Chapter 6

Electrochemical Properties of gsNOS by Protein Film Voltammetry

6.1 Abstract

The accurate measurement of a protein's electrochemical properties is an important part of understanding its function. Several methods have been developed to facilitate communication between deeply buried protein metal centers and electrodes. One such technique, protein film voltammetry (PFV), involves the immobilization of proteins on the surface of electrodes by various means. Such techniques can result in clear signals from proteins, allowing the measurement of not only reduction potentials but kinetics as well. Two types of PFV have been employed in the study of the nitric oxide sythase from *Geobacillus stearothermophilus*. First, a mutant of this NOS was covalently connected to a gold electrode. It was hypothesized that the use of a hydrophilic linker would maintain a normal aqueous environment around the enzyme and avoid the shifting of potentials (a common problem in PFV). When it was found that this technique still resulted in measuring responses with significantly shifted potentials (as compared with those measured by redox titration in solution), a more traditional film was employed. The kinetics of gsNOS was studied in DDAB films and compared with the mammalian inducible isoform. It was found to show similar behavior, and experiments are still underway to further characterize the kinetics of wild type and three mutants of gsNOS (W70H, W70F, and W70Y, introduced in Chapter 3).

6.2 Introduction and Background

Key to the function of most metalloenzymes is the redox activity of transition metals. These metals have the unique ability, as distinct from most organic molecules, to easily access multiple oxidation states. This electrochemical property of metal sites enables the catalysis of difficult but essential chemical reactions, e.g., the oxidation of unactivated C-H bonds, the reduction of dinitrogen to ammonia, and the generation of hydrogen from water. Clearly, the accurate measurement of a protein's electrochemical properties is an important part of understanding its function. The protein matrix often separates active sites from the surface of the protein by many Ångstroms, rendering the standard electrochemical techniques used by inorganic chemists useless.[1-3] In many cases, no electrochemical response can be observed.

Several methods have been developed to facilitate communication between deeply buried protein metal centers and electrodes. One such technique, protein film voltammetry (PFV), involves the immobilization of proteins on the surface of electrodes by various means.[4] Such techniques can result in clear signals from proteins, allowing the measurement of not only reduction potentials but kinetics as well.[5-7] However, such methods have recorded potentials vastly different from those measured by other methods, as shown in **Table 6.1** for the case of a heme protein called cytochrome P450 (cyt. P450).

Table 6.1. The $Fe^{III/II}$ reduction potential of a cytochrome P450-BM3 as measured by different methods.[8-10]

Method	Potential	Reference
PFV – DDAPSS	+4 mV vs. NHE	Blair 2004
PFV – SDS	-133 mV vs. NHE	Udit 2006
Redox titration	-303 mV vs. NHE	Sligar 1979

Discrepancies in measured reduction potentials are not limited to cyt. P450s or even iron enzymes in general. A chemically modified electrode, cysteamine on Au(111), was used to measure the $Cu^{II/I}$ potential of the enzyme copper nitrite reductase. The authors neglect to specifically mention the measured potential in the report; however, the included cyclic voltammograms show a quasi-reversible wave slightly negative of 0 mV vs. SCE.[11] The same group published later voltammetric studies of the same copper-containing nitrite reductase, this time using gold electrodes modified not with cysteamine but with self-assembled monolayers of alkane thiols.[12] They again fail to mention the $E_{1/2}$ they measured, but the couple clearly lies at nearly +100 mV vs. SCE. Two different monolayers on the same electrode resulted in two different reduction potentials. Curiously, these differing potentials go undiscussed but for one mention of differing dielectric constants between films and aqueous solution.[4] The cause of these shifts remains unknown.

Interestingly, varied electrochemical approaches have produced consistent results in other cases. Film voltammetric methods have been used for several small electron transfer (ET) proteins, such as cytochromes *c* and cupredoxins, **Figure 6.1**. The reduction potential cupredoxin azurin, for example, falls near +300 mV vs. NHE regardless of electrochemic method.[13-15] A trend seems apparent: technique-based discrepancies in reduction potential are endemic to larger metalloproteins, but measured values tend to converge as molecular size decreases.

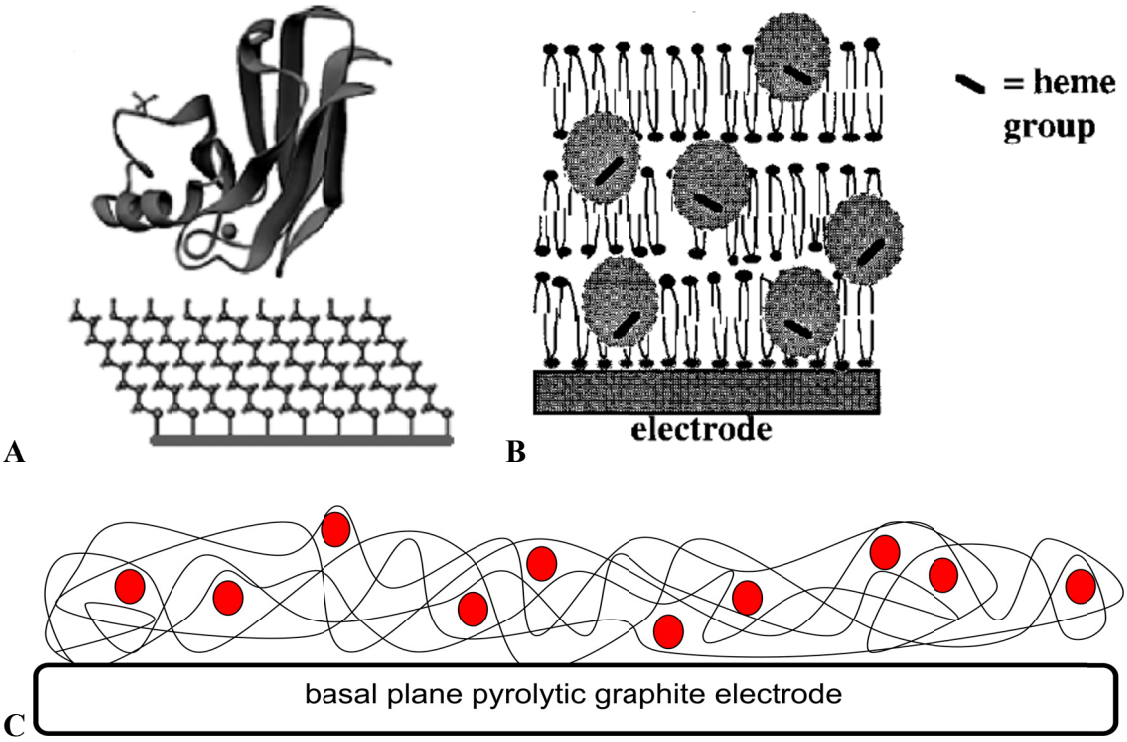
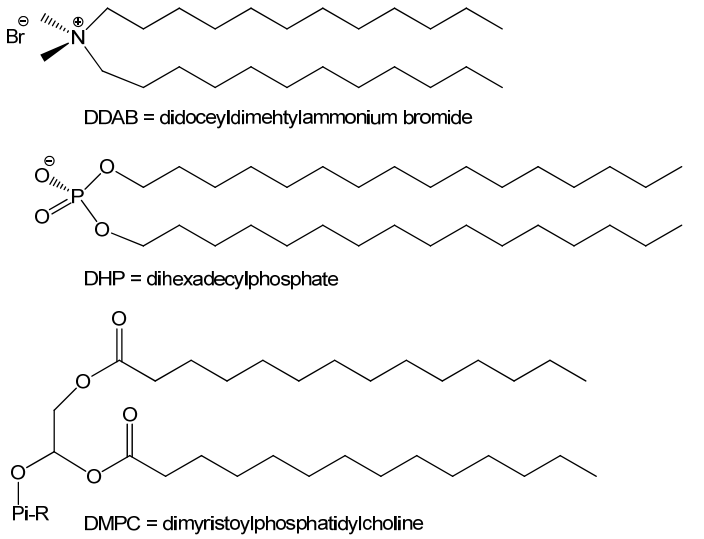


Figure 6.1. Depictions of proteins immobilized on electrodes (**A)** using SAMs (azurin),[16] (**B)** using phospholipid-like films (cytochrome *c*),[4] and (**C**) using a DDAB film and nitric oxide synthase.

When simple Coulombic interactions will not suffice, alternative methods of attachment have been employed in order to promote electronic coupling between protein active sites and electrodes. Self-assembled monolayers (SAMs) on gold surfaces have been studied in detail.[17] SAMs have been used extensively by Gray and coworkers to characterize the electrochemical properties of azurin and cytochrome *c*, among other small ET proteins. Monolayers of mixed hydroxyl and alkyl-terminated thiols on gold are soaked in protein solutions, then rinsed, and used in voltammetric expierments. It is unclear, to date, what exactly the nature of this interaction is (surface characterization methods have provided sparse insights),[11] but clear signals can be recorded.

Another non-covalent method of attaching a protein sample to an electrode involves surfactant films. Characterization of a protein sample within this matrix is very

difficult; only when using a specially designed cell and extensive reflections can one take a UV-visible or IR spectrum of a protein within a film.[18] Films have successfully been employed with a number of enzymes including cyt. P450, myoglobin, and nitric oxide synthase.[19] A selection of some of the surfactants that have been used for film voltammetry is shown in **Scheme 6.1**.



Scheme 6.1. Surfactants commonly used for protein film voltammetry.

These surfactants typically contain a polar head group and a long alkyl chain, making them similar to lipids and, presumably, cell membranes. Solutions of these surfactants in organic solvents are dropped onto polished electrode surfaces and the solvent is allowed to evaporate.[20] An interlocking network of the surfactant, or a film, is left behind. When soaked in an aqueous solution of protein sample, some protein is taken up into the film or otherwise interacts with it. This technique has demonstrated the largest deviation from solution reduction potentials amongst the many electrochemical techniques. The reason for this is hypothesized to be the lipid-like nature of the film. Perhaps lipid-like films provide a more accurate measure of the potential of membrane-bound proteins, although no concrete data support this hypothesis. Unfortunately,

detailed characterization of biological samples on solid surfaces is difficult as most characterization techniques are solution-based.

Covalent attachment of the samples of interest can be used to couple the active site to the electrode. Mutagenesis to install a single, solvent-exposed cysteine will allow for attachment of the protein to a gold electrode directly through the cysteine sulfur atom. Alternatively, this cysteine can be used to functionalize the protein with some other group for attachment to the surface, as demonstrated by Liu and coworkers.[21] Investigators have proposed that these methods block movement of the protein and lock it out of potentially necessary conformations, particularly with respect to protein-electrode ET pathways.[4, 7] This lack of motion is a concern with all methods that tether the sample to the electrode surface. Comparison of the results of voltammetry with both covalent and non-covalent attachment to gold electrodes using SAMs should provide insight into the issue of sample diffusion on the electrode surface.

Electrochemistry was carried out using covalent attachment of NOS to standard SAMs, but the potential measured was +195 mV vs. NHE, about 450 mV positive of other NOS enzymes.[22] A hydrophilic SAM terminated in a maleimido functionality was then used to attach gsNOS to the surface in a covalent fashion rather than the traditional hydrophobic SAMs. It was hypothesized that the hydrophilic SAMs on a gold electrode would promote a normal hydration sphere around the protein and prevent the shift in potential previously observed in PFV of mammalian iNOS. Cyclic voltammograms were collected on gsNOS. This bacterial enzyme displayed similar properties as iNOS, with the observable dissociation of water upon reduction of the iron center. Unfortunately, the measured potential was again shifted very positive of the potential measured in solution.

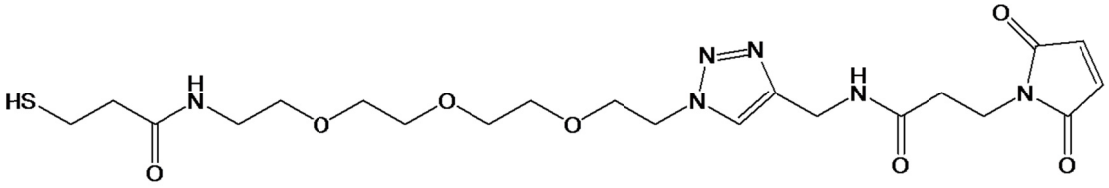
Given that this technique using covalent attachment still showed altered reduction potentials for the iron site, the complicated synthesis and electrode prep were deemed unnecessary and further investigations were made using traditional films. Films of DDAB and wild type gsNOS were co-deposited onto the surface of basal plane pyrolytic graphite electrodes and their electrochemical properties were investigated.

6.3 Materials and Methods

Electrodes were purchased from Pine Instruments. Chemicals, such as arginine and TrisHCl buffer, were purchased from Sigma-Aldrich. The enzymes used in this study were expressed and mutated as described previously in Chapter 3.

A mutant form of gsNOS was expressed, containing a single, solvent-exposed cysteine residue for use in covalent attachment. The native enzyme has four cysteine residues at positions 76, 161, 227, and 269. Cys76 ligates the iron center and is necessary for activity and for heme incorporation. Position 161 is completely buried within the protein interior and hidden from solvent. These residues were left un-mutated as they should not interfere in any way with the protein labeling process. Positions 227 and 269, however, are exposed to solvent at the surface of the protein, so these two residues were mutated to serines so as not to interfere with covalent labeling reactions which rely on a nucleophilic thiolate. A cysteine was installed near the heme (in order to facilitate communication with the electrode) at position 115, where a lysine residue is found in the wild type and is fully solvent exposed. SAMs were made with polyethylene glycolated (PEG-ylated) thiols in order to produce a hydrophilic surface. This PEG-ylated thiol was terminated in an azide to facilitate “click” chemistry. A small molecule containing an

alkyne and a maleimide was "clicked" to the surface using a Cu(bathophenanthroline) catalyst. The complete molecule used for the film is shown in **Scheme 6.2**.



Scheme 6.2. The maleimide-terminated thiol used to attach gsNOS to the surface of a gold electrode for voltammetry.

For DDAB films, a solution containing DDAB in DMSO was co-spotted onto BPG electrodes with aqueous solutions of NOS. Not only was the wild type studied, but the mutant enzymes described in Chapter 3, W70H, W70F, and W70Y were as well, to investigate the effect of the proximal hydrogen bonding network on the electronics of the heme site. The buffer used for these studies was 150 mM NaCl, 50 mM Tris, pH 7.5.

6.4 Results

First, "click" chemistry was used to attach gsNOS covalently to standard SAMs (alkane thiols) on gold electrodes, as shown in **Figure 6.2**. When the electrochemical response of NOS was measured using cyclic voltammetry, strong signals were observed. The potentials that were measured, however, were far positive of those found in Chapter 3 by redox titration (**Figure 6.3**).

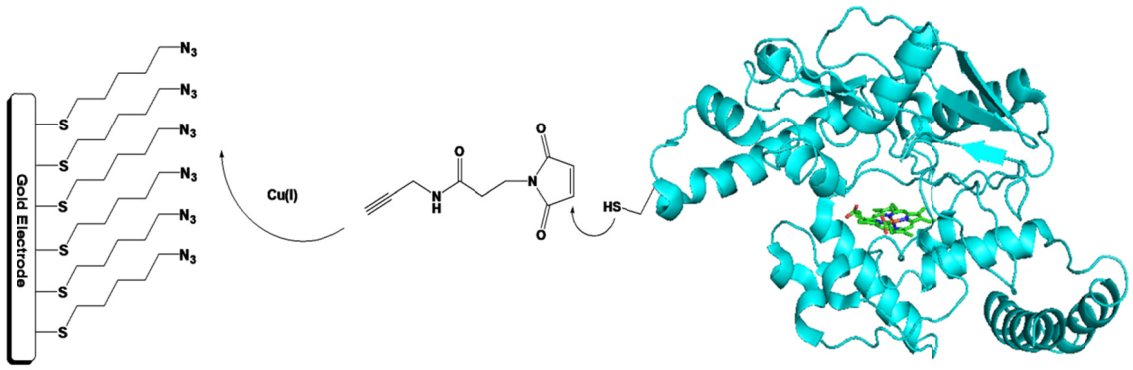


Figure 6.2. Method of attachment of gsNOS to gold electrodes.

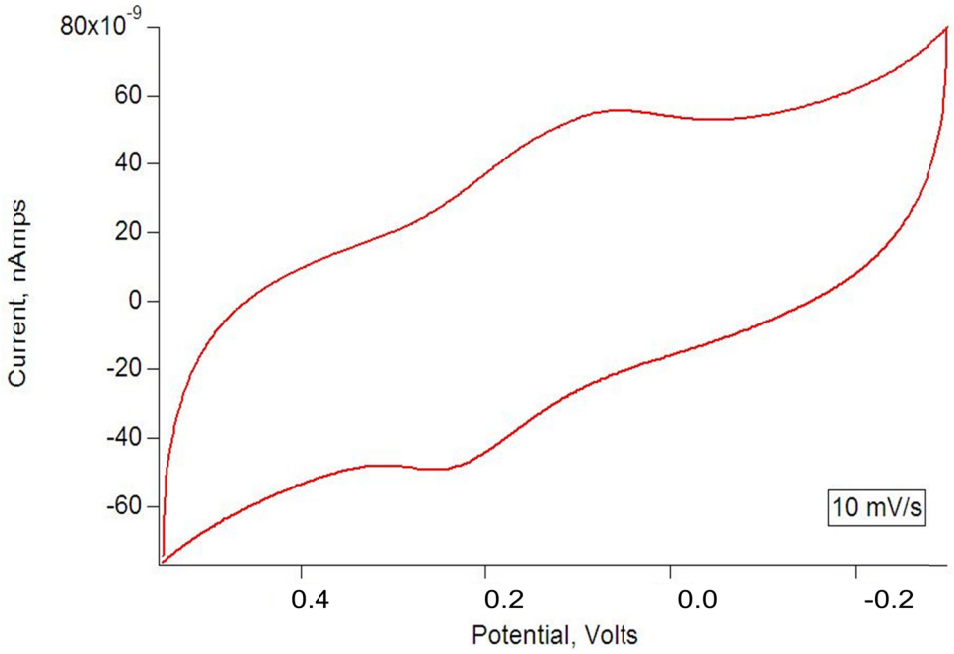


Figure 6.3. Cyclic voltammogram of K115C/C227S/C269S gsNOS when covalently attached to a gold electrode by the molecule shown in **Figure 6.2**, 10 mV/s scan rate. The surface coverage was determined to be 3 pmol/cm^2 (50% coverage) as determined by integrating the area of each peak.

These samples, while giving very elevated potentials, did give good electrochemical responses. To verify that the protein was in fact bound to the surface, the scan rate dependence of the signals were studied. The electrochemistry of surface-bound species varies with scan rate in a predictable manner.[23-24] The scan rate dependence can be fit to Equation 6.1 if the samples are indeed sequestered on the surface and not

diffusing through solution. **Figure 6.4** shows the broadening of the voltammetric signal in response to an increased rate of scanning the potential. **Figure 6.5** shows the fit of the observed scan rate dependence to Equation 6.1. Voltammograms were simulated using CH Instruments software assuming $\alpha = 0.5$, allowing the calculation/approximation of the electron transfer rate between the iron center and the electrode as well as the reorganization energy: average $k^o \sim 10^{-1}$ s^{-1} and $\lambda \sim 1$ eV.

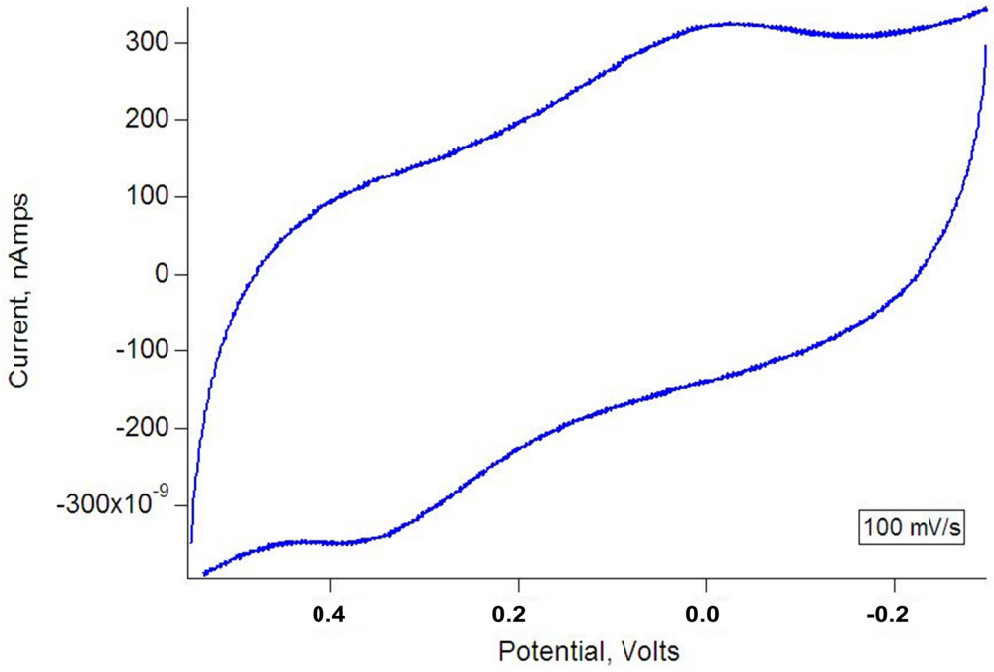


Figure 6.4. Voltammetric response at an increased scan rate, 100 mV/s.

$$k_{red,ox} = \mu\rho k_B T \int \frac{exp[-(x-(\lambda \pm \eta)/(k_B T))^2 * k_B T/(4\lambda)]\, dx}{[1+exp(x)]} \qquad (6.1)$$

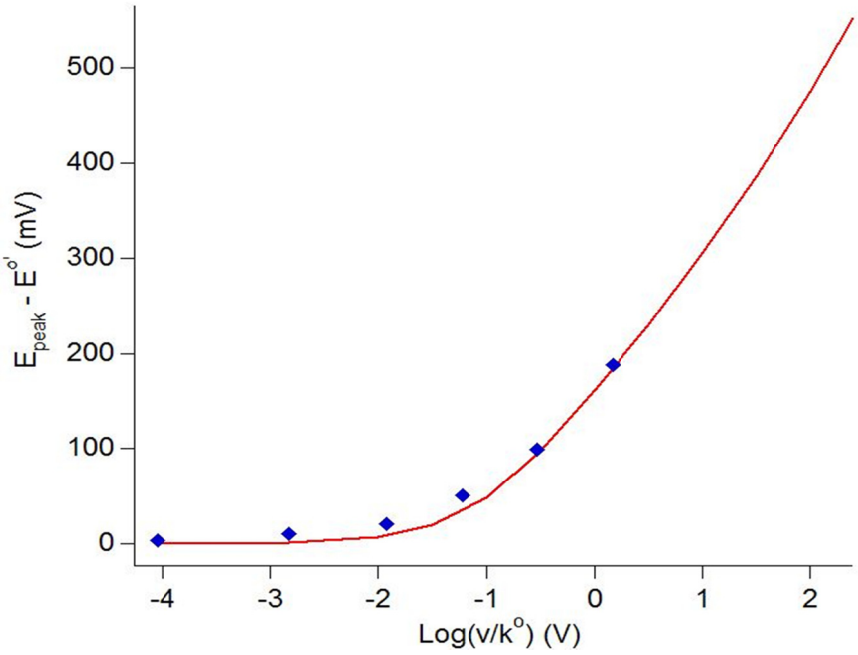


Figure 6.5. The red line in the theoretical fit for scan rate dependence for surface-bound species, Equation 6.1. The blue diamonds are the actual peak separations measured in gsNOS samples on gold-thiols as the scan rate is varied.[23]

The potentials measured with this method are shifted nearly half a volt positive of the known value of the redox couple under investigation. In order to observe a more reasonable potential for this couple, SAMs were made using the hydrophilic molecule shown in **Scheme 6.1**. It was hypothesized that the presence of water around the PEG groups and near the surface of the protein would shift the potential closer to that measured in aqueous solution. The voltammetric response using the molecule from **Scheme 6.2** to form the SAM and connect NOS to the electrode can be seen in **Figures 6.6** and **6.7**.

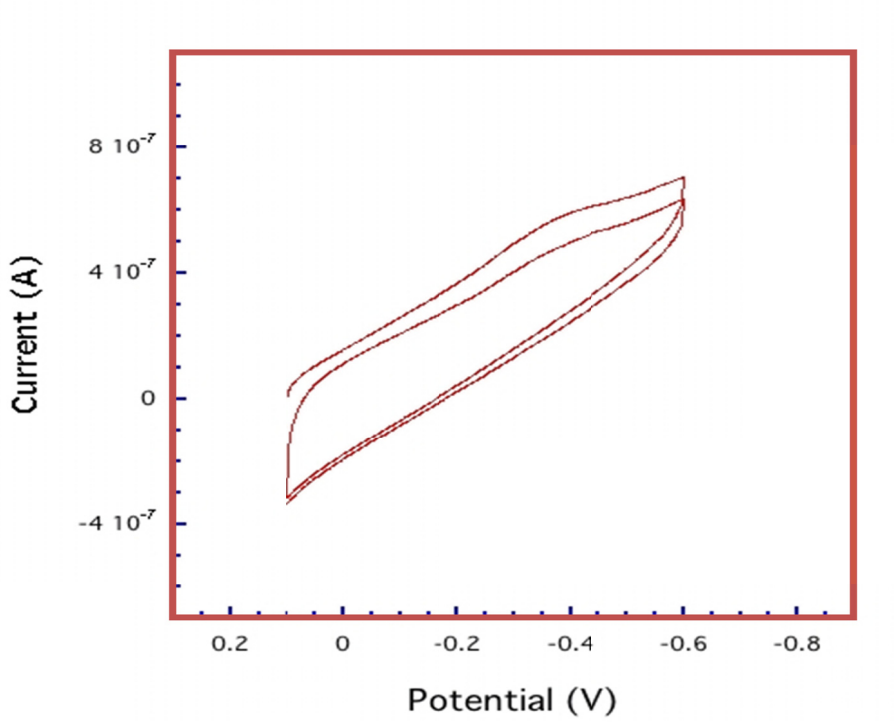


Figure 6.6. Cyclic voltammogram of K115C/C227S/C269S gsNOS on PEG-ylated SAMs on a gold electrode.

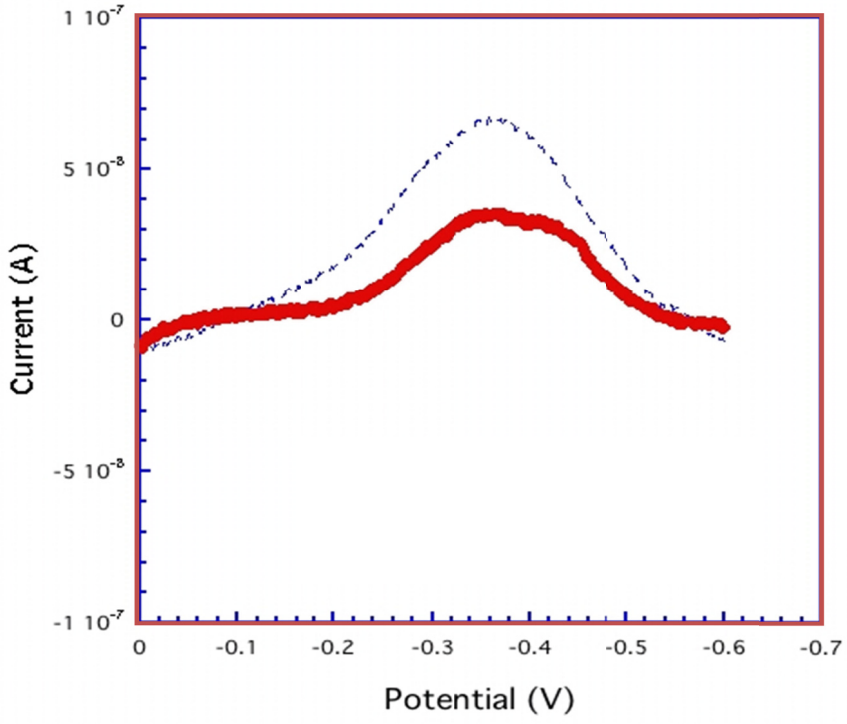


Figure 6.7. The same data as shown in **Figure 6.6**, but with the background signal subtracted to allow a clearer view of the details of each peak. The first scan is shown as solid line, and the second scan as dashes.

From **Figure 6.7** it can be seen that the peak shows similar fine structure as iNOS. A water molecule is loosely coordinated to the iron in the resting ferric state. Upon reduction, the water molecule rapidly dissociates. This explains the presence of two peaks for the reduction wave, but only a single peak for the re-oxidaiton wave of the voltammogram. However, while the potential is less positive (about -220 mV vs. Ag/AgCl or 0 vs. NHE) it is still well positive of the potential measured in aqueous solution. Due to the complicated synthesis of the molecule used for this SAM and its inability to improve upon issues seen with other films, this method was abandoned in favor of DDAB films as used previously with iNOS. The voltammetric response of wild type gsNOS in a DDAB film on a basal plane graphite (BPG) electrode is shown in **Figure 6.8**, with clearly two distinct peaks in the reduction wave similar to results seen with mammalian iNOS.[19]

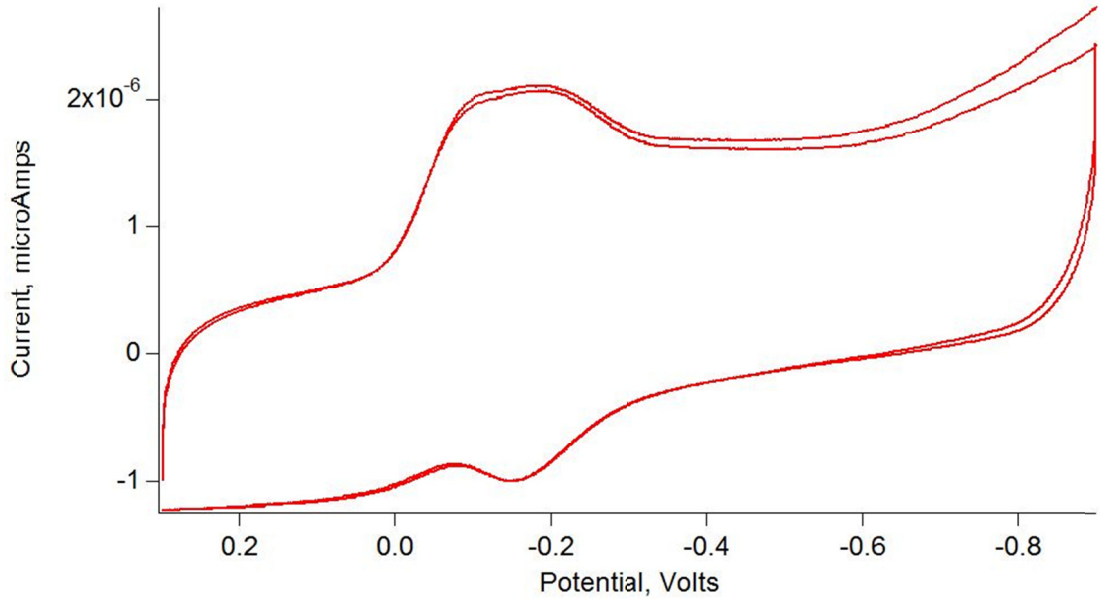


Figure 6.8. Cyclic voltammogram of wt gsNOS in DDAB film on a BPG electrode, showing two peaks in the reduction wave as was seen with iNOS.

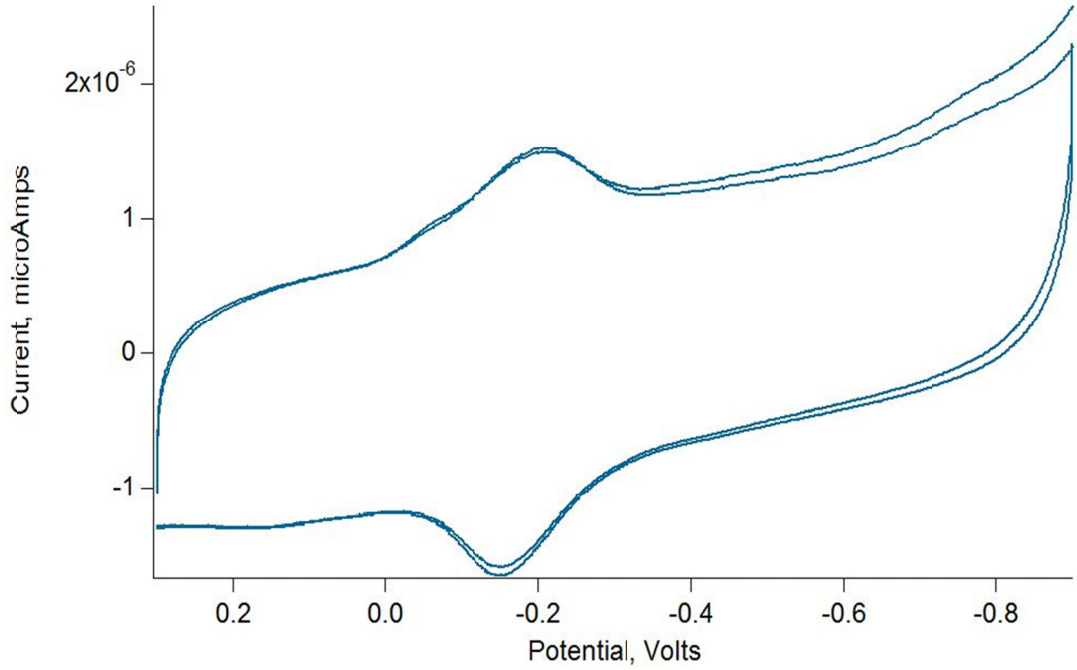


Figure 6.9. Cyclic voltammogram of W70F gsNOS in DDAB film on a BPG electrode, showing the presence of only one peak in the reduction wave.

The use of DDAB films on BPG electrodes gave large signals from gsNOS. Due to the ease of fabrication and the large signals, on-going studies are investigating the electrochemistry of mutant forms of gsNOS by this method. Initial values for the measured reduction potentials are shown in **Table 6.2**.

Table 6.2. The measured reduction potentials of 4 mutants (at position 70) of gsNOS.

Sample	$E_{(1/2)}$, mV vs. Ag/AgCl
WT	-178
His	-172
Phe	-216
Tyr	-209

Interesting features can be observed in the voltammograms, such as differing interactions with the axial water ligand (see **Figure 6.9**) due to altered electronics of the heme site. The trend in measured reduction potentials matches that observed by redox titration. Experiments were also carried out with the wild type enzyme at increased pH and indicated a shift of approximately 60 mV per pH unit, as is consistent with a proton-coupled electron transfer event. Given the presence of the water molecule that coordinates the heme in the Fe(III) state, but not the Fe(II) state and the possible protonation of the axial thiolate ligand, this result was expected. Studies are on-going in an effort to characterize the equilibrium for water ligation in each enzyme and the effect of the substrate arginine on these kinetics.

6.5 References

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