Voltammetric studies on vitamin K1 in organic solvents

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**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**VOLTAMMETRIC STUDIES ON VITAMIN K₁ IN
ORGANIC SOLVENTS**

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Chapter 1

Introduction

1.1 Voltammetry of Quinones

1.1.1 General Introduction

Quinone/hydroquinone (Q/QH₂) compounds are one of the most important and widely-studied examples of organic redox systems, because they often play very important roles in biological reactions.¹⁻⁵ In addition, Q/QH₂ couples have been extensively studied because they display fundamentally interesting chemically reversible redox properties in solution.^{1,3,6-13} According to the findings of many researchers, Bailey and colleagues constructed pH- $E_{1/2}$ diagrams for Q/QH₂ systems.^{1,14-17} The redox reaction of Q/QH₂ system occurs *via* complex electron- and proton-transfer processes. Eggins and Chambers' investigations showed that the species which participated in the redox reaction of Q/QH₂ in acetonitrile (CH₃CN) were strongly influenced by the concentration of protons.^{3,7-12} Parker investigated the role of protons in the redox behavior of Q/QH₂ in CH₃CN by using various concentrations of HClO₄ and 2,6-lutidine.¹⁸ Robertson and Pendley also performed research on the redox behavior of Q/QH₂ in H₂SO₄ solution by varying the concentration of both the quinone and H₂SO₄.¹⁹ Laviron constructed the nine-member square scheme to theoretically treat the kinetics of the electrochemical redox reaction of the Q/QH₂ couple in aqueous

media.²⁰

The applications of quinones are extensive; for example, quinones can be used as dyes and redox agents in chemical synthesis. Furthermore, medicinal properties, such as antibiotic, antimicrobial, and anticancer activity have been found from a few quinones.²¹⁻²³

Scheme 1. Quinone redox reactions.

Studies of the Q/QH₂ system over the past 100 years have deduced that the electrochemical behavior can be divided into two major categories: behavior in aqueous (buffered and unbuffered) solutions and in aprotic non-aqueous solvents. The differences between the different media have been rationalized on the basis of proton-transfer and hydrogen-bonding mechanisms.²⁴ The redox chemistry of quinones in buffered aqueous solutions at pH < ~7 can be described as shown in Scheme 1a,

where quinones are reduced in a $+2e^-/+2H^+$ chemically reversible process to form the hydroquinones.^{1,25} The observed potential (E_{obs}) of the process varies according to the pH because protons are involved in the reduction reaction. Hence, the measured E_{obs} shifts from the formal potential (E_f^0) according to the Nernst equation;²⁶

$$E_{\text{obs}} = E_f^0 + \frac{2.303RT}{nF} \times \log \frac{[Q][H^+]^2}{[QH_2]} \quad (1)$$

Where n is the number of electrons transferred (2), R is the gas constant (8.3143 J K⁻¹ mol⁻¹), T is the temperature (in kelvin), and F is the Faraday constant (96485 C mol⁻¹). When $[Q] = [QH_2]$, then $E_{\text{obs}} = E_{1/2}^r$ (the reversible half-wave potential). According to eq 1, at 25 °C the $E_{1/2}^r$ optimally shifts by -59.2 mV per one unit increase in pH (providing the E_f^0 does not significantly vary with the change in pH). Although the reaction is written as $2e^-/2H^+$ in one step (concertedly), it is probable that the electron transfer and proton transfer steps occur consecutively or by a mixed concerted/consecutive mechanism.

In buffered solutions, as the pH increases above ~7, the semiquinone ($Q^{\cdot-}$) and dianion (Q^{2-}) have increased lifetimes, meaning that the acid dissociation constants of their associated protonated forms need to be taken into account in the Nernst equation.²⁴ Thus, the relationship between the E_{obs} of the quinone and the proton concentration can be given by eq 2, where K_{a1} and K_{a2} are the acid dissociation constants of QH_2 and QH^- , respectively.

In unbuffered aqueous solutions, the initial reaction occurs via $2e^-$ to form the hydrogen-bonded dianion.^{1,24-37} Due to its basicity and depending on the exact solution pH, the dianion will exist in solution as a mixture of Q^{2-} , QH^- , QH_2 and $Q^{2-}(H_2O)_n$.

$$E_{\text{obs}} = E_f^0 + \frac{2.303RT}{nF} \times \log \left(1 + \frac{[H^+]}{K_{a2}} + \frac{[H^+]^2}{K_{a1}K_{a2}} \right) \quad (2)$$

In non-aqueous solvents in the absence of added acids, the quinone undergoes two one-electron reductions to form the semiquinone and the dianion (Scheme 1c).^{1-3,24-28,38-56} The first one-electron reduction is generally electrochemically reversible (fast electron transfer) and the second one-electron reduction is at least quasi-reversible. The solvents, supporting electrolyte, protonation equilibria and the presence of acids can affect the potentials of the reductions.^{1-3,38,41,43,45,46,57} For example, by reducing anthraquinone (AQ) in dimethylformamide (DMF) in the presence of increasing concentrations of a weak acid, which react only with the dianion, leads to a positive shift of the second reduction peak potential, due to the protonation of the Q^{2-} , until the second wave merges with the first reduction peak.^{2,3,12,58}

1.1.2 Hydrogen Bonding between One- and Two-electron Reduced Forms of Quinones

Hydrogen bonding is the attractive interaction that occurs between the hydrogen atoms and electronegative atoms, such as nitrogen, oxygen or fluorine. Because of the strength and directionality of hydrogen bonds, they are the most important non-covalent interactions existing in nature which can occur intermolecularly or

intramolecularly.⁵⁹ The hydrogen bond occurs in both inorganic molecules and organic molecules, which is stronger than a van der Waals interaction, but weaker than covalent or ionic bonds.

Hydrogen-bonding interactions in aprotic solvents with the one- and two-electron reduced products of quinones has been demonstrated by changes in the reduction potentials and by changes in their optical spectra.⁶⁰ Wilford and Archer observed smooth shifts in the redox waves of *p*-benzoquinone as small increments of water were added to aprotic solvents.⁴⁵ Peover pointed out that the one-electron reduction potentials of quinones in aqueous solutions are much more positive than in aprotic solvents and this discrepancy increases with the basicity of the semiquinone.³

There is much experimental evidence that indicates that hydrogen bonding alone, without proton transfer, can have a greater effect on the Q^-/Q^{2-} redox potential. By consideration of the appropriate pK_a values of the protonated radical anions and the continuous shift in potential with no change of peak height and reversibility (without the appearance of any new waves), indicates that hydrogen-bonding occurs between the reduction products of quinones and the hydroxylic additives.^{40,42,45,48,51-54} In reports on the electrochemistry of quinones in aprotic solvents, hydrogen-bonding between additives and quinone species have been found to occur in the presence of low concentrations of hydroxylic additives. The degree of hydrogen-bonding interactions is caused by the different effects of the agents.⁴⁸

The cyclic voltammograms shown in Figure 1 show that the potentials of the two

one-electron reduction reactions of quinones are both heavily influenced by water in an aprotic solvent. The potential of the second reduction process (E_2/V of Q^-/Q^{2-}) in particular shifts positively with the addition of water to the solutions of quinones.^{30,42,45,58,61-63} The potential of the first reduction process (E_1/V of Q/Q^-) also moves positively, but not as much as E_2 ($\Delta E_2 > \Delta E_1$). Eventually, the two waves merge together at high water concentrations, corresponding to the reversible transfer of two electrons per molecule.^{41,42,48}

Figure 1. CVs of quinone in 0.1 M Bu_4NPF_6/DMF after addition of water: (a) 0% H_2O , (b) 0.5% H_2O , (c) 1% H_2O , (d) 2% H_2O , (e) 4% H_2O , (f) 8% H_2O . $v = 100$ mV/s with Au working electrode. Excerpt from *J. Am. Chem. Soc.* **2007**, 129, 12847–12856.

Hydrogen-bonding interactions are occurring between the anionic quinones and water molecules to cause the shift in E_1 and E_2 .^{1-3,24,25,27,28,38-54} UV-Vis and EPR spectroscopic experiments support that the hydrogen-bonding occurring between the

water and the semiquinone and dianion of quinones.^{60,64,65} The maximum potential shift is related to the ratio of binding constants in the oxidized ([Ox]) and reduced states ([Red]) as given in eq 3 (at 25 °C).⁶⁶

$$\Delta E_{\max}^0 = \frac{59\text{mV}}{n} \times \log \frac{K_{\text{Red}}}{K_{\text{Ox}}} \quad (3)$$

1.2 Vitamin K

1.2.1 General Introduction of Vitamin K

Vitamin K comprises a series of fat-soluble compounds which are based on 2-methyl-1,4-naphthoquinone derivatives with different side chains at the 3-position.⁶⁷ They are synthesized by plants and bacteria, which contain two kinds: vitamin K₁ and vitamin K₂. Vitamin K₁ (VK₁) is the only important molecular form in plants, also called phyloquinone, which has a phytyl side chain (Scheme 2a). Vitamin K₂ (VK₂) is a family of compounds which are synthesized by bacteria, also named as menaquinone with a side chain based on variable numbers of unsaturated prenyl units (Scheme 2b). VK₁ and VK₂ are from nature and are not toxic, but there is a synthetic type of vitamin K: vitamin K₃ (also known as menadione), without the phytyl chain which is very toxic (Scheme 2c).

Vitamin K₁ (phylloquinone) Vitamin K₂ (menaquinone) Vitamin K₃ (menadione)**Scheme 2.** Different forms of vitamin K.

Vitamin K is an essential fat-soluble micronutrient which is needed for the post translational modification of vitamin K-dependent proteins and required for blood coagulation. It was also discovered that vitamin K plays a role in bone growth and the maintenance of bone density, and also displays anticancer effects. The vitamin K-dependent coagulation proteins are synthesized in the liver and comprise factors II, VII, IX, and X, which have a haemostatic role, and proteins C and S, which have an anticoagulant role.⁷⁰⁻⁷² Several other vitamin K-dependent proteins have been isolated from bone, cartilage, kidney, lungs, and other tissues as well, for example, osteocalcin and matrix Gla protein (MGP), which is to prevent over calcification of the bone and cartilage.⁷³⁻⁷⁸

VK₁ is the main source of dietary vitamin K which is absorbed from the intestine.⁷⁹ Then the VK₁ is incorporated into chylomicrons within the intestinal mucosa into the lymph, and enters the blood via the lacteals.^{80,81} Vitamin K is mostly stored in the liver and comprises about 90% VK₂ and 10% VK₁.^{82,83} But the major circulating form is invariably VK₁. Vitamin K deficiency will cause bleeding; named as vitamin K deficiency bleeding (VKDB), which represents a significant public health problem in infants up to around age 6 months compared to adults, although rare.⁸⁴⁻⁸⁶ Therefore, vitamin K supplements is given to the infants in the immediate perinatal period to protect them because VKDB is hard to test.

1.2.2 Properties of Vitamin K

The important biological properties of vitamin K involve proton and electron transfers which are controlled by the quinone structure (2-methyl-1,4-naphthoquinone).⁸⁷ The function of vitamin K in the cell is to convert glutamate (Glu) in proteins to gamma-carboxyglutamate (Gla). Within the cell, vitamin K undergoes reduction reactions to form the vitamin K hydroquinone by the enzyme. Then the vitamin K hydroquinone is oxidized by another enzyme to allow the carboxylation of Glu to Gla (Scheme 3). Consequently, the mechanism of blood clotting of vitamin K is believed to involve the reversible formation of the hydroquinone in a two-electron, two-proton process which is analogous to the electrochemical mechanism that occurs for most Q/QH₂ systems.^{1,25}

Scheme 3. The cyclic metabolism of vitamin K in relation to the conversion of glutamate (Glu) to γ -carboxyglutamate (Gla) residues.

VK₁ is a water-immiscible yellow oil, which is found mostly in green leafy vegetables and acts as an electron-transfer agent to form the semiquinone radical anion in the photosynthetic membrane in photosystem I.⁸⁸⁻⁹⁴ Therefore, electrochemical investigations concerning the electron- and proton-transfer properties of VK₁ have been conducted in aqueous and non-aqueous media for a better understanding of its behavior in a biological environment.⁹⁵⁻¹⁰⁵ The electrochemical properties of vitamin K in biomimetic membrane systems have been investigated because vitamin K and its reduced forms exist inside cell membranes. For such purposes, a lipophilic

environment is used to interrogate the voltammetry of vitamin K because it can reflect the biological environment more realistically.^{101-103,105,106} Micro-droplets or a self-assembled monolayer of vitamin K has been immobilised on the surface of the working electrode with the presence of aqueous electrolytes.^{105,107-111}

1.3 Aim of Present Project

The electrochemical properties of vitamin K in aqueous and non-aqueous solutions have been studied because of its importance in biological processes. In this thesis, the investigations of VK₁'s electrochemical properties were conducted in a low water content environment (aprotic organic solvents) because VK₁ exists inside hydrophobic cell membranes in the human body. Adsorbed phospholipids or supported lipid bilayers were also chosen and immobilized on the top of the working electrodes in an attempt to study the electrochemistry of VK₁ in a membrane-like environment. Even when the VK₁ is immobilized inside a membrane, it will be demonstrated that electron- and proton-transfer reactions are still able to occur.

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Chapter 2

Experimental Section

2.1 Introduction to Voltammetry

Voltammetry is based on the measurement of the current in an electrochemical cell under conditions of polarization in which the rate of oxidation and reduction of the analyte are limited by the rate of mass transfer of the analyte to the electrode surface.¹ Cyclic voltammetry and square-wave voltammetry are two types of voltammetric methods.

2.1.1 Theory of Cyclic Voltammetry: Reversible and Irreversible Kinetics

Cyclic voltammetry is an important and widely used electrochemical technique for studying redox processes at an electrode surface by the application of a triangular potential sweep, allowing one to sweep back through the potential region just covered.²⁻⁴ Cyclic voltammetry is carried out under stationary conditions, therefore, the mode of mass transfer to the electrode is diffusion only.

The different values of the standard electrochemical rate constant, k^0 , lead to different limiting behavior of the heterogeneous electron transfer processes. The ‘fast’ and ‘slow’ electrode kinetics are characterized by the labels ‘electrochemically

reversible' and 'electrochemically irreversible' respectively. Figure 1 shows the voltammograms with different heterogeneous rate constants.

Figure 1. Cyclic voltammograms for electrochemically reversible reaction (Blue line), electrochemically quasi-reversible reaction (Red line) and electrochemically irreversible reaction (Green line) with different rate constants.

2.1.2 Electrochemical Cell for Cyclic Voltammetry

The experimental facility for recording cyclic voltammograms consists of an electrochemical cell (Figure 2) that has three electrodes: reference electrode (RE), working electrode (WE), and auxiliary or counter electrode (CE), all immersed in a solution and connected to a potentiostat. The potentiostat⁵ allows the potential difference between the reference and working electrode to be controlled by adjusting the potential between the working and auxiliary electrodes with minimal interference from IR (ohmic) drop.

Figure 2. Electrochemical cell for cyclic voltammetry with three electrodes.

The purpose of positioning the reference electrode close to the working electrode is to reduce the IR drop between the reference and working electrode due to the resistivity of the solution phase. The electrodes commonly used for cyclic voltammetric experiments are:

(1) *Reference electrode*: to provide a constant potential with which to monitor the working electrode. Aqueous Ag/AgCl or calomel half-cells^{6,7} are commonly used reference electrodes, which can be obtained commercially or easily prepared in the laboratory. However, the common reference electrode is not suitable for some conditions, e.g. for some dry organic solvents or room temperature ionic liquids, or when they introduce problems with salt leakage or junction potentials, then

pseudo-reference electrodes, such as a simple silver or platinum wire, can be used in conjunction with an internal potential reference provided by ferrocene.^{8,9} Experimentally, ferrocene is added into the cell at the end of a series of measurements, and the reversible voltammetric response for the $\text{Fc}^{+/0}$ couple is taken as the reference point on the potential scale.

(2) *Working electrode*: where the electrochemical reaction of interest occurs. Inlaid disc electrodes (Pt, Au, glassy carbon (GC)) of well-defined area are most commonly used as working electrodes.

(3) *Auxiliary electrode*: where the opposite reaction occurs to the reaction occurring at the working electrode. Platinum wire or mesh is most commonly used as the auxiliary electrode, which is a non-reactive high surface area electrode.

2.1.3 Theory of Square-wave Voltammetry

Square-wave voltammetry (SWV) is a powerful electrochemical technique that can be applied in both electrokinetic and analytic measurements.¹⁰⁻¹⁴ Because of the widespread use of computer controlled instruments, a well-developed theory and most importantly, its high sensitivity to surface-confined electrode reactions, the application of SWV has boomed in the last decade.

SWV was developed by combining the high-amplitude, high-frequency square wave with the fast staircase waveform and by using computer-controlled instruments. Figure 3 shows the potential-time waveform of modern SWV. Each square wave period

occurs during one staircase period τ . Hence, the frequency of the excitation signal is $f = \tau^{-1}$, and the pulse time is $t_p = \tau/2$. The square-wave amplitude, E_{sw} , is one-half of the peak-to-peak amplitude, and the potential increment ΔE is the step height of the staircase waveform. The currents are measured at position 1 and 2.

Figure 3. Waveform for square-wave voltammetry. Excerpt from *Fundamentals of Analytical Chemistry (8th Edition)*, 2004, Chapter 23, p692.

A single SWV potential scan generates three voltammograms showing i_1 , i_2 and Δi as a function of the staircase potential, as sketched in Figure 4.¹⁵ Here i_1 is the forward current, i_2 is the reverse current, and the current difference, Δi is directly proportional to concentration.

Figure 4. Schematic voltammetric profiles of the current measured during the forward and reverse pulses and resultant difference, Δi , plotted against the staircase potential E . Excerpt from *Fundamentals of Analytical Chemistry (8th Edition)*, 2004, Chapter 23, p693.

2.2 Introduction to Karl Fischer Titration

Karl Fischer (KF) titration is a classic titration method in analytical chemistry that uses coulometric or volumetric titration to determine the trace amounts of water in a sample.

The KF titration is the standard method for obtaining the water content of various substances.¹⁶ It is based on the solution-phase reactions given in eqs 1 and 2 and can be performed in coulometric or volumetric mode. ROH in eq 1 is an alcohol, usually methanol or ethanol, and RN in eq 1 is an amine such as imidazole.

Equation 2 shows that there is a 1:1 reaction between I_2 and H_2O ; thus, if the amount of I_2 is accurately known, then the amount of water can be determined. In KF coulometric titrations (as performed in this study), the I_2 is generated electrochemically by the oxidation of iodide (eq 3).

According to eqs 2 and 3, 2 mol of electrons is produced for every mole of water that reacts with iodine. Therefore, 2×96485 coulombs (C) are produced for 1 mol of water present, or stated another way: 1 μg of water corresponds to 0.01072 C passed. The end point of the titration is measured by a change in voltage across two indicator electrodes due to the presence of free iodine.

The great advantage of the KF titration is that it does not need calibration, since the charge can be directly correlated with the amount of water present. However, some limitations exist; in particular, there is a minimum charge that can be accurately measured by the coulometer, meaning that low concentrations of water require higher masses of the analyte to achieve the greatest accuracy. With care the KF titration can accurately give measurements down to 1 ppm water. In this study, most measurements were conducted between 200 and 2000 ppm water, a range over which reliable readings can be easily obtained. In certain situations, the charge measured during the oxidation of iodine is not necessarily selective for water because other oxidizable species are present,

or the reaction in eq 2 between I_2 and H_2O does not occur in a 1:1 ratio.¹⁶ Nevertheless, the solvents/electrolytes used in this thesis work represent straightforward media within which to calculate the water content without any known special difficulties.

KF titrations were conducted with a Mettler Toledo DL32 coulometer using (Riedel-deHaën) HYDRANAL[®]-coulomat CG for the cathode compartment and HYDRANAL[®]-coulomat AG for the anode compartment. The instrument was first allowed to stabilize until the drift was $0 \mu g \text{ min}^{-1} H_2O$ which usually required 30 minutes of operation. The samples were injected into the coulometer using plastic disposable syringes through a silicon/Teflon septum. Each measurement took less than 1 minute, meaning that the drift from atmospheric water entering the coulometer was negligible.

2.3 Introduction to Spectrochemical Methods

Spectroscopy is a technique that uses the interaction of radiation with matter to obtain a spectrum, which is a plot of the intensity of energy detected versus the wavelength or frequency. Information about atomic and molecular energy levels, molecular geometries, chemical bonds, interactions of molecules, and related processes can be obtained from a spectrum. Spectra can be used to qualitatively analyse the components of a sample or quantitatively measure the amount of material in a sample. Ultraviolet-visible (UV-Vis) spectroscopy and Fourier transform infrared (FTIR) spectroscopy are two kinds of spectrochemical methods, which are widely used

in chemistry, biology, engineering and many other fields.

The spectroscopic instruments of UV-Vis and FTIR spectroscopy contain five components: (1) a stable source of radiant energy; (2) a wavelength selector; (3) one or more sample containers; (4) a radiation detector; and (5) a computer to process and readout the signal.

2.3.1 Theory of Ultraviolet-visible Spectroscopy

Ultraviolet-visible (UV-Vis) spectroscopy refers to absorption spectroscopy or reflectance spectroscopy in the UV-Vis spectral region, which is the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. This means it uses light in the visible and adjacent (near-UV and near-infrared (NIR)) ranges. The absorption or reflectance in the visible range directly affects the perceived color of the chemicals involved. In this region of the electromagnetic spectrum, molecules undergo electronic transitions. UV-Vis spectroscopy is useful to characterize the absorption, transmission, and reflectivity of a variety of technologically important materials.

The Beer-Lambert law (eq 4) is used to quantitatively determine the concentrations of the absorbing molecules in solution.

$$A = \log(I_0/I) = \epsilon bc \quad (4)$$

where A is the measured absorbance, I_0 is the intensity of the incident light at a given wavelength, I is the transmitted intensity, b the path length of the absorbing

medium, and c the concentration of the absorbing species, ϵ is a proportionality constant called the molar absorptivity. The molar absorptivity is a fundamental molecular property in a given solvent at a particular temperature and pressure, and has units of $\text{L mol}^{-1} \text{ cm}^{-1}$.

A deuterium discharge lamp and a tungsten-halogen lamp are usually used as the light source for UV and visible measurements in UV-Vis spectroscopy, respectively. The instruments automatically swap lamps when scanning between the UV and visible regions. Holographic gratings¹⁷ are used to disperse the wavelengths of these continuous light sources in a monochromator or spectrograph. The wavelength band is then determined by the monochromator slit width. UV-Vis spectrometers utilize a combination of a photomultiplier tube (PMT) and a Peltier-cooled PbS IR detector. The light from the lamp is dispersed before reaching the sample cell. Figure 5 shows the light pathways of single-beam and double-beam instruments used for UV-Vis spectroscopy.

Figure 5. The light pathways of (a) single-beam instruments and (b) double-beam instruments.

For both the single-beam and double-beam instruments, the radiation from the lamps passes through an entrance slit into the monochromator. A holographic grating diffracts the radiation, and the selected wavelength band passes through the exit slit into the sample compartment. A detector converts the light intensity into a related electrical signal which is enlarged and displayed on a computer.

2.3.2 Theory of Fourier Transform Infrared Spectroscopy

Infrared (IR) spectroscopy is a vibrational spectroscopy technique which is a powerful tool for identifying molecules' structural information in terms of their functional groups, the orientation of those groups and information on isomers which can be termed a molecular "fingerprint". Most molecular species absorb IR radiation, and each molecule has a unique IR absorption spectrum, except chiral molecules in the crystalline state.¹⁸ IR radiation is insufficient in energy to excite electronic transitions, but it can excite vibrational and rotational transitions of the molecules. The molecules can undergo a number of vibrations and rotations.

There are two types of IR instruments used to obtain complete spectra for qualitative identification: dispersive spectrometers and Fourier transform infrared (FTIR)¹⁹ spectrometers. The latter is the commonly used type now. FTIR instruments use an ingenious device called Michelson interferometer (Figure 6) to obtain precise measurements.²⁰

Figure 6. Diagram of a Michelson interferometer.

A beam from the light source is split into two beams by the beam splitter. One of the two beams, A' is reflected to the stationary mirror and the other beam, B is transmitted to the movable mirror. The mirrors then reflect the beams back to the beam splitter, and A' and B converge in the same region of space and form an interference pattern. The movable mirror is moved to generate different interference patterns to modulate the optical signal. The resulting interferogram of the wavelength in the beam is recorded and used for measurement. A sample is then inserted into the beam, and the sample interferogram is then recorded. The two interferograms are used to obtain the

spectrum of the sample. The beam is transmitted through or reflected off the surface of the sample in IR analysis, depending on the type of analysis being performed.

There are many different ways for sample preparation. For liquids, cells of sodium chloride (NaCl) or potassium bromide (KBr) are used for non-aqueous media, and calcium fluoride (CaF_2) for aqueous media which are all free of interferences. For solids, some can be dissolved in suitable solvents or cast onto films with a transmitting medium. Other solids can be ground into a mineral oil. For small samples, a microscope attachment can be used to obtain reflectance or transmittance spectra on particles as small as 20 microns. In addition, while preparing the sample for transmission mode it is sometimes difficult to get the correct sample-to-matrix ratios as well as ensuring the homogeneity of the sample, and these factors affect the reproducibility of the IR data. A special sampling technique, attenuated total reflectance (ATR), is used to address the concerns regarding sample preparation and reproducibility of spectra. ATR functions by measuring the changes that occur in a totally internally reflected IR beam when the beam comes into contact with a sample (Figure 7).

Figure 7. Diagram of an attenuated total reflectance (ATR) cell.

In ATR the sample is brought into close contact with the surface of the prism which

is made of a crystal with a high refractive index. An IR beam is directed at a certain angle onto the crystal resulting in total internal reflectance, thereby creating an evanescent wave that extends beyond the crystal surface and into the sample. If the sample absorbs IR radiation, an IR spectrum can be obtained.

It is to be noted that there are conditions to fulfill in order for the ATR technique to be successful. The first condition is that the ATR crystal needs to be in direct contact with the sample since the evanescent wave protrudes only a few microns ($0.5\ \mu\text{m} - 5\ \mu\text{m}$) beyond the crystal surface; the second condition is the refractive index of the crystal must be considerably larger than that of the sample otherwise the IR beam will be transmitted instead of being internally reflected within the crystal.

A Mettler Toledo ReactIR™ iC10 (Figure 8(a)) is used in the ATR experiments in this thesis, which is a real-time *in-situ* reaction analysis and reaction monitoring system. Measurements are carried out using the iC IR™ software which permits the measurement of a range of frequencies over time. Hence changes in the character or quantity of a specific bond can be clearly observed while simultaneous coulometric measurements are being taken. Diamond is used for the ATR crystal because it has the best durability, robustness and chemical inertness. The probe consists of a flexible IR transmission fiber which is composed of silver chloride/silver bromide. The flexible nature of the probe facilitates its use and easy integration with various experimental setups (Figure 8(b)).

Figure 8. Photograph of (a) Mettler Toledo ReactIR™ iC10 instrument and (b) ATR probe with diamond as the crystal.

2.4 Humidity Controlled Chamber

A humidity controlled chamber is used to control interior humidity levels above or below ambient conditions. The chamber is equipped with a humidity sensor technology and ultrasonic humidifier and a dry gas purge system. The automatic controller provides monitoring and controlling of the relative humidity. It activates the necessary system (dehumidification or humidification) when the moisture level deviates from the adjustable set point. Since this is a closed loop circulation system, an inert gas environment can be maintained in the glove box while controlling the humidity.

The ultrasonic humidifier vibrates the water in the reservoir to provide a cloud of mist that is pushed into the glove box from the pump system. At the same time air is pulled out of the glove box and circulated through the water reservoir, completing the humidification system's closed loop circuit. The water reservoir is equipped with a self-sealing quick disconnect inlet for refilling under sealed conditions. The chamber is equipped with gloveless sleeves to allow handling of interior samples.

2.5 General Experimental Section

2.5.1 Chemicals

Vitamin K₁ (VK₁) (98%) (Sigma-Aldrich) was obtained from Sigma-Aldrich and stored in refrigerator. Bu₄NPF₆ was prepared by reacting equal molar amounts of aqueous solutions of Bu₄NOH (40%, Alfa Aesar) and HPF₆ (65%, Fluka), washing the precipitate with hot water, recrystallizing 3 times from hot ethanol followed by drying under vacuum at 160 °C for 24 hours and storing under vacuum.²¹ All solvents were HPLC or analytical grade and used directly from the bottles, after drying over 3 Å molecular sieves. Lecithin (refined) was obtained from Alfa Aesar, and Nafion[®] (5 wt. % in a mixture of lower aliphatic alcohols and containing 15 – 20% water) was obtained from Sigma-Aldrich. Water, with a resistivity $\geq 18 \text{ M}\Omega \text{ cm}$ from an ELGA Purelab Option-Q was used for experiments at different pH-values. Britton–Robinson buffer solutions (pH 3, 5, 7, 9, 11, and 13) were prepared using 0.04 M acetic, phosphoric, and boric acids (Merck), and adjusted to the required pH using NaOH (Merck).

2.5.2 Voltammetry

Cyclic voltammetry (CV) and square-wave voltammetry (SWV) experiments were conducted with a computer controlled Eco Chemie μ Autolab III potentiostat. In non-aqueous solutions, working electrodes were 1 mm diameter planar Pt and glassy carbon (GC) disks (Cypress Systems), used in conjunction with a Pt auxiliary electrode (Metrohm) and an Ag wire miniature reference electrode (Cypress Systems) connected

to the test solution *via* a salt bridge containing 0.5 M Bu_4NPF_6 in CH_3CN . Accurate potentials were obtained using ferrocene as an internal standard. A pseudo reference electrode consisting of a Pt wire was used for experiments at water concentrations < 0.1 M. Coulometry experiments were performed in a divided controlled potential electrolysis cell separated with a porosity no. 5 ($1.0 - 1.7 \mu\text{m}$) sintered glass frit.²² In aqueous solutions, working electrodes were 3 mm diameter Au, GC and Pt disks (Metrohm), or a 1 mm diameter GC disk (Cypress Systems) used in conjunction with a Pt auxiliary electrode and an Ag/AgCl (3 M KCl) reference electrode. All voltammetric experiments were conducted at $22 \pm 2^\circ\text{C}$.

2.5.3 Method for Drying Solutions for Electrochemistry

The solvent containing 1 mM VK_1 and a variable concentration of supporting electrolyte were placed inside a 25 mL vacuum syringe (SGE Analytical Science) containing $3\text{\AA}$ molecular sieves (that were dried under vacuum at 513 K for 12 hours) and the syringe was stored under a nitrogen atmosphere for at least 48 hours. The VK_1 was stable under these conditions in each of the solvents tested, even after several days directly in contact with the molecular sieves, since the peak currents measured by voltammetry were the same before and after drying. The syringe was wrapped in aluminum foil during the drying process to prevent photochemical reactions of VK_1 . The contents of the syringe were then injected into a glass electrochemical cell that had been dried at 100°C in an oven and then allowed to cool under an argon atmosphere.

After deoxygenating the solution for 10 minutes with argon gas, the voltammetric scans were commenced, with an aliquot of solvent being simultaneously tested by KF titration. For experiments at the lowest water concentrations (< 0.1 M), the trace water level was allowed to increase from the lowest value under natural humidity conditions (the mean relative humidity in Singapore is 84%, typically ranging from $> 90\%$ in the early morning to 60% in the late afternoon). For concentrations of water > 0.1 M, the water content was calculated based on the accurately known volume of added water.

2.5.4 Controlled Potential Electrolysis

Experiments were performed in a divided controlled potential electrolysis (CPE) cell separated with a porosity no. 5 ($1.0 - 1.7 \mu\text{m}$) sintered glass frit.²² The working and auxiliary electrodes were identically sized Pt mesh plates symmetrically arranged with respect to each other with an Ag wire reference electrode (isolated by a salt bridge) positioned to within 2 mm of the surface of the working electrode. The volumes of both the working and auxiliary electrode compartments were approximately 10 mL each. The solution in the working electrode compartment was simultaneously deoxygenated and stirred using bubbles of argon gas. The number of electrons transferred during the bulk oxidation process was calculated from

$$N = Q/nF \quad (6)$$

where N = no. of moles of starting compound and Q = charge (coulombs).

2.5.5 In-situ UV-Vis Spectroscopy

A Perkin-Elmer Lambda 750 spectrophotometer was used in conjunction with an optically semi-transparent thin layer electrochemical (OSTLE) cell (pathlength = 0.05 cm) using a Pt mesh working electrode.^{23,24} Variable temperature (253 – 293 K) experiments were controlled with a Thermo Electron Neslab RTE 740 circulating bath containing propan-2-ol. The temperature of the OSTLE cell was controlled using a PerkinElmer flow cell connected to the circulating bath. The temperature difference between the circulating bath and the cell was calibrated using a thermocouple inside the OSTLE cell. The cavity of the spectrometer was purged from the atmosphere with a high volume flow of nitrogen gas.

2.5.6 Theoretical Calculations

Digital simulations of the CV data were performed using the DigiElch software package.²⁵⁻³⁰

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Chapter 3

Voltammetry of Vitamin K₁ in CH₃CN Containing Varying Concentrations of Water

3.1 Introduction

Vitamin K is a series of natural compounds that are based on 2-methyl-1,4-naphthaquinone derivatives, with an aliphatic side chain in the 3-position.¹ Vitamin K₁ (VK₁; also known as phyloquinone) contains four isoprenoid groups in its side chain, one of which is unsaturated (Scheme 1a). Vitamin K₂ (also called menaquinone) has a side chain with a variable number of unsaturated isoprenoid groups. The important biological properties of vitamin K involve proton and electron transfers and are controlled by the naphthaquinone functional group, while the side chain provides compatibility with the hydrophobic membrane, similar to the function of the phetyl chain in vitamin E and other lipid soluble compounds.²

Vitamin K and its reduced forms exist inside hydrophobic cell membranes, so it is beneficial to study its electrochemical properties in a low water content environment, such as aprotic organic solvents, where the electrochemistry differs from that observed in aqueous systems.³ It is also important to examine the interactions between reduced forms of vitamin K and potential hydrogen-bonding donors (including trace water) which may significantly influence its biological redox properties.

Vitamin K₁ (phylloquinone) Vitamin K₂ (menaquinone)

Scheme 1. Different forms of vitamin K and their electrochemical reduction mechanism.

The differences in the electrochemical behavior of quinones in aqueous (buffered and unbuffered) and non-aqueous solvents have been rationalized on the basis of proton

transfer and hydrogen-bonding mechanisms.^{4a} In buffered aqueous solutions at pH < ~7, quinones (Q) are reduced in a $2e^-/2H^+$ process to reversibly form the hydroquinones (QH₂) (Scheme 1b).³ Although the reaction is written as $2e^-/2H^+$ in one step (concertedly), it is probable that the electron transfer and proton transfer steps occur consecutively or by a mixed concerted/consecutive mechanism. As the pH increases above ~7, the semiquinone (Q^{•-}) and dianion (Q²⁻) have increased lifetimes, meaning that the acid dissociation constants of their associated protonated forms need to be taken into account in the Nernst equation.^{4a}

In aprotic organic solvents containing no added acid, the quinone undergoes one-electron reduction to the semiquinone, which can be further reduced by one-electron at more negative potentials to form the dianion (Q²⁻) (Scheme 1c).^{3,4,6-17} The voltammetry has been shown to be heavily influenced by water in the solvent as the potential of both the first reduction process (E_1/V) and especially the second reduction process (E_2/V) shift to more positive potentials with increasing water concentration ($\Delta E_2 > \Delta E_1$). Eventually at high water concentrations the second process merges with the first process, corresponding to the reversible transfer of two-electrons per molecule. It is believed that the shift in potential of the two processes is too great to be caused by protonation effects (because the pH of the solution does not significantly change). Instead it has been proposed that the shift in E_1 and E_2 with increasing water is due to an equilibrium that exists between the anionic quinones (Q^{•-} and Q²⁻) and their hydrogen

bonded forms.^{3,4,6-17} UV-Vis and EPR spectroscopic experiments support the hydrogen bonding mechanism during the reduction of quinones.¹⁸

Although hydrogen bonding is known to occur between water and quinone anions/dianions in non-aqueous solvents,^{3,4,6-17} we are not aware of any studies that accurately report the residual water content of the organic solvents. While it is commonly known that the water content of a "dry" solvent is not negligible, it is often assumed that the voltammetric responses are unaffected by very low levels of water. This is because it could be expected that the solvent-substrate interactions would be more significant than trace water-substrate interactions, which is true in situations where the non-aqueous solvents undergo strong hydrogen bonding with the substrate. However, it will be demonstrated in this study that differences of a few millimolar H₂O in the solvent CH₃CN are surprisingly sufficient to change the appearance and potential of the reduction processes of VK₁, and the differences are most pronounced at the lowest water concentrations (0 – 0.01 M).

Unless scrupulous care is taken in preparing the cells for electrochemical experiments, it is likely that the water content of the solvent will be much greater than that of the substrate,¹⁹ especially in high humidity environments.²⁰ Solvents that are taken from distillation apparatus or from above molecular sieves will rapidly begin adsorbing water from the atmosphere as soon as they are added to the cells. This is due to limitations in the design of electrochemical cells that require numerous ports for electrodes and purging devices, and require the working electrode to be easily removed

for polishing.²¹ Aqueous reference electrodes will immediately add moisture to the analyte solution and even non-aqueous reference electrodes are a significant source of water. The supporting electrolytes used for non-aqueous electrochemistry are often hygroscopic and should be dried by heating under vacuum immediately prior to use.²¹

In the present study the water concentration in the solvent in the electrochemical cell was accurately measured by performing Karl Fischer (KF) titrations. Voltammograms of VK₁ were recorded and the water content simultaneously adjusted over relatively small concentration changes (~0.005 M). It was determined that at water concentrations < 0.1 M, the peak separation between E_1 and E_2 was sensitive to a 0.001 M change in (water) concentration. The electrochemical results were complemented with results from *in-situ* UV-Vis spectroscopy over a range of water concentrations in order to identify the hydrogen bonded anions.

3.2 Results and Discussion

3.2.1 Electrochemistry at Intermediate (0.05 M) to High (10 M) Water Concentrations

Figure 1 shows CVs of VK₁ in CH₃CN obtained at GC and Pt electrode surfaces at variable scan rates in the presence of 0.05 (±0.01) M H₂O. On GC, two one-electron reduction processes were detected corresponding to the chemically reversible transformation of VK₁ to the monoanion (VK₁^{•-}) (eq 1) and dianion (VK₁²⁻) (eq 2).

$E_{f(1)}^0$ and $E_{f(2)}^0$ refer to the formal electrode potentials, which for quinones are strongly dependant on the water content of the solvent; thus absolute values can only be reported if the water content is accurately known. The E_f^0 -values can be approximated from the $E_{1/2}^f$ -values obtained during CV experiments, which for electrochemically reversible processes can be measured from the mid-point of the cathodic (E_p^{red}) to anodic (E_p^{ox}) peak potentials in situations where the cathodic (i_p^{red}) and anodic (i_p^{ox}) peak current ratios are equal to unity.²²

On GC surfaces at a scan rate (ν) of 100 mV s⁻¹, the anodic to cathodic peak-to-peak separation (ΔE_{pp}) for both processes was 75 mV, similar to the value obtained for ferrocene under identical conditions. However, as the scan rate was increased, the ΔE_{pp} -values for the two processes diverged, so that at $\nu = 50$ V s⁻¹, the ΔE_{pp} -values for the first and second processes were 115 mV and 150 mV respectively. The increase in ΔE_{pp} -value for the first process at faster scan rates can be accounted for by the effects of uncompensated solution resistance, and was similar to that observed for ferrocene at an identical scan rate (which is known to undergo fast heterogeneous electron transfer²³).

Figure 1. Variable scan rate CVs recorded at 293 K at 1 mm diameter planar GC and Pt working electrodes in CH_3CN containing 1 mM VK_1 , 0.2 M Bu_4NPF_6 and 0.05 (± 0.01) M H_2O . The current data were normalized by multiplying by $v^{-0.5}$ (v = scan rate). Data at 10 V s^{-1} and 50 V s^{-1} are offset by -20 and -40 μA respectively. (---) Simulations recorded with the following parameters (see equations 1 – 4): $E_1 = -1.23$ V ($k_{s(1)} = 1 \text{ cm s}^{-1}$), $E_2 = -1.80$ V ($k_{s(2)} = 0.1 \text{ cm s}^{-1}$), $D = 2.0 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$, $R = 320 \Omega$, $C_d = 1 \times 10^{-7} \text{ F cm}^{-2}$, $k_{f(1)} = 1 \times 10^7 \text{ L mol}^{-1} \text{ s}^{-1}$, $k_{b(1)} = 1 \times 10^3 \text{ s}^{-1}$, $K_{\text{eq}(1)} = 1000 \text{ L mol}^{-1}$, $k_{f(2)} = 1 \times 10^{-3} \text{ s}^{-1}$, $k_{b(2)} = 1.6 \times 10^{-9} \text{ L mol}^{-1} \text{ s}^{-1}$, $K_{\text{eq}(2)} = 6.4 \times 10^5 \text{ mol L}^{-1}$.

The large ΔE_{pp} -value observed for the second process at $v = 50 \text{ V s}^{-1}$ is greater than can be accounted for by the effects of solution resistance (which was not experimentally compensated for, but was incorporated into the digital simulations), and is instead likely to be caused by a relatively slow rate of electron transfer. The effects of slow heterogeneous electron transfer are even more pronounced on Pt surfaces where the

second process is difficult to detect even at slow scan rates, while the ΔE_{pp} -value for the first process at $\nu = 50 \text{ V s}^{-1}$ was much greater than observed on GC at the same scan rate. The second process is also difficult to detect on Pt surfaces at intermediate to high water concentrations because of the kinetic ease of reduction of water on Pt electrodes (hence the large background current shown in Figure 1 on Pt at potentials more negative than -2 V). Because simpler voltammetric behavior was obtained on GC compared to Pt surfaces, the remaining discussion in this chapter is restricted to the GC electrode.

The CVs on GC in Figure 1 illustrate that the current observed for the second process becomes smaller in relation to the first process as the scan rate is increased. It has previously been observed in quinone electrochemistry that the height of the second peak is often less than the first and it has been explained based on a complexation reaction between the dianion and the starting material (eq 3).¹⁵

For eq 3 to be observable voltammetrically, it requires that the thermodynamically favorable monomerization reaction to form the anion radicals (eq 4, $k_{f(2)}$) to be relatively slow compared to the complexation reaction (eq 3, $k_{f(1)}$). Furthermore, the dimerization reaction of the anion radicals (eq 4, $k_{b(2)}$) (which is a mechanism known to occur for the anion radicals of 9-substituted anthracenes²⁴ and aromatic esters²⁵) must be slower than the monomerization reaction. The simulations indicate that the complexation reaction

(eq 3) is feasible, although the kinetic values are only approximate because the observed $E_{1/2}^r$ -values (and hence the kinetic data) are heavily influenced by the exact solvent composition.

Controlled potential electrolysis experiments were conducted on VK_1 in order to determine the lifetimes of the reduced states and to confirm the number of electrons transferred during the reduction processes over longer times. Figure 2 shows CVs and coulometry data obtained at various intervals during the reduction of VK_1 in CH_3CN containing 0.10 (± 0.01) M water. Under electrolysis conditions it was very difficult to further reduce the amount of water present because of the requirement of multiple compartments in the electrolysis cell.

Figure 2. Voltammetric and coulometric data obtained at 293 K during the controlled potential electrolysis of 5 mM VK_1 in CH_3CN with 0.2 M Bu_4NPF_6 and 0.10 (± 0.01) M H_2O (a) CVs recorded at a scan rate of 0.1 V s^{-1} with a 1 mm diameter GC electrode. (Black line) Prior to the bulk reduction of VK_1 . (Red line) After the exhaustive reduction of VK_1 at an applied potential of $-1.4 \text{ V vs. Fc/Fc}^+$. (Blue line) After the reduction of $VK_1^{\cdot-}$ at an applied potential of $-1.8 \text{ V vs. Fc/Fc}^+$. (b) Current/coulometry vs. time data obtained during the exhaustive reduction of VK_1 at $-1.4 \text{ V vs. Fc/Fc}^+$. (c) Current/coulometry vs. time data obtained during the exhaustive reduction of $VK_1^{\cdot-}$ at $-1.8 \text{ V vs. Fc/Fc}^+$. N is the number of moles of VK_1 .

Figure 2a shows CVs obtained before (black line) and after the one-electron (red line) and two-electron (blue line) reduction of VK₁. For both processes (E_1 and E_2) the total current measured between the i_p^{red} and i_p^{ox} -values remained constant indicating that the reduced forms were stable on the time-scale of the electrolysis experiments. The current values observed during CV experiments were offset in the positive current direction after the electrolysis experiments were commenced due to the change in oxidation state of the VK₁ species present in the bulk solution. Figures 2b and 2c show the corresponding current and coulometry data obtained during the electrolysis of VK₁ and VK₁^{•-} respectively, confirming that one-electron per molecule was transferred in each electrochemical step.

It was found that the electrochemistry of VK₁ was affected by low levels of dissolved molecular oxygen. Oxygen can be electrochemically reduced by one-electron to superoxide at $-1.25\text{ V } (\pm 0.2)$ vs. Fc/Fc⁺ in CH₃CN, with a half-life of around 1 hour at 20 °C.²⁶ Figures 3a(i) and 3b(i) show a SWV and CV respectively of the reduction of dissolved molecular oxygen present in atmospheric concentrations (2 – 3 mM). When 0.5 mM of VK₁ was added to solutions containing atmospheric concentrations of O₂, SWV experiments indicated that the first cathodic peak shifted to more positive potentials by $\sim +30\text{ mV}$ and the peak current increased (Figure 3a(ii)). Thus, the SWV results indicate that the first reduction process (E_1) for VK₁ in CH₃CN (containing relatively low levels of H₂O) is sufficiently close to the formal reduction potential of O₂

to render their reduction processes indistinguishable from each other in CH_3CN containing both species (VK_1 and O_2).

It was also observed that the second reduction process of VK_1 (E_2) appeared chemically irreversible when CVs were performed in solutions of VK_1 in the presence of O_2 (Figure 3b(iii) and (iv)). It was only when the solution had been extensively deoxygenated that the first and second reduction processes both appeared fully chemically reversible during CV experiments (Figure 3b(v)). The amount of time needed for deoxygenating depended on the concentration of VK_1 , the rate of purging of the argon gas and the solution volume. The higher the concentration of VK_1 the less purging time was required because the amount of dissolved oxygen was relatively lower compared to the VK_1 concentration. Under our conditions it was found that 10 minutes purging was required to remove molecular oxygen.

At very low concentrations of VK_1 ($< 0.1 \text{ mM}$) it was found that the second process appeared much smaller than the first process (ratio $< 0.2 : 1$), which is possibly caused by residual O_2 (or $\text{O}_2^{\cdot-}$) reacting with $\text{VK}_1^{\cdot-}$ (or VK_1), which could not be satisfactorily removed with purging. Furthermore, during SWV experiments at VK_1 concentrations $> 0.5 \text{ mM}$, the second reduction process always appeared smaller than the first (ratio of $0.8 : 1$), which is likely to be caused by the slower heterogeneous electron transfer rate for the second process ($k_{s(2)} \approx 0.1 \text{ cm s}^{-1}$) and the complexation reaction of the dianion with the quinone (eq 4) affecting the peak height of the SWV.

Figure 3. Voltammograms recorded at a 1 mm diameter planar GC electrode in CH_3CN containing $0.2 \text{ M Bu}_4\text{NPF}_6$ and $0.05 (\pm 0.01) \text{ M H}_2\text{O}$ at 293 K showing the effect of dissolved molecular oxygen on the voltammetry of 0.5 mM VK_1 . (a) SWVs recorded with a pulse period $(\tau) = 25 \text{ Hz}$, a potential step $= 2 \text{ mV}$ and a pulse amplitude $= 20 \text{ mV}$. (b) CVs recorded at a scan rate of 100 mV s^{-1} .

Figure 4 shows CVs obtained for VK_1 in carefully deoxygenated solutions of CH_3CN in the presence of varying H_2O concentrations. It can be observed that both the

first and second processes shift to more positive potentials with increasing water concentrations, but the second process shifts by a greater amount ($\Delta E_2 > \Delta E_1$) so that the processes eventually merge into one at a H₂O concentration of approximately 7.2 M (corresponding to a two-electron reduction where the two one-electron transfers occur in rapid succession at similar potentials).

Figure 4. CVs recorded of VK₁ (initial concentration = 1.0 mM, final concentration = 0.83 mM) at a scan rate of 0.1 V s⁻¹ at a 1 mm diameter planar GC electrode in CH₃CN containing 0.2 M Bu₄NPF₆ at 293 K, with varying concentrations of water.

Based on the CV results it can be concluded that both the monoanion and dianion undergo hydrogen bonding with water in the solvent, but the very large shift observed for the second process would suggest that the dianion undergoes much stronger hydrogen bonding. It would be expected that the more negatively charged oxygen atoms in the dianion are more likely to interact with water molecules than the less negatively charged oxygen atoms in the monoanion. Theoretical calculations²⁷ on quinone anion radicals and dianions have indicated that the oxygen atoms do have an increasing negative charge density in moving from the neutral molecule to the semiquinone and then dianion.^{27c} Due to the relatively large shift in potential of E_2 with small additions of water, it is questionable whether the dianion actually exist in "non-aqueous" solvents in a non-hydrogen bonded form, since the water content of the solvent is usually much greater than that of the analyte.²¹

3.2.2 In-situ UV-Vis Spectroscopy at Intermediate (0.05 M) to High (10 M) Water Concentrations

In order to further investigate the degree of hydrogen bonding in the monoanion radical and dianion, *in-situ* electrochemical-UV-Vis spectroscopy experiments were conducted on VK₁ in CH₃CN in the presence of varying concentrations of water (0.05, 0.55, 2.14, 5.05 and 9.26 M H₂O). Figure 5 shows the spectra obtained (a) prior to reduction, after the (b) first and (c) second reduction steps and (d) after the reduced compounds had been oxidized back to the starting material. Irrespective of the

concentration of water, it was found that the UV-Vis spectrum of the starting material could be obtained after the reoxidation of the anion/dianion, indicating that the reduced forms were stable on the time-scale of the experiments (~30 minutes).

The cyclic voltammograms shown in Figure 4 illustrate that there is a relatively small change in E_1 for water concentrations between 0.05 – 0.5 M. Therefore, it would be expected that with a H₂O concentration of 0.05 M, the anion radical would exist in a predominantly non-hydrogen bonded form. Thus, the spectrum of the one-electron reduced form of VK₁ with 0.05 M water is likely to be the non-hydrogen bonded semiquinone radical (Figure 5b, red line). As the concentration of water was increased to 0.55 M, a relatively small change in the UV-Vis spectrum of the singly reduced species was observed (Figure 5b, green line). As the concentration of water was increased > 0.55 M, the spectra began to change substantially, due to increased hydrogen bonding of the semiquinone. It was observed that the band at 485 nm decreased in intensity faster than the band at 400 nm as the water concentration increased, while the sharp band at 300 nm (that appeared characteristic of the non-hydrogen bonded semiquinone) decreased in intensity with increasing water concentration. The UV-Vis results in Figure 5b are consistent with equilibrium between the hydrogen bonded and non-hydrogen bonded semiquinone.

The UV-Vis spectra obtained after the second one-electron reduction step (two-electrons overall) are given in Figure 5c. Three bands were detected which all moved to lower wavelength with increasing concentration of water, with the greatest

shift observed for the band at the highest wavelength. The position of the bands remained close to constant as the water content increased above 5 M H₂O. Based on the large shifts in potential observed with increasing water concentrations (Figure 4), it is likely that under the present experimental conditions the dianion *only* exists in the bulk solution in a hydrogen bonded form (unlike the situation observed for the semiquinone radical). The UV-Vis spectra did not display any substantial differences in appearance when the temperature was varied between 293 – 253 K, indicating that any equilibria between the hydrogen bonded species responsible for the absorbances in Figure 5 were not strongly sensitive to changes in temperature.

It is interesting that even at very high water concentrations (9.26 M), when only one voltammetric wave was observed, small amounts of the non-hydrogen bonded semiquinone can still be detected in the UV-Vis spectrum, *via* the band at 300 nm. Because only one voltammetric process was detected in the presence of 9.26 M water, the UV-Vis spectra of the species responsible for the first and second electron transfer steps could not be controlled by adjusting the applied potential. Instead the spectra were assigned based on the observation that the band at 300 nm initially increased in intensity during the first electron transfer, and then diminished in intensity during the second electron transfer. Thus the spectrum in Figure 5b obtained in the presence of 9.26 M water (black line) was assigned by observing the intensity of the absorbance at 300 nm during the *in-situ* electrolysis.

Figure 5. *In-situ* UV-Vis spectra obtained in an OTTLE cell during the reduction (and reoxidation) of 1 mM VK₁ at 293 K in CH₃CN with 0.2 M Bu₄NPF₆ and varying concentrations of water.

Based on the electrochemical and spectroscopic results in Figures 4 and 5, a mechanism can be proposed for the fates of the reduced species in CH_3CN containing varying concentrations of water, which is essentially a "square scheme" mechanism³ where the normal proton transfers are replaced with hydrogen bonding interactions (Scheme 2). The initial one-electron reduction of VK_1 produces $\text{VK}_1^{\cdot-}$ which undergoes little hydrogen bonding at water concentrations < 0.5 M. As the water concentration increases above 0.5 M, $\text{VK}_1^{\cdot-}$ reacts to form the hydrogen bonded species, $\text{VK}_1^{\cdot-}(\text{H}_2\text{O})_{a,b}$. The number of water molecules undergoing hydrogen bonding to the oxygen atoms increases with increasing water concentrations, according to the equilibrium between the hydrogen and non-hydrogen bonded forms. Since the two oxygen atoms in VK_1 are non-equivalent, it is possible that the oxygen atoms undergo differing degrees of interaction with H_2O (hence the existence two hydrogen bonded forms; $\text{VK}_1^{\cdot-}(\text{H}_2\text{O})_a$ and $\text{VK}_1^{\cdot-}(\text{H}_2\text{O})_b$).

$\text{VK}_1^{\cdot-}$ (or $\text{VK}_1^{\cdot-}(\text{H}_2\text{O})_{a,b}$) can be further reduced by one-electron to form VK_1^{2-} which *immediately* undergoes hydrogen bonding, even at intermediate water concentrations (0.05 M). The electron transfer step could occur consecutively (horizontal lines) with the hydrogen bonding (Scheme 2).²⁸ The spectroscopic experiments indicate that the amount of hydrogen bonding is influenced by the concentration of water, thus the UV-Vis spectra display different extremes. At low water concentrations (≤ 0.05 M) $\text{VK}_1^{2-}(\text{H}_2\text{O})_{n,m}$ exists, while $\text{VK}_1^{2-}(\text{H}_2\text{O})_{x,y}$ exists at high water concentrations (≥ 5 M) with the H_2O coefficients $n,m < x,y$ (Scheme 2). An

equilibrium exists between the hydrogen bonded forms at intermediate water concentrations. A summary of the spectra of the different species are given in Figure 6, which were obtained under conditions that most favored their formation. A pure spectrum of $\text{VK}_1^-(\text{H}_2\text{O})_{a,b}$ was not obtained since it always existed in equilibrium with VK_1^- . The hydrogen bonding between water and the VK_1^- anions is likely to involve a fluctuating network of flickering (not static) hydrogen bonds. Therefore, it is difficult to determine the exact number of water molecules involved in the interactions, but the numbers certainly vary depending on the water concentration as shown by how the voltammetry and UV-Vis spectra vary as the water content is changed.

Scheme 2. Electrochemical reduction and hydrogen bonding mechanism for VK_1 in aprotic organic solvents containing water. The counter ion for the charged species is the supporting electrolyte cation, Bu_4N^+ .

Figure 6. *In-situ* UV-Vis spectra obtained in an OSTLE cell during the reduction of 1 mM VK₁ in CH₃CN with 0.2 M Bu₄NPF₆ and varying concentrations of water. The UV-Vis spectra of VK₁^{•-} and VK₁²⁻(H₂O)_{n,m} were obtained *via* the one- and two-electron respective reductions of VK₁ in the presence of 0.05 (± 0.01) M H₂O. The UV-Vis spectrum of VK₁²⁻(H₂O)_{x,y} was obtained *via* the two-electron reduction of VK₁ in the presence of 9.26 M H₂O.

Figure 7. Electrochemical and spectroscopic data obtained during the electrolysis of 1 mM VK_1 in CH_3CN containing 0.2 M Bu_4NPF_6 and 9.26 H_2O at 293 K. (a) Cyclic voltammograms obtained at a 1 mm diameter planar GC electrode. (Black line) Prior to the electrolysis of VK_1 . (Red line). After the 2-electron exhaustive reductive electrolysis of VK_1 (to form $\text{VK}_1^{2-}(\text{H}_2\text{O})_{x,y}$) and the addition of 0.05 M of $\text{CF}_3\text{SO}_3\text{H}$ (to form VK_1H_2). (b) UV-Vis spectrum of VK_1H_2 that was prepared by electrolyzing VK_1 and then adding $\text{CF}_3\text{SO}_3\text{H}$.

In order to confirm that the reduction process produced the hydrogen bonded species and not the protonated forms, electrolysis experiments were conducted at high water concentrations and the reduced species were then reacted with the strong non-aqueous acid, $\text{CF}_3\text{SO}_3\text{H}$. Figure 7a (black line) shows a CV of 1 mM VK_1 in CH_3CN containing 9.26 H_2O . Figure 7a (red line) shows the CV obtained of the same

solution after the VK₁ had been reduced by two electrons per molecule under bulk controlled potential electrolysis conditions and the resulting dianion was reacted with CF₃SO₃H to form the hydroquinone (VK₁H₂) (eq 5).

The two voltammograms in Figure 7a differ in that the CV for VK₁ (black line) was initially scanned in the negative potential direction (reduction), while the CV for VK₁H₂ was initially scanned in the positive potential direction (oxidation). The voltammetry observed for VK₁H₂ is similar to that observed for other hydroquinones (such as dopamine) in acidic conditions; that is, they can be reversibly oxidized in a two-electron two-proton process with a wide separation between the forward and reverse peaks.^{3a,29} The minor additional processes detected at 0.2 – 0.3 V in the CV of VK₁H₂ are possibly associated with a small amount of an additional reaction product formed during the electrolysis/protonation reaction.

The UV-Vis spectrum of VK₁H₂ is given in Figure 7b, which displays relatively strong and sharp bands at 207 nm and 241 nm and broader weaker bands at 320 and 331 nm. The spectrum is similar to that observed for the closely related 1,4-dihydroxynaphthalene.³⁰

The complete mechanism for the interaction of VK₁ and reduced forms (VK₁^{•-} and VK₁²⁻) (and any other quinone) with H₂O molecules can also be given in the form of Scheme 3. The reaction is an example of an electrochemical square-scheme mechanism,³¹ with electron transfer steps occurring vertically and homogeneous

interactions with H_2O occurring horizontally, each with a corresponding equilibrium constant ($K_{\text{eq,H}_2\text{O}}$). In theory, additional reactions can be added to the right of Scheme 3 if more H_2O molecules are incorporated into the mechanism, since the number of H_2O molecules bonding to $\text{VK}_1^{-\bullet}$ and VK_1^{2-} is not limited to two.

Scheme 3. Electrochemical “square-scheme” mechanism showing hydrogen bonding interactions between VK_1 and its reduced forms with H_2O . “A”, “B” and “C” can represent any quinone, and are not limited to just VK_1 .

The electrochemical mechanism was examined in detail by modeling the experimental data according to the reactions in Scheme 3, using digital simulation software.³² Figure 8 (black lines) shows the experimental cyclic voltammograms of VK_1

in MeCN that could be obtained over a wide water content range and the corresponding digital simulations (red lines). The parameters used in the simulations are provided in Table 2, with the electron transfer and homogeneous chemical reactions given in Scheme 3. Each electron transfer reaction in Scheme 3 has an associated E^0_{f} -value and each homogeneous reaction a corresponding equilibrium constant.

The parameters given in Table 1 represent the best average fits over the entire water concentration range. However, it can be observed from the CVs in Figure 8 that there is substantial deviation between the experimental and simulated voltammograms as the water concentration is decreased or increased either side of the 1.0 M water concentration. It was found that adding additional electron transfer and chemical steps, such as to the right of Scheme 3, did not result in a substantial improvement to the match between the experimental and simulated voltammograms. It was possible to more accurately fit the simulated voltammograms to the experimental data over narrow water concentrations ranges using a particular set of simulation parameters. However, no unique set of simulation parameters fit over the whole water concentration range shown in Figure 8.

Figure 8. CVs of 1×10^{-3} M VK_1 in CH_3CN with 0.2 M Bu_4NPF_6 at 22 ± 2 °C, at a scan rate of 100 mV s^{-1} , at a 1 mm diameter GC electrode and in the presence of varying amounts of H_2O . Digitally simulated voltammograms are based on the mechanism in Scheme 3 and the parameters given in Table 1.

Table 1. Parameters used for digital simulations of voltammetric data.^{a,b}

Heterogeneous electron transfer reactions		Homogeneous chemical reactions		
Reaction	E / V^c	Reaction	K_{eq}	$k_{\text{f}} / \text{L mol}^{-1} \text{s}^{-1}$
$\text{A}_1 + \text{e}^- \rightleftharpoons \text{B}_1$	-0.84	$\text{A}_1 + \text{H}_2\text{O} \rightleftharpoons \text{A}_2$	1.0×10^{-5}	1
$\text{B}_1 + \text{e}^- \rightleftharpoons \text{C}_1$	-1.44	$\text{A}_2 + \text{H}_2\text{O} \rightleftharpoons \text{A}_3$	1.0×10^{-6}	1
$\text{A}_2 + \text{e}^- \rightleftharpoons \text{B}_2$	-0.66	$\text{B}_1 + \text{H}_2\text{O} \rightleftharpoons \text{B}_2$	1.0×10^{-2}	1×10^7
$\text{B}_2 + \text{e}^- \rightleftharpoons \text{C}_2$	-1.12	$\text{B}_2 + \text{H}_2\text{O} \rightleftharpoons \text{B}_3$	1.0×10^{-3}	1×10^7
$\text{A}_3 + \text{e}^- \rightleftharpoons \text{B}_3$	-0.49	$\text{C}_1 + \text{H}_2\text{O} \rightleftharpoons \text{C}_2$	2.5×10^3	1×10^7
$\text{B}_3 + \text{e}^- \rightleftharpoons \text{C}_3$	-0.82	$\text{C}_2 + \text{H}_2\text{O} \rightleftharpoons \text{C}_3$	1.0×10^3	1×10^7

^aThe experimental data and simulations are given in Figure 8 and reaction mechanism in Scheme 3. ^bThe diffusion coefficients of all species were set to $2.0 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ and the heterogeneous electron transfer rate (k_{s}) set to 1 cm s^{-1} . ^cPotential versus silver wire separated from the test solution with a salt bridge containing 0.5 M Bu_4NPF_6 in MeCN.

The reason for the divergence between the experimental data and simulated voltammograms is because of the complexity of the hydrogen bonding mechanism. The reactions in Scheme 3 represent the hydrogen bonding interactions with VK_1 and its reduced forms in MeCN, while other equilibria are also likely to be important. For example, (i) interactions between the solvent and water, (ii) ion-pairing interactions between the VK_1 anions and the supporting electrolyte cations, (iii) ion-pairing interactions that occur between the electrolyte ions themselves, which are likely to be more important in low-dielectric constant solvents,^{33,34} (iv) solvation effects of the

organic solvent with the reduced forms of VK₁. Therefore, due to the large number of interactions and the uncertainties associated with their respective chemical parameters (equilibrium and rate constants) it is extremely difficult to accurately simulate the voltammetric data over a wide water concentration range.

Nevertheless, the trends in simulated values in Table 1 are consistent with values that can be obtained over narrow water concentration ranges. In particular, the equilibrium constants for the hydrogen bonding interactions increase as the negative charge on VK₁ increases. The potential of the electron transfer step shifts to more positive potentials as the number of water molecules undergoing hydrogen bonding increases for each species, indicating that the hydrogen bonded anions are easier to reduce than their analogous non-hydrogen bonded forms.

3.2.3 Electrochemistry at Low (0.001 – 0.05 M) Water Concentrations

Having established the electrochemical and spectroscopic behavior of VK₁ in intermediate to high water concentrations, voltammetric experiments were next conducted at very low water concentrations by carefully drying the solvents and accurately measuring the water content by KF titration (see Chapter 2 for details).

For water concentrations between 0.001 – 0.05 M, H₂O was allowed to increase under natural humidity conditions from the lowest level.²⁰ Even under an argon atmosphere, the water content increased from 0.01 M to 0.05 M within approximately 2 hours for CH₃CN solutions. In order to have the initial water content below 0.05 M, it

was necessary to use a pseudo Pt wire as the reference electrode, located in the same compartment as the working electrode. Therefore, accurate potentials could not be obtained for the first and second processes. However, this was not considered problematic because the low concentration experiments were concerned with measuring the *potential difference* between the first and second processes and these potentials were unlikely to drift on the time-scale of a voltammetric scan (a few seconds). Figure 9a shows a SWV of 1 mM of VK₁ in CH₃CN containing 0.011 (± 0.002) M water. At this concentration the voltammogram shows two clear processes separated by 0.635 V. The second process was always smaller than the first process in SWV experiments because of relatively slow heterogeneous electron transfer.

Figure 9. Voltammetric data recorded at a 1 mm diameter planar GC electrode in CH_3CN with 0.2 M Bu_4NPF_6 at 293 K. CVs were recorded at a scan rate of 0.1 V s^{-1} and SWVs were recorded with a pulse period (τ) = 25 Hz, a potential step = 2 mV and a pulse amplitude = 20 mV. (a) SWVs of 1.0 mM VK_1 with different concentrations of water. (b) CVs of (Red line) 1.0 mM VK_1 and 0.005 (± 0.002) M H_2O , and (Black line) 5.0 mM VK_1 and 0.005 (± 0.002) M H_2O . (c) *In-situ* (—) CV and (----) SWV obtained in a solution containing 1.4 mM VK_1 and 3 Å molecular sieves. (d) Plot of cathodic peak separations measured by SWV versus water content during the electrochemical reduction of 1.0 mM VK_1 .

Interesting voltammetric features were observed when the water concentration was below 0.010 M. Figure 9b (red line) is a CV obtained of 1 mM VK₁ in the presence of 0.005 (± 0.002) M H₂O. While the first process appears as a chemically reversible one electron process, the second process has a wider than normal peak-to-peak separation (200 mV) with broad and non-ideal shaped peaks, and with additional complicated features detected on the reverse oxidation sweep at ~ -0.8 V *vs.* Pt wire. The unusual peak shapes are possibly caused by interactions of the dianions with the electrode surface, due to the low concentrations of water reducing the amount of hydrogen bonding and lowering the solubility of the dianion. Figure 9b (black line) shows the CV of VK₁ at a higher concentration (5 mM) but in the presence of a lower water content ratio (0.005 (± 0.002) M). Similar features were observed to the lower concentration experiment with the second process showing additional features and an extra process evident at -0.8 V *vs.* Pt wire when the scan direction was reversed.

Another feature that is evident in the voltammograms in Figure 9b is that the $|E_1 - E_2|$ -values measured for a 5 mM solution of VK₁ are greater than those for a 1 mM solution of VK₁, for close to equivalent H₂O concentrations ($|E_1 - E_2| = 680$ and 645 mV, respectively). This effect is only observable at concentrations of H₂O $< \sim 0.03$ M, while at H₂O concentrations $> \sim 0.03$ M, the $|E_1 - E_2|$ -values are the same (within experimental error) for different concentrations of VK₁ in CH₃CN measured at equivalent concentrations of H₂O.

Experiments were also conducted at very low water concentrations by performing *in-situ* voltammetric experiments in the presence of 3 Å molecular sieves. Under these conditions it was possible to lower the water content to 0.0015 (± 0.0005) M, almost the same as that of VK₁ (Figure 9c). Interestingly, both the CV and SWV showed the first process, which seemed unchanged from other experiments, but the second process was difficult to detect. This result can be interpreted in two ways; (i) the electron transfer step occurs concertedly with the hydrogen bonding, so if there is insufficient water present the electron transfer does not occur, or (ii) the very low water content results in an unusual adsorptive affect where the reduction of the semiquinone is somehow masked. A small reductive process can be detected close to the potential where the semiquinone is normally reduced, but with a wide peak-to-peak separation (Figure 9c). In light of the results shown in Figure 9b, it is more likely that scenario (ii) occurs, so that the lack of the second process in very low water concentration environments is due to specific interactions of the dianion with the electrode surface.

The results in Figures 9b and 9c indicate that low levels of water make a large difference to the voltammetric behavior of VK₁, especially the second reduction process. It is possible that some of the unusual voltammetric behavior reported for quinones in organic solvents can be explained by low levels of water in the solvent, or by the effects of dissolved molecular oxygen as shown in Figure 3. Lehmann and Evans examined a number of quinones in aprotic media and observed anomalous voltammetric behavior, some of which was of unknown origin.^{17a}

Based on the electrochemical results on VK_1 , it seems appropriate that studies that report on the electrochemical behavior of quinones in non-aqueous solvents should also report the trace water content of the solvent, to enable unambiguous comparisons with different data sets. This is particularly true for studies that examine interactions between reduced quinones and a range of hydrogen bonding additives (such as alcohols and amines), because the trace water content of the solvent could significantly affect the measured $|E_1 - E_2|$ -values.^{15,16} With this point in mind, we have constructed calibration curves of the potential separation between the first and second processes ($E_1 - E_2$) against the water content of the solvent, which allows a straightforward method for determining the concentration of water. Figure 9d shows the aforementioned plot over a wide water content range (0.01 – 9.26 M). The plot illustrates that there is an abrupt increase in the potential separation ($E_1 - E_2$) as the water content approaches zero. From an analytical perspective, the more useful range for measuring the water content is between 0 – 0.1 M, since it is straightforward to exclude water at higher concentrations and in many instances where "dry" solvents are reported the water content could still be in the mM range (more detailed experiments on the electrochemistry of VK_1 in the presence of very low concentrations of water are described in Chapter 4).

The primary error in measuring the water content in this study is systematic and arises from transferring a sample of the test solution from the electrochemical cell into the KF titration cell. During the transfer it is likely that some atmospheric water will enter into the syringe needle, but this is only avoidable if both reactions are performed in

a dry box. However, the observation that there is not an excessive scatter in the slope of the plot in Figure 9d would suggest that the systematic error is not severe. This is because the data in Figure 9d is a combination of a least 3 experimental runs for the solvent, under conditions where the atmospheric humidity varied, but there is no obvious change in slope between the different experiments. With the low water concentration measurements it was not possible to perform more than one measurement at a fixed water concentration (in order to obtain a statistical average) because the water content was continually changing over time.

3.3 Conclusions

This work has demonstrated that differences of a few mM water can have a pronounced affect on the reductive electrochemistry of a quinone in non-aqueous solvents, especially at low water levels (< 0.05 M). Therefore, it is important that electrochemical studies on quinones in non-aqueous solvents report the initial water content of their solvents, since trace amounts of water are likely to interfere with measurements made in the presence of other hydrogen bonding donors (such as alcohols and amines).

The voltammograms and UV-Vis spectra obtained during the one- and two-electron reduction of VK₁ in CH₃CN in the presence of varying H₂O concentrations allowed estimations of the degree of hydrogen bonding. The semiquinone anion radical exists in a predominantly non-hydrogen bonded form at concentrations of water < 0.5 M,

since the UV-Vis spectra and the voltammetric potential (E_1) changed by a small amount when the water concentration was varied below this level. In contrast, the quinone dianion undergoes extensive hydrogen bonding at even very low water concentrations, indicated by how the second voltammetric peak (E_2) shifts substantially over a wide range of water concentration (0.01 – 7.2 M) and by how the bands in the UV-Vis spectra shift uniformly with water concentrations between 0.05 – 5 M. It is unlikely that the UV-Vis spectrum of the non-hydrogen bonded dianion can be obtained unless extremely dry solvents can be used, where $[\text{H}_2\text{O}] \leq [\text{VK}_1]$. The voltammetric behavior became very complicated when the concentration of water began to approach that of the substrate. At these very low water concentrations ($[\text{H}_2\text{O}] < 0.01 \text{ M}$), there is insufficient water for favored hydrogen bonding of the dianion, likely resulting in a decrease in solubility of VK_1^{2-} and hence the appearance of adsorptive processes in the voltammograms.

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Chapter 4

Voltammetric Method for Determining the Trace Moisture Content of Organic Solvents Based on Hydrogen Bonding Interactions between Reduced Vitamin K₁ and H₂O

4.1 Introduction

In aprotic organic solvents, the quinones are able to be electrochemically reduced in two chemically reversible one-electron reduction processes to form first the anion radical ($Q^{\bullet-}$), followed by further reduction to the dianion (Q^{2-}).^{1,2} It has been shown *via* electrochemical³⁻¹² and spectroscopic^{11,14-16} measurements that $Q^{\bullet-}$ and Q^{2-} undergo hydrogen bonding interactions with water or other hydrogen bonding additives (such as alcohols and amines) present in the solvent. The potential of both the first reduction process (E_1) and especially the second reduction process (E_2) shift to more positive potentials with increasing water concentration ($\Delta E_2 > \Delta E_1$), so that at high enough water concentrations the two processes can merge together into one two-electron process, providing that the water content of the solvents can be increased to a sufficiently high ratio. The shift in potential is caused by an equilibrium that exists between $Q^{\bullet-}$ and Q^{2-} , and their hydrogen bonded forms.⁵⁻¹²

The voltammetric behavior of vitamin K₁ (VK₁) in MeCN has previously been described in the presence of varying concentrations of water (Chapter 3).^{11,13} It was established that the greatest change in the difference between E_1 and E_2 ($|E_1 - E_2|$)

occurred at very low levels of water (between 0 – 0.1 M).^{11,13} In this chapter the number of solvents studied have been extended and calibration data that are used to estimate the water content of a solvent are presented. It will be demonstrated that the degree of hydrogen bonding with reduced forms of VK₁ and trace water varies significantly in the different solvents, which requires that the voltammetric data be interpreted based on a complex mixture of solvent-water-substrate-electrolyte interactions. The results have consequences on the way that formal potentials (E^0_f) for semiquinones and perhaps other compounds are reported and evaluated, because the presence of trace water can have an effect on the measured redox potentials.

We were also interested in determining whether it was possible to attach the VK₁ to an electrode surface and thereby use the voltammetry of the attached form as a method for estimating the water content of a solvent (after suitable calibration experiments). Self-assembled monolayers (SAMs) of alkanethiolates on gold have been used as a method to modify electrode surfaces.¹⁷⁻¹⁹ Voltammetry experiments have previously been performed by self-assembling alkanethiols on Au electrodes in aqueous solutions, and incorporating vitamin K inside the alkanethiol layers.^{20,21} An alternative method to attach vitamin K to an electrode surface, is *via* synthesizing a vitamin K derivative containing an alkyl chain with sulfhydryl head group in the 3-position.²⁰ Scheme 1 shows the structures of VK₁ and the VK₁ derivative (VKSH), which is proposed to have similar electrochemical behavior as VK₁.^{22,23,24}

Scheme 1. Forms of vitamin K₁ (VK₁) and derivative of VK₁ with alkanethiol linkage (VKSH).

4.2 Results and Discussion

4.2.1 The Effect of Water on Reduction Processes of Vitamin K₁

Figure 1 shows cyclic voltammograms obtained of VK₁ in a range of organic solvents with different dielectric constant values. Process E_1 corresponds to the one-electron reduction of VK₁ to the semiquinone anion radical (VK₁^{•-}) (eq 1) and process E_2 is the further one-electron reduction of the semiquinone radical to the dianion (VK₁²⁻) (eq 2).^{11,13} $E_{f(1)}^0$ and $E_{f(2)}^0$ refer to the formal electrode potentials, which for quinones are strongly dependant on the water content of the solvent; thus absolute values can only be reported if the water content is accurately known. As water is added to the organic solvents, the potential of $E_{f(1)}^0$ and $E_{f(2)}^0$ shift to more positive values due to hydrogen bonding interactions.^{11,13}

The differences in reductive peak current (i_p^{red}) for the first process (E_1) in the CVs shown in Figure 1 are mainly due to differences in diffusion coefficient of VK₁ in the different solvents. The peak currents measured in DMSO and 1,1,2,2-tetrachloroethane (TCE) are relatively small compared to the other solvents due to their high viscosities. The CV performed on VK₁ in TCE showed only one chemically irreversible reduction process, indicating that the reduced form was short-lived. Surprisingly, only one reductive process was observed in propionitrile (EtCN) (even at water concentrations < 0.01 M) with a relatively high i_p^{red} -value compared to MeCN and butyronitrile (PrCN). Coulometry experiments on VK₁ in EtCN indicated the transfer of 1.5 electrons per molecule, suggesting that the electrochemical reduction mechanism was more complicated than simple electron transfer.

Figure 1. CVs obtained of 1 mM solutions of VK₁ (with 0.2 M Bu₄NPF₆ as the supporting electrolyte) at a 1 mm diameter glassy carbon (GC) electrode at 22 ± 2 °C and at a scan rate of 100 mV s⁻¹. Water contents of the solvents are between 0.01 – 0.05 M.

The mechanism for the interaction of VK₁ and reduced forms (VK₁^{•-} and VK₁²⁻) with H₂O molecules is given in Scheme 3 in Chapter 3 (Page 68). For the solvents in Figure 1, where two one-electron reduction processes were detected, SWV experiments were performed and the $|E_1 - E_2|$ -values were measured over a range of water concentrations (E_1 and E_2 were measured as the cathodic peak potentials (E_p^{red})²⁶). Water is highly soluble in MeCN, DMF and DMSO; therefore, voltammetric data could be obtained over a very wide range of H₂O concentrations (Figure 2a). The results in

Figure 2a show a large steepening of the plots as the H_2O concentration approaches zero. In MeCN, the E_1 and E_2 processes cannot be distinguished from each other at a water concentration of approximately 7 M.^{11,13}

For DMSO and DMF the voltammetry showed additional complicated features at high H_2O concentrations possibly caused by adsorption effects, therefore, the data collection was stopped before the $|E_1 - E_2|$ -values reached zero. A plot giving the log transformation of the water concentrations in Figure 2a is provided in Figure 2b, which has the effect of spreading the low H_2O concentration values over a wider range and improving the linearity at low concentrations (although the log plots are not linear over the entire concentration range).

The water concentration of the solvent/electrolyte/VK₁ mixture contained within the syringe could be lowered to approximately 0.001 M (depending on the solvent). However, as soon as the solvent/electrolyte/VK₁ was added to the electrochemical cell, the water levels increased (even with the cell maintained under an argon atmosphere), with the rate of increase dependent on the particular solvent. Water solubility in PrCN, dichloromethane (DCM) and 1,2-dichloroethane (DCE) is much lower than in MeCN, DMF and DMSO. It was found that when PrCN, DCM or DCE were added to the electrochemical cell, the measured water concentrations increased from approximately 0.002 M (0.01 M in PrCN) to a maximum of 0.025 M in around two hours. DMF and DMSO are considerably more hygroscopic and absorb water quickly within the electrochemical cell; hence the starting concentrations of H_2O were approximately 0.020 M.

Figure 2. Plots of $|E_1 - E_2|$ (measured by SWV at a 1 mm diameter GC electrode) versus the water concentration (measured by KF titration) at 22 ± 2 °C for 1 mM solutions of VK_1 in different solvents. For (a), (b) and (c) the concentration of Bu_4NPF_6 is 0.2 M, while for (d) the data were obtained in DCM with varying concentrations of Bu_4NPF_6 .

The $|E_1 - E_2|$ -values obtained for all of the solvents plotted against the log of the water concentration (over a low concentration range of H_2O) are given in Figure 2c. For

each of the solvents tested, the change in the $|E_1 - E_2|$ -values with change in water concentration mainly came about due to the positive potential shift of the E_2 process. The E_1 process was considerably less sensitive to changes in the water concentration.

The plots in Figure 2c are summarized in equation form in Table 1. The data in Table 1 provide a simple procedure to estimating the water content of the solvent by measuring the $|E_1 - E_2|$ -values and applying the equation. It is preferable that the VK_1 concentration is close to 1 mM to avoid concentration dependant deviations of the straight lines at very low water concentrations.¹¹ $|E_1 - E_2|$ -values above or below the usable range given in Table 1 are either drier or wetter, respectively, than the given water content range (some extrapolation outside the usable range may be permissible). Recording a single voltammogram in the presence of VK_1 and applying the constants in Table 1 provides a simple method for determining the water content of solvents used during electrochemical experiments, in a similar fashion that ferrocene is often used as an internal calibrant for the potential. As well as measuring the water content during the reduction of quinones,¹⁻¹⁶ the method may be valuable for any process where the redox active species are suspected of undergoing reactions with trace water, such as during the reduction of aromatic esters^{27,28} and the oxidation of vitamin E.^{29,30}

The uncompensated solution resistance is not expected to reduce the accuracy of the measured $|E_1 - E_2|$ -values because each individual potential will be uniformly effected. Furthermore, providing low scan rates are used for the CV or SWV experiments, there will be minimal distortion of the voltammograms due to resistance effects.

Table 1. Solvent parameters and experimentally derived constants (a and b) that can be used to estimate the water content of a solvent (between specified ranges) according to the equation:^a

$$\text{water content (mol L}^{-1}\text{)} = 10^{(a \times |E_1 - E_2|) + b}$$

Solvent	Dielectric constant	Normalized solvent basicity ^b	$a / (\log_{10} M) \text{ V}^{-1}$	$b / \log_{10} M$	Calibrated potential range $ E_1 - E_2 / \text{V}$	Calibrated water content range / mol L ⁻¹
DMSO	46.7	0.647	-13.107	8.433	0.770-0.675	0.020-0.400
DMF	36.7	0.613	-10.096	6.045	0.770-0.640	0.020-0.400
PrCN	20.3	0.365	-6.300	2.375	0.685-0.630	0.010-0.025
MeCN	37.5	0.286	-6.183	2.118	0.660-0.410	0.010-0.400
DCM	9.1	0.178	-7.269	1.748	0.610-0.470	0.002-0.020
DCE	10.4	0.126	-8.976	2.092	0.530-0.410	0.002-0.025

^aThe concentration of VK₁ used for obtaining the $|E_1 - E_2|$ -values should equal 1.0×10^{-3} ($\pm 0.2 \times 10^{-3}$) M and the supporting electrolyte (Bu₄NPF₆) should equal 0.2 M. ^bFrom reference 32.

A useful feature of the proposed voltammetric method for determining the water content of the solvents is that the procedure is independent of the nature of the reference electrode, since a potential difference is measured between two redox processes within the same compound. Furthermore, it was found that the $|E_1 - E_2|$ -values were not strongly influenced by the solution resistance effects, since experiments performed with and without *IR* compensation yielded the same values for $|E_1 - E_2|$ at equivalent water concentrations. The reason for this is because the voltammetric effects of solution resistance would be expected to affect both E_1 and E_2 processes equally, so the difference between the two processes remains the same as the solution resistance increases, at least for the slow scan rates used in this study. The effects of solution resistance are also reduced by performing the voltammetry using relatively low concentrations of VK_1 and high concentrations of the supporting electrolyte.

The results in Figure 2 illustrate that there is a large difference in the $|E_1 - E_2|$ -values between the different solvents for equivalent concentrations of water. It can be reasoned that any interactions between $VK_1^{\cdot-}/VK_1^{2-}$ and the solvent molecules are very low, because small additions of H_2O are able to substantially decrease the $|E_1 - E_2|$ -values even in the presence of vastly outnumbering solvent molecules. The differences in the data shown in Figure 2 between the different solvents can be explained based on how interacting the solvent is with water, which indirectly effects how H_2O is able to undergo hydrogen bonding with $VK_1^{\cdot-}$ and VK_1^{2-} . For example, DMSO and DMF are known to interact relatively strongly with water,^{31,32} while solvents with low dielectric constants (ϵ) such as DCM and DCE do not readily interact

with water (being poorly soluble). Therefore, $\text{VK}_1^{-\bullet}$ and VK_1^{2-} are able to undergo more hydrogen bonding with equivalent concentrations of water in DCE compared to in DMSO, because in DMSO the free water concentration is effectively reduced because of extensive interactions with the solvent.

The plots in Figure 2 show that a $|E_1 - E_2|$ -value of 0.5 V occurs for a water concentration of 0.005 M in DCE, and for a water concentration of 3.0 M in DMSO. If the $|E_1 - E_2|$ -values are used as an absolute measure of the degree of hydrogen bonding, it can be interpreted that H_2O is 600 times more effective at hydrogen bonding with $\text{VK}_1^{-\bullet}/\text{VK}_1^{2-}$ in DCE compared to in DMSO (due to the DMSO more strongly interacting with H_2O than DCE). An interesting consequence of the differing reactivities is that even though DCE is less hygroscopic than DMSO and so easier to keep dry, the water that exists in DCE is more reactive (at least to hydrogen bonding) than higher concentrations of water in DMSO.

An alternative parameter that can be used to estimate the degree of interactions between the solvent and water is the experimentally derived normalized solvent basicity scale, whose values for the different solvents follow the order DCE (0.126) < DCM (0.178) < MeCN (0.286) < PrCN (0.365) < DMF (0.613) < DMSO (0.647).³³ It would be expected that the higher the solvent on the basicity scale, the more it is likely to undergo hydrogen bonding reactions itself with water, and consequently the less interactions between reduced forms of VK_1 and water occur. In support of this, the data in Figure 2c show that the solvent basicity scale does appear to correlate inversely with the order of interactions between reduced forms of VK_1 and water, and a similar

correlation exists with the solvent donicity or donor number (DN) scale.³⁴ An additional complication to interpreting the data is the extent that the solvent interacts with the water molecules can vary in the presence of the radical anions and dianions. Because the water molecules are reacting as Lewis acids for H-bonding with radical anions and dianions, the amphoteric (Lewis acid and Lewis base) properties of the solvents may also be important.³⁵

In addition to interactions between the solvent-water-analyte, it would also be expected that the Bu_4N^+ cation would interact as an ion-pair with the charged compounds and thereby reduce the water interactions with $\text{VK}_1^{\cdot-}$ and VK_1^{2-} , especially in solvents with low dielectric constants.^{36,37} Thus the data in Figure 2d demonstrate that as the concentration of Bu_4NPF_6 increases, the $|E_1 - E_2|$ -values also increase, due to the supporting electrolyte ions reducing the hydrogen bonding interactions between water and $\text{VK}_1^{\cdot-}/\text{VK}_1^{2-}$. Therefore, in order to use the calibration data in Table 1, it is important that the supporting electrolyte concentration equals 0.2 M.

4.2.2 Reduction Mechanism

The shift in observed potential (E_{obs}) for both the E_1 and E_2 processes with increasing water concentration can be accounted for by the equilibrium that occurs between the reduced forms of the VK_1 and the H_2O molecules. The E_{obs} can be measured from the mid-point of the E_p^{red} and E_p^{ox} peaks during CV experiments, or from the E_p^{red} peak during SWV experiments.²⁶ If the equilibrium reaction between the reduced forms of VK_1 with H_2O strongly favors the hydrogen bonded forms, then the

Nernst equations for the reduction reactions can be given by equations 3 and 4;

$$E_{1(\text{obs})} = E_{f(1)}^0 - \frac{2.303RT}{nF} \times \log \frac{[(VK_1(H_2O)_x)^{-}]}{[VK_1][H_2O]^x} \quad (3)$$

$$E_{2(\text{obs})} = E_{f(2)}^0 - \frac{2.303RT}{nF} \times \log \frac{[(VK_1(H_2O)_{x+y})^{2-}]}{[(VK_1(H_2O)_x)^{-}][H_2O]^y} \quad (4)$$

where n is the number of electrons transferred in each step (1), R is the gas constant ($8.3143 \text{ J K}^{-1} \text{ mol}^{-1}$), T is the temperature (in K) and F is the Faraday constant (96485 C mol^{-1}). However, the electrochemical experiments indicated that the potential of the first reduction process was weakly affected by the addition of water, while the second reduction process was strongly affected by the addition of even very small quantities of water. Therefore, process E_2 is likely to be more accurately represented by equation 5 at low water concentrations,

$$E_{2(\text{obs})} = E_{f(2)}^0 - \frac{2.303RT}{nF} \times \log \frac{[(VK_1(H_2O)_z)^{2-}]}{[VK_1^{-}][H_2O]^z} \quad (5)$$

where the equilibrium expression involves the one-electron reduction of the non-hydrogen bonded VK_1^{-} to form VK_1^{2-} , which then undergoes immediate hydrogen bonding with z molecules of H_2O (at high water concentrations, the equilibrium expressions for the hydrogen bonding reactions need to be incorporated into the Nernst equation^{3,9,36,39,40}).

At low water concentrations, such as for the data given in Figure 2c, the change in the $|E_1 - E_2|$ -values with varying water concentration are entirely due to the shift in E_2 , therefore, equation 5 can be used to estimate the number of water molecules involved in the reaction. Equation 5 predicts that the slope of the plots in Figure 2c should increase (become more negative) by -0.059 V for every molecule of water involved in hydrogen bonding. The slopes of the plots in Figure 2c are given as the inverse of the a values in

Table 1 and vary from -0.076 in DMSO to -0.162 in CH_3CN , suggesting between 1 – 3 molecules involved in the reaction. The values are only approximate because eq 5 does not account for the interactions between the solvent and water, and ion-pairing effects, which are also known to occur. The data in MeCN show a substantial steepening of the plots at higher water concentrations suggesting more water molecules are involved in hydrogen bonding, although the interpretation of the data becomes more complicated because the monoanion also undergoes hydrogen bonding with H_2O .

4.2.3 Effect of Water on Reduction Processes of 7,7,8,8-Tetracyanoquinodimethane (TCNQ)

We were interested in determining whether the reduction potentials of other compounds were as affected by the presence of trace water as VK_1 (and other quinones). TCNQ was chosen as a comparison molecule because it undergoes two chemically reversible one-electron reduction processes, because its reduced forms are known to be long-lived in the presence of water, and because it contains nitrogen atoms which could undergo hydrogen bonding interactions.³⁷ SWVs obtained during the reduction of TCNQ in MeCN are given in Figure 3a which show that the second reduction process (E_2) does shift positively with increasing water concentrations, although the first process (E_1) was barely observed to shift. The change in the $|E_1 - E_2|$ -values with varying water content are considerably less than observed during the reduction of VK_1 , as shown in the plot in Figure 3b for TCNQ and in Figure 2a for VK_1 , plotted over the same scale. The peak currents in the SWVs shown in Figure 3a appear to initially

increase in intensity as water is added, but this is brought about by a small concentration change due to some evaporation of the solvent. The decrease in peak current values of the SWVs at high concentrations of water shown in Figure 3a are due to dilution effects as more water is added to the MeCN solution.

Figure 3. (a) SWVs obtained during the reduction of 1.0 mM TCNQ in MeCN containing varying amounts of water and 0.2 M Bu_4NPF_6 at a 1 mm diameter GC electrode at 22 ± 2 °C. Recorded with a pulse period (τ) = 25 Hz, a potential step = 2 mV and a pulse amplitude = 20 mV. (b) and (c) Plots of $|E_1 - E_2|$ (measured by SWV at a 1 mm diameter GC electrode) versus the water concentration (measured by KF titration) at 22 ± 2 °C for 1 mM solutions of TCNQ in MeCN or DCM containing 0.2 M Bu_4NPF_6 .

Experiments were also performed on the reduction of TCNQ in the presence of very low levels of water and the plots of the $|E_1 - E_2|$ -values with varying water content for the solvents MeCN and DCM are shown in Figure 3c. In contrast to the results obtained for VK₁ (Figure 2c), the plots for TCNQ show no change (within experimental error) in $|E_1 - E_2|$ -values at low water concentrations. The results in Figure 3c also show that the $|E_1 - E_2|$ -value at a particular water concentration is slightly greater for DCM compared to MeCN, which is the reverse effect observed for VK₁. The difference between the data obtained for TCNQ and VK₁ can be rationalized if the interactions between TCNQ and the solvents (i.e. solvation effects) are more important than interactions between TCNQ (and its reduced forms) with trace water. Therefore, the effects of trace water on the reduction or oxidation potentials of compounds need to be assessed on a case-by-case basis, although it is likely that for most compounds the presence of trace water has a small effect on the measured redox potentials.

4.2.4 Method for Coating Gold Electrode and Voltammetry of Vitamin K-SH

The following discussion refers to the procedure that was used in order to attach VK₁ to an electrode surface. The aim of this approach was to see whether the immobilized VK₁ gave a voltammetric response that was similar to the diffusional controlled voltammetric behavior. The gold electrode was first polished carefully with 3 μm and 1 μm grit alumina oxide powder on polishing pad and then sonicated in deionised water for 15 min followed by EtOH for 3 min. After the electrode was cleaned with hot sulfuric acid and rinsed with water, 5 scans were taken in the range of

-0.3 V to 1.5 V (vs. Ag/AgCl) in freshly prepared deoxygenated 0.5 M H₂SO₄. The electrode was then rinsed with water and EtOH, dried in air, and immersed finally into an ethanolic solution with 2mM VKSH overnight. Before electrochemical measurements, the electrode was rinsed with EtOH and dried in a stream of nitrogen. Or a 2 μ L aliquot of the ethanolic solution of 2 mM VKSH was deposited on top of the Au electrode surface and the EtOH allowed to evaporate under vacuum or in air, to produce a SAM.

It was believed that an alternative voltammetric method that could be used to measure the water content of a solvent (after appropriate calibration studies), was to attach the VK₁ to an electrode surface and then immerse the electrode into the solution (rather than adding the VK₁ directly to the solution). Therefore, to produce SAMs on top of the Au electrode surface, three methods were tried. One method involved immersing an Au electrode in an ethanolic solution of 2 mM VKSH overnight. The second method consisted of dropping 2 μ L of an ethanolic solution of 2 mM VKSH onto the surface of the Au electrode, and drying the electrode under vacuum. The third method consisted of dropping 2 μ L of an ethanolic solution of 2 mM VKSH onto the surface of the Au electrode surface and drying in air.

Figure 3 shows the CVs recorded in CH₃CN with 0.2 M Bu₄NPF₆ as supporting electrolyte with an Au electrode that had been modified with a SAM by coating it from an ethanolic solution containing 2 mM VKSH (according to the three methods). In each case, the first (least negative) process corresponds to the one-electron reduction of VKSH to the semiquinone anion radical (VKSH^{•-}) (eq 6), and the second process is the

further one-electron reduction of the semiquinone radical to the dianion (VKSH^{2-}) (eq 7).^{11,13} $E_{f(3)}^0$ and $E_{f(4)}^0$ refer to the formal electrode potentials, that are analogous to the $E_{f(1)}^0$ and $E_{f(2)}^0$ processes for solution phase VK_1 (eqs 1 and 2). The mechanism for the interaction of VKSH and reduced forms ($\text{VKSH}^{\cdot-}$ and VKSH^{2-}) with H_2O molecules is given in Scheme 3 (essentially the same as in Scheme 2 in Chapter 3).

The CVs in Figure 3 illustrate that the anodic and cathodic peak current ratio ($i_p^{\text{ox}}/i_p^{\text{red}}$) of the first process are close to unity for the second and subsequent scans for each method. However, the second process appears only partly chemically reversible because only the reduction peak is observed. The CVs in Figure 3 indicate that the results from each coating method were similar. The peak current for the first process observed on the first scan was larger than the subsequent scans, while the voltammograms observed on scans 2 – 5 closely overlapped, indicating that the coated electrodes were fairly robust. The reason for the apparent chemical irreversibility of the second process is not clear, but it does not appear to relate to chemical stability of the tethered dianion otherwise the voltammograms would be expected to show large differences when multiple scans were performed. The CVs in Figure 3 were obtained using a pseudo Pt wire reference electrode; therefore, the potentials differences observe between the different methods are not significant.

Figure 3. Cyclic voltammograms (5 scans) recorded at a scan rate of 0.1 V s^{-1} of films of VKSH deposited on a 3 mm diameter planar Au electrode at $22 \pm 2 \text{ }^{\circ}\text{C}$. (a) Immerse Au electrode in an ethanolic solution of 2 mM VKSH overnight. (b) Drop $2 \text{ }\mu\text{L}$ of ethanolic solution of 2 mM VKSH on top of the Au electrode surface, dry under vacuum. (c) Drop $2 \text{ }\mu\text{L}$ of ethanolic solution of 2 mM VKSH on top of the Au electrode surface, dry in air. Red is the first scan, black are the intermediate scans and blue is the final scan.

Scheme 3. Mechanism shows hydrogen-bonding interactions between VKSH and its reduced forms with H_2O .

Figure 4. CVs recorded at a scan rate of 0.1 V s^{-1} of films of VKSH deposited on a 3 mm diameter planar Au electrode at $22 \pm 2^\circ\text{C}$. (a) CH_3CN with $0.2 \text{ M Bu}_4\text{NPF}_6$ as the supporting electrolyte (Fifth scan of 5 scans). (b) CH_2Cl_2 with $0.2 \text{ M Bu}_4\text{NPF}_6$ as the supporting electrolyte. Red is the lowest water concentration, black is intermediate water concentration and blue is the highest water concentration.

Figure 5. Plots of $|E_3 - E_4|$ (measured by SWV at a 3 mm diameter Au electrode) vs. the water concentration (measured by KF titration) at 22 ± 2 °C for 2 mM solution of VKSH in different solvents with 0.2 M Bu_4NPF_6 as the supporting electrolyte. Red is CH_3CN and blue is CH_2Cl_2 .

Voltammetric experiments were performed by adding water to organic solvents that contained a gold electrode coated with VKSH, in order to determine whether the coated electrode displayed the same trend (in terms of the position of the E_3 and E_4 processes) as when the VK_1 was in solution. Figure 4 shows CVs obtained for SAMs of VKSH in carefully deoxygenated solutions of CH_3CN and CH_2Cl_2 in the presence of varying H_2O concentrations. It can be observed that both the first and second processes shift to more positive potentials with increasing water concentrations, which is similar to the results obtained from VK_1 solutions.^{11,13} For each scan, a new SAM on the Au working electrode surface was prepared (and the second scan used), followed by KF titration to obtain the water concentration. There was a large amount of potential drift observed in the voltammograms in Figure 4 due to the fact that a pseudo reference

electrode was used. The drift was not critical to the measurements because we were only interested in measuring the difference between E_3 and E_4 .

SWV experiments were performed to measure the $|E_3 - E_4|$ -values over a range of water concentrations. Figure 5 shows the $|E_3 - E_4|$ -values against the water concentrations of the solvents CH_3CN and CH_2Cl_2 . The data in Figure 5 are a combination of several experiments, and show a similar trend as was observed for the data obtained when VK_1 is dissolved in solution. However, the results show more scatter and the shift in $|E_3 - E_4|$ was not as great as when VK_1 was used in solution.

4.3 Conclusions

The data in this study has demonstrated that the presence of what is often considered trace amounts of water (mM levels) has a pronounced influence on the E^0_{f} -values of semiquinones, especially in solvents with low relative basicity. The degree of hydrogen bonding of VK_1^{2-} with water in organic solvents was found to increase in the order $\text{DMSO} < \text{DMF} < \text{PrCN} < \text{MeCN} < \text{DCM} < \text{DCE}$, for equivalent concentrations of water and supporting electrolyte. The extent that $\text{VK}_1^{\cdot-}$ and VK_1^{2-} undergo hydrogen bonding with low levels of water is indirectly determined by how interacting the solvent is with water (inversely correlating with the relative solvent basicity) and the concentration of the supporting electrolyte.

This study has demonstrated that VKSH, a derivative of VK_1 , can be attached to Au electrode surfaces via a sulfur linkage and the voltammetry of immobilized VKSH can be used to estimate water concentrations in organic solvents by performing

voltammetry experiments. Two reduction peaks were observed in CVs, which indicated $\text{VKSH}^\bullet$ and VKSH^{2-} were produced at the surface of Au electrode. The reduced form of VKSH, $\text{VKSH}^\bullet$ and VKSH^{2-} , both undergo hydrogen-bonding with H_2O in solutions and the peaks shift to more positive potentials.

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Chapter 5

The Absorption of Water into Organic Solvents used for Electrochemistry under Conventional Operating Conditions

5.1 Introduction

Voltammetric experiments often need to be performed in non-aqueous solvents because of the solubility properties of the analytes, as well as to prevent reactions of the oxidized or reduced species with water.^{1,2} The Karl Fischer coulometric (or volumetric) titration is the major method used to determine the water content of non-aqueous solvents. However, despite it being commonly known that water is a major impurity in all organic solvents, a literature search in ISI Web of Science combining the keywords “Karl Fischer” with “voltammetry” found just 10 publications over the last 20 years! Although there are likely to be other publications using voltammetry where the water content is measured, the vast majority of studies that perform electrochemistry in non-aqueous solvents do not report the water content of the electrochemical solutions under the conditions that the experiments were carried out.

The problem of interference from trace water is exacerbated by the requirement that voltammetry be conducted with low concentrations of analytes;^{1,2} thus the water content can be much greater than the analyte concentration. An analyte concentration of between 0.5×10^{-3} M to 2×10^{-3} M is typically used for cyclic voltammetry experiments, which is high enough to provide adequate signal-to-noise ratios, but

sufficiently low so as not to distort the voltammograms due to resistance effects (IR-drop) or migration effects.³ It can be argued that the water content of the test solution should always be reported in order for the formal potentials (E^0_f) to be meaningful. Furthermore, unless the initial water content of the solvent is known, it is not possible to know whether the effects of trace water on the electrochemical reaction are important or unimportant.

While, it is relatively straightforward to dry many solvents to < millimolar levels of water (which is less than the normal analyte concentration for voltammetry measurements),⁴⁻⁷ once the solvent is added to the electrochemical cell, the water levels will immediately significantly increase unless the experiments are conducted in a glove box, which is not usually convenient for electrochemical measurements due to the positioning of the potentiostat and the requirement of having to frequently polish the electrodes. Other major sources of water that need to be considered and controlled are from the supporting electrolyte, the reference electrode (especially if it is separated from the test solution by a junction containing an internal filling solution), the surface of the electrochemical cell and the substrate itself.¹⁻³ Pre-saturation containers that are used to compensate for evaporation of volatile solvents are another major source of water. Therefore, it is far more important to know the water content of the test solution under operating conditions, rather than the water content of the dried solvent in its storage vessel. The purpose of the research presented in this Chapter is to provide guidelines for estimating the water content of organic solvents under conventional electrochemical operating conditions.

5.2 Results and Discussion

It is not the purpose of this study to perform measurements under ultra-dry conditions, which generally requires a glove box or high vacuum conditions. Instead, interest was in measuring the water content of organic solvents under normal conditions where low water content is preferred, but the vigorous requirements of an ultra-dry environment are not necessary. It is not possible to cover all the possible cell designs, but the conditions represented in this work are typical of many experiments. Namely, a solution containing solvent, electrolyte and analyte are placed in a glass electrochemical cell under an argon or nitrogen atmosphere and voltammetry experiments are conducted over a period of a few minutes to a few hours. The most significant local variation is the humidity conditions, which can change substantially in some locations on an hourly basis, thus were carefully controlled for these measurements using a constant humidity chamber that contained all of the apparatus (Figure 1).

Figure 1. Constant humidity chamber containing apparatus for performing water content measurements.

One major source of water, which is not accounted for in this study, is from reference electrodes that use internal filling solutions. If an aqueous reference electrode is placed in a non-aqueous solvent, then the water content of the solvent will immediately increase. Trial experiments indicated that the water content of a “dry” ($\sim 1 \times 10^{-3}$ M H_2O) solvent will quickly increase within a few minutes to > 0.1 M H_2O if an aqueous reference electrode is used. Reference electrodes that contain organic filling solutions will also add substantial amounts of water to the solvent, since it is difficult to load the internal filling solution without exposing the solution to the atmosphere. The longer the non-aqueous reference electrode is stored, the more water it will absorb. Therefore, experiments at low water content should only be performed using a pseudo wire reference electrode, which is the *de facto* assumption for this study.

The drying procedure described in the Experimental Section (page 38) lowered the water content of the solution inside the syringe to a base level that depended on the properties of the solvent (Table 1). For dichloromethane (DCM), acetonitrile (MeCN), dichloroethane (DCE) and butyronitrile (PrCN) the drying procedure lowered the water content of the solvent/electrolyte inside the syringe to $< 1 \times 10^{-3}$ M (column 2 in Table 1). For *N,N*-dimethylformamide (DMF) and propionitrile (EtCN) the water content of the solvent/electrolyte inside the syringe could be lowered to approximately 2.5×10^{-3} M, while for dimethylsulfoxide (DMSO) the water content remained $\geq 20 \times 10^{-3}$ M. DMSO is known to be very difficult to dry because of strong hydrogen bonding interactions between the solvent and water.^{8,9} For the solvents used in this study, it has been found that pre-drying the solvents by distilling over chemical drying agents (such

as CaH_2 or P_2O_5) or passing the solvent through a column of activated alumina, does not improve the drying procedure beyond using the molecular sieves.⁴⁻⁷

For some solvents, such as DMSO, improved drying has reported to be possible by using 4 Å molecular sieves (rather than 3 Å), but under these conditions the solvent also needs to be dried and stored inside a glove box to maintain its ultra-dry state.⁶ The possibilities of using larger amounts of molecular sieves or drying the samples for longer periods of time (many days) were not investigated. Some molecular sieves can introduce impurities into solution that can affect the electrochemical results. It was found that the brand of molecular sieves listed in the experimental section had good drying characteristics that did not appear to introduce impurities into solution.^{10,11}

An advantage of using the molecular sieves for drying the solutions compared to other methods is that it enables the solvent and electrolyte to be dried together, because the electrolyte often contains waters of crystallization which can be difficult to remove by heating. Furthermore, if the electrolyte is weighed in air and then added to the electrochemical cell, it would have already absorbed water from the atmosphere (depending on the local humidity conditions). If possible, adding the analyte to the solvent and electrolyte in the syringe is also useful in obtaining a starting solution with reasonably low water content (providing it does not react with the molecular sieves). For the measurements discussed in this Chapter, it was found that within experimental error, the presence of the electrolyte did not increase the rate that water was absorbed from the atmosphere compared to results obtained using the pure solvent, but for consistency all reported measurements were performed in the presence of 0.2 M supporting electrolyte.

Table 1. Concentration of water in dried organic solvents measured by Karl Fischer coulometric titrations at 22 ± 2 °C.

Solvent ^a	^b Water concentration / mmol L ⁻¹			
	Solvent/electrolyte stored over 3 Å molecular sieves	Solvent/electrolyte transferred into electrochemical cell under a nitrogen atmosphere ($t = 0$)		
		30% humidity	50% humidity	70% humidity
DCM	0.2 ± 0.1	0.7 ± 0.2	0.7 ± 0.2	1.0 ± 0.2
MeCN	0.5 ± 0.3	0.7 ± 0.3	0.7 ± 0.3	1.2 ± 0.3
DMF	2.5 ± 0.5	3.0 ± 1.0	3.0 ± 1.0	5.0 ± 1.5
DMSO	25 ± 5	30 ± 5	30 ± 5	30 ± 5
DCE	0.15 ± 0.1			
EtCN	2.5 ± 0.5			
PrCN	0.2 ± 0.1			

^aSolvent contains 0.2 M Bu₄NPF₆. ^bThe error is the standard deviation from measurements on at least three different samples of each solvent.

The concentration of water obtained by KF titrations are usually calculated in units of ppm ($\mu\text{g g}^{-1}$) for the mass of water divided by the mass of solvent. However, for cyclic voltammetry measurements the units of concentration are typically reported in mol L⁻¹. Therefore, in this study all measured water concentrations have been converted to mol L⁻¹ (or mmol L⁻¹ in Table 1). As a comparison, 1×10^{-3} M H₂O in DCM, MeCN, DMF and DMSO correspond to 14, 23, 19 and 16 ppm H₂O, respectively. In order to confirm the accuracy of the KF titrations for the present measurements, accurately controlled μL volumes of H₂O were added to test solutions and KF titrations conducted to determine if the added amount agreed with the measured amount. It was found that the KF titrations were accurate up to 0.5 M total concentration of water, which is considerably higher than the amounts measured for the dried solvents used in this study.

Columns 3 – 5 in Table 1 give the water content of the dried DCM, MeCN, DMF and DMSO solutions (containing electrolyte) immediately after they were added to the electrochemical cell under different humidity conditions. It can be observed from the data in Table 1 that in each case there was an immediate increase in the water content as the solvent was added to the electrochemical cell. For DCM and MeCN the initial water content inside the electrochemical cell remained $\leq 1 \times 10^{-3}$ M (except for MeCN at the highest humidity). The results in Table 1 indicate that if an identical drying and solvent/electrolyte transfer procedure is used as in this study, it is possible in most instances to obtain water concentrations close to that of the analyte concentrations (except for DMSO), at least for experiments that are conducted immediately after adding the solvent/electrolyte to the electrochemical cell.

Figure 2 gives plots showing how the water content of the solvents varied over time when stored in an electrochemical cell under a nitrogen atmosphere in different relative humidity environments at 22 ± 2 °C. The plots are all on the same scale to aid comparison purposes and demonstrate that the electrochemical cell does not exclude the entry of water, even when under a nitrogen atmosphere. For DCM, the water content inside the electrochemical cell over all humidity values was $< 1 \times 10^{-3}$ M at $t = 0$ min. and increased to $< 5 \times 10^{-3}$ M at $t = 120$ min. For MeCN, DMF and DMSO differences in water content were detected depending on the relative humidity, with the highest humidity levels resulting in measurably larger increases in the solvent water contents over time. For MeCN, the water content increased from $\sim 1 \times 10^{-3}$ M at $t = 0$, up to $\sim 10 \times 10^{-3}$ M (30% humidity) and $\sim 25 \times 10^{-3}$ M (70% humidity) at $t = 120$

min. For DMF the water content increased by approximately double the amount observed for MeCN over similar humidity levels and times. For DMSO the water content increased by a lesser amount than DMF, but because the starting water concentration was much higher ($\sim 30 \times 10^{-3}$ M), it recorded the highest overall concentrations of H₂O after 120 min. ($\sim 60 \times 10^{-3}$ M).

In Singapore, where these measurements were performed, the mean relative humidity is 84%, typically ranging from > 90% in the early morning to 60% in the late afternoon. The laboratory where the experiments were performed has 24 hour air-conditioning with the temperature maintained at 22 ± 2 °C, and the relative humidity measured to be 55 (± 2)%. Therefore, when working in the open laboratory it would be expected that the water content of the pre-dried solvents would increase at a rate closest to that observed for 50% humidity. Researchers conducting electrochemical experiments under environments with < 30% humidity or > 70% humidity would be expected to observe the water contents of the solvents increasing at a lesser or greater amount, respectively, than given in Figure 2.

Figure 2. Plots showing how the solvent water contents (measured by KF titrations) change over time when pre-dried solvents (containing 0.2 M Bu_4NPF_6) are stored in an electrochemical cell under a N_2 atmosphere at a fixed flow of $100 \text{ cm}^3 \text{ min}^{-1}$ at different relative humidity values (30, 50 and 70%) at $22 \pm 2^\circ \text{C}$. Plotted measurements are from one experimental run.

Figure 3 shows the effect that the nitrogen atmosphere has on the rate that water is absorbed from the atmosphere at different humidity levels. As expected, having the solvent maintained under the dry nitrogen atmosphere (lower plot) does significantly reduce the rate that the atmospheric water is absorbed into the solvent compared to the results obtained without a nitrogen atmosphere (upper plot). Because the KF titrations involve aliquots of the solvent being removed from the electrochemical cell, the solution volume does not remain constant over the time scale of the experiments. Therefore, the upwards curvature that is observed in the plots in Figure 3 (and Figure 2) is most likely caused by changes in the solvent volume over time increasing the apparent rate that water is absorbed from the atmosphere.

The sampling procedure can itself potentially increase the rate that water is absorbed from the atmosphere because it involves having to repeatedly insert a needle into the electrochemical cell to extract an aliquot of the solvent for injection into the Karl Fischer titrator. It was found that in the case of MeCN, when the dried sample was added to the electrochemical cell under nitrogen and left for a period of one hour, the amount of water detected in the MeCN was within 5% of the amount obtained after one hour when samples were taken every 10 minutes (suggesting that the sampling method was not affecting the accuracy of the results).

The error associated with the measurements given in Figures 2 and 3 depends on the absolute amount of water in the sample and the mass of the sample. There is a minimum charge that can be accurately measured by the coulometer; meaning that low concentrations of water require higher masses of the analyte in order to achieve the

greatest accuracy.¹² The error is also highest at the lowest water concentrations and at the highest relative humidities. This arises from the sampling procedure where some water from the atmosphere could enter the syringe needle and some solvent can be lost (evaporate) during the transfer of the solution to the KF coulometric titrator. However, it is believed that any error due to water being introduced during the sampling is small; otherwise the results obtained for all the solvents would be very similar, since an identical sampling procedure is used in each case. The biggest percentage error (seen by the amount of scatter in the water content vs. time plots) was observed for DCM because it contains the least amount of water.

Figure 3. Plots showing how the solvent water contents (measured by KF titrations) change over time when pre-dried solvents (containing 0.2 M Bu₄NPF₆) are stored in an electrochemical cell at 50% relative humidity at 22 ± 2 °C in the presence or absence of a N₂ atmosphere at a fixed flow of $100 \text{ cm}^3 \text{ min}^{-1}$. Plotted measurements are from one experimental run.

5.3 Conclusions

The results in this study demonstrate that for many organic solvents it is possible to reduce the water content to below or slightly above the analyte concentration by storing the solvent/electrolyte over 3 Å molecular sieves. Once the dried solvent is added to the electrochemical cell under an inert atmosphere, the water content of the solvent increases over time with the rate of increase depending on the properties of the solvent and the relative humidity. Experiments with very low water concentration ($\sim 1 \times 10^{-3}$ M) are still possible in many solvents in a conventional electrochemical cell if the experiments are conducted in the presence of the molecular sieves, or if the measurements are done as soon as the solvent/electrolyte is added to the cell.

One important reason for making the effort to accurately measure and report the water content of the organic solvent used during voltammetric measurements is that relative changes in the observed (E_{obs}) or formal (E_f^0) peak potentials will be the largest at the lowest (mM) water concentrations if the product of the electron transfer reaction undergoes a reaction with water. For example, equations 1 and 2 (ignoring the charge on the species and using concentrations in place of activities) represent the situation where an oxidized species, [Ox], is reduced by x electrons to form [Red], and [Red] further reacts with y molecules of trace water in the solvent to form [Red(H₂O)_y]. If the equilibrium reaction in equation 2 strongly favors the product, the Nernst equation can be given by equation 3, which predicts that the shift in potential will be dependent on $\ln(1/[\text{H}_2\text{O}]^y)$. An analogous situation is observed for electron transfer reactions that are

coupled to proton transfer steps, where there is a linear dependence of the reversible half-wave potential ($E_{1/2}^r$) on the pH.¹³

$$E_{\text{obs}} = E_{\text{f}}^0 - \frac{RT}{nF} \times \ln \frac{[\text{Red}(\text{H}_2\text{O})_y]}{[\text{Ox}][\text{H}_2\text{O}]^y} \quad (3)$$

An interesting observation that needs to be considered when working in dry conditions is the effect that the solvent has on the availability of water to react with the solute. Although it appears that the solvent DCM is easier to keep dry than the other solvents (see Figures 2 and 3), it does not mean that solutes in DCM are less likely to react with water. For example, it has been observed that quinone anions are far more reactive with water in DCM compared to DMSO, because in DMSO the water itself undergoes very strong hydrogen-bonding reactions with the solvent (essentially reducing the water reactivity in that media).¹¹ Since DCM only interacts very weakly with water, the water in DCM is freer to interact with the solute than the water in DMSO. Parameters that can be used to estimate the degree of interactions between the solvent and water are the experimentally derived normalized solvent basicity scale¹⁴ and the donor number scale (see Chapter 4).¹⁵

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Chapter 6

***In-situ* Infra-red Spectroscopy of Reduced Forms of Vitamin K₁**

6.1 Introduction

Based on the understanding of vitamin K₁ (VK₁) from the voltammetric and *in-situ* UV-Vis spectroscopic data (Chapters 3 and 4), the degree of hydrogen bonding between the reduced forms with water in the solvent were further investigated using *in-situ* electrochemical infra-red (IR) spectroscopy with an attenuated total reflection (ATR) probe (see Experimental section, Chapter 2). IR spectroscopy is extremely useful because it can give information on the molecular structure of a molecule and allows for compounds to be characterized, identified and also quantified.¹ The IR spectra of VK₁ and its reduced forms were studied by combining both electrochemical and spectroscopic techniques. In an attempt to identify how the positions of the IR bands of the reduced compounds varied depending on the solvent water content, *in-situ* IR spectroscopy was conducted simultaneously with the electrolysis of VK₁ solutions. The solution containing VK₁ was dried very carefully and Karl Fischer (KF) titration was used to determine the precise water content. Subsequently, different volumes of water were added into the dry VK₁ solution and their spectra (after reduction) were compared. A two compartment electrochemical electrolysis cell (Figure 1) was designed to enable FTIR spectra to be simultaneously collected. This

cell enabled the quantitative and reversible formation of the radical monoanion and dianion, allowing the simultaneous recording of electrochemical and IR spectroscopic data in a single set-up that includes an electrochemical cell, potentiostat, and a spectrophotometer (Figure 1).

Figure 1. Electrochemical cell for electrolysis and *in-situ* FTIR spectroscopy.

6.2 Results and Discussion

6.2.1 IR Spectroscopy for Vitamin K₁ in Non-dried CH₃CN

The electrolysis experiments described in Chapter 3 indicated that the monoanion and dianion survived in solution for many minutes, therefore should be observable by *in-situ* FTIR spectroscopy. CH₃CN was used as the solvent in the electrolysis experiments that were carried out concurrently with IR spectroscopy. It was discovered that by using non-dried CH₃CN, i.e. using it directly from the bottle without drying it, the electrolysis of VK₁ solution could proceed to completion and the

reduced states could be successfully reoxidized back to the starting material. KF titration was used to measure the initial concentration of water content in the wet CH_3CN which was found to be 0.100 M.

Upon the completion of the electrolysis, KF titration was carried out separately on the solution contained in the cathodic and anodic compartments. It was revealed that although the volume of the anodic compartment solution was four times that of the solution volume in the cathodic compartment; the water content in the cathodic compartment solution where the reduced species are generated was approximately twice the water content found in the anodic compartment solution. This finding is in agreement with what we know regarding the interaction between the reduced species and water found in solvents where the dianion VK_1^{2-} undergoes hydrogen bonding with water molecules (radical monoanion $\text{VK}_1^{\bullet-}$ undergoes little hydrogen bonding at water concentrations $< 0.5 \text{ M}$)². Hence, it is possible that the water molecules from the anodic compartment are transferred into the cathodic compartment during the course of the second reduction process, due to the ability of the strongly electronegative oxygen atoms in VK_1^{2-} to form strong hydrogen bonding with water. An equilibrium exists between the rate at which the water enters and moves out the cathodic compartment and based on the KF titrations, it seems to suggest that the rate at which water moves out of the cathodic compartment after reoxidization is much slower than the rate of water entering it during the second reduction step, and consequently the water content of the solution in cathodic compartment is greater than that of the solution in anodic compartment.

Figure 2 shows the IR spectra of VK₁ and its reduced forms obtained during the electrolysis in non-dried CH₃CN. The IR spectra of the neutral starting material VK₁ (shown as red lines in Figure 2) show characteristic bands between 1700 and 1200 cm⁻¹. The strongest bands arise from the C=C vibrations that are due to ring stretching band of the aryl group as well as from the C=O vibrations. These bands are located between 1692 and 1560 cm⁻¹ based on theoretical calculations,³ experimental data,⁴ and results from using isotopically labeled 2,3-dimethoxy-5-methyl-6-decaisoprenyl-1,4-benzoquinone.⁵ The sharp and intense band at 1663 cm⁻¹ is assigned to the C=O and the band at 1598 cm⁻¹ to the symmetric C=C vibration of the aryl group in VK₁. The assignment of the C=O and C=C bands to single IR bands is facilitated since both these modes are allowed to be closely approximated as group vibrations.³ It is mostly the electronic effects of the substituent on the quinone ring in VK₁ that will determine the exact positions of the C=O and C=C bands, and only to a little extent by interactions with other molecules, e.g. hydrogen bonding to water found in solvent. Since the quinone structure in VK₁ is asymmetrically substituted, a total of four bands are expected to be observed in these spectra, i.e. two C=C and two C=O bands. However, it is usually difficult to clearly differentiate the four individual bands due to band overlapping. The assignment of bands in the remaining region between 1500 and 1200 cm⁻¹ is less certain and will be neglected here since it does not contain valuable information to aid in the understanding of the IR spectra.

Figure 2. *In-situ* IR data recorded during the electrolysis of 5 mL 20 mM VK₁ solution using wet CH₃CN as solvent arranged in increasing water concentration of the solution tabulated using KF titration after electrolysis. (a) 0.0089 M. (b) 0.0395 M. (c) 0.0414 M. Starting material VK₁ (Red lines). Radical monoanion of VK₁ obtained after first reduction at applied potential of -1.1 V (Green lines). Dianion of VK₁ obtained after second reduction at applied potential of -1.8 V (Blue lines). Products obtained after oxidation at applied potential at 0 V (Black lines).

Upon the first reduction at an applied potential of -1.1 V to generate the radical monoanion (Green lines of Figure 2), the colour of the solution changed from yellow to dark green, and the intense band at 1663 cm^{-1} in the spectrum disappeared. A new strong peak appears at 1710 cm^{-1} which initially looks to be rather broad but on closer inspection, the peak actually consists of two bands that are overlapping with each other. The original $\text{C}=\text{C}$ band at 1598 cm^{-1} is also observed in the monoanion spectrum and similarly, the band appears to have two overlapping bands. Recall that in the original VK_1 spectrum, two $\text{C}=\text{O}$ and two $\text{C}=\text{C}$ bands are expected although due to band overlapping, it was not possible to distinguish them. But for the radical monoanion which in fact exists as two resonance forms (Figure 3), the spectrum revealed that the degree of band overlapping has decreased as shoulder peaks could be seen from the bands at 1710 cm^{-1} and 1598 cm^{-1} . Hence, it is inferred here that the resonance forms of the monoanion are actually causing the overlapping bands to be more easily distinguished in the monoanion spectrum. As such, the intense peak and its shoulder peak at 1710 cm^{-1} and 1702 cm^{-1} respectively are assigned to the two $\text{C}=\text{O}$ bonds of the monoanion resonance structures. And the band at 1598 cm^{-1} together with its shoulder peak at 1582 cm^{-1} are assigned to the two $\text{C}=\text{C}$ bonds in the $\text{VK}_1^{\cdot-}$ structure where they belong to both the symmetric and asymmetric stretching modes of the benzoquinone radical anion group.

Figure 3. Resonance structures of the radical monoanion.

Comparing the positions of the C=O bands belonging to VK₁ and its monoanion, it is apparent that the C=O peak in the monoanion is located at a higher wavenumber than the C=O band in VK₁. This suggests that the C=O bond in the monoanion is stronger because a stronger bond would require greater energy to stretch the bond; and consequently, the higher the energy (or wavenumber, in cm⁻¹) of the corresponding band. The stronger C=O bond can be attributed to the increased electron density being distributed in the aromatic ring. This observation is consistent with the CV results obtained in a study which showed that the potential of the first reduction peak did not change much with varying water concentration, given that a strong C=O bond would imply that the radical monoanion is less likely to interact with water molecules *via* hydrogen bonding.

Due to the resonance structures of the monoanion, there should also exist two C–O bands in its IR spectrum. Two separate distinct peaks were found at lower wavenumbers at 1492 cm⁻¹ and 1465 cm⁻¹ and these are assigned to the C–O single bonds, since according to a study which reported on the IR bands of various quinones, they located the C–O band belonging to the VK₁ radical monoanion at 1488 cm⁻¹ although the solvent used in the study was dichloromethane (CH₂Cl₂).⁵ The positions of the C–O IR bands can still be found in approximately the same region even though CH₃CN is used as the solvent.

At an applied potential of –1.8 V, the second reduction process is carried out, forming the dianion with the solution changing colour from dark green to dark maroon. From the dianion IR spectra shown in Figure 2 (Blue lines), it can be seen that the two

C=O bands at 1710 cm^{-1} and 1702 cm^{-1} have disappeared. This signifies that all, if not most of the radical monoanion have been reduced to form the dianion since the dianion structure does not contain any C=O bonds (Figure 3b). In the dianion spectrum at lower wavenumbers, two peaks are located at 1373 cm^{-1} and 1343 cm^{-1} and these are tentatively assigned to the two C–O single bonds present in the dianion structure.

Recall that in the radical monoanion spectrum, two peaks found at 1492 cm^{-1} and 1465 cm^{-1} were assigned to C–O bonds. Using that as a comparison, it is evident that the C–O bands belonging to the dianion are found at fairly low wavenumbers which could suggest that the C–O bonds in the dianion are weaker than the C–O bonds in the monoanion. The weaker C–O bond can be explained by the observation that the dianion is capable of forming much stronger hydrogen bonding interactions with water molecules as its structure contains strongly negatively charged oxygen atoms. As a result of the stronger hydrogen bonding with water, the bond strength of the C–O bond will weaken; meaning that less energy is needed to stretch the bond and that is why the C–O bands belonging to the dianion are located at lower wavenumbers than in the monoanion.

When the dianion was electrochemically oxidized by applying 0 V to the electrode surface, the colour of the solution changed from dark maroon to yellow, indicating that the VK_1 was being regenerated from the reduced forms in the solution. Furthermore, it was observed that in all the IR spectra, the characteristic peaks representing the starting material of VK_1 at 1663 cm^{-1} and 1598 cm^{-1} appeared after the reoxidation (Black lines in Figure 2) of the dianion VK_1^{2-} , thereby reaffirming that

the reduced states were stable on the timescale of the experiment, which was approximately 60 minutes.

6.2.2 IR Spectroscopy of Vitamin K₁ in Ultra-dry CH₃CN

Having established the electrochemical and spectroscopic behavior of VK₁ in intermediate water concentration of 0.100 M, electrolysis experiments were next performed at low water concentrations by carefully drying the solvents. The water content of the dry solution could reach to as low as 0.0010 M which was determined by KF titration. Although the solution used is dry, once it is in the electrochemical cell under a nitrogen atmosphere, the water content will increase slowly. Previous studies has shown that even under an argon atmosphere, the water content of the dry VK₁ solution increased steadily from 0.01 to 0.05 M within 2 hours when using CH₃CN as the solvent.⁶ But in these electrochemical-ATR-FTIR experiments, the concentration of VK₁ solution is much higher at 20 mM, which is 20 times higher than used for the purely voltammetric experiments. Therefore, it is reasonable to assume that the trace water will have a lesser effect on the hydrogen-bonding with the VK₁ anions. The IR spectra in Figure 4 were obtained from the electrolysis of VK₁ in dry CH₃CN. The IR spectra obtained for the first reduction step to produce the anion radical were similar to obtained in the non-dried CH₃CN. However, it can be seen that the second reduction spectrum (Blue line) obtained is similar to the spectrum of the first reduction (Green line), indicating that the second reduction was not progressing even though the applied voltage was at -1.8 V and the solution was being stirred continuously to ensure the

homogeneity of the solution. Furthermore, the solution in the cathodic compartment remained dark green, confirming that the second process was not proceeding. The electrolysis of dry VK_1 solution was repeated several times and the same results were obtained each time where the second reduction would appear not to proceed.

Figure 4. *In-situ* IR spectra recorded during the electrolysis of 5 mL 20 mM VK_1 solution using dry CH_3CN as solvent. Starting material VK_1 (Red line). Radical monoanion of VK_1 obtained after first reduction at applied potential of -1.1 V (Green line). Dianion of VK_1 obtained after second reduction at applied potential of -1.8 V (Blue line).

This interesting feature observed could be due to the fact that the water content in the solution was insufficient to allow hydrogen bonding of the dianion with water molecules, and consequently reducing the solubility of the dianion. As a result of the lowered solubility of the dianion, the dianion is likely to be adsorbed on the electrode surface and therefore the second reduction process could not be observed. One piece of evidence for this interpretation is that a dark green residue was found coating the

surface of the platinum plate when the working electrode was removed after the experiment. This reasoning is also supported by the CVs that were carried out on dry VK₁ solution in a study where unique voltammetric characteristics were observed when the water content was below 0.010 M (Chapter 3).⁶ In the study, the CV performed showed the second reduction process gave wide and oddly shaped peaks which were rationalized to be due to the lack of water for hydrogen-bonding of the dianion and causing it to interact with the electrode surface (by absorbing).

The water content of dry VK₁ solution which was used to perform the electrolysis experiment is very low at 0.0010 M as compared with the substrate concentration, in this case 0.0200 M (20 mM) of VK₁. And therefore at such low water concentrations, the most suitable explanation for the lack of the second reduction process is that inadequate hydrogen-bonding led to the reduced solubility of the dianion and as a result, the dianion interacts with the electrode surface in a specific manner causing an adsorptive effect.

6.2.3 IR Spectroscopy of Vitamin K₁ in CH₃CN with the Addition of Large Amounts of Water

Because the IR spectra obtained during controlled potential electrolysis experiments showed that the second reduction step for VK₁ cannot proceed when dry CH₃CN solution was used, experiments were performed by adding large amounts of water into the CH₃CN solutions. Electrolysis experiments were then carried out with the aim of observing any shift of the IR bands, especially for the second reduction step

where the dianion is understood to form strong hydrogen-bonds with water. IR spectra shown in Figure 5 indicate that with the addition of water to the dry VK₁ solution, the electrolysis experiments that were carried out were able to go to completion, which indirectly affirms the fact that hydrogen-bonding between the dianion and water molecules is an important interaction that is required for the second reduction step to proceed. Figure 5 shows FTIR spectra obtained during the electrolysis of VK₁ in the presence of increasing amounts of water. Unfortunately in the presence of extremely high concentrations of water (> 0.2 M) the bands associated with VK₁ suffer from excessive interference from the bands associated with free water, even when background subtracted spectra were used.

Figure 5. (a) VK₁ with wet CH₃CN; water content 0.0414 M. (b) Dry VK₁ added with 9 μ L of water; water content 0.0565 M. (c) Dry VK₁ added with 50 μ L of water; water content 0.1950 M. Starting material VK₁ (Red line). Radical monoanion of VK₁ obtained after first reduction at applied potential of -1.1 V (Green line). Dianion of VK₁ obtained after second reduction at applied potential of -1.8 V (Blue line). Products obtained after oxidation at applied potential at 0 V (Black line).

In order to find out the affect on the IR spectra for the first and second reductions through the addition of different volumes of water to the VK₁ solution, IR spectra for different concentrations of water were compared. Figure 6 shows the bands between 1740 cm⁻¹ and 1420 cm⁻¹ for the first reduction process, and the bands between 1500 cm⁻¹ and 1300 cm⁻¹ for second reduction process in the presence of different amounts of water.

Figure 6. IR spectra for the (a) first and (b) second reduction processes of VK₁. (Solid lines) Dry VK₁ solution with 50 μL of water; water content = 0.1950 M. (Dotted lines) Dry VK₁ solution with 9 μL of water; water content = 0.0565 M. (Dashed lines) VK₁ solution with wet CH₃CN; water content = 0.0414 M.

Looking at the main IR bands belonging to the radical monoanion in Figure 6(a), which includes the overlapping bands for both C=O (1710 cm^{-1} , 1702 cm^{-1}) and C=C (1598 cm^{-1} , 1565 cm^{-1}) as well as the two C–O bands at 1492 cm^{-1} and 1465 cm^{-1} , it is observed that there are no noticeable changes to the positions of those IR bands even though water was added to the dry VK₁ solution. This is expected since it is understood that the oxygen atoms on the monoanion are not sufficiently electronegative for the radical monoanion to be able to undergo significant hydrogen-bonding with water. Therefore even with the addition of water, there will be insignificant changes to the behavior of monoanion since there is little hydrogen-bonding occurs between the monoanion and water molecules.

The CV results obtained in the previous studies have shown that when the water concentration in VK₁ solution was gradually increased, the potential of the second reduction process shifts by a more significant amount as compared to that of the first reduction potential (Chapters 3 and 4).⁶ Therefore, the IR spectra obtained for the second reduction step would be expected to show significant changes when the water concentration of the VK₁ solution is varied by adding water. Because the C–O bonds in the dianion are considerably weakened due to the strong hydrogen-bonding interactions between the dianion and the water molecules, the two C–O bands in the dianion which were located at lower wavenumbers (1371 cm^{-1} , 1343 cm^{-1}) as compared to the two C–O bands in the radical monoanion (1492 cm^{-1} , 1465 cm^{-1}). Therefore, it is predicted that the two C–O bands would shift towards lower wavenumbers as a result of the greater degree of hydrogen bonding when the water

concentration increases.

The two C–O IR spectra for the second reduction step shown in Figure 6(b) were inspected carefully showed no difference in wavenumber as the water content was varied. This finding may be due to how the water content was not able to be varied over a significantly wide range in order to observe an effect, and due to uncertainty in the true position of the C–O bands.

6.2.4 Effect of Water on the Apparent Absorbance Intensity of Vitamin K₁

An interesting effect was observed when increasing amounts of water were added to the CH₃CN solutions. Looking at the IR spectra obtained in Figure 7(a), it was discovered that there is a dramatic increase in the intensities of the VK₁ bands even though the volume of water added to the solution was very small as compared to the total volume of the VK₁ solution. Only 0.01 mL (10 µL) of water was added to 5 mL of the solution each time, meaning that the volume of the VK₁ solution is 500 times that of the volume of water added. However, it is clear from the spectra that even with the addition of such a low volume of water; the IR bands for VK₁ became much stronger and more intense as though somehow the ATR probe is detecting more of the VK₁.

Figure 7. *In-situ* IR spectra obtained during the sequential addition of water to 5 mL of 20 mM VK_1 solution prepared (a) with wet CH_3CN ; (b) with dry CH_3CN without Bu_4NPF_6 ; (c) IR spectra obtained in transmittance mode during the sequential addition of water to 5 mL of 20 mM VK_1 solution prepared with dry CH_3CN without Bu_4NPF_6 . 10 μL of water was added each time until a total volume of 100 μL was added into the solution. (Red line) VK_1 solution with 0 μL water added. (Black line) VK_1 solution with 10 – 90 μL water added. (Blue line) VK_1 solution with 100 μL water added.

In order to verify this unique result obtained, this experiment was repeated again but with dry CH_3CN as solvent and without the presence of supporting electrolyte to see if the same effect can be attained. Figure 7(b) shows that the same result was obtained and it can be seen that the overall increment in the absorbance (by comparing the red and the blue lines) is slightly greater than that when the wet CH_3CN is used. This is an indication that the increased sensitivity of the ATR probe towards VK_1 is most likely due to the presence of water that is being added. The fact that with the addition of only 100 μL of water, the intensity of the VK_1 absorbance is able to increase almost twenty-fold seems to be suggesting that there exists a unique form of interaction between VK_1 and water.

IR spectroscopy in the transmittance mode was used to repeat the same experiment to see if the same results can be obtained or that such an observation was only exclusive to ATR. Figure 7(c) shows the IR data obtained when transmittance mode is used and the spectra shows that the VK_1 peaks did not increase; only the water peak at 1637 cm^{-1} increased correspondingly with the sequential addition of water into the VK_1 solution. The results shown in Figures 7 indicate that only the ATR probe is capable of sensing the unique manner in which VK_1 is interacting with water.

The structure of VK_1 contains two $\text{C}=\text{O}$ bonds and it is understood that the $\text{C}=\text{O}$ bonds are able to form hydrogen bonds with water molecules. There, it could be argued that hydrogen bonding interactions are somehow allowing the ATR probe to be more sensitive to the VK_1 present, and thereby increasing the intensities of the IR bands. To test this theory out, the same procedure was produced on the compound,

Dimethyl terephthalate (DMT) (Scheme 2(a)), which has two C=O bonds like VK₁.

Scheme 2. The structures of (a) Dimethyl terephthalate and (b) VK₁ model compound.

Figure 8 shows that for DMT, the band at 1725 cm^{-1} is assigned to C=O and it is obvious from the IR data that with the addition of water to the solution, the main C=O band shows no increase in intensity at all; only the intensity belonging to the water peak at 1637 cm^{-1} is increasing. This result suggests that hydrogen bonding may not be the factor that is responsible for the effect of water on the VK₁ IR bands.

Figure 8. *In-situ* IR spectra obtained during the sequential addition of water to 5 mL of 20 mM DMT solution prepared with dry CH_3CN without Bu_4NPF_6 . 10 μL was added each time until a total volume of 100 μL was added into the solution. (Red line) DMT solution with 0 μL water added. (Black line) DMT solution with 10 – 90 μL water added. (Blue line) DMT solution with 100 μL water added.

FTIR experiments were performed on the VK_1 model compound (without the phytol tail) (Scheme 2(b)) to determine whether the presence of the phytol tail was somehow influencing the ATR-FTIR results. Cyclic voltammetry experiments were first carried out with the VK_1 model compound and it was found that it displayed the same voltammetric behavior when water was added sequentially to the solution as observed for VK_1 (Figure 9). Using the peak separation data obtained for the model compound, calibration graphs of peak separations versus water concentration were also plotted and they were found to be similar to those of VK_1 (Figure 10).

Figure 9. (a) CVs recorded of 1 mM VK₁ solution at scan rate of 0.1 V s⁻¹ by using a 1 mm diameter planar glassy carbon electrode in CH₃CN containing 0.2 Bu₄NPF₆ with sequential addition of water. (b) CVs recorded of 1 mM VK₁ model compound solution at scan rate of 0.1 V s⁻¹ by using a 1 mm diameter planar glassy carbon electrode in CH₃CN containing 0.2 Bu₄NPF₆ with sequential addition of water. (Red line) Original VK₁/VK₁ model compound. (Blue line) CV merged to become a single two-electron process. (Black line) Intermediate levels of water before merging of reduction processes.

Figure 10. Calibration plots of $|E_1 - E_2|$ -values for (a) VK₁ and (b) VK₁ model compound (measured by SWV with 1 mm diameter glassy carbon electrode) versus the water concentration which was measured by KF titration. Data was obtained using CH₃CN as the solvent with 0.2 M Bu₄NPF₆.

From Figures 9 and 10, it is firmly established that the electrochemical properties of VK₁ model compound are similar to that of VK₁. Like the reduced forms of VK₁, the reduced species of VK₁ model compound are also capable of forming hydrogen-bonding interactions with water and likewise, as Figure 9(b) shows, the potential of the second reduction process is found to shift by a larger amount than the first reduction potential, such that at adequately high water concentrations, the two processes merge into a single two-electron process. As for the calibration plots, the trend of peak separations decreasing with increasing water concentrations for both VK₁ and VK₁ model compound are also demonstrated to be similar as shown in Figure 10. Therefore, if we were to attribute the effect water has on the absorbance of VK₁ peaks to hydrogen-bonding interactions with water, then hypothetically the same effect should also be seen on the VK₁ model compound since it is evident that they both share similar electrochemistry.

The distinct peak at 1663 cm^{-1} located on the red line in Figure 11 is assigned to the C=O bond in the VK₁ model compound structure. From the IR spectra obtained, it is basically the water peak at 1637 cm^{-1} that is increasing in intensity in response to the increasing volume of water in the solution, whereas the intensities of the C=O IR peaks are not shown to be affected by the extra volumes of water being added sequentially. Hence, judging from the IR results that have been acquired for both DMT and VK₁ model compound, it can be ascertained that the increased intensities of VK₁ peaks (Figure 7) due to the adding of water into the solution cannot be caused by hydrogen-bonding interactions.

Figure 11. *In-situ* IR data obtained during the sequential addition of water to 5 mL of 20 mM VK₁ model compound solution prepared with dry CH₃CN without Bu₄NPF₆. 10 μ L was added each time until a total volume of 100 μ L was added into the solution. (Red line) VK₁ model compound solution with 0 μ L water added. (Black line) VK₁ model compound solution with 10 – 90 μ L water added. (Blue line) VK₁ model compound solution with 100 μ L water added.

VK₁ exists in as yellow liquid oil at 293 K (the temperature at which all experiments is carried out) and it is understood that oil and water are immiscible when added together. In addition, it was observed that the solution would turn cloudy whenever water was added to the solution using a micropipette but after stirring and then allowing the mixture to settle, the solution became clear again. Building on this observation and the understanding regarding the immiscibility of oil and water, it could be deduced that by introducing more water into the CH₃CN solution containing

VK₁ causes the VK₁ to form small oil droplets. As a result of this solubility effect, it is likely that the VK₁ forms micro-particles, which in turn are interacting more with the ATR probe at the inter-facial surface of the solution. This effect consequently leads to a heightened sensitivity of the ATR probe towards VK₁, which translates into a dramatic increase in the intensity of the IR bands in the VK₁ spectrum.

6.3 Conclusions

In-situ ATR-FTIR experiments were performed during the electrochemical reduction of VK₁ in CH₃CN in the presence of varying concentrations of water. It was found that FTIR spectrum VK₁^{•-} showed intense bands at 1710 and 1702 cm⁻¹ that were associated with the carbonyl resonances and bands at 1598 and 1582 cm⁻¹ that were associated with symmetric and asymmetric C=C ring stretches. The FTIR spectrum of the dianion showed no bands in the carbonyl region and no recognizable ring stretching bands, although bands associated with the C-O group were possibly observed at 1373 and 1343 cm⁻¹. ATR-FTIR measurements of the anion radical and dianion at varying quantities of water did not lead to show any differences in the position of the absorbance.

Some interesting features were observed during the *in-situ* electrolysis experiments. Firstly, it was found that at very low concentrations of water, the electrolysis reaction to form the dianion did not progress. The reason is thought to be due to the solubility of the (non-hydrogen-bonded) dianion being so low that it precipitates from solution and blocks the electrode surface. The second observation is

that during the electrolysis experiments the water content of the cathode compartment increases over time, while the water content of the auxiliary electrode compartment decreases (with the water content measured by KF titrations). The reason for this observation is thought to relate to the anion radical and dianion interacting with water, thereby increasing the transfer of water across the glass membrane interface separating the working electrode and auxiliary electrode.

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Chapter 7

An Electrode-Supported Biomembrane for Examining Electron Transfer and Ion Transfer Reactions of Vitamin K₁

7.1 Introduction

Many low molecular weight ($< 1000 \text{ g mol}^{-1}$) lipid soluble naturally occurring biological molecules, such as vitamin K, are thought to undergo electron transfer reactions as part of their natural function.¹⁻³ While the procedures for examining electron transfer reactions of solution phase species are well-established,⁴ there are experimental difficulties in studying the electrochemical properties of vitamin K in an environment similar to where it exists naturally; that is, inside lipid bilayer membranes. The primary difficulties relate to the ion-transfer processes that must occur simultaneously to the electron-transfer steps in order to maintain overall charge neutrality, and the non-ideal solvent properties (in an electrochemical sense) of the biomembranes. In this Chapter a procedure is described that enables voltammetry experiments to be performed directly on bilayer membrane bound molecules.

Vitamin K₁ (VK₁), phylloquinone, chemically known as 2-methyl-1,4-naphthoquinone; acts as a crucial cofactor for the formation of coagulation factors II (prothrombin), VII, IX and X by the liver which are required for blood-clotting. VK₁ acts as a cofactor through electrochemical reactions where naphthoquinone, the key functional group of the entire molecular structure; undergoes

proton and electron transfers. Since the quinone structure is found in all the compounds of the vitamin K family, the redox reaction results observed in this experiment will most likely be similar for all K-vitamins. Being a fat-soluble compound, VK₁ and its reduced forms exist inside the hydrophobic cell membranes. This property is due to the lipophilic side chain of the vitamin structure that allows it to be soluble in lipid mediums such as lecithin, squalene and cholesterol.

In this Chapter, the redox reactions of VK₁ in an emulsion layer consisting of lecithin and water will be discussed. Lecithin forms a bilayer membrane structure and VK₁ can be embedded inside the membrane. The reason for using lecithin is to keep VK₁ attached to a GC electrode surface at all times and at the same time; providing a lipid environment, which has some similarities to its biological environment when in the human body.

In recent years there have been considerable advances made in examining the electrochemical properties of high molecular weight biological molecules such as proteins and enzymes usually by chemically modifying the surface of a solid electrode to encourage an interaction between the solution phase macromolecule and the surface-modified electrode.⁵⁻¹⁰ For protein-sized molecules, maintaining electroneutrality during the electron transfer process is not usually problematic, because the bulk of the macromolecule lies outside of the surface confined layers, thus ions from solution are easily able to balance the charge brought about by the electron transfer reactions initiated at the electrode surface. However, for small molecules located completely inside the bilayer membranes, the movement of ions is more difficult

because of the hydrophobic nature of the membranes which hinders ion transfer across the membrane-aqueous solution interface. Nevertheless, there have been important advances made in using electrochemistry to initiate and study ion transfer reactions across interfaces where ions would be expected to have very low solubility. For example, voltammetric techniques have been used to reversibly insert ions into microcrystals across a solid-solution interface,¹¹ and ion-transfer processes have been initiated at liquid-liquid (solvent) interfaces,¹² where in some cases one of the liquids is a viscous oil type droplet,¹³⁻¹⁵ with similar solubility properties to a hydrophobic membrane.

There are two major methods that have been developed to study electron-transfer reactions of low molecular weight molecules incorporated inside membrane environments using supported bilayer membranes (s-BLMs). In one instance, a solid electrode is first coated with a self-assembled monolayer (SAM), such as an alkanethiol,¹⁶⁻²¹ and then a solution containing the molecule of interest and another bilayer forming molecule are allowed to spontaneously interact and form a membrane by attaching themselves to the tethered portion [a tethered bilayer membrane (t-BLM)].²²⁻³⁵ An alternative procedure simply involves placing a droplet of a volatile organic solvent (such as chloroform) that contains membrane forming molecules onto the surface of the electrode and allowing the bilayer to form during the evaporation process,^{7-9,35-39} or allowing a clean electrode surface (such as a freshly cut Pt electrode) to come into contact with bilayer forming molecules in aqueous solution.⁴⁰⁻⁴³ In order to improve the conductivity properties of the membrane, there are several reports where low molecular weight molecules such as TCNQ are incorporated into the s-BLM in

order to promote the transfer of an electron through the membrane to a solution phase species (a mediated electron transfer).^{17,35,44-51}

One major issue with interpreting the results from electrochemical studies on s-BLMs is that defect free bilayer membranes will only form on glass substrates, with metallic and carbon surfaces being poor substrates to obtain ideal membrane coverage.⁵² Similarly, even with tethered molecules attached to the electrode surface, such as self-assembled layers of alkanethiols, it is highly likely that pin-holes will exist in the layers.^{16,53} The presence of pin-holes or defects in the SAMs or s-BLMs means that it is often difficult to determine whether the Faradaic current that is observed during voltammetric measurements is due to the electroactive molecules confined inside the membrane layers, or due to electron transfer reactions that occur between the electrode surface and solution phase molecules that reside in the defects (or channels) in the membrane coating.

In order to overcome the uncertainty of the presence of pin-holes in the s-BLM, this study has developed an alternative approach where the biological molecules are first incorporated into liposomes, and then the liposomes are deposited on the surface of a chemically modified electrode to spontaneously form multilayers. Using this procedure it is not critical for the electrode to be perfectly coated, since any pin-holes or defects will only incorporate the electrolyte ions, whereas the electroactive molecules will remain completely encapsulated within the membrane structure. VK_1 ⁵⁴⁻⁵⁸ that exists naturally within lipid bilayer membranes was chosen to examine because it undergoes redox reactions as part of its *modus operandi* and there exists extensive literature reports

of the voltammetric properties of it in both organic solvents^{54,57-68} and as an oil attached to electrodes in aqueous solutions.^{54-56,69}

Chart 1. Structure of membrane forming molecules.

7.2 Results and Discussion

7.2.1 Preparation of Electrode Surfaces

The electrodes were polished consecutively with P400 (35 μm) P1200 (15.3 μm) P2000 (10.3 μm) and P4000 (6.5 μm) grades of SiC paper followed by polishing with 3 μm and 1 μm grit alumina oxide powder on Buehler Ultra-Pad polishing cloths.

The procedure for preparing the vitamins inside the lipid (lecithin) multilayers was based on literature methods.^{54,70} The VK₁ (0.01 g) and lecithin (0.09 g) were combined in 2 mL of chloroform in a vial and placed in a vortex mixture for 1 minute (a clear solution was obtained). The chloroform was slowly removed (~20 minutes)

with a rotary evaporator and the VK₁/lecithin mixture dried under vacuum at room temperature (22 ± 2 °C) for 2 hours. 4 mL of ultrapure water was added to the VK₁/lecithin mixture and the slurry heated to 40 °C. The slurry was placed in a vortex mixer for 30 minutes, periodically reheating the solution so that it remained at 40 ± 5 °C, and then the solution was finally homogenized in an ultrasonic bath at 40 °C for 10 minutes and appeared as a milky white consistency.

In order to provide an optimal droplet size to completely coat the electrode, different μL -sized volumes of the solutions that contained the materials for coating the electrodes were used depending on the size of the electrode to be coated. A 1- μL coating drop volume was used for the 1 mm diameter electrodes and a 2- μL coating drop volume was used for the 3 mm diameter electrodes. A 1- μL or 2- μL droplet of the Nafion solution was placed on the electrode surface with a micropipette and the solvent allowed to evaporate in air for 5 minutes. A 1- μL or 2- μL aliquot of the homogenized VK₁/lecithin solution was deposited on top of the Nafion coated electrode surface and the water allowed to evaporate in air (around 5 minutes), to produce a multilayer film containing 22.5 – 45 μg of lecithin and 2.5 – 5 μg of the VK₁. Three further 1- μL or 2- μL droplets of the Nafion solution were deposited on top of the VK₁/lecithin multilayer, with the solvent allowed to evaporate for 5 minutes after each addition. The optimal coating procedure described above was determined by a number of trial and error experiments.

7.2.2 Preparation of Membranes and Electrodes for Electrochemistry

Previous studies described experiments where TCNQ³⁵ or vitamin E⁵⁴ were incorporated inside lecithin layers and deposited on Au or GC electrode surfaces. Refined lecithin was chosen for the lipid bilayers because it is a naturally occurring phospholipid present in plant and animal tissues. Lecithin consists of a range of lipid acyl chain lengths (in addition to the phosphatidylcholine shown in Chart 1) which favors the formation of bilayer membranes that are perfectly aligned and defect free.⁷¹⁻⁷⁴ Thus, once the homogenized liposome solutions are placed on the electrode surface, they spontaneously form highly ordered multilamellar phases interleaved with layers of solvent molecules. Based on the amount of lecithin deposited on the electrode surface, and comparing previous FTIR measurements of lecithin coated electrodes,³⁵ it can be estimated that the electrodes used in this work contained on average between 5 – 10 lecithin bilayers making up the multilamellar lipid phases on the electrode surface.

Figure 1. Schematic diagram showing proposed structure of electrode|Nafion|lecithin-vitamin|Nafion|water interfaces.

In order to improve the voltammetric responses, the electrode coating procedure described above was modified by the addition of a Nafion layer onto the electrode surface, with another Nafion layer deposited on top of the lecithin multilayers, to form a sandwich structure (Figure 1). Nafion is a cation-exchange polymer with a highly acidic sulfonate group (Chart 1). Electrodes coated with Nafion (and other surface confined molecules) have frequently been used to trap or concentrate solution phase species and thereby improve their analytical detection.⁷⁵⁻⁷⁷ In this work the Nafion layers are thought to have two functions; (i) to improve the ion conduction between the electrode surface and the lecithin multilayers and, (ii) to provide a readily exchangeable source of H^+ to

molecules within the membranes in order to balance the charge brought about by electrochemical reactions.

7.2.3 Voltammetry of Vitamin K₁

Figure 2a (solid line) shows a CV of the quinone, VK₁, in acetonitrile (CH₃CN) solutions containing approximately 50 mM of water. Two reduction processes are observed corresponding to the one-electron reduction of the quinone into its radical anion (semiquinone) (E_1) and at more negative potentials the further one-electron reduction of the semiquinone into its dianion (E_2).⁵⁷ When water is increasingly added to the CH₃CN, both E_1 and E_2 shift to more positive potentials, but E_2 shifts more than E_1 , so at high enough water concentrations the two one-electron reduction processes merge into one two-electron process (E_3) (Figure 2a, dashed line).⁵⁸ The reason that E_1 and E_2 shift in potential is due to hydrogen-bonding reactions of the reduced forms of VK₁ with water in the solvent.^{57,58,78}

Figure 2. Cyclic voltammograms recorded at a scan rate of 0.1 V s^{-1} at $22 \pm 2^\circ \text{C}$. (a) 1 mM VK_1 in CH_3CN containing $0.2 \text{ M Bu}_4\text{NPF}_6$ at a 1 mm diameter planar Pt electrode, (—) in the presence of 0.05 M water, and (---) in the presence of 7 M water. (b) $5 \mu\text{g}$ of VK_1 inside Nafion|lecithin|Nafion layers deposited on a 3 mm diameter planar GC electrode in a pH 3 buffered solution.

In the presence of acid, VK_1 can be electrochemically reduced in a chemically reversible two-electron process to form the vitamin K_1 hydroquinone (VK_1H_2), typical of the reduction mechanism of quinones in acidic conditions.⁷⁸ Therefore, two one-electron reduction processes are only observed in organic solvents containing limited amounts of water, while in the presence of acid or in aqueous systems, one two-electron reduction process is always observed.^{78,79} The “square-scheme” mechanism containing the individual electron transfer and chemical steps for the

reduction of quinones (Q) in acidic conditions and in the presence of water are given in Schemes 2 and 3 respectively. The vertical arrows in Schemes 2 and 3 involve electron transfer steps, while the horizontal arrows involve homogeneous reactions with H^+ or H_2O . At $pH < 7$ the reactions in Scheme 2 will mainly occur, while at $pH > 7$ the reactions in Scheme 3 will mainly occur, depending on the pK_a 's of all the individual species. In unbuffered solutions of approximately neutral pH, the protons released during the oxidation of the hydroquinones (QH_2) can sufficiently decrease the localized pH at the electrode surface to give similar voltammetric results to those obtained in more acidic bulk conditions.⁷⁹

Scheme 2. Electrochemical square-scheme mechanism showing series of possible consecutive electron-transfer and proton-transfer reactions associated with the chemically reversible transformation between quinones (Q) and hydroquinones (QH₂) in acidic conditions.

Scheme 3. Electrochemical square-scheme mechanism showing series of possible consecutive electron-transfer and hydrogen-bonding reactions associated with the chemically reversible transformation between quinones (Q) and hydrogen-bonded dianions, $[Q(H_2O)_2]^{2-}$, in the presence of water and the absence of acid.

7.2.4 Voltammetry of Membranes Containing Vitamin K_1

Figure 2b shows the CV of VK_1 incorporated inside the Nafion|lecithin|Nafion layers, where the forward scan shows one reduction process and one oxidative process was observed when the scan direction was switched. The voltammograms remained the

same as multiple scans were performed, indicating that the reductive and oxidative processes were chemically reversible.

Figure 3. Cyclic voltammograms (second scan) recorded at a scan rate of 0.5 V s^{-1} of films of VK_1 deposited on a 3 mm diameter planar GC electrode at $22 \pm 2^\circ \text{C}$. (a) $5 \mu\text{g}$ of VK_1 inside Nafion|lecithin|Nafion layers. (b) $5 \mu\text{g}$ of VK_1 inside lecithin layers.

Figure 3a shows the CVs obtained for the Nafion|lecithin- VK_1 |Nafion layers at different bulk solution pH values and Figure 3b show CVs of VK_1 -lecithin layers

deposited on a GC electrode over a range of pH values in the absence of Nafion. It can be observed that in the absence of Nafion (Figure 3b), the peak potentials obtained for both the forward (E_p^{red}) and reverse (E_p^{ox}) processes vary substantially as the bulk pH was changed, especially at $\text{pH} < 7$, due to protonation of the reduced forms of the quinones effecting the observed peak potentials (E_{obs}) according to the Nernst equation. As the pH increased above ~ 7 , the semiquinone ($Q^{\cdot-}$) and dianion (Q^{2-}) have increased lifetimes, meaning that the acid dissociation constants of their associated protonated forms need to be taken into account in the Nernst equation.⁷⁸ Thus, the relationship between the E_{obs} of the quinone and the proton concentration can be given by eq 1 (which applies in buffered aqueous media), where K_{a1} and K_{a2} are the acid dissociation constants of QH_2 and QH^- respectively, E_f^0 is the formal potential, and $n = 2$.

$$E_{\text{obs}} = E_f^0 + \frac{2.303RT}{nF} \times \log \left(1 + \frac{[H^+]}{K_{a2}} + \frac{[H^+]^2}{K_{a1}K_{a2}} \right) \quad (1)$$

The main difference between the data in Figures 3a and 3b is that the peak potentials for the CVs of VK_1 in the Nafion|lecithin|Nafion layers remain almost constant as the pH is changed, indicating that the Nafion layers are providing a constant pH environment for the VK_1 inside the membranes, so the VK_1 is unaffected by the bulk solution pH. Furthermore, the peak potential of the reduction process observed for the Nafion|lecithin- VK_1 |Nafion layers (Figure 3a) is less negative than the peak potentials obtained for the lecithin- VK_1 layers without Nafion (Figure 3b). Since the reduction potential (or observed peak potential) of VK_1 is dependent on the acid concentration according to eq 1, the shift towards positive potentials of the E_p^{red} peak of VK_1 in the Nafion|lecithin|Nafion layers (Figure 3a) implies that the effective pH experienced by

VK_1 is < 3 , regardless of the bulk solution pH. Thus, all the processes shown in Figure 3a are likely to involve a chemically reversible $2e^-/2H^+$ reaction and follow the mechanism in Scheme 2 (eqs 2 and 4). In contrast, the voltammograms obtained of lecithin- VK_1 films (without Nafion) in Figure 3b are likely to follow the reactions in Scheme 2 at lower pH (eqs 2 and 4), and the reactions in Scheme 3 at higher pH (eqs 3 and 5).

Variable scan rate CVs of Nafion|lecithin- VK_1 |Nafion layers are given in Figure 4, which display a particularly wide cathodic to anodic peak-to-peak potential separation (ΔE_{pp}) at all scan rates, which is also observed when films of VK_1 are directly deposited on the electrode surface⁵⁶ or when VK_1 is attached to an electrode surface by an alkanethiol linkage (and the voltammograms conducted in aqueous solutions).^{80,81} It is not believed that the resistance property of the membranes is responsible for the wide separation between the forward and reverse peaks. Instead, the reason for the wide separation between the reductive and oxidative peaks with increasing scan rate could relate to either slow heterogeneous electron transfer of one or more of the electron transfer steps in Scheme 2, or slow homogeneous chemical reactions with H^+ . It is also likely that the relative high ΔE_{pp} -value at slow scan rates is because the forward

(reductive) and reverse (oxidative) reactions of VK₁ occur by partly different routes (Scheme 2).

Figure 4. Cyclic voltammograms (second scan) recorded at variable scan rates of 5 μg VK₁ inside Nafion|lecithin|Nafion layers deposited on a 3 mm diameter planar GC electrode and placed in a pH 3 buffered solutions at 22 ± 2 °C.

Based on the observation that the pH experienced by VK₁ within the Nafion|lecithin|Nafion layers is low, it can be assumed that the forward and reverse reactions occur by two-electrons per molecule and also involve the transfer of two-protons. In an acidic environment it is unlikely that the semiquinone can survive for long enough to be detected using moderate scan rates. The reason that only one process is observed for both the forward and the reverse reactions even though two one-electron steps are involved, is because in both directions (reduction and oxidation), the second one-electron transfer process occurs at a lesser potential than the first one-electron transfer process, so the two electrons appear to transfer in one step.

CV experiments were also conducted on the Nafion|lecithin-VK₁|Nafion layers in pH 13 buffers, with the results being substantially different from the data obtained between pH 3 – 11. At a scan rate of 0.1 V s⁻¹ (Figure 5a), two voltammetric processes were observed on the first scan when the potential was applied in the negative direction, one at approximately -0.4 V (process D₁') and one at -0.7 V (process D₁'') vs. Ag/AgCl. The process at -0.4 V occurs at a similar potential to the reductive process observed when experiments were conducted with bulk solution pH values between 3 – 11. An oxidative peak was detected at close to 0 V vs. Ag/AgCl when the scan direction was reversed (process D₂'). Processes D₁' and D₂' are thus associated with the reduction of the quinone (VK₁) and oxidation of the hydroquinone (VK₁H₂), respectively (Q and QH₂ in Scheme 2). As multiple scans were performed, processes D₁'/D₂' diminished and processes D₁''/D₂'' increased in magnitude. When CV experiments were conducted at pH 13 using VK₁ directly attached to the electrode surface or incorporated into lecithin layers (without Nafion), it was found that only processes D₁''/D₂'' were observed (Figure 5a, dashed line). Processes D₁' and D₁'' both involve reduction of the VK₁, but the shift in potential is due to the quinone existing in a different environment. The shift in potential to more negative potentials of process D₁'' compared to process D₁' indicates that the VK₁ exists in a higher pH environment.

At high pH, processes D₁''/D₂'' most likely involve the transformation between the quinone (VK₁) and the hydrogen bonded dianion, [VK₁(H₂O)_x]²⁻ (Scheme 3). Although the peak-to-peak separation for processes D₁''/D₂'' is relatively small compared to the wide ΔE_{pp} -values obtained for processes D₁'/D₂' at lower pH values, it is unlikely that it

is because the electron transfer process occurs *via* only one-electron, such as a reversible transformation between the quinone and semiquinone ($Q/Q^{\cdot-}$). In the presence of water the reduced forms of VK₁ ($VK_1^{\cdot-}$ and VK_1^{2-}) will undergo immediate hydrogen bonding with water.^{57,58,78} So it is likely that processes D₁"/D₂" also involve the transfer of two-electrons (eqs 3 and 5).

Figure 5. Cyclic voltammograms of 5 μg of VK₁ inside Nafion|lecithin|Nafion layers deposited on a 3 mm diameter planar GC electrode and placed in pH 13 buffered solutions at $22 \pm 2^\circ\text{C}$.

The results in Figure 5a indicate that the VK₁ inside the Nafion|lecithin|Nafion layers must undergo a change in environment as multiple CV scans are performed. Initially the VK₁ exists in a low pH environment controlled by the high acidity of Nafion. However, in order for VK₁H₂ to convert into [VK₁(H₂O)_x]²⁻ (as indicated by the appearance of processes D₁''/D₂''), the pH within the Nafion layers must substantially increase. At very high pH, the reduction cycle must be accompanied by a transfer of hydroxide through the Nafion membranes, which could occur *via* pores in the Nafion membrane structure.

Evidence of a relatively slow transfer of hydroxide into the membrane following reduction of VK₁ is supported by experiments conducted at pH 13 at variable scan rates (Figure 5). As the scan rate increased (Figure 5a – 5d), there was a decrease in the rate of conversion of the VK₁ in an acidic environment (shown by processes D₁'/D₂') compared to VK₁ in a basic environment (shown by the appearance of processes D₁''/D₂''). Once the scan rate reached 2 V s⁻¹ (Figure 5d), only processes D₁'/D₂' were observed, indicating that the quinone was chemically reversibly converted into the hydroquinone, without being further converted into the hydrogen bonded dianion (the conversion process from VK₁H₂ back to VK₁ was sufficiently fast that it did not undergo deprotonation).

7.3 Conclusions

This study has demonstrated that it is possible to construct membranes on electrode surfaces that allow CV experiments to be performed over a wide potential range on

molecules confined inside the membranes. The method should be applicable to all low molecular weight biological molecules that exist in lipid bilayer membranes. The membranes when constructed of Nafion|lecithin|Nafion layers allow reproducible voltammograms to be obtained at moderately fast scan rates. The voltammetric results indicate that the membranes have solvent like properties that allow both electron transfer and ion transfer processes to occur. In all the experiments that were conducted on VK₁ within the Nafion|lecithin|Nafion layers, there was no evidence that the organic molecule left the membrane. Instead, the balance in charge that was brought about by the reduction and oxidation processes was controlled by the transfer of protons through the Nafion layers. The function of Nafion is to interface with the hydrophilic groups of the bilayer membranes and thereby provide an improved pathway for ion transfer between the membranes and aqueous solutions.

VK₁ undergo a series of electron and proton transfer reactions that can be voltammetrically detected within the membranes. The processes are complicated but can be interpreted based on previous voltammetry experiments of the compounds in non-aqueous solvents^{54,57-68} and where the compounds are attached to the electrodes in aqueous solutions.^{54-56,69} For bulk solution pH values between 3 – 11, the Nafion layers were able to shield the membranes from the solution phase electrolytes and thereby provide a constant and low pH environment for the molecules within the membranes during the voltammetric experiments. At pH 13, hydroxide ions were able to enter the membrane structure during slow voltammetric scanning experiments, while at faster scan rates ($\nu > 2 \text{ V s}^{-1}$), the hydroxide was excluded from the membranes.

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Chapter 8

Summary

Vitamin K₁ (VK₁) was shown by voltammetry and coulometry to undergo two chemically reversible one-electron reduction processes in acetonitrile (CH₃CN) containing Bu₄NPF₆ as the supporting electrolyte. The potential separation between the first and second electron transfer steps diminished sequentially with the addition of water, so that at a H₂O concentration of approximately 7 M (~13% v/v) only one process was detected, corresponding to the reversible transfer of two-electrons per molecule. The voltammetric behavior was interpreted based on the degree of hydrogen-bonding between the reduced forms of VK₁ with water in the solvent. It was found that the potential separation between the first and second processes was especially sensitive to water in the low molar levels (0.001 – 0.1 M), therefore; by measuring the peak separation as a function of controlled water concentrations (accurately determined by Karl Fischer coulometric titrations) it was possible to prepare calibration curves of peak separation versus water concentration. The calibration procedure is independent of the type of reference electrode and can be used to determine the water content of CH₃CN between 0.01 – 5 M, by performing a single voltammetric scan in the presence of 1.0 mM VK₁. The reduction processes were monitored by *in-situ* electrochemical–UV–Vis spectroscopy in CH₃CN over a range of

water concentrations (0.05 – 10 M) in order to spectroscopically identify the hydrogen bonded species.

The voltammetry of VK₁ was investigated in a range of other organic solvents of varying dielectric constant that are commonly used for electrochemical measurements [dimethyl sulfoxide (DMSO), *N,N*-dimethylformamide (DMF), propionitrile (EtCN), butyronitrile (PrCN), 1,2-dichloroethane (DCE), dichloromethane (DCM) and 1,1,2,2-tetrachloroethane (TCE)]. The water content of the solvents were accurately measured using KF coulometric titrations and the voltammetric data were used to estimate the degree of hydrogen-bonding interactions between the reduced forms of VK₁ and variable levels of water, thereby allowing a ranking of water-substrate interactions in the different solvents. The voltammetric data were analyzed based on interactions that occur between reduced forms of VK₁ and the water, the solvent and the supporting electrolyte. Calibration data were obtained which are independent of the nature of the reference electrode, and allow the water content of the solvents to be calculated by performing a single voltammetric scan in the presence of VK₁ and 0.2 M supporting electrolyte (Bu₄NPF₆).

Vitamin K-SH (VKSH), a derivative of VK₁, was used to investigate the electrochemical properties of surface bound vitamin K by performing voltammetry in CH₃CN and CH₂Cl₂. VKSH was chemically attached to the surface of a Au working electrode through the sulfur linkage. Two one-electron processes were observed when a Pt wire was used as a reference electrode in solutions with 0.2 M Bu₄NPF₆ as the supporting electrolyte. The water concentrations were accurately measured by KF

titration, and the peak separations between the two processes diminished when the water concentration increased. The voltammetric behavior of VKSH was similar to that of VK₁, which was based on the hydrogen-bonding between the reduced forms of VKSH and the water in the solvents.

A robust model membrane environment was developed to enable voltammetry experiments to be performed on low molecular weight biological molecules completely incorporated inside artificial lipid bilayer (or multilayer) membranes. The artificial supported membranes were prepared by sandwiching multilayers of lecithin between layers of Nafion that were deposited on the surface of a glassy carbon electrode. The Nafion films acted as a conduit to aid proton transfer across the lecithin solution interface, and thereby balance the charge brought about by the electrochemical reactions. VK₁ was separately incorporated inside the Nafion|lecithin|Nafion layers and the coated electrodes were immersed in aqueous solutions between pH 3 – 13. The electrode coating procedure produced multilayer membranes with solvent like properties enabling highly reproducible diffusion controlled voltammetric processes to be observed. VK₁ underwent multiple electron-transfer and proton-transfer reactions inside the membranes.

Many solvents used for electrochemistry can be dried to $< 1 \times 10^{-3}$ M water content by storing the solvents over 3Å molecular sieves in a nitrogen or argon atmosphere. However, as soon as the solvents are placed in an electrochemical cell, the water content increases significantly. KF coulometric titrations were conducted on several pre-dried solvents commonly used for electrochemistry (CH₃CN, CH₂Cl₂, DMF and DMSO) in a

controlled humidity environment (30%, 50% and 70% relative humidity) to determine the rate of moisture uptake into the organic solvents when used under typical electrochemical conditions (either in an electrochemical cell under a nitrogen atmosphere or in an electrochemical cell directly exposed to the atmosphere). The results in this study give guidelines for estimating the water content of organic solvents under conventional electrochemical operating conditions.

Publications

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Hydrogen Bonding Interactions between Water and the One- and Two-Electron Reduced forms of Vitamin K₁: Applying Quinone Electrochemistry to Determine the Moisture Content of Non-Aqueous Solvents

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