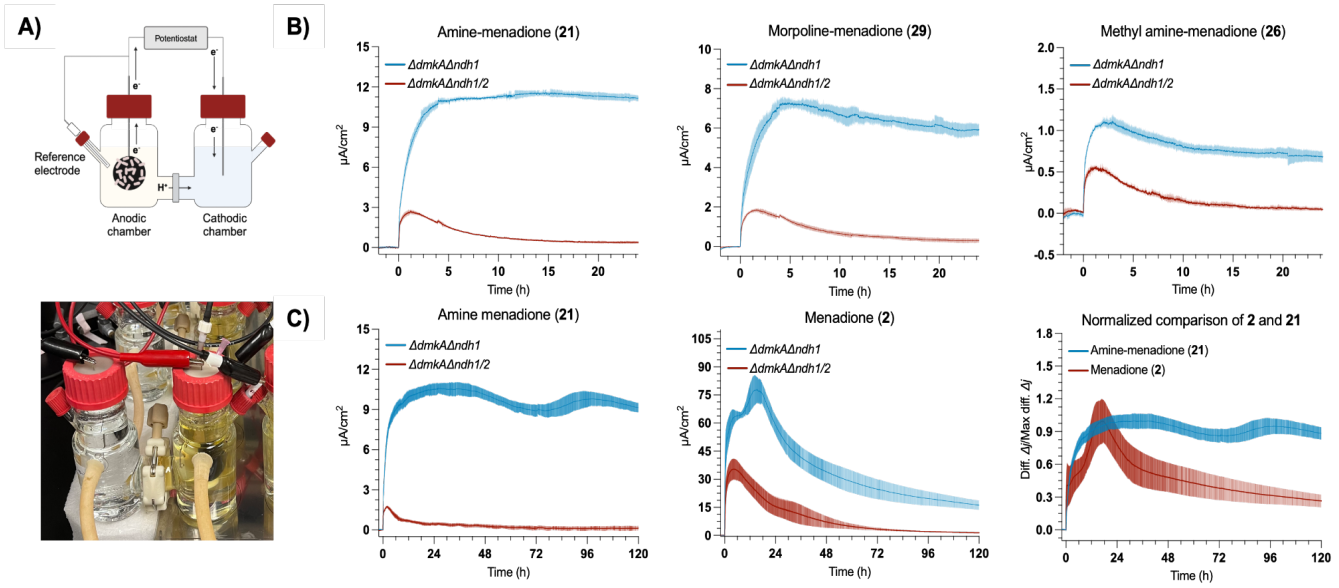


Figure 4. Ndh2-mediated activity of amine modified compounds **21**, **26**, and **29** in BES experiments. **A**) Schematic depicting electron flow in a two-chambered three-electrode BES. Bacterial cells (pink) transfer electrons to the working electrode (black circle). Electrons flow through the circuit to the cathode, enabling current measurements to be taken by a potentiostat. The accompanying image shows a BES reactor during an experiment. **B**) Current density generated by Ndh2-competent (blue line) and Ndh2-deficient (red line) *L. plantarum* in the presence of **21**, **26**, and **29** in a BES over 24 h (Created with Biorender.com; Agreement number: JO26TB46BX). **C**) Current density generated by Ndh2-competent (blue line) and Ndh2-deficient (red line) *L. plantarum* in the presence of Ndh2-competent *L. plantarum* over 120 h in the presence of **21** and **2**. Current density for both **21** and **2** normalized to the peak current density. Normalized data was calculated by dividing each mediator's current density attributed to Ndh2 by their respective peak current densities (see experimental details). All BES data are representative of multiple experiments and error bars show 1 standard deviation for n=3 biological replicates at a final concentration of 1 µM mediator.

analogous ether analogs, **10** and **25**, respectively. This difference in activity cannot be explained by other chemical properties, such as reduction potential or polarity as they all have comparable reduction potentials [-1.19 - (-1.46)] and cLogP properties. In addition, no distinguishable differences in EET activity were observed between types of hydrogen bond donors as mediators with either hydroxyl or amine groups promoting Ndh2-dependent EET. The only exception to this trend was the EET properties of **23** and **24**, however stability studies suggest both are prone to degradation through oxidation of the sulfur to the sulfoxide and subsequent sulfone (**Fig. S53**). We hypothesize this result is due to one or a combination of the following factors: 1) the ability of the mediator to change state under different physiological conditions, i.e., uncharged in a lipid membrane and ionic in aqueous environments, and/or 2) potential favorable interactions in the active site.

A third phase of quinones were designed to further probe the promising activity of **21** and included both secondary and tertiary amine analogs. Following previous literature precedent, primary (i.e., methyl and ethyl amine) and secondary amines (i.e., piperidine and morpholine) were added to both **2** and **3** through a perchloric acid catalyzed reaction.[42] This yielded methylamine-menadione (**26**), ethylamine-menadione (**27**), piperidine-menadione (**28**), morpholine-menadione (**29**), methylamine-naphthoquinone (**30**) and morpholine-naphthoquinone (**31**) in moderate yields (20 - 57%). Evaluation of these compounds in the iron nanoparticle reduction assay revealed that all were capable of mediating Ndh2-dependent EET, with the primary and secondary amines all outperforming the tertiary amines (**Table 1**).

Evaluation of the entire library of mediators for EET in the nanoparticle reduction assay provided insights into important chemical properties that drive Ndh2-dependent EET. Over half of the mediators were capable of performing Ndh2-mediated EET, but to varying degrees (**Fig. 3A** and **3B**) and this activity is most correlative to cLogP values and binding affinity

(**Fig. 3C** and **3D**). There appears to be no significant linear relationship between activity profile and reduction potential or tPSA (**Fig. S54**). In general, the amine- and hydroxyl-substituted mediators exhibited the best EET properties, which supports the correlation between polarity and activity profile. However, there were seven exceptions to this trend. Four mediators (**4**, **5**, **11**, and **18**) exhibited strong EET profiles but were not selective to Ndh2 as was evident by the generation of high amounts of Fe^{2+} in both strains of *L. plantarum*, including the Ndh2 deficient strain (**Fig. S55**). This can be explained partially by low binding affinity by **11** (-2.5) and **18** (-3.1), while both **4** and **5** are believed to have stability issues while performing EET. The other three mediators that we would have predicted to have strong Ndh2-dependent EET but were inactive (**1**, **23**, and **24**) all exhibited stability issues, and this likely impacted their activity profiles. This is supported by previous literature showing that **1** degrades within hours in complex media, likely due to the reactivity of the unoccupied 3-position on the naphthoquinone.[30] Therefore, design of future mediators should include functionality at both the reactive sites (2- and 3-, respective of the quinone) with one site possessing a hydrogen bond acceptor, maintain a cLogP below 2, have a favorable binding affinity (below -4) and possess a reduction potential between -0.22 eV and the desired TEA.

Ndh2 binding affinity of the mediators was strongly correlated to activity (**Fig. 3C**) with a significant ($p = 0.0047$) non-zero slope, however there were six noticeable outliers - compounds **1** - **5** and **7**. As mentioned above, three of these outliers have stability issues that make their activity profiles unreliable. However, the other three compounds are all of our core structures that were used for derivatization - **2**, **3**, and **7**. This result was not entirely unexpected as it was previously reported that both **2** and **3** do not promote Ndh2-dependent EET when the TEA is iron(III) nanoparticles but can mediate EET when the TEA is an electrode within a BES.[17] Thus, it seems likely that **2**, **3**, and **7** may be unable to interact efficiently with iron(III) nanoparticles for unknown reasons.

Since screening mediators via iron(III) reduction is much higher throughput than BES experiments, we used the iron reduction data to select lead compounds. We selected five structurally distinct mediators for a more thorough analysis in a BES to gain a better understanding of their potential use in biosensing applications. This included three amine analogs (**21**, **27**, and **30**), and two hydroxyl analogs (**8** and **15**; **Fig. S56**). We performed BES experiments following previously published protocols (**Fig. 4A**),[17,43] evaluating the current generation from each mediator in the presence of the same two *L. plantarum* strains previously described in biological triplicates. All five mediators enabled Ndh2-dependent EET; however, the hydroxyl analogs (**8** and **15**) produced limited signal above the background current generated by the Ndh2-deficient strain (**Fig. S57**). In contrast, the amine analogs (**21**, **27**, and **28**) produced steady-state current in the Ndh2-competent strain that was significantly greater than the background signal and the background signal from the Ndh2-deficient strain was very low, settling to a Δj value close to 0 $\mu A/cm^2$ within 8 to 12 h (**Fig. 4B**). . Moreover, the temporal evolution of current across the three analogs was qualitatively similar, reaching the peak within ~4 h and remaining near that peak level for the duration of the experiment. These data show that these amine analogs stably mediate Ndh2-dependent EET over 24 h, strongly suggesting they could effectively mediate signals for bioelectronic sensing.

Since **21** produced the strongest signal in the Ndh2-competent strain, we conducted additional experiments to investigate this mediator's performance in a BES over longer time scales. Indeed, **21** stably mediated EET with Ndh2 for 5 days (120 h), yielding incredibly stable, predictable current output over the full experiment (**Fig. 4C**). We conducted these experiments in comparison against the EET performance of **2**, previously characterized to be a strong natural substrate for Ndh2 in BES experiments.[17] While **2** produced a much greater current magnitude than **21**, **2** also produced a high background signal in the Ndh2-deficient strain. Additionally, current generation for **2** reaches its maximum within a short period of time after mediator addition and current output continually drops over the course of the experiment under these experimental conditions. Comparison of normalized chronoamperometry traces for **2** and **21** (see experimental details), clearly illustrates the differences in current profile between the two compounds, specifically the stability of **21** (**Fig. 4C**). Taken together, these results highlight the potential of **21**, **26**, and **29** for use in biosensing applications, where stable current responses with low background are ideal properties. As such, these compounds have the potential to be used in whole-cell *L. plantarum* bioelectronic devices, expanding the molecules available for EET-based biosensing.

In summary, we generated a library of 30 structurally diverse 1,4-naphthoquinone-based mediators to gain a better understanding of the important physicochemical properties and biochemical interactions driving Nhd2-dependent EET in *L. plantarum*, a commensal gut bacterium. The library was inspired by naturally-occurring naphthoquinones and consisted of eleven natural products, sixteen semi-

synthetic, and three commercially sourced compounds. A particular focus of the library was to assemble mediators with diverse polarity (cLogP), reduction potential (E^o), and binding affinity. Correlation of these properties to the Ndh2-dependent EET activity in an iron(III) nanoparticle assay revealed that EET is linearly related to cLogP and binding affinity. Analysis of some of the top performing mediators in a BES confirmed that they are each capable of generating a detectable current, and the current of one of the mediators generated was stable over 5 d. This study has significantly increased our library of known mediators, provided insights into the properties that make a mediator successful, and has led to a mediator that generates stable current outputs for potential use in a variety of biosensing applications.

Supporting Information.
The following files are available free of charge.
Supporting Information - Experimental details, media recipes, computational data, 1H and 13C NMR spectra, toxicity and degradation studies, BES experiments (PDF)

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Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding Sources
DOD ARO W911NF-22-1-0239 and Virginia Tech Startup funds (EM)

ACKNOWLEDGMENT

This research was sponsored by the Army Research Office and was accomplished under Grant Number W911NF-22-1-0239 (EM, SH, and CAF) and Virginia Tech Startup funds (EM). We thank the Virginia Tech NMR facility and Virginia Tech Mass Spectrometry Incubator for analytical services.

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