

Developing applications of bioelectrochemistry: interfaces and electrocatalytic synthesis

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Abstract

The use of biomolecules in chemical applications, such as biosensors, fuel cells and chemical synthesis, is becoming widespread, as improvements are made in biomolecule production, engineering and stability, and cofactor provision. This allows chemists to exploit the selectivity and high activities often observed with biological molecules such as antibodies and enzymes. This thesis concerns the characterisation of (i) an alkanethiol monolayer interface for the immobilisation of antibodies for biosensing, and (ii) the development of an electrochemical cofactor regeneration system for an application in bioelectrosynthesis.

Firstly, experiments are performed to confirm the capacitance characteristics of alkanethiol films on gold surfaces. These films have been used as antibody supports for proximal biosensing of antigens, though most examples have required the use of a redox transducer to measure an electrochemical signal. A more specific model for the capacitance of a molecular film has been developed and the empirical observation of these parameters is confirmed in this thesis.

Secondly, an electrochemically-driven NADH regeneration system is presented with NAD⁺-reductase modified carbon electrodes, offering perfect selectivity for the active NADH cofactor. It is shown that the NAD⁺-reductase enzyme can be immobilised onto carbon interfaces and that it is electrochemically active at much more modest potentials than using unmodified electrodes. NADH-dependent oxidoreductase enzymes offer exquisite selectivity under mild reaction conditions, but have proved difficult to exploit in electrosynthesis. These enzymes require stoichiometric hydride transfer from the expensive biological NAD(P)H cofactor, which means that their use is only viable when they are coupled with an efficient cofactor recycling method. This bioelectroenzymatic system is demonstrated as a modular approach to biocatalytic reductions, coupling NADH-dependent reductases for efficient C=X reductions. Application of this bioelectrocatalytic interface to an electrochemical flow cell increases catalytic activity, with turnover frequencies up to 8.19 s⁻¹, improves stability and allows for in line optimisation analysis.

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List of Abbreviations

A	Absorption.
A	Electrode area.
A_{geometrical}	Geometrical surface area.
A_{total}	Total surface area.
Å	Ångström = $\times 10^{-10}$ m.
a.c.	Alternating current.
ACN	Acetonitrile.
aCP	Activated carbon paper.
aCP(H⁺)	Activated carbon paper pre-treated with concentrated HCl.
alaDH	Alanine dehydrogenase.
ATR	Attenuated total reflectance.
BET	Brunauer-Emmett-Teller specific surface area.
BIS-TRIS-HCl	Bis-tris methane buffered with concentrated HCl.
c	Concentration.
c_∞	Concentration in the bulk solution.
C	Concentration.
C'/C''	Real/imaginary capacitance.
C_A	Area-averaged capacitance.
C_d	Discharge capacitance.
CE	Counter electrode.
CP	Carbon paper.
CPE	Constant phase element.
CS	Capacitance spectroscopy.
d	Doublet.
δ	Chemical shift.

δ	Reciprocal of the inter-plate distance.
D	Diffusion coefficient.
DMF	Dimethylformamide.
DMSO	Dimethyl sulfoxide.
dt	Doublet of triplets.
e	Charge of an electron = -1.602×10^{-19} C.
e^-	Electron.
ϵ	Molar extinction coefficient.
ϵ_0	Permittivity of free space.
ϵ_r	Relative permittivity.
E	Potential.
$E'_{\text{NAD}^+/\text{NADH}}$	Equilibrium potential for the NAD^+/NADH couple.
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide.
e.e.	Enantiomeric excess.
EIS	Electrochemical impedance spectroscopy.
F	Faraday's constant = $eN_A = 96\,485$ C mol $^{-1}$.
ϕ	Electrochemical potential.
ϕ	Phase (angle).
FMN	Flavin mononucleotide.
FR	Fermi resonance.
FTIR	Fourier transform infrared spectroscopy.
G	Gibbs free energy.
η_{Faradaic}	Faradaic efficiency.
HoxFU(I ₂)	NAD^+ -reducing subunit of the SH from <i>R. eutropha</i> .
HoxHY	H_2 -oxidising subunit of the SH from <i>R. eutropha</i> .
HPLC	High-performance liquid chromatography.
i	Current.
i_d	Discharge current.
i_L	Levich current.
i_{max}	Maximum catalytic current.
j	Imaginary number = $\sqrt{-1}$.

List of Abbreviations

J	Coupling constant.
κ	Debye length.
k_B	Boltzmann constant = $1.38 \times 10^{-23} \text{ J K}^{-1}$.
k_{cat}	Turnover number (Michaelis-Menten model).
K_M	Michaelis constant (Michaelis-Menten model).
l	Length.
L	Inter-plate distance.
λ_{max}	Wavelength of maximum absorption.
L_D	Monolayer thickness.
LDH	Lactate dehydrogenase.
LOD	Limit of detection.
m	Mass.
$\mathbf{m}$	Multiplet.
MMSE	Mercury-mercurous sulfate electrode, $E = +0.64 \text{ V}$ vs SHE.
n	Number of electrons.
N_0	Ionic concentration.
N_A	Avogadro number = 6.02×10^{23} .
ν	Frequency.
ν	Kinematic viscosity.
ν	Scan rate.
NAD^+	Nicotinamide adenine dinucleotide.
$\text{NAD}^+\text{-red}$	Variant of the SH from <i>R. eutropha</i> with a deactivated hydrogenase module.
NADH	Nicotinamide adenine dinucleotide hydride.
NHS	N-Hydroxysuccinimide.
$\text{NMR}/^1\text{H NMR}$	^1H Nuclear magnetic resonance spectroscopy.
ω	Angular rotation rate.
ω	Electronic perturbation frequency.
ω	Radial frequency.
OCP	Open circuit potential.

O/R C=X	Oxidoreductase substrates containing C=X and their reduced equivalent molecules.
Ox	Oxidised species.
π	Pi = 3.141 . . .
PCTFE	Polychlorotrifluoroethylene.
PEEK	Polyether ether ketone.
PDB	Protein Data Bank.
PGE	Pyrolytic graphite edge.
pK_a	Dissociation constant for an acid.
ppm	Parts per million.
py	1-Pyrenebutyric acid
<i>Q</i>	Charge.
<i>Q</i>	Reaction quotient.
<i>Q_t</i>	Constant phase element.
<i>R</i>	Resistance.
<i>R</i>	Molar gas constant = 8.314 J K ⁻¹ mol ⁻¹ .
<i>R. eutropha</i>	<i>Ralstonia eutropha</i> .
<i>R</i>²	Coefficient of determination.
RDE	Rotating disk electrode.
RE	Reference electrode.
Red	Reduced species.
rpm	Rotations per minute.
s	Singlet.
SAM	Self-assembled monolayer.
SCE	Saturated calomel electrode, <i>E</i> = +0.242 V vs SHE.
SH	Soluble hydrogenase.
SHE	Standard hydrogen electrode, <i>E</i> = 0 V vs SHE.
<i>t</i>	Time.
<i>T</i>	Temperature.
τ	Transition time.
τ_c	Angular time constant.

List of Abbreviations

t_R	Residence time.
<i>T. thermophilus</i> .	<i>Thermus thermophilus</i> .
Tris-HCl	Tris(hydroxymethyl)aminomethane buffered with concentrated HCl.
UV/UV-Vis	Ultraviolet-Visible spectroscopy.
V_i	Initial potential.
$V_{\max}$	Maximum achievable rate (Michaelis-Menten model).
V_{onset}	Onset potential.
$v/v\%$	Volume/volume percentage.
WE	Working electrode.
Y	Constant phase element.
z	Valence number of ions.
Z	Impedance.
Z'/Z''	Real/imaginary impedance.

1

Introduction

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The process of electron transfer is an integral part of all biological systems primarily because it is the method by which biological cells store and transfer energy.

Bioelectrochemists are interested in the study of these electron transfer pathways to better understand the fundamental processes of biological molecules. With this information, scientists have been able to develop biological electrochemical devices, such as biosensors, biofuel cells and bioelectrodes for catalysis. This Thesis concerns the development of two of such devices: the characterisation of a molecular monolayer support for antibody biosensors, and the development of a bioelectrocatalyst for selective reductions in flow.

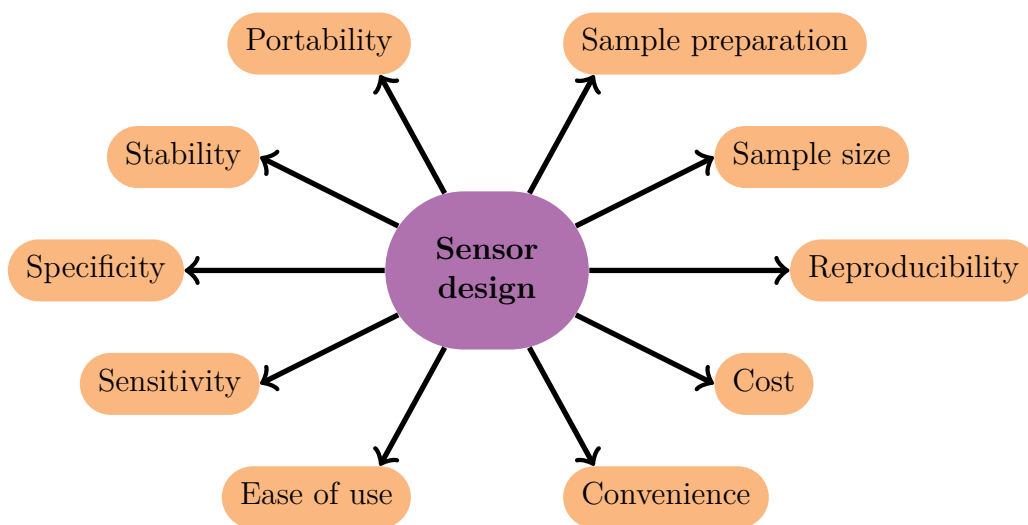
1.1 Electrochemical immunoassays and molecular self-assembling monolayers as an antibody support

The electrochemical biosensor is a topic of considerable interest in the bioelectrochemistry community. Using biological constructs such as enzymes, antibodies or whole cells that demonstrate specific interactions, and developing a method to transduce their activity into an electrochemical signal, creates bioelectrochemical devices that can detect small proteins and molecules. The advantage of combining biological molecules with electrochemical transduction methods is the high specificity that comes with reactions at biological sites, combined with the sensitivity, convenience and ability to miniaturise electrochemical devices.

1.1.1 Electrochemical sensor design principles

Scheme 1.1 indicates some of the fundamental principles of electrochemical sensor design.^[1] These ten factors are the main driving forces for good biosensor design, which could be successfully implemented as a useful device. This section explains the reason reasoning behind these design principles.

Sample preparation Ideally samples would not have to be prepared and could be used directly from the source, otherwise this would increase the amount of time and skill required to take measurements.



Scheme 1.1: Aspects of sensor design for successful application.

Sample size A small sample size is often desirable, to minimise discomfort to a patient, in the case of medical diagnostic devices or minimise intrusion on items such as foodstuff etc.

Reproducibility Problems in reproducibility can arise from user error or from the device itself. Firstly the incorrect preparation or operation of the sensor device could affect the reproducibility of the sensor. In particular, ensuring the correct calibration method is performed often leads to large errors in the reading. There are often reproducibility errors when measurements are performed in different media, where other molecules could interfere with the sensor itself. Another source of poor reproducibility could be manufacturing errors. Generally industrial producers of sensors perform calibrations on batches of devices to minimise the error found between each device, however if the sensor is designed to measure very low concentrations (down to femtomolar, for example), calibrations may need to be performed on each device before use, which would increase diagnostic time and would require trained scientists.

Cost Particularly in the case of medical sensors, where the aim is to pick up early symptoms in a large proportion of the population, low production cost is essential

to ensure they are widely available, especially in countries with low GDP per capita.

Convenience The sensor should be a convenient, quick and reliable method for routine diagnostic assessments.

Sensitivity A key aspect of sensors is their sensitivity. For example, diagnostic proteins often exist in very low concentration in the body, picomolar or lower. Therefore the sensor interface is required to have a *limit of detection*, LOD, low enough to be diagnostic.

Specificity Sensor devices need to be specific for the analytes they are designed to detect. If not, false positives can occur. Biological molecules such as antibodies and enzymes are often natively highly specific and so are often incorporated into sensor design as receptors.

Stability After manufacture, sensors may not be used immediately. The device interface needs to be mechanically and chemically stable with a reasonable shelf-life so that it can be stored and used on demand. If the sensing interface is not chemically stable, there may be a decrease in sensitivity over time, or false positives or negatives may occur.

Ease of use The operation of the sensor should be straightforward, so that the use of the device is not limited to trained scientists.

Portability The size and weight of the sensor device should not restrict it to a fixed position, or restrict it to use in a laboratory.

The application of electrochemistry to sensing can address many of these aspects, in particular sensitivity, convenience, cost, portability, ease of use and sample size. However, electrochemical sensors may falter in their reproducibility and sample preparation. For example, in the cases where an external redox probe is needed in the sample to measure a charge transfer signal.

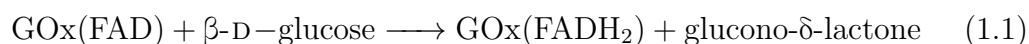
1.1.2 Types of electrochemical enzymatic biosensors

There are many different approaches to biosensor design, however the glucose biosensor, which employs the use of enzymes on an electrode, is probably the most well known. Since the introduction of the glucose biosensor, there have been other enzymes used for electrochemical sensing applications. Microorganisms can also be immobilised onto electrodes in a membrane contained environment with an electrochemical transducer molecule. Another sensor design is to use immobilised antibodies on a molecular surface as the analyte receptor and as a proximity immunosensor. It is the electrochemical design of this molecular surface which is explored further in this Thesis in Chapter 3 on page 71.

1.1.2.1 Glucose biosensors

The glucose determining biosensor is probably one of the most famous examples of electrochemical biosensor. The concept of the glucose enzyme electrode was first published by Clark and Lyons in 1962^[2] and has revolutionised blood glucose monitoring of millions of medical patients around the world.

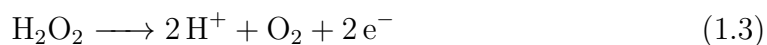
The fundamental reaction behind the glucose enzyme biosensor is based on the fact that the enzyme *glucose oxidase*, GOx, immobilised as a thin film at an oxygen electrode, catalyses the oxidation of β -D-glucose to produce a gluconic acid and hydrogen peroxide via the reduction and reoxidation of the FAD cofactor, as shown in equations (1.1)–(1.2).



Historically, the depletion of the oxygen was measured as a way of determining the concentration of glucose within the blood sample. However *amperometric biosensors* have been developed to measure the oxidation or reduction currents of any of the electrochemically active species in the above equations (1.1)–(1.2). These work by measuring the charge passed through the working electrode used

to oxidise or reduce the species in question, which can be used to calculate the concentration of glucose in the sample

For example, the H_2O_2 produced as a result of these reactions can then be oxidised, most often, at a Pt electrode, as shown in Equation (1.3). The charge transferred through the electrode in order to oxidise the H_2O_2 can be measured and the number of moles of glucose molecules in the blood sample can be calculated.^[3]



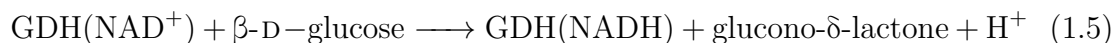
1.1.2.2 Other opportunities for enzyme electrochemical biosensors

One area that is under exploited in sensor technology is the application of enzymes dependent on NAD(P)H cofactors. These molecules act as biological mediators which can reversibly oxidise or reduce by a two electron transfer, as shown in Equation (1.4).



The NADH-dependent enzyme *glucose dehydrogenase*, GDH, offers an alternative way for the electrochemical sensing of glucose.

With GOx sensors, it was often found that the concentration of O_2 within the constructed electrodes was at a much lower concentration than that of glucose in the blood samples. Therefore the O_2 may be depleted before all the glucose in the sample had reacted with GOx. In addition to this, any O_2 contamination could give false readings in the true concentration of glucose in the blood sample. Therefore there is active investigation into the use of GDH as an alternative enzyme, which does not require O_2 for the oxidation of glucose. Instead, a hydride is transferred from the glucose to an NAD^+ cofactor (a 2e^- transfer), as shown in Equation (1.5).



However, the thermal instability of GDH as compared to GOx means that the construction of these sensors is more challenging.

The use of GDH fed with glucose for NADH regeneration is a common method in industrial reactions where NADH-dependent enzymes are used for specific reductions. This topic is discussed later in Section 1.2.4.

If the reaction shown in Equation (1.5) is coupled with a reversible NAD^+ -reductase enzyme, which exhibits electron transfer to a working electrode, direct amperometric measurement can be made for the reduction of NAD^+ . This can be used to calculate a concentration for glucose in the blood sample.

Microbes have evolved to express enzymes which are very specific at catalysing one reaction only. Those that catalyse redox reactions are called *oxidoreductase* enzymes, discussed in more detail in Section 1.2.2 on page 12. Since enzymes are often very specific for their substrates, they make for very effective receptors. For oxidoreductases, if the charge passed due to their oxidation or reduction reactions can be transduced to an electrode, an electrochemical amperometric biosensor can be designed.

Of all the oxidoreductase enzymes, around 50% are dependent on the NAD(P)^+ or NAD(P)H cofactors. This offers a huge range of opportunities for sensing different molecules if $\text{NAD(P)}^+/\text{NAD(P)H}$ dependent enzymes can be effectively interfaced with electrodes.

1.1.3 Proximity immunosensors

1.1.3.1 Background and ELISA

The study and use of the binding interaction between an antibody and the antigen was first proposed by Yalow and Berson^[4] for insulin detection. The binding interaction is transduced via the radioactivity of insulin which is labelled with radioactive iodine-131. Soon after another article was published by Ekins,^[5] who described a similar immunoassay for the detection of thyroxine using radioactive iodine-131 labelled thyroxine. These two articles led the way for the development of electrochemical immunosensors using an electrochemical signal as the transduction method instead of radioactivity assays.

The use of antibodies as a receptor for the analyte is very desirable due to their high sensitivity and high specificity for the specific antigen. Typically, antibodies have affinity constants in the range of $10 \times 10^8 \text{ L M}^{-1}$ to $10 \times 10^{10} \text{ L M}^{-1}$ for their target antigen. These values are generally three orders of magnitude higher than the average affinity constants for enzymes with their substrates, therefore antibody sensors have the capability of having stronger binding responses.

Another well-known example of the application of immunoassays is for pregnancy testing. These devices test for the presence of the human chorionic gonadotropin hormone, hCG, the levels of which rise quickly in the first few weeks of pregnancy. The device contains two different antibodies for hCG: one that binds to the hCG in the mobile phase of the device and another antibody which is fixed in position on the test strip area. The hCG hormone becomes sandwiched between the two antibodies in the test strip area. An enzyme conjugated to the mobile antibodies then induces a colour change in the dye included in the test area, indicating a positive response.

The ELISA assay, or the *enzyme-linked immunosorbent assay* was developed from the pregnancy testing device, which makes use of monoclonal antibodies to detect a wide range of antigens. ELISA is the standard diagnostic tool in biotechnology, as well as for medical diagnostics^[6,7] and for use as a quality control measure, for example in the food and drink industry. The fundamental principle of the ELISA assay is the interaction between an antibody and an antigen. The binding interaction is transduced by a colour changing substrate that is converted by an enzyme conjugated with the antibody, often *horseradish peroxidase*, HRP, or *alkaline phosphatase*, AP. Monitoring the absorbance of these substrates in the presence of these enzymes can be used to construct a calibration curve, and then determine the concentration of the analyte in the sample.

There are a few different ELISA methods, such as direct ELISA, sandwich ELISA (similar to the pregnancy test device), competitive ELISA and reverse ELISA. However they all rely upon the proximal binding interaction between one or two antibodies with the antigen itself. Although these protocols are robust

and established methods in the diagnostic community, there are a few limitations with the procedure.

Firstly, the ELISA protocols are multistep and can be time consuming. This has a slight negative effect on the convenience of such a diagnostic device. The procedure requires laboratory equipment and trained scientists to perform the protocol, therefore ELISA is not easy to use for untrained people. Also, the LOD is restricted by the detection limits on the platereader, which is often used for measuring the absorbance of the dye. Colorimetric or fluorimetric transduction methods for ELISA are normally limited to an LOD of 1 ng mL^{-1} to 20 ng mL^{-1} ,^[8-10] as compared to electrochemical measurements that have been reported to achieve LODs of 0.1 ng mL^{-1} .^[8,11-14] However, this is highly dependent on the affinity constants for the antibodies used in the ELISA study as well as the sensitivity of the plate reader.

1.1.3.2 Electrochemical proximity immunosensors

Antibodies can be attached to conductive interfaces. There are generally five main electrochemical methods for the transduction of the binding event between antigen and antibody. These are:

Voltammetric The most commonly used method is an active technique where a potential is applied to a conductive surface modified with antibodies. The applied potential forces a change in the concentration of an electroactive species in the solution, as a result of a redox event at the electrode. Sometimes other species in the sample are also electroactive at the applied potential, contributing to errors in the measurement.

Potentiometric A method where the potential of an electrode modified with antibodies is measured. Upon binding of the antigen with the antibodies, there is a change in the charge held at the electrode, as a result of changing net electrical charge polarity at the electrode surface. The sensitivity of these assays are limited

due the low changes in potential observed for the binding events, often between 1 mV to 5 mV.

Conductometric The conductivity of an electrolyte is measured, which is affected by the variance of the concentration of antigens bound to the relevant antibodies immobilised on an electrode. An advantage is that these devices can be very cheaply manufactured, as no reference electrode is required, and differential methods negate the effect of some interferences.^[15] However these devices often have high LODs of about 1 μM ^[16] limiting their sensitivity.

Impedimetric The real and imaginary resistance and capacitance properties of a surface modified with antibodies are measured upon perturbation by a small amplitude sinusoidal excitation signal.^[17] This can be by monitoring a charge transfer mechanism between the electrode and a mediator, in solution or at the surface of the molecular interface (Faradaic), or intrinsically with no mediator (non-Faradaic). It is an incredibly powerful electrochemical technique, and could be used as a label and mediator-free electrochemical method for substrate detection, as well as having very low detection limits^[17,18] however it can often provide too much information on the impedimetric characteristics of the modified electrode. This has brought problems in specificity and reproducibility, as often the impedimetric response may not only arise from antibody-antigen interaction.^[19–21]

Capacitive This monitors the change in the thickness or the dielectric properties of the dielectric layer at the electrolyte-dielectric interface as a result of the binding interaction between the antibody and the antigen. This can provide a direct, label-free and sensitive transduction method for interpreting the antibody-antigen interaction.^[16,22,23] This method can provide very low sensitivities in the 10^1 ng mL^{-1} range,^[23] and has been reported as low as 0.5 pg mL^{-1} .^[24] However problems arise as specificity can be quite poor^[25] and there are often large problems in reproducibility.^[26]

1.1.4 Self-assembled monolayers as an antibody support

The spontaneous chemisorption of alkanethiols onto gold surfaces was first observed by Whitesides and coworkers.^[27–31] These so-called *self-assembled monolayers*, SAMs, offer an easy method for the functionalization of gold surfaces and therefore have been exploited to associate diagnostic antibodies to gold electrodes for the development of electrochemical immunosensors.^[32,33] The presence of the SAM as a spacer group between the gold surface and the antibody also avoids the deactivation of the protein, if it were associated directly with the gold surface.

There are overwhelming numbers of examples of these electrochemical proximity immunosensors that use self-assembled monolayers as an antibody support. Most of the electrochemical techniques mentioned in Section 1.1.3.2 have been used for these electrochemical sensors, but there are relatively few using impedimetric techniques for the analytical determination of analyte concentration.^[21] This is mainly due to this electrochemical technique being incredibly sensitive, which leads to inadequate resolution of the electrochemical signal as a result of the proximal

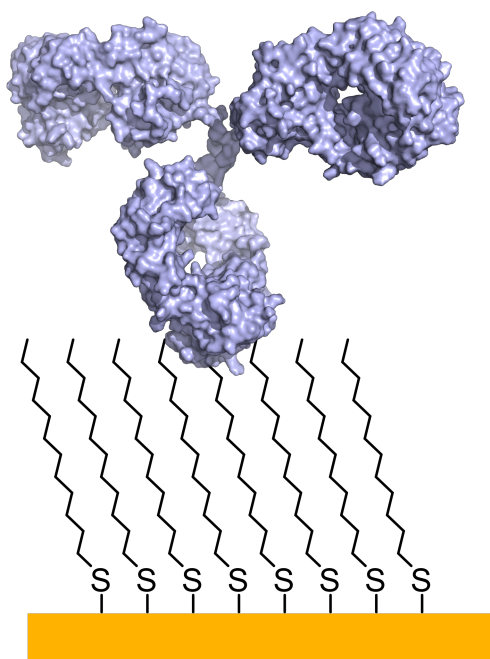


Figure 1.1: Schematic representation of an antibody immobilised onto a molecular SAM on a gold electrode (not to scale).

association of the analyte to the antibody. However, the benefit of impedance spectroscopy being so sensitive is that analytical LODs could reach incredibly low limits using this electrochemical technique. Therefore it is important to gain a more detailed understanding of the electrochemical properties of the SAMs which form the foundation of the immunosensor, from which there will be a more accurate understanding and resolution of the impedimetric response.

The project discussed in Chapter 3 aims to measure and interpret the resolved capacitance terms of a SAM film revealed through the development of a more general electrochemical mode of these films.

1.2 Redox biocatalysis

1.2.1 Types of redox biocatalysis

The use of enzymes for molecular redox transformations have become established industrial synthetic routes over the last couple of decades. In 2018, Turner and Kumar claimed that we are now in the “golden age of biocatalysis”.^[34] Enzymes, either isolated, or as part of whole cells, can offer significant advantages in chemoselectivity, regioselectivity, diastereoselectivity and enantioselectivity over traditional non-biological catalysts. Not only does this offer improvements in Green Chemistry credentials for industrial synthesis,^[35] but the use of oxidoreductases for redox reactions have opened up a wide variety of reaction routes previously unachievable and are particularly attractive for the synthesis of chiral alcohols and amines.

1.2.2 NAD(P)H dependent oxidoreductases for biocatalysis

The group of redox enzymes called *oxidoreductases* perform reversible redox transformations on their substrates. Typically they catalyse the reduction of one species with the oxidation of another in tandem;^[36] this reaction is also often reversible. Oxidoreductases account for roughly a third of all enzymatic activities recorded in the Braunschweig Enzyme Database, BRENDA, and are able to act on a wide variety of organic substrates, for example alcohols,^[37] amines^[38] and ketones,^[39] and

inorganic substrates, such as sulfite and metals such as mercury.^[36] The smaller subsection of oxidoreductases that are dependent on a nicotinamide cofactor, NAD(P)H-dependent, constitute roughly 50% of all oxidoreductase enzyme activities registered on BRENDA. There are roughly 150 000 different protein sequences that are defined to be, or predicted to be, NAD(P)H-dependent in their reactions.^[40]

In biological cells, NAD(P)^+ and the reduced forms, NAD(P)H, act as the pool of two-electron redox cofactors, with labile hydrides providing the electrons. In NADP^+ and NADPH cofactors, the presence of a phosphate group on the 2'-hydroxyl group of the adenine ring only modifies the structure of the cofactor, thereby allowing different enzymes to have different specificities for NAD^+/NADH and $\text{NADP}^+/\text{NADPH}$, separating the two pools into two effectively equivalent redox systems. The two different pools of NAD(P)H cofactors allows for different redox potential poises, for example in *Escherichia coli* (*E. coli*) growing by aerobic respiration, anaerobic respiration or anaerobic fermentation, the prevalence of O_2 affects the steady-state NADH/NAD^+ ratio.^[41] At the same time, the $\text{NADPH}/\text{NADP}^+$ pool is generally biased in the more reduced state, providing electrons for biocatalytic reduction.^[42] The separate pools of NADH and NADPH cofactors allows for different enzymes to catalyse key reaction steps in opposite directions, without cancelling the action of each other.^[43]

There are many different types or groups of oxidoreductases which can be used for redox catalysis, including alcohol dehydrogenases, oxygenases and monooxygenases. There is great toolbox of reactions that can be performed with NAD(P)H oxidoreductases that is open to exploitation for synthetic chemists.

1.2.3 Industrial biocatalysis

The 2018 market for industrial use of enzymes was estimated to have a value of \$10.48 bn,^[44] with pharmaceutical and biocatalytic transformations contributing to 21% of the total revenue (Figure 1.2). Industrial biocatalysis is a very lucrative business and much research has gone into refinement of the redox biocatalytic processes.

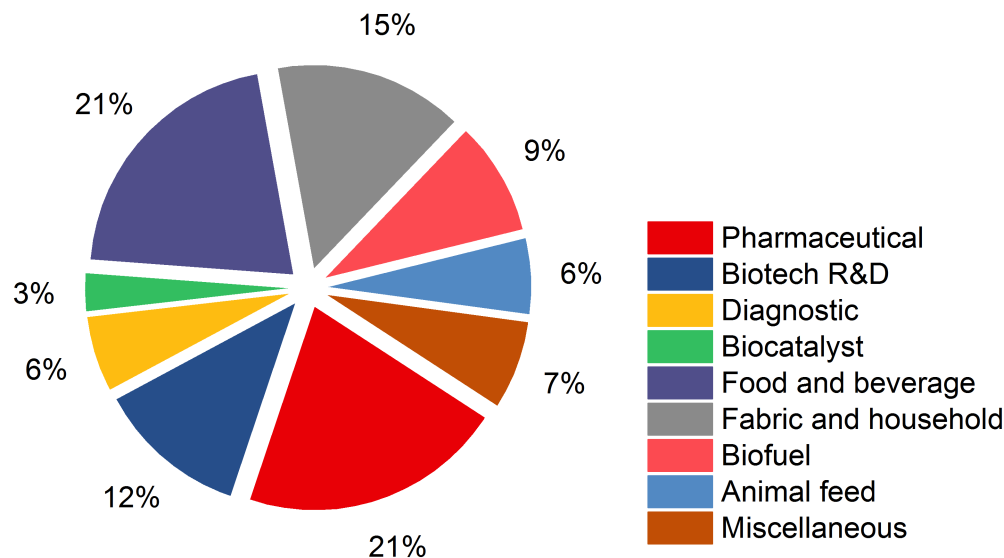


Figure 1.2: Breakdown of the World Market of industrial enzyme use in 2010 by sector.^[44]

1.2.4 Common NAD(P)H-dependent reductive biocatalysis in industry and common cofactor regeneration techniques

The use of NADH-dependent enzymes for catalysis is only viable when the cofactor is recycled. This is due to the prohibitively high cost of having stoichiometric amounts of NAD(P)H cofactors in solution with the reagents as shown in Table 1.1. Therefore most industrial biotransformations are coupled with a second enzyme to regenerate the cofactor.^[45–48] The main enzymes used in industrial biocatalysis are formate dehydrogenases, FDHs, which are generally more NAD^+ -specific, and glucose dehydrogenases, GDHs, which are generally more NADP^+ specific.^[39] Advantages in using these two enzymes for cofactor recycling is that they reduce

Table 1.1: Typical cost per mole of cofactor.

Cofactor	Cost / £mol^{-1a}
NAD^+	2320
NADP^+	6696
NADH	5320
NADPH	51 511

^aPrices from Prozomix, October 2019.

the amount of cofactor required for the reaction, are relatively cheap and easily available with high activities and stabilities.^[39]

For example, the manufacture of (*R*)-2-hydroxy-4-phenylbutyric acid by Ciba Spezialitätenchemie AG uses an NAD⁺-dependent α -ketone reductase, LDH, from *Staphylococcus epidermidis* in tandem with an FDH from *Candida boidinii*, as shown in Scheme 1.3a.^[39] The use of the enantiomerically pure chiral building block (*R*)-2-hydroxy-4-phenylbutyric acid is vital in the production of different angiotensin converting enzymes (ACE) inhibitors. These drugs are important in the treatment of hypertension and congestive heart failures.^[49] The traditional chemical method

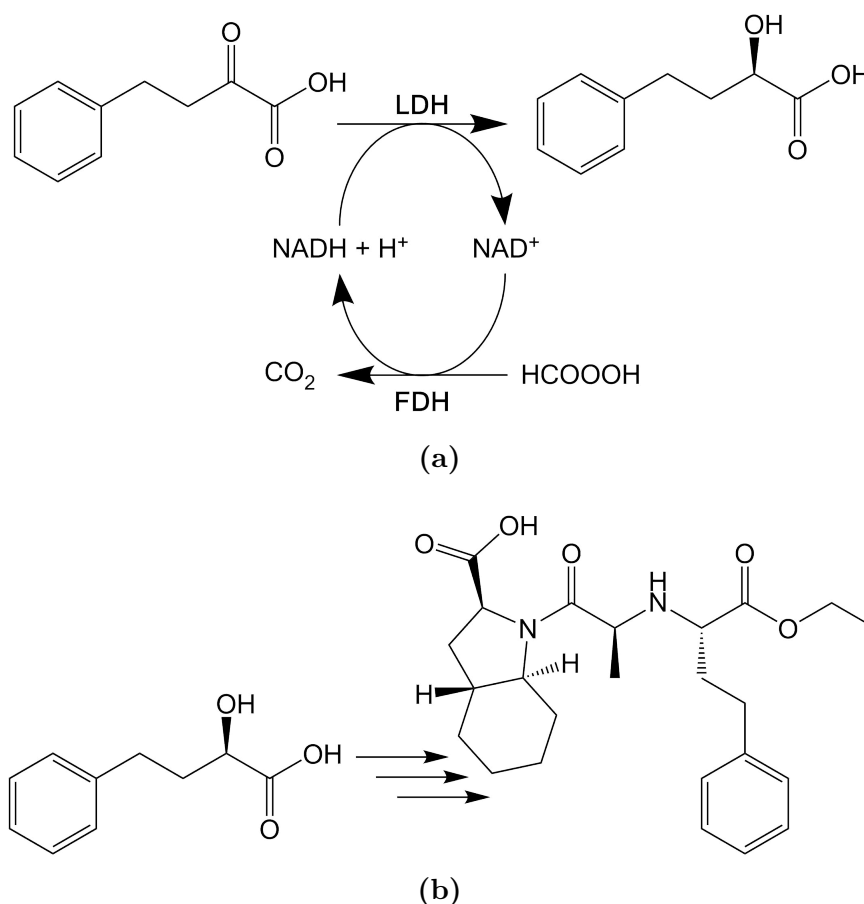


Figure 1.3: Biosynthesis of a Trandolapril precursor. **(a)** Example of NADH recycling by use of formate dehydrogenase (FDH) coupled with a lactate dehydrogenase (LDH) for enantiomeric-specific α -ketone reduction to generate (*R*)-2-hydroxy-4-phenylbutyric acid. **(b)** An example of the use of the chiral building block (*R*)-2-hydroxy-4-phenylbutyric acid, used in the manufacture of Trandolapril.

made use of a Pt–Al₂O₃ catalyst, which produced enantiomeric excesses (e.e.) of > 80%.^[49] The introduction of the LDH, coupled with an FDH to regenerate the NADH cofactor, together in a stirred tank reactor gave a superior enantiomeric excess of 99.9% e.e., reaching a space-time yield of 410 g L⁻¹ d⁻¹.^[39,49]

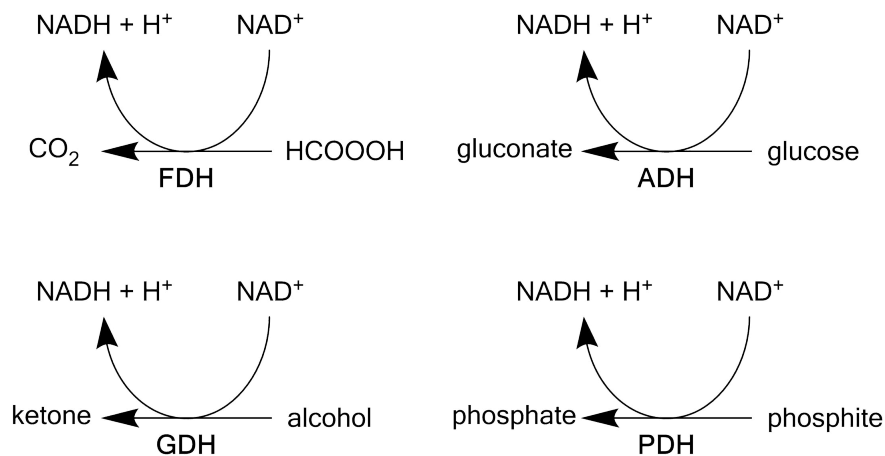
Scheme 1.3b shows an example of use of the chiral (*R*)-2-hydroxy-4-phenylbutyric acid in the production of Trandolapril. The chiral centre at the 2-hydroxyl group, generated by the use of the LDH, is retained, indicating the high importance of the enantioselective carbonyl reducing step in the manufacture of high-value pharmaceutical products.

The cofactor regeneration techniques frequently employed by industry are shown in Scheme 1.2 and summarised below.

Formate dehydrogenase FDH, was first demonstrated as a method of NADH regeneration by Shaked and Whitesides.^[50] The advantage of using FDH as the cofactor regeneration enzyme is that the waste product, CO₂, is a gas which is beneficial to the thermodynamics of the forward reaction, however this may acidify the solution on large scales. Formate as a substrate is cheap, though FDH enzymes are generally lower in specific activity and have generally been less popular in recent years.

Glucose dehydrogenase GDH, oxidises glucose to gluconate, which is immediately and irreversibly hydrolysed in aqueous solutions. Depending on the pH, the waste product could acidify the solution at large scales, and there is typically high sacrificial carbon waste, as a large excess of the cheap and readily available substrate glucose is often used with this method. However, GDH enzymes are highly active and are used often in industrial cofactor regeneration systems.

Alcohol dehydrogenase ADH, is the most common method of cofactor generation in industry, where a simple alcohol, such as isopropanol, is used as a substrate to reduce the cofactor. Due to the inherent reversibility of these enzymes, large



Scheme 1.2: A summary of common cofactor regeneration techniques in biocatalytic reductions. FDH: formate dehydrogenase; GDH: glucose dehydrogenase; ADH: alcohol dehydrogenase; PDH: phosphite dehydrogenase.

concentrations of alcohol are required which could begin to denature the enzyme as the solvent phase moves from aqueous to organic.

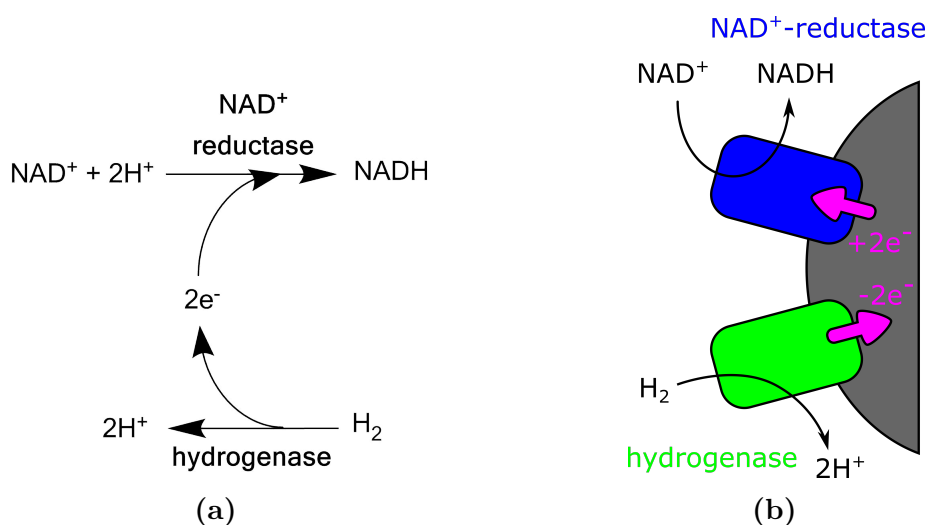
Phosphite dehydrogenase PDH, can be used as a useful cofactor regeneration, especially in phosphate buffer systems, in which the oxidation product would not interfere. However due to the low atom efficiency, the use in industrial processes is rather limited.

The main problem with these methods of cofactor regeneration is the use of a sacrificial substrate for hydride donation, and consequently a poor atom efficiency. Ideally, the enzyme used for cofactor regeneration should produce little or no waste. However in terms of powering enantioselectively specific oxidoreductase-catalysed reductions, the use of an enzyme for NADH regeneration with NADH-dependent oxidoreductase enzymes paves the way for enantioselective specific reductions with high enantiomeric excess, without suffering from the high expense of stoichiometric addition of cofactor.

1.2.5 Hydrogenase as a clean cofactor regeneration technique

Members from the Vincent Group^[51–53] have developed a cofactor recycling system where H_2 is used as the energy source for the reduction of NAD^+ to NADH. They observed that the NAD^+ -reducing moiety of *Ralstonia eutropha* (*R. eutropha*) soluble hydrogenase, immobilised on a graphite electrode, is an efficient electrocatalyst for NAD^+ reduction and NADH oxidation.^[54] This enzyme was combined with a robust hydrogenase, *E. coli* Hyd2,^[55] on a commercially available carbon surface (Black Pearls 2000, from Cabot) and supplied with H_2 . The H_2 was stripped of its electrons by the hydrogenase, in turn producing two protons (Scheme 1.3a), whilst the two electrons transferred to the NAD^+ reductase enzyme through the conductive carbon particles (Scheme 1.3b).

These enzyme-modified particles were found to operate at pressures as low as 0.02 bar H_2 and their heterogeneous nature meant that they could be separated from the reaction solution, with the possibility of being reused.^[52] An O_2 -tolerant hydrogenase, *E. coli* Hyd1, was also demonstrated to enable NADH regeneration at 1% to 2% O_2 levels in H_2 .

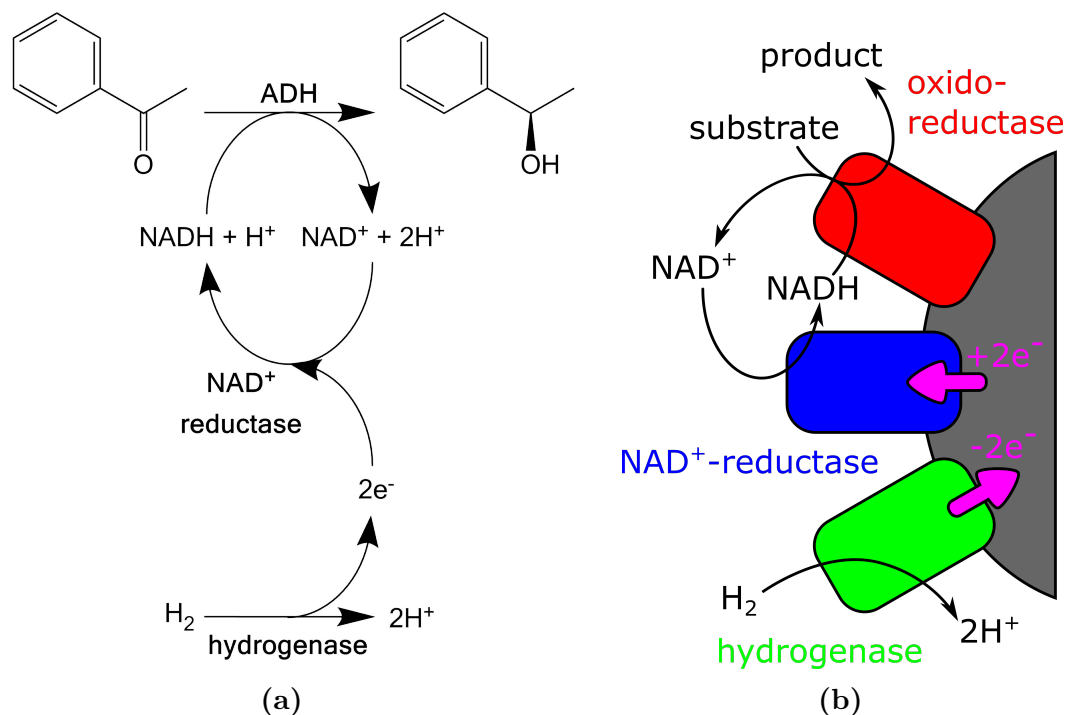


Scheme 1.3: (a) Using electrons generated by *E. coli* Hyd2 to fuel the reduction of NAD^+ to NADH with NAD^+ -reductase. (b) Diagram to show the movement of electrons between the immobilised hydrogenase (green) and NAD^+ -reductase (blue) through the conductive Black Pearls 2000 carbon nanoparticles (grey).

The reason why these hydrogenases work to provide electrons to power an NAD^+ reductase enzyme in the reduction of NAD^+ is that the reduction potentials for the H^+/H_2 (-0.41 V vs SHE at pH 7) and NAD^+/NADH (-0.32 V) couples are very close together. Therefore, any applied overpotential can very easily have an effect on the bias for the reduction or the oxidation steps. These reduction potentials do change with concentration of each of the reaction species, in line with the *Nernst equation* (see Section 2.1.3) and at high levels of H_2 and a high amount of NAD^+ compared to NADH , the reduction of NAD^+ by H_2 is favoured.

There have also been examples of using a combined chemo-bio system for the reduction of NAD^+ . Wang and Yiu^[56,57] have used Pt on an Al_2O_3 support for H_2 -driven NAD^+ reduction to NADH , which they used to power the reduction of propanal to propanol using an ADH. Although some NADH recycling was observed, it was seen that some incorrect structures of the cofactor were being generated by this catalyst. Some NADH recycling was also observed by Reeve *et al.* with a 20% catalytic loading of Pt on carbon particles with an NAD^+ reductase immobilised.^[51,53]

Reeve *et al.* have also demonstrated the coupling of this H_2 -driven cofactor recycling system with NADH -dependent oxidoreductase enzymes, for the specific reduction of pyruvate to L-lactate with an L-lactate dehydrogenase, LDH,^[51,58] and for the reduction of acetophenone to phenylethanol with an ADH (Figure 1.4), under mild reaction conditions.^[52,58] This third enzyme can be coimmobilised onto the carbon surface, maintaining the heterogeneous biocatalyst design. Advantages to this system include that the reduction reaction is fuelled by a clean renewable energy source, H_2 , and there is no carbon-based chemical waste as a result of the sacrificial reduction. It was also shown that the enzymes operate at high stability, with the NAD^+ reductase having total turnover number of $> 150\,000$ and the NADH cofactor having a high turnover number of > 600 . Depending on the ADH chosen for acetophenone reduction, it was shown that the reduction can be either (*R*)- or (*S*)- selective.



Scheme 1.4: (a) Using electrons generated by *E. coli* Hyd2 to fuel the reduction of NAD⁺ to NADH with NAD⁺-reductase, which is then used to provide the electrons to the specific reduction acetophenone to (*R*)-1-phenylethanol. (b) The cofactor regeneration system coupled with a co-immobilised NADH-dependent oxidoreductase (red) for specific reduction of a substrate.

1.2.6 Enzyme immobilisation

It has been observed in recent years that immobilisation of enzymes can improve their activity and stability across a broad range of conditions and can be tuned to enhance their desired properties, such as the physical, chemical, electrochemical, or mechanical characteristics.^[59] The effect on the functionality of the enzyme depends on the method of immobilisation.^[60–64] Table 1.2 shows the advantages and disadvantages exhibited by immobilised biocatalysts.^[65–69] Generally it is found that the correct immobilisation of enzymes onto surfaces improves the catalytic and process efficiency of industrial bio-processes.

Although these points apply generally to enzyme immobilisation, for the work presented in this Thesis, there is one more consideration for the material used for protein immobilisation. The surface onto which the enzyme is immobilised on must be conductive to allow for electron transfer between the electrode and the enzyme.

Table 1.2: Advantages and disadvantages of enzyme immobilisation.^[59]

Advantages	Disadvantages
Improved stability in a broad range of conditions (pH, temperature, pressure, electrochemical potential etc.)	Immobilisation of enzyme in wrong orientation leading to loss of activity due to blocked active site or electron transfer pathways
Heterogenous catalyst can be removed easily from the product	Additional costs required for the enzyme carrier
Reusability of heterogeneous biocatalyst	Longer and laborious immobilisation steps
Heterogenous biocatalysts can be used in batch or flow processes (e.g. packed column)	Limitations in mass transport
Enzyme immobilisation orientation can sometimes be controlled for enhanced activity	The chemical properties of the enzyme carrier material itself could decrease enzyme activity

Literature shows that there have been limited forays into enzyme immobilisation onto conductive surfaces, rather the support materials trialled are dominated by polymers and hydrogels, silica, and metal based nanoparticles.^[65,70] Therefore this review focuses on using carbon for the dual purpose of enzyme immobilisation and as a electrochemical electrode material.

For example, Green *et al.*^[71] have demonstrated the use of single-walled carbon nanotubes as a semiconductor for the immobilisation of a variety of metalloproteins and enzymes on oxidised single-walled carbon nanotubes^[72] in aqueous solution, at comparable levels with and without a coupling reagent. The high conductance and chemical stability of carbon nanotubes means they are a promising material for use as high surface area electroenzymatic electrode surfaces. There have also been demonstrations of π - π stacking on single-walled carbon nanotubes as a method of enzyme immobilisation, via modification of the nanotubes with 1-Pyrenebutanoic acid.^[73] This method has been utilised by Krishnan and Armstrong^[74] to observe an order of magnitude enhancement in performance of an enzymatic H₂-air fuel cell.

Wang *et al.*^[75] have demonstrated the specific and reversible covalent attachment of NADH-oxidase using NHS/EDC coupling chemistry onto N_α , N_α -bis(carboxymethyl)-L-lysine hydrate modified single-walled carbon nanotubes. Specific activity of the NADH oxidase was in line with free enzyme, but with improved stability when stored at 4 °C and with high reusability and reloading.

More recently, there has been a review of carbon surfaces by Quinson *et al.*^[76] for the adsorption of hydrogenase 1 from *E. coli*, for direct electrochemical applications. A range of novel and commercially available carbon materials, namely nanoparticles, nanopowders and nanotubes, were examined for electrochemical H₂ oxidation activity and it was found that carbon black particles (Black Pearls 2000) gave the highest catalytic current. These particles have been used in the Vincent Group to immobilise a number of different enzymes for bioreductions such as the aforementioned hydrogenase 1 from *E. coli* and a range of commercially available NADH-dependent oxidoreductases.^[51–53,58]

1.2.7 Batch vs flow processes

The advantage of immobilising enzymes and developing a heterogeneous catalyst, which can be easily removed from solution and reused, have been described in Sections 1.2.5 and 1.2.6. This section focusses on the advantages of using flow processes over batch processes for immobilised enzymes.

Amongst the growing topics in the field of biocatalysis, of particular interest is the development of enzyme catalysed reactions in continuous flow. In the case of immobilised-enzyme catalysis, this involves the continuous pumping of substrates and solvent, over an immobilised enzyme. In general, flow chemistry provides a very attractive field, for improvement in reaction productivity, scale-up efficiency and downstream processing, as compared to batch processes. Biotransformations performed on immobilised enzymes in continuous flow, rather than in batch, offer these benefits:^[77–79]

- Fewer problems with enzyme inhibition as the products are continuously removed,

- Further processing in another catalytic reaction module allows for facile further transformations,
- Improved total turnover numbers (TTNs),
- Continuous monitoring of the reaction, in particular with in-line spectroscopy, allowing for real-time optimisation of reaction conditions,
- Easier scale-up and safer process intensification,^[80]
- Higher loading in a packed column leading to shorter reaction times,
- No mechanical mixing leads to less attrition of the catalyst and a longer lifetime.

With the immobilisation of an enzyme often comes a lowering of catalytic activity. However this is alleviated within a flow cell, as the ratio of enzyme to substrate is relatively higher than that for a batch reactor, offsetting this decrease in bioactivity.^[77]

An example where where this is not the case is with the immobilisation of a ketoreductase from *Pichia glucozyma* and a GDH from *Bacillus megaterium* to aldehyde activated agarose, where the total enzyme activity decreased by 60%. Dall'Oglio *et al.*^[81] used this enzyme matrix to perform stereoselective reduction of various ketones at residence times between 7 min and 180 min. For example, they demonstrated the stereoselective reduction of ethyl secodione to make 60 mg of the hydroxy ketone reduction product after 15 d of continuous operation, with a space-time yield of $4.8 \text{ g L}^{-1} \text{ d}^{-1}$. But the immobilised protein constituted 1.4 wt% of the matrix, thereby exhibiting the poor volumetric activity.

The Vincent Group have demonstrated their enzymatic hydrogenation system (Section 1.2.5) in use within a continuous flow reactor, with the hydrogenase from *E. coli* and an NAD^+ -reductase variant from *R. eutropha* with various oxidoreductases immobilised on a carbon nanotube-lined quartz column.^[58] Conversion of pyruvate to L-lactate using this H_2 -fuelled NADH regeneration system using an L-alanine dehydrogenase produced conversions up to 90% and a total turnover number of

up to 54 000 product per NAD⁺-reductase. However, the flow solution was recycled through the catalytic column rather than being single pass, and there were limitations on the catalytic rate due to H₂ availability.

The use of continuous flow bioreactors is an ever growing field in the development of industrial biotransformations, with the number of examples of such experiments increasing. There is large scope for developing ‘plug-and-play’ biocatalytic modules for continuous flow processes in the manufacture of pharmaceuticals. However there is room for improvement, particularly in maintaining enzyme activity upon immobilisation, and maintaining high substrate availability.

1.2.8 Green credentials of continuous flow biocatalysis

Continuous flow processing is increasingly being viewed as a more sustainable method for chemical processing. Aspects such as an enhanced heat and mass transfer, precise residence time control, shorter processing times, increased safety, reproducibility, better product quality and facile scale-up are all benefits of using continuous flow setups.^[79] Combining this with biocatalytic methods, which bring high enantiomeric specificity, high turnover numbers and, in the case of immobilised enzymatic systems, reuseability, biocatalysis in continuous flow promises to meet many of the twelve Green Chemistry principles offered by Anastas and Warner.^[82] Some of the important advantages are:

Increased safety^[83] This relates to the typically smaller reactor volumes meaning a smaller severity if an accident does occur. For example, the concentration of gases can be controlled accurately in flow and mixed efficiently with the liquid stream without a buildup of pressure, providing a safe and reliable biocatalyst performance.

Improvement in product yields Enhanced mixing and heat transfer efficiency provides precise and efficient control of reaction conditions, such as temperature and residence time. Conditions can also be controlled to maximise yield and selectivities and minimise impurities and solvent waste. Purifications for intermediates are also simplified as these can react in the next stage of a multi-step process without need

for purification. This also improves the atom efficiency of reactions performed in continuous flow. The possibility of in-line analysis allows for continuous monitoring of a process in order to identify undesired products or changes in productivity.

Increased reaction speed^[84] As reactions rates are generally intensified by an increase in temperature, pressure or substrate concentration, the efficient heat-transfer offered by flow chemistry, together with small reactor volumes, which can also provide higher reagent-to-catalyst ratios, means reaction rates can often be improved.

Cost savings Generally there is higher throughput in continuous flow reactions as compared to batch processes, thereby requiring a smaller equipment footprint. Therefore, higher space-time yields are often achieved. However, initial equipment setup can be costly as most industrial plants are already setup to accommodate batch reactions.

1.3 Oxidoreductases coupled with a bioelectrochemical cofactor recycling system for electroenzymatic synthesis

Non-catalytic reduction of the biological cofactor NAD(P)^+ , or the oxidation of NAD(P)H , is generally impractical at common electrodes, due to the requirement of a large overpotential. Also, most enzymes that handle NAD(P)^+ and NAD(P)H are difficult to interface with an electrode. Therefore, there have only been a few examples of bioelectrochemical NAD(P)H recycling, and coupling this with an enzymatic redox process. Of most relevance is the NADPH regeneration system developed by Armstrong and coworkers.^[85–87] In this work, ferredoxin- NADP^+ reductase, FNR, which is a redox flavoenzyme in photosynthetic CO_2 assimilation, is immobilised onto indium tin oxide, ITO, electrodes. Direct electron transfer is observed between the electrode and the immobilised FNR, which fast and reversible reduction of NADP^+ to NADPH is observed. With the FNR immobilised onto ITO, it was observed at an analytical pyrolytic graphite edge, PGE, rotating disk electrode,

RDE, that there is a catalytic current of approximately $-22 \mu\text{A cm}^{-2}$. The total surface area is not reported for this electrode. It was observed that this reduction current was very much mass transport limited. The presence of a ‘non-turnover’ electrochemical signal, attributed to a two-electron ‘charging’ of the flavin adenine dinucleotide, FAD, cofactor residing within the active site of the FNR enzyme, occurs at a similar potential to the potential for NADP^+ reduction, therefore it was necessary for this to be subtracted from the measured cyclic voltammogram.

This bioelectrocatalytic NADPH recycling system, first demonstrated in 2017,^[85] was coupled with an NADPH-dependent glutamate dehydrogenase enzyme, which catalyses the conversion of 2-oxoglutarate to L-glutamate, in the presence of the cofactor and NH_4^+ . The reduction current observed at a similar PGE RDE under similar conditions, with $50 \mu\text{M}$ NADP^+ , $6 \mu\text{M}$ of the dehydrogenase and 10 mM 2-oxoglutarate substrate in solution (i.e. not immobilised), reduction currents were observed to increase to $-100 \mu\text{A cm}^{-2}$ compared to the FNR-only experiment.

After a chronoamperometry experiment performed at -0.46 V vs SHE, an overpotential of -0.11 V , with the FNR immobilised onto a larger ITO on glass electrode, the reduction current fell from $-40 \mu\text{A cm}^{-2}$ to $0 \mu\text{A}$ at 20 h, with complete conversion of 1.5 mg L-glutamate. This electrocatalyst was seen to be useable immediately afterwards but at 50% current recovery and at 66% Faradaic efficiency. The product was analysed by NMR and the system was seen to provide good Faradaic efficiency. However, there was no control experiments performed with the 2-oxoglutarate substrate in solution without the dehydrogenase, therefore the reduction cannot be confidently attributed to the dehydrogenase alone.

Since then, larger ITO electrodes with immobilised FNR have been created, with similar electrochemical activities to before.^[86] Later, it was observed that there is a slow accumulation of oxidation current after an injection of another NADPH-dependent oxidoreductase, alcohol dehydrogenase, which was attributed to an adsorption effect into the ITO pores, the effect of which enhances the catalytic current.^[87] This effect was seen with other enzymes.

Minteer and coworkers have used an electrode with a cobaltacene-modified poly(allylamine) redox polymer, incorporated with a diaphorase for the mediated electron transfer to the diaphorase and for the oxidation of NAD^+ to NADH .^[88] Faradaic efficiencies of bioelectrochemical NAD^+ reduction were found to be consistently above 80%. This system was used with an alcohol dehydrogenase in solution for the bioelectrocatalytic reduction of formaldehyde or propanal at concentrations of 30 mM.

The relative sparseness in the field of a bioelectrochemical catalyst for the reduction of $\text{C}=\text{X}$ containing molecules, indicates that there is scope for novel research in this field of bioelectrochemistry. To date, there is only one system with a bioelectrochemical NAD(P)H regeneration system, which is then used in tandem with NADPH -dependent oxidoreductases for the enantiospecific reductions of $\text{C}=\text{X}$ containing substrates.^[85–87] However, there has not been extensive analysis of the products to ensure enantiomeric purity. The work in this Thesis develops a system with some similarities to that reported by Armstrong and coworkers, but using a bioelectrochemical NADH recycling system, instead of NADPH recycling and develops the bioelectrocatalyst for use in a flow cell with a cheap carbon support.

1.4 Comparison to chemo-electrosynthesis

Despite there being relatively few examples of purely bioelectrochemical systems where NAD(P)H -dependent oxidoreductases are used in tandem with a bioelectrochemical cofactor recycling system, there are some examples of chemo-electrochemical reductions. This section describes the present state of work on chemo-electrochemical NADH regeneration and chemo-electrochemical redox processes on organic substrates.

1.4.1 Chemo-electrochemical NADH regeneration

There have been examples of chemo-electrochemical NADH regeneration, ranging from the use of mediator polymer modified electrodes to precious metal nanoparticles. This section summarises a few examples reported in the literature.

There is a vast amount of literature relating to mediated NADH regeneration. Katakis and Domínguez^[89] have listed a wide wide range of mediators for the electrochemical reduction of NAD^+ .

Song *et al.*^[90] use $[\text{Cp}^*\text{Rh}(\text{bpy})\text{Cl}]^+$, $\text{Cp}^* = \text{C}_5\text{Me}_5$, $\text{bpy} = 2,2'$ -bipyridine, as a primary mediator between glassy carbon or gold working electrodes and NAD^+ for the generation of NADH. A current enhancement at the applied potential for NAD^+ reduction, -0.6 V vs SHE, was observed with the addition of platinum nanoparticles in solution. Reduction currents increased in magnitude from $-2\text{ }\mu\text{A}$ to $-120\text{ }\mu\text{A}$.

The reduction of NAD^+ with Ru(III) mediators have been shown by Salimi *et al.* using glassy carbon electrodes modified with single-walled carbon nanotubes.^[91] The reduction potentials using these Ru-modified surfaces for NAD^+ reduction at a maximum current of $-6\text{ }\mu\text{A}$. No geometrical surface area of the electrode is reported, therefore the current per geometrical surface area is unknown, however the electrochemically active surface area of the glassy carbon electrode modified with single-walled carbon nanotubes is calculated to be 0.15 cm^2 , meaning that the current per electrochemically active surface area is $-40\text{ }\mu\text{A cm}^{-2}$. This improves to $-130\text{ }\mu\text{A cm}^{-2}$ when coupled with an ADH in solution.

Electrochemical regeneration of NADH has had extensive research by Minteer and coworkers. For example, they have demonstrated the modification of a glassy carbon electrode with a redox polymer and a diaphorase, for the reversible reduction of NAD^+ to NADH, for applications to a redox flow cell batteries,^[92–94] or electrosynthetic reductions.^[88,95,96] Of particular interest, poly(neutral red)-modified glassy electrodes were shown to reduce NAD^+ reversibly.^[92] In addition, they have demonstrated the use of toluidine blue mediator in solution as a redox mediator for the reduction of NAD^+ .^[95] This was also coupled with an ADH to demonstrate bioelectrocatalytic reduction of a range of aldehydes. They have also demonstrated the electro-oxidation of ethanol to CO_2 using a enzyme cascade with an ADH and aldehyde dehydrogenase in solution. These electron transfers are mediated by TEMPO and the system is described as a ‘hybrid’ electrocatalyst.

Thiolic NAD⁺-derivatives have been immobilised onto gold surfaces and have been used as cofactors for the ADH driven bioreductions of ethanol, as shown by Qian *et al.*^[97] It is assumed that there is direct electron transfer between the gold working electrode and the immobilised NAD⁺ derivative and there was some successful ethanol reduction observed.

An amperometric NADH sensor has been developed by Eryiğit *et al.* using Prussian blue as the electron mediator, which was electrochemically grafted onto a gold electrode with reduced graphene oxide.^[98] Successful NADH oxidation was observed.

The use of electrochemically grafted Pt and Ni nanoparticles onto glassy carbon electrodes for NAD⁺ reduction was shown by Ali *et al.*^[99] It was shown that up to 100% of NADH can be generated and tested to be biologically active with a lipoamide dehydrogenase as a control test. However, these were performed at potentials between -0.76 V and -1.16 V. At these very negative potentials, H₂ evolution occurs, which the authors take into consideration by subtracting a current recorded with no cofactor.

In general, there are a few limitations in studies of chemo-electrochemical NADH regeneration. Very commonly, the authenticity of generating the biologically active form of the NADH cofactor product is not measured, especially over many cycles of cofactor use. Even a loss of 1% of cofactor regeneration is significant, as the cost of cofactor is significant (Table 1.1). Sometimes UV-Vis spectroscopy is used as an analytical method to calculate the conversion of the NADH product, but this technique does not give enough information on the structure of the reduction product. If mediators are used in the chemo-electrosynthesis, metal-based catalysts are often used that are toxic to enzymes. In addition, sometimes rare metals like Co, or extremely expensive metals like Pt are used as a mediator, which would make scale up for industrial use cost-inefficient. Despite examples of high yielding reduction of NAD⁺ to NADH, a large overpotential is still required, or low Faradaic efficiencies are reported. Also, studies into chemo-electrochemical NADH regeneration rarely perform long periods of electrosynthesis, therefore it is difficult to gauge the stability in constant use of these electrosynthesis systems.

1.4.2 Enantioselective chemo-electrochemical reductions

The use of electrochemical catalysts in asymmetric synthesis have been reviewed recently by Lin, Li and Luo,^[100] in which they describe the general methods for enantioselective ketone reductions. Generally chemo-electrochemical reductions are dominated by the use of an electrode to create a radical or unstable intermediate, before successive radical exchange reactions. For example, Baron and coworkers have recently demonstrated the use of carbon working electrodes to generate carbocations en route to producing sterically hindered ethers, from the oxidation of carboxylic acids.^[101] However, this section focuses on the examples of enantiomeric selectivity in chemo-electrochemical reductions.

In general, the enantioselectivity is driven by design of a chiral supporting electrolyte, solvent, mediator, electrode or catalyst. The design of a chiral supporting electrolyte has been seen to improve enantiomeric excesses in reductions from acetophenone to 1-phenylethanol.^[100] The enantiomeric excess of 1-phenylethanol by the direct electrochemical reduction of acetophenone was found to increase from 4.6% to 8.4% with the introduction of chiral tetraalkylammonium salt as an electrolyte in the organic solvent. It is assumed that chiral induction from the salt proceeded through an ion pair interaction in the double layer.

In another example, electrochemical oxidation of enol esters is shown to be possible using tetraethylammonium camphorsulfonate as the electrolyte salt.^[102] Upon removal of an electron from the enol, the chiral electrolyte protects one face of the molecule, allowing for further chemical oxidation to take place on the other face of the molecule.

There are only a few examples of the use of a chiral solvent for enantioselective reductions. Seebach and Oei^[103] demonstrated the dimerisation of an acetophenone, with some enantiomeric control. It is assumed that the reduced acetophenone radical is formed at the double layer and then the dimerisation would occur within the chiral solvent (a chiral amino-ether) sphere.

TEMPO is a common electrochemical radical initiator, which acts as a redox mediator in organic electrosynthesis redox reactions. For example Rafiee *et al.*^[104]

have demonstrated TEMPO is a useful electrochemically generated radical initiator for the oxidation of primary alcohols and aldehydes. Minter and coworkers have shown that TEMPO can be electrochemically oxidised by direct electron transfer for the oxidation of H_2O_2 for use in bioelectronics.^[105] The electrochemical oxidations using TEMPO as a mediator have been recently reviewed.^[106] It has been demonstrated that chiral TEMPO-type mediators can be used for the kinetic resolution of secondary alcohols or amines, with enantiomeric excesses between 33% and 91%.^[100]

Chiral transition metal complexes have been used as electron transfer mediators for the asymmetric carboxylation of methylbenzyl chloride.^[107] A Co(II)-salen complex was used to substitute the chloride with carbon dioxide, provided as a gas. Enantiometric excesses performed with this electrochemical mediator consistently provided enantiomeric excesses over 80%.

Methyl viologen has been demonstrated as use as a chiral catalyst for the reduction of benzoylformic acid.^[108] Enantiomeric excesses were seen to be between 14% and 19%, when used in tandem with β -cyclodextrins which is higher than the 6% observed with NaBH_4 and β -cyclodextrin.

Finally, the use of a chiral Lewis acid transition-metal catalyst has been demonstrated for the oxidative cross-coupling of 2-acyl imidazoles with silyl enol ethers by Huang *et al.*^[109] The catalyst behaves similarly to traditional chiral Lewis acid transition-catalysts, however the Rh complex is oxidised with a one electron transfer, first with the coordinated imidazole, and a second time with the coordinated enol ether. The enantioselective coupling takes place with enantiometric excesses consistently above 95% and the chirality is driven by the chiral ligand surrounding the Rh.^[110]

There have been many attempts at developing enantioselective chemo-electrochemical synthesis systems. However, reported enantiomeric excesses are frequently low and often do not benefit in enantioselectivity from traditional, non-electrochemical, synthesis. This highlights the power of exploiting oxidoreductase enzymes as reduction catalysts, which provide inherently excellent enantioselectivity.

1.4.3 Flow electrosynthesis

There have been an increasing number of examples of using flow electrosynthesis in organic synthetic reactions, with the aim of taking advantage of the safer and more efficient process inherent in flow synthesis cells.^[111] Generally electrochemical flow cells flow the reaction mixture between a cathode and anode plate sandwiched together, with the working electrode mostly performing one electron transfers directly to the organic reagents, or with an electrochemical mediator, such as TEMPO, which creates reactive intermediate radical species.^[112] The rest of the reaction, such as a direct or mediated coupling, cyclisation or redox reaction with a second organic reagent, then occurs away from the surface and influence of the electrodes.

In addition to the advantages presented by chemical flow reactors as described in Sections 1.2.7, there are other advantages in the design of electrochemical flow cells.^[112–114] With the cathode and anode plates positioned very close together, the potential drop as a result of the resistance of the reaction solution is minimised. In addition to this, in the design of microelectrochemical reactors, the flow is almost entirely laminar. This means that the convective mass transport is almost absent, leaving diffusion (and migration, which is largely minimised) as the main driver for mass transport to the electrodes, described by the following equation.

$$d = \sqrt{2Dt} \quad (1.6)$$

With a diffusion constant, D , in the region of $10^{-5} \text{ cm}^2 \text{ s}^{-1}$, a molecule can diffuse 50 μm in 1 s, a common inter-electrode distance in microelectrochemical flow reactors as compared to 1.3 cm in 1 day, a distance often used in electrochemical batch cells. Therefore, the smaller the flow channels in the microelectrochemical cell, the better the diffusion and the faster the electrochemical reactions.^[115] Microelectrochemical flow reactors can also be designed so that the working electrode surface area in contact with the electrolyte is hugely increased, maximising the amount of time the reaction solution has in contact with the electrode.^[112,116]

As discussed before, oxidations are common for organic flow electrosynthesis reactions,^[112,117–120] especially as these often do not need to be enantiomerically controlled. These electrochemical oxidations can be coupled with another process further down the line. For example, Tanaka *et al.*^[121] have been able to generate an unstable *ortho*-quinone within a microelectrochemical cell, which is immediately drip fed into a batch reactor for a Diels-Alder reaction with a dieneophile. The yield of the adduct increased from 13% using entirely batch chemistry to 75% with the flow microelectrochemical reactor.

The ability to have in-line product analysis using an electrochemical flow reactor is demonstrated by Folguez-Amador *et al.*^[122] In this study, an electrochemical flow reactor was used to initiate a radical-induced cyclisation with TEMPO as the initiator and electron mediator. The synthesis of isoindolinone was monitored by in-line mass spectrometry. The rate of the overall product generated could be monitored and was shown to correlate with charge passed through the electrodes.

Chemo-electrochemical flow reactors remain in limited use and development however, especially due to their lack of chemo and enantiomeric selectivity. The existent literature examples are dominated by studies of relatively simple model compounds, rather than compounds for real applications.^[123]

1.5 Aims and structure of this Thesis

This project describes the development of two different applications of bioelectrochemistry in the areas of biosensing and bioelectrocatalysis.

Firstly, electrochemical impedance spectroscopy and capacitance spectroscopy is used to probe the dielectric of self-assembling monolayers. Taking advantage of recently derived equations for the capacitance terms of the monolayer, it is anticipated that these electrochemical techniques will be able to probe and confirm the individual capacitance contributions of the dielectric film. With this more detailed understanding of the electrochemical characteristics of self-assembling monolayers, it is hoped that impedance and capacitance spectroscopy can be used as

a much more sensitive signal transducer than previous electrochemical techniques for antibody-proximity biosensors.

Secondly, a biological-electrochemical reduction catalyst is developed, combining a bioelectrochemical NADH regeneration technology with oxidoreductase enzymes, with the anticipation of efficient and enantiomerically specific reductions of prochiral C=X-containing substrates. The structure of this Thesis is discussed below and outlined in Scheme 1.5.

Chapter 3 elucidates on recently developed mathematical models for the empirical measurement of resolved capacitance and resistance terms of self-assembling monolayers on gold substrates. The effect on monolayer crystallinity and the contribution of ionic ingress to the film capacitance as a function on the film dipole is investigated. This work was published in 2017.^[124]

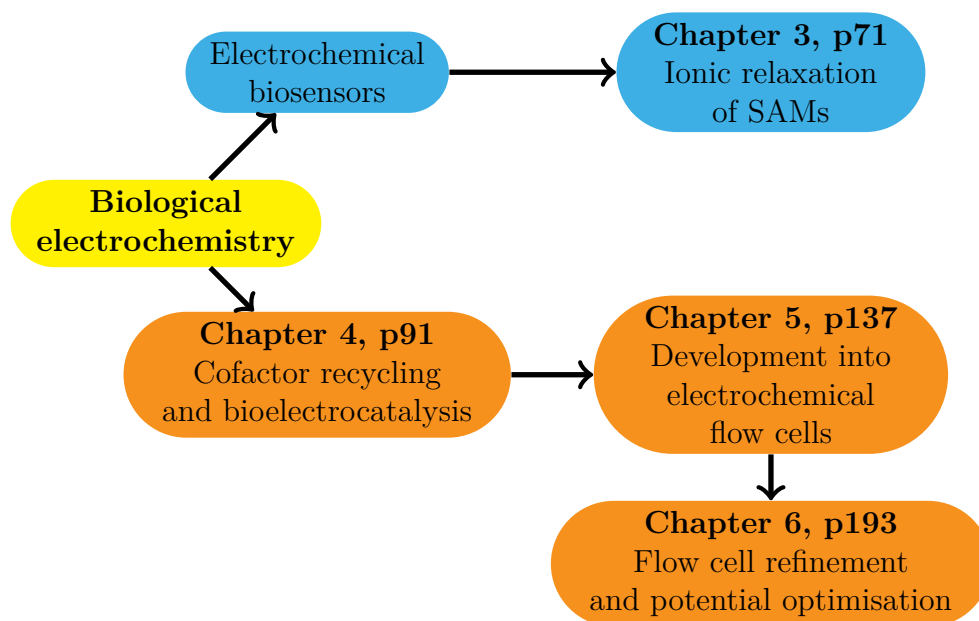
Chapter 4 discusses the investigation into the development of an electroenzymatic NADH recycling method at analytical electrodes. The electrochemical behaviour of carbon working electrodes and their ability to support enzyme immobilisation is discussed. High surface area electrodes are developed to increase the catalytic activity. The effect of coimmobilising an NADH-dependent enzyme on the electrochemical response is investigated. In addition, the effect on the electrochemistry of changing certain reaction conditions such as pH and the effect of covalent immobilisation of the enzymes onto the surface of carbon electrodes is investigated.

Chapter 5 develops the results and ideas presented in Chapter 4 by application to an electrochemical flow cell for a series of electrosynthesis reactions. The effect on the electrocatalytic response of changing the internal dimensions of the electrochemical flow cell is investigated. In particular the effect of relative carbon nanoparticle mass for enzyme immobilisation is compared, as well as the effect of enzyme immobilisation onto carbon nanoparticles compared to carbon paper working electrodes. Preparation of the carbon paper before enzyme immobilisation is discussed, as well

as the development of a high surface area counter electrode. Finally, an in-line product analysis method is developed and applied to the electrocatalytic flow reaction.

Chapter 6 is a case study following on from the experiments described in Chapter 5. Identical electrocatalytic flow experiments are setup, one without and one with an NADH-dependent enzyme in solution. Multi-step chronoamperometry is performed at each electrode to measure the catalytic activity dependency on the applied potential. This was compared using different product analysis techniques, UV-Vis, HPLC and NMR. The reduced cofactors were analysed after electrocatalytic reduction at each electrode, and at much more reducing potentials.

Overall, Chapter 3 contributes a more resolved understanding on the electrochemical behaviour of self-assembled monolayers to the field of electrochemical biosensor development. Chapters 4 to 6 contribute a viable approach to electrosynthesis using NADH-dependent oxidoreductases at carbon electrodes.



Scheme 1.5: Chapter outline of this Thesis.

2

Methods and experimental theory

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The experiments described in this Thesis use a wide range of experimental techniques and materials in order to probe molecular films and enzyme catalysis, to drive electrosynthesis or to analyse chemical products. In this Chapter the theory and general concepts of these experimental methods are discussed. This information sets the foundation for the procedures and discussion within the experimental Chapters 3 to 6, and is referenced back to this chapter as relevant.

2.1 Theory of electrochemistry

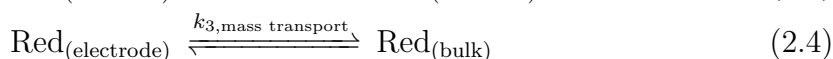
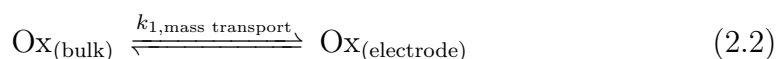
Electrochemistry is a powerful analytical method for probing the kinetics and activities of electrocatalysts. For an electrochemical system where there is an oxidation occurring at an *anode* and a complementary reduction occurring at a *cathode*, a single redox event can be isolated and characterised precisely using electrochemical methods. This section gives a brief insight into the important theory and background in electrochemistry.

2.1.1 Electrochemical mechanisms for a half cell reduction

In general, a half cell potential is described as the activity of 1 mol of oxidant, ‘Ox’, being reduced to 1 mol of its resulting reduced state, ‘Red’. This is described in scheme (2.1).



In reality however, assuming the two species are both stable and entirely soluble in electrolyte, this reaction is made up of at least three fundamental steps if performed in solution.



Therefore, the rate of the electron transfer which is to be measured as a current at the anode or cathode, is limited by either the *mass transport* of redox species to the electrode, or the *electron transfer* from the electrode to the electroactive species.

Electrochemical experiments can be designed so that the rate of reduction is either mass transport or electron transfer controlled. Although mass transport can readily be improved at an ideal analytical rotating disk electrode, it becomes more challenging at the higher surface area electrodes used in this Thesis for electrosynthesis. There are generally three different contributions to mass transport within the electrochemical cell.

- Diffusion — Close to a reducing electrode surface, $c_{\text{Ox}} < c_{\text{Ox}}^{\infty}$, (c_{Ox}^{∞} being the concentration in the bulk) therefore a concentration gradient is set up which draws more ‘Ox’ towards the surface of the electrode. The opposite is true for ‘Red’, at an oxidising electrode surface (where close to the electrode surface, $c_{\text{Red}} < c_{\text{Red}}^{\infty}$)
- Migration — The flow of electrons from the anode to the cathode through the external circuit induces a potential gradient through the cell solution, which charged species in the electrolyte move along. However, this contribution to mass transport can be ignored if a large excess of inert electrolyte is used (the forces involved are purely electrostatic, so any ionic species in the cell, including the electrolyte, can carry the charge).
- Convection — Convection currents are induced via outside mechanical forces, for example with heat, from stirring or flowing the mixture, or by using a *rotating disk electrode*, RDE. If these methods are utilised, these convection currents have large influences on the current density, overriding the diffusion and migration contributions to mass transport.

The utilisation and dynamics from using an RDE is discussed in Section 2.4.4.1 on page 56.

2.1.2 The three electrode system

In a simple electrochemical setup, there is one *working electrode*, WE, on which the redox process of interest is studied, and there is one combined *counter electrode*, CE, and *reference electrode*, RE. The applied *potential difference*, V , between the two electrodes can be split into three contributing parts as shown in Equation (2.5).

$$V = (\phi_{\text{WE}} - \phi_{\text{sol}}) + iR + (\phi_{\text{sol}} - \phi_{\text{CE/RE}}) \quad (2.5)$$

The first term, $(\phi_{\text{WE}} - \phi_{\text{sol}})$ represents the potential difference between the WE and the bulk electrolyte solution. Measurement of this parameter is the purpose behind electrochemical experiments.

The other two terms, iR and $(\phi_{\text{sol}} - \phi_{\text{CE/RE}})$, respectively model the potential drop across the solution between the WE and the CE/RE much like a resistor ($V = iR$), and the potential difference between the solution and the CE/RE. In any voltametric experiment, the aim is to be able to measure the *current* response, i , as the $(\phi_{\text{WE}} - \phi_{\text{sol}})$ is varied. However at high currents, the iR and $(\phi_{\text{sol}} - \phi_{\text{CE/RE}})$ terms are affected by changes in i . Therefore the potential cannot be effectively controlled if using only two electrodes.

A three electrode model has been developed in order to minimise the i -dependency of the latter two terms in Equation (2.5) simplifying the equation to a form shown in Equation (2.6).

$$V = (\phi_{\text{WE}} - \phi_{\text{sol}}) + \text{constant} \quad (2.6)$$

This is achieved by splitting the CE and RE roles of the CE/RE electrode to generate a three electrode system. A RE is designed to have high resistance, so that the potential is measured without any, or very little, current passing through; it has a defined potential. The CE is highly conducting to complete the cell circuit, but is also often very high in surface area, so that high current processes are not limited by the CE surface area.

2.1.3 The Nernst equation

The *Nernst equation* allows for the determination of the reduction potential of an electrochemical reaction, when not performed under standard conditions. It relates the variation of the reduction potential of an electrochemical reaction to the reaction quotient and also combines the temperature contribution as part of the calculation. It is derived from the *Gibbs free energy*, ΔG , associated with the reaction in Equation (2.1), which is related to the Gibbs free energy under standard conditions, ΔG° , through the reaction quotient, Q .

$$\Delta G = \Delta G^\circ + RT \ln Q \quad (2.7)$$

The Gibbs free energy is also dependent on the electrochemical reduction potential and defined in Equation (2.8) for an electrochemical reduction.

$$\Delta G_{\text{red}} = -nFE \quad (2.8)$$

Substituting the definition for ΔG into Equation (2.7) reveals the Nernst equation,

$$E = E^\circ - \frac{RT}{nF} \ln Q \quad (2.9)$$

where E is the thermodynamic reduction potential, E° is the standard reduction potential, R is the molar gas constant, T is absolute temperature, F is Faraday's constant and n the number of moles of electrons required for reduction of 1 mol of the oxidised species.

2.2 a.c. electrochemical techniques

The use of alternating current (a.c.) electrochemical techniques is a very powerful approach for the measurement of *impedance*. A rich array of information can be retrieved about the resistive and capacitance terms of an electrochemical system. Chapter 3 makes use of *electrochemical impedance spectroscopy*, EIS, as a method for probing the ionic characteristics of molecular films.

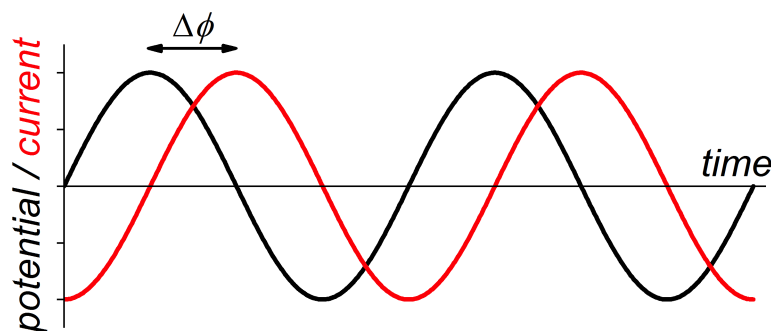


Figure 2.1: Phase shift between an applied a.c. potential (black) and the a.c. current response (red).

2.2.1 Impedance spectroscopy

Ohm's law defines resistance as a ratio between voltage, E and current, i .

$$R = \frac{E}{i} \quad (2.10)$$

However, this only applies to ideal resistors, that is:

- the resistance is not frequency dependent,
- the voltage and current terms are in phase, and
- Ohm's law is obeyed at all potential and current values.

However, molecular films at conductive interfaces do not behave ideally and have complex behaviours that resist the flow of a circuit current. Impedance is the general measurement of the ability of a circuit element to resist an applied a.c. potential, and EIS is used to measure the complex behaviour exhibited by these systems.

Electrochemical impedance of a system is determined by applying a small a.c. potential to the electrochemical system and measuring the phase shift in the a.c. current response, as shown in Figure 2.1. A small excitation potential is applied to ensure that the electrochemical cell response is pseudo-linear, so the frequency of the a.c. current response is the same as the applied excitation a.c. potential. The respective excitation (potential) and response signal (current) at a given time in a linear system are described as,

$$E = E_0 \sin(\omega t) \quad (2.11)$$

$$i = i_0 \sin(\omega t + \phi) \quad (2.12)$$

where ω is the radial frequency and ϕ is the difference in phase. Therefore a more general expression for the impedance of the electrochemical system, analogous to Ohm's law, can be described:

$$Z = \frac{E}{i} = \frac{E_0 \sin(\omega t)}{i_0 \sin(\omega t + \phi)} = Z_0 \frac{\sin(\omega t)}{\sin(\omega t + \phi)} \quad (2.13)$$

Plotting the a.c. current response against the applied a.c. potential generates an oval, called a *Lissajous Figure*. If the system is not linear, then this would not be the case. This method was also used for EIS measurements before more modern methods were developed.

Using *Euler's formula*,

$$e^{j\phi} = \cos \phi + j \sin \phi \quad (2.14)$$

the impedance of the electrochemical system can be written as a complex function, as shown in equations (2.16) to (2.17).

$$E = E_0 e^{j\omega t} \quad (2.15)$$

$$i = i_0 e^{j(\omega t - \phi)} \quad (2.16)$$

$$Z = \frac{E}{i} = Z_0 e^{j\phi} = Z_0 (\cos \phi + j \sin \phi) \quad (2.17)$$

The imaginary impedance can be plotted against the real impedance for each frequency to generate a *Nyquist plot*. Separating the real and imaginary parts of the impedance and plotting each against the frequency of the applied a.c. potential generates *Bode plots*.

The use of EIS in this Thesis is used to measure the electrochemical impedance characteristics of molecular alkanethiol films on gold surfaces. In particular, the molecular monolayer capacitance is of academic interest and is discussed further in Chapter 3.

2.2.2 Capacitance spectroscopy

Capacitance spectroscopy is a term used for measuring the complex capacitance terms of an electrochemical system. In Chapter 3, EIS is used to measure the impedance terms of the electrochemical system and the complex capacitance terms are derived from these values. EIS experiments were carried out on prepared surfaces (see Section 2.9) in 0.15 M NaClO₄ (99.99% purity, Fischer) electrolyte in ultrapure water. EIS measurements were then carried out between frequencies of 1 MHz and 10 Hz at open circuit potential. The capacitance terms are converted from the real and imaginary impedance terms as described in Equation (2.18). The development of capacitance spectroscopy is discussed further in Chapter 3.

$$C'(\omega) = \frac{Z''}{\omega|Z|^2} \qquad C''(\omega) = \frac{Z'}{\omega|Z|^2} \qquad (2.18)$$

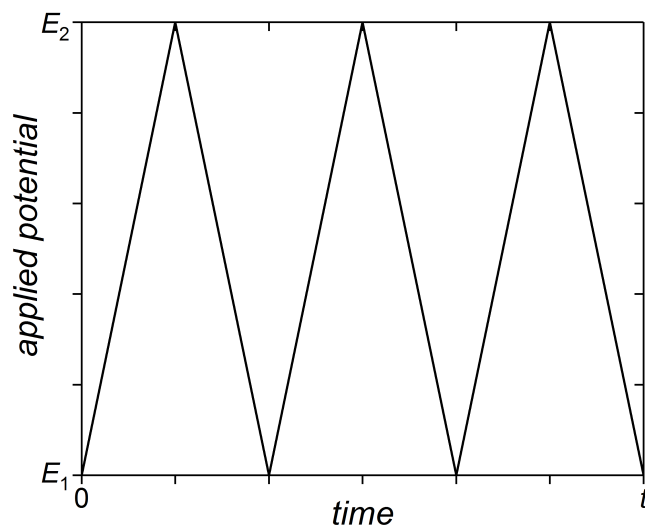
2.3 Other electrochemical methods

A range of other electrochemical methods were used to probe the electrocatalytic activity of NAD⁺-reductase enzymes, as described in Chapters 4 to 6. *Cyclic voltammetry* was used to characterise the electrocatalytic behaviour of the electrodes modified with NAD⁺-reductase. *Chronoamperometry* and *chronopotentiometry* were used to monitor electrocatalytic activity and to drive the electrocatalytic reduction reactions.

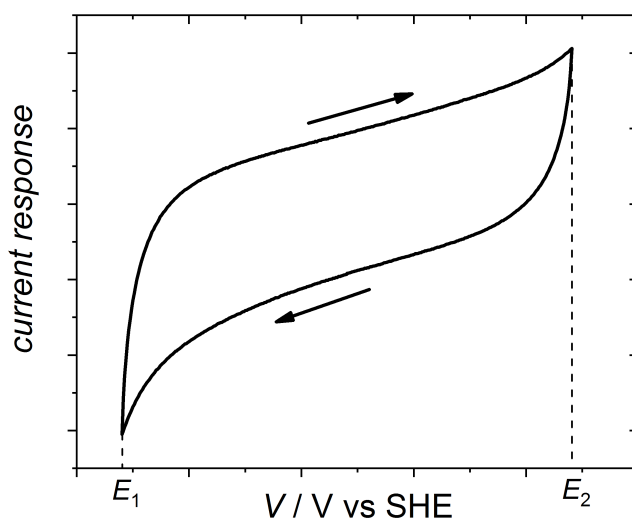
2.3.1 Cyclic voltammetry

During cyclic voltammetry experiments, an applied potential to the working electrode is swept between two potentials, E_1 and E_2 . Figure 2.2a shows the potential profile of a cyclic voltammetry experiment. The resultant current output of the working electrode is recorded and displayed against voltage as a cyclic voltammetry plot, as shown in Figure 2.2b.

Figure 2.2b shows the response of cyclic voltammetry performed at a pyrolytic graphite edge electrode (Section 2.4.1) with no redox species in an electrolyte solution. The parallelogram shaped current response is a result of the non-Faradaic



(a)



(b)

Figure 2.2: Cyclic voltammetry electrochemistry profiles. (a) Applied potential profile for cyclic voltammetry experiments as a function of time. (b) Current response at a pyrolytic graphite edge electrode as a result of the variation in applied potential.

phenomenon of charging and discharging the double layer, known as the *discharge capacitance*, C_d . Upon changing the potential of the working electrode, there is a rearrangement of ions within the double layer of the electrode, which gives rise to a change in the current. The discharge capacitance is described by Equation (2.19).

$$C_d = \frac{i_d}{\nu} \quad (2.19)$$

The value of C_d is dependent on the scan rate, ν . The higher the scan rate, the higher the discharge current, i_d contribution to the cyclic voltammogram. If it is too high, the non-Faradaic discharge current will distort the voltammogram from other redox phenomena being visible. This is particularly true with very high surface area electrodes, which are used in this Thesis. Therefore for all cyclic voltammetry experiments, a small scan rate of 10 mV s^{-1} is applied and for the pryoletic graphite electrode, it was polished until the discharge capacitative current was less than $0.1 \mu\text{A}$. The first cycle of each set of voltammograms was discarded to allow the electrode and solution to equilibrate, as the first scan showed a high initial capacitative current.

Whilst cyclic voltammetry measures the non-Faradaic phenomenon of the double layer discharge capacitance, the measurement of Faradaic current, the exchange of electrons due to redox processes, is the main reason for using cyclic voltammetry in this Thesis. The contributions to the total current measured are shown in Equation (2.20).

$$i_{\text{total}} = i_{\text{Faradaic}} + i_{\text{non-Faradaic}} \quad (2.20)$$

Therefore the cyclic voltammograms displayed in this Thesis contain contributions from both non-Faradaic and Faradaic current in the system. It is for this reason that blank cyclic voltammetry experiments are measured first with no redox active species within the potential range of interest in solution. If there are variances from this voltammogram for redox processes, it can be determined that this is entirely contributed by Faradaic phenomena.

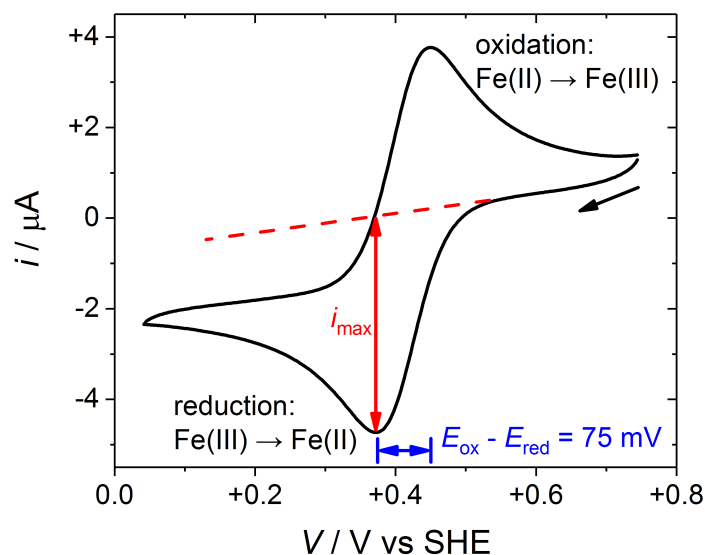


Figure 2.3: Cyclic voltammogram recorded at a stationary pyrolytic graphite edge working electrode in the presence of 1 mM $[\text{Fe}(\text{CN})_6]^{3-}$. Rotation rate = 1000 rpm; electrolyte: 100 mM Tris-HCl buffer, pH 8.

Figure 2.3 shows a cyclic voltammogram recorded at a pyrolytic graphite edge electrode in the presence of 1 mM $[\text{Fe}(\text{CN})_6]^{3-}$ and plotted following the IUPAC convention. This demonstrates a typical voltammetric response for a diffusion-controlled reversible redox process in solution. The start of the cyclic voltammogram is indicated by the black arrow. In order to calculate the maximum reduction or oxidation current, the discharge capacitance must be considered. Therefore the maximum current for the reduction of Fe(III) is calculated by taking a baseline from an area of no Faradaic activity immediately before the reduction peak, as indicated by the red lines in Figure 2.3, to have a reduction current of $-4.8 \mu\text{A}$.

The difference in potential of the reductive and oxidative peaks indicated on the cyclic voltammogram can be used to indicate the reversibility of the redox species. For a system to be truly reversible, it should satisfy the relation in Equation (2.21) at 25 °C.

$$E_{\text{ox}} - E_{\text{red}} = \frac{59}{n} \text{ mV} \quad (2.21)$$

For this demonstration, the inter-peak separation is calculated to be 75 mV, which indicates relatively good reversibility. The inter-peak potential values are also

used to calculate the reduction potential for the Fe(III)/Fe(II) couple under these conditions, $E_{1/2}$, as described as the mean of the values of E_{ox} and E_{red} .

$$E_{1/2} = \frac{E_{\text{ox}} + E_{\text{red}}}{2} \quad (2.22)$$

For the Fe(III)/Fe(II) redox couple used for demonstration here, the $E_{1/2}$ value is calculated to be +412 mV.

The technique of cyclic voltammetry is used in this Thesis to characterise the electrochemical properties of NAD⁺-reductase, which is immobilised onto the electrode. The onset potential for catalysis, that is the value at which a reduction or oxidation catalytic wave first appears, is used to calculate the overpotential, the additional energy required beyond that from the half-reaction thermodynamic potential, required to power the redox process of the enzyme. The shape of the redox peaks give information on whether the current is limited by mass transport of the substrate. The maximum reduction currents achieved within the potential windows of study indicates a suitable potential or current to apply to the electrocatalyst for electrocatalytic reductions. These electrocatalytic reactions are driven by chronoamperometry or chronopotentiometry experiments.

2.3.2 Chronoamperometry and chronopotentiometry

In this Thesis, time based electrochemical experiments were used to drive electrocatalysis. During chronoamperometry experiments, the potential of the working electrode is held constant as the current response is measured, and during chronopotentiometry experiments, the current of the working electrode is held constant as the potential response is measured. Whilst driving electrocatalysis, there is a continuous supply of electrochemical information (current and potential) of the electrocatalyst, which can provide a prediction of the extent of the reaction. By calculating the charge passing through the electrode (Equation (2.23)), a prediction on the concentration of the electrocatalytic products can be made (Equation (2.24)), assuming all the electrons passing through the electrode have been used for electrosynthesis.

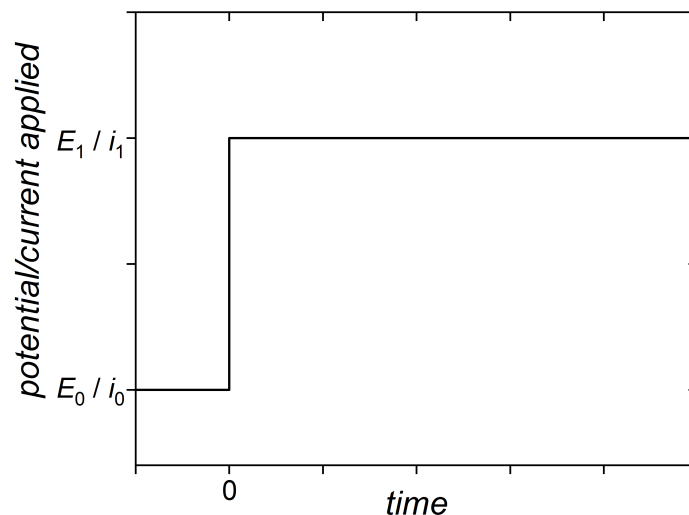


Figure 2.4: Applied potential and current profile for chronoamperometry and chronopotentiometry experiments respectively as a function of time.

$$Q = \int_{t_1}^{t_2} i dt \quad (2.23)$$

$$c_{\text{products}} = \frac{Q}{nFV} \quad (2.24)$$

In Equation (2.24), Q is the charge passed through the electrode between t_1 and t_2 , n is the stoichiometric number of electrons required for the redox process and V is the total volume of the solution. F is the Faraday constant, defined as the magnitude of electric charge per mole of electrons, $F = eN_A = 96\,485 \text{ A s mol}^{-1}$, where e is the charge of an electron and N_A is the Avogadro number.

Figure 2.4 shows the potential and current profiles for the time based chronoamperometry and chronopotentiometry experiments. Before $t = 0$, at which a potential or current is applied, the cell is at open circuit potential and the current is at 0 A. At $t = 0$, a fixed potential or current is applied to the cell by the potentiostat. At this moment, there is a spike in the current or potential response, as a result of the relaxation of the double layer. However at the scale of these electrosynthesis experiments, often 24 h, this moment is insignificant.

During chronoamperometry, the current response at a planar electrode is described by the *Cottrell equation*:

$$|i| = \frac{nFD^{1/2}c_{\infty}}{\pi^{1/2}t^{1/2}} \quad (2.25)$$

where all symbols have their usual definitions, D is the diffusion coefficient and c_{∞} is the concentration of the species undergoing oxidation or reduction in the bulk solution. The Cottrell equation describes the magnitude of the current response for chronoamperometry decreasing as $t^{-1/2}$.

Chronopotentiometry on the other hand is a slightly different technique. Whilst it is the analagous galvanostic analogue to chronoamperometry, chronopotentiometry is not often used by analytical electrochemists. This is due to the discharge current of the double layer.

The electrode response during a chronopotentiometry experiment is described by the *Sand equation*:

$$|i\tau^{1/2}| = \frac{nFD^{1/2}\pi^{1/2}c_{\infty}}{2} \quad (2.26)$$

where all symbols have their usual definitions and τ is the transition time between the two periods of double layer discharge, as shown in Figure 2.5. The product $i\tau^{1/2} = Q$ is independent of the applied current density and is proportional to c_{∞} . The potential response is described by Equation (2.27) and sketched out in Figure 2.5.

$$E = E_{\tau/4} + \frac{RT}{nF} \ln \frac{\tau^{1/2} - t^{1/2}}{t^{1/2}} \quad (2.27)$$

The result of the double layer discharging effects means that the transition time, τ , is not well defined. This is because, similarly to chronoamperometry experiments, there is a period of double layer rearrangement following the initial application of a current, and at the end of the experiment, where the concentration of the redox species in the solution to be oxidised or reduced, c_{∞} , disappears. The discharging of the double layer requires a time as described in Equation (2.28).

$$\int_0^t \frac{i}{C_d} dt = \Delta E \quad (2.28)$$

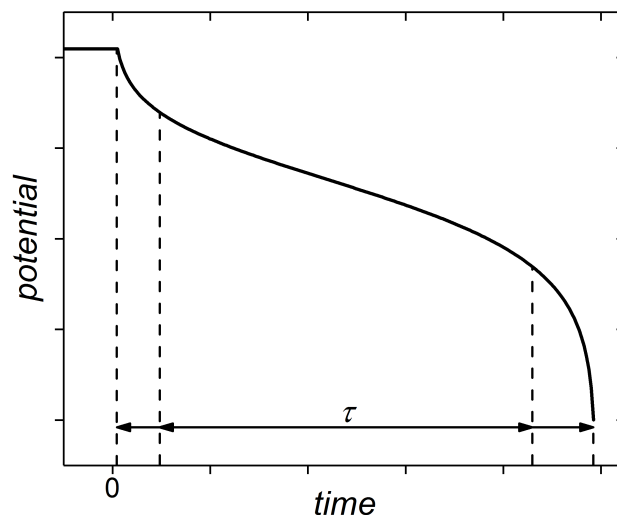


Figure 2.5: Potential response for chronopotentiometry experiments as a function of time. τ is an undefined transition time between double layer discharging events.

Despite these limitations, chronopotentiometry is a relatively cheap way of powering electrosynthesis, proving a useful business case for industrial applications^[125] and it is used widely in organic synthesis in recent years.^[120,123,126–140] The problems with an undefined transition time does not affect the experiments presented in this Thesis, as the length of the total electrosynthesis experiment is often a much larger scale than that for the initial double layer discharge effect and the experiment is never run to completion, avoiding the double layer discharge effect observed when c_{∞} reaches 0.

2.4 Electrochemical setup

2.4.1 Working electrodes

Several materials were utilised as a *working electrode*, WE, for the experiments presented in this Thesis. Gold electrodes were used as a suitable conductive substrate for self-assembled monolayer immobilisation for sensing applications. Carbon electrodes were used for enzyme immobilisation for electrosynthesis applications.

2.4.1.1 Gold electrodes for self-assembled monolayer immobilisation

In Chapter 3, which discusses the ionic characteristics of *self-assembled monolayers*, SAMs, gold disk electrodes were used as the WE for SAM immobilisation. The Whitesides Group have been interested in dynamic self-assembly,^[141] in particular of thiols at single crystal and polycrystalline gold surfaces since the late 1980s.^[27–29,31,142] For the experiments described in this Thesis, polycrystalline gold stationary disk electrodes were used, of 1.6 mm diameter (BASi, 99.99% purity) connected with a brass rod, both of which are contained within a PCTFE casing. These electrodes were polished and prepared for thiol immobilisation as described by Tkac *et al.*^[143]

2.4.1.2 Carbon electrodes for enzyme immobilisation

Various types of carbon electrodes were utilised for enzyme immobilisation in Chapters 4 to 6. For *rotating disk electrode*, RDE, experiments in Chapter 4, a *pyrolytic graphite edge*, PGE, electrode was used. It was prepared by using a pyrolytic graphite rod (Momentive Performance Materials) with an ‘edge’ surface area of 0.03 cm² (circular diameter 2 mm), sealed into a Delrin rotating disk electrode case. The outside space surrounding the extruding graphite rod was filled with insulating epoxy resin.

The PGE RDE was prepared by polishing on P400 abrasive paper, and sonicated in ultrapure water for 60 s. The electrode was shaken to remove water from the surface of the electrode and allowed to dry in the N₂ atmosphere of an anaerobic glovebox (Glove Box Technology), before enzymes were drop cast on the PGE RDE, as described when necessary in Chapter 4.

For an ideal disc electrode, if the mass transport is the limiting factor in the current response, the current should respond as a function of $\omega^{-1/2}$, as described by the *Levich equation*.

$$i_L = 0.620nFAD^{2/3}\nu^{-1/6}C\omega^{1/2} \quad (2.29)$$

This equation assumes that the amount of ‘active’ enzyme in the catalytic layer is constant and that the catalytic layer is stable. Secondly, the Levich equation would not be applicable if there is a loss of catalyst from the electrode, for instance if there is a loss of the enzymes into the bulk solution with increasing rotation rate. Also, for protein films immobilised onto the PGE RDE and the high surface area carbon nanoparticle working electrodes, the network of protein or nanoparticles complicate the ‘ideality’ of a completely flat surface, drifting away from the $i \propto \omega^{1/2}$ relation.

Carbon black nanoparticles, namely *Black Pearls 2000*, BP, (Cabot Corporation), were utilised for the creation of high surface area nanoparticle electrodes in Chapters 4 and 5. These have a typical range of diameters between 17 nm to 31 nm and a specific surface area (BET) of $(1332 \pm 22) \text{ m}^2 \text{ g}^{-1}$ as determined by Quinson *et al.*^[76] The pores have dimensions of diameter 4.7 nm and volume $1.36 \text{ cm}^3 \text{ g}^{-1}$. The use of these BP nanoparticles as a conductive surface onto which NAD^+ -red can be immobilised has been demonstrated to work for heterogeneous H_2 -driven NAD^+ reduction by Reeve.^[51,52] The preparation for these particles change somewhat between different experiments, and the precise preparation of NAD^+ -red immobilised on BP, NAD^+ -red/BP, is described in each section of results as necessary. Other NADH-dependent enzymes, such as L-lactate dehydrogenase, LDH, can also be co-immobilised with NAD^+ -red to create NAD^+ -red/LDH/BP particles.

Another electrode material, mainly used as a WE within the electrochemical flow cells in Chapters 4 and 5, is *carbon paper*, CP. CP is made from interwoven carbon fibres and is most commonly used in a range of enzyme fuel cells and some precious metal fuel cells.^[144–146] As described in this Thesis, it is used as WE for the direct adsorption of NAD^+ -red, or for the adsorption of NAD^+ -red/BP or NAD^+ -red/LDH/BP particles. Another type of carbon paper used in this Thesis is *activated carbon paper*, aCP (AvCarb), which is characterised by AvCarb having a geometrical volume of $0.01 \text{ m}^3 \text{ g}^{-1}$ and a through-plane resistivity of $7.8 \text{ m}\Omega \text{ cm}^{-2}$. This is also manufactured by interweaving high purity carbon fibres.

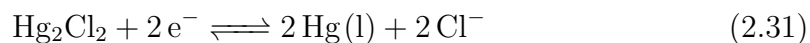
2.4.2 Reference electrode

For all biological electrochemical experiments, a *saturated calomel electrode* (ALS or homemade), SCE, was chosen as a reference electrode. The recorded potential was corrected to *standard hydrogen electrode*, SHE, using the following equation.

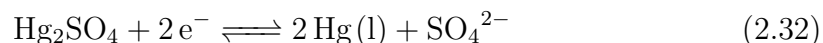
$$E_{\text{SHE}} = E_{\text{SCE}} + 0.242 \text{ V} \quad (2.30)$$

This equation applies for an electrochemical experiment performed at 298 K. For other temperatures, a linear correction of -67 mV K^{-1} was applied.

For the electrochemical experiments investigating the ionic properties of self-assembling monolayer films, namely in Chapter 3 on page 71, an SCE cannot be used. This is because of the risk of leakage of Cl^- ions from the electrode through the half cell reaction shown in Equation (2.31).



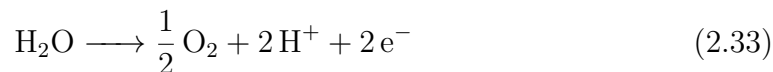
This is problematic as chloride ions are known to ingress into SAM films^[147–152] and adversely affect the results from the ultra-sensitive electrochemical impedance spectroscopy, EIS, technique. Therefore a mercury-mercurous sulfate electrode (ALS), MMSE, was used as the RE which only generates sulfate ions, Equation (2.32), which are generally too large to ingress into the molecular films that are prepared in this Thesis.



2.4.3 Counter electrodes

A range of *counter electrodes*, CEs, were used for electrochemical and electrosynthesis experiments. The role of the CE is to complement the redox event occurring at the WE. It matches the current from the WE with a similar redox event occurring at the surface of the CE. Therefore it has to be highly conducting and of suitable surface area, in order that the redox event at the WE is not limited by the one at the CE.

For a WE at low current, one possibility is that the CE is oxidising the solvent in the following experiments demonstrated in this Thesis. This would entail oxidising water to protons and electrons as shown in Equation (2.33).



It is also observed that gas is generated at the surface of the CE during electrochemical experiment, which is assumed to be O_2 .

For electrochemical experiments performed in the RDE cell at low catalytic currents, a Pt wire was utilised as the CE. This was washed with water before every use. For experiments where a large charge is being passed through the WE, for example for electrosynthesis reactions in the electrochemical flow cell, where there is a sustained current for many hours, the Pt electrode was put in a fritted compartment to avoid re-oxidation of the reduced product from the WE. This is likely as the concentration of the reduction product increases in the bulk solution. The CE compartment was made by putting the Pt wire in a segment of flow tubing. Then a piece of heat shrink tubing was placed around the flow tubing, and heated up. Whilst doing so, a piece of molecular sieve was placed at the end of the heatshrink, so as the heatshrink shrunk, the molecular sieve was held in place, and therefore is able to act as a frit. The compartment was filled with the same buffer as the bulk solution and the top was closed with a rubber bung.

For a very high surface area CE, for use in the electrochemical flow cell, carbon wool was used as the counter electrode material. A flow cell screw was hollowed out and carbon wool was put inside, after a protonation step of being soaked in concentrated HCl to make the wool hydrophilic. The cap was sealed on the outer end with silica resin around a graphite rod, connecting the carbon wool with the outside. The inside of the screw was filled with electrolyte and screwed into place in the electrochemical flow cell. A piece of Nafion[®] 117, a well known proton conductor, was placed between the cap and the main body of the electrochemical flow cell to act as a barrier that separates the WE compartment from the CE compartment.

Although the discussion on the bioelectrosynthesis reactions presented in this Thesis is focussed on the cathode, for the scale-up of these electrochemical reactions, it will be important to consider the anode reactions too. Possible anode reactions that can be utilised include the H_2 oxidation reaction, releasing 2H^+ , which will maintain pH neutrality, or could include an electroenzymatic cofactor oxidation with a reversible NAD^+ -reductase or NADP^+ -reductase.

2.4.4 Electrochemical cells

Four types of electrochemical cells are utilised in this Thesis: two disk electrode cells and two flow cells.

2.4.4.1 Disk electrode electrochemical cells

For the EIS measurement of the electrochemical characteristics of molecular films on gold electrodes, an undivided cell was used. As these measurements were performed at open circuit potentials, there should be no redox reactions happening in the cell. Therefore, there is no need to separate the anode and cathode from each other, which is only necessary to avoid re-oxidation or re-reduction of an electrochemical redox product.

Figure 2.6a shows the setup for the undivided electrochemical cell. A glass vial (maximum volume 10 mL) was prepared by cleaning with hot piranha solution (concentrated $\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2$, v/v 3:1) for 10 min and then washed with copious amounts of ultrapure water. Holes were drilled into the vial cap in order to hold the three electrodes in places. Once filled with electrolyte, the vial was secured into place with a clamp inside a Faraday cage, in order to suppress any interfering electromagnetic radiation. Electrodes were put into place (the CE and RE being washed first) before closing the Faraday cage and performing EIS and capacitance measurements.

For bioelectrocatalysis experiments performed at a disk electrode, it was necessary to perform experiments in an RDE cell. This is because the electrocatalysts demonstrated in this Thesis are limited by mass transport and therefore require

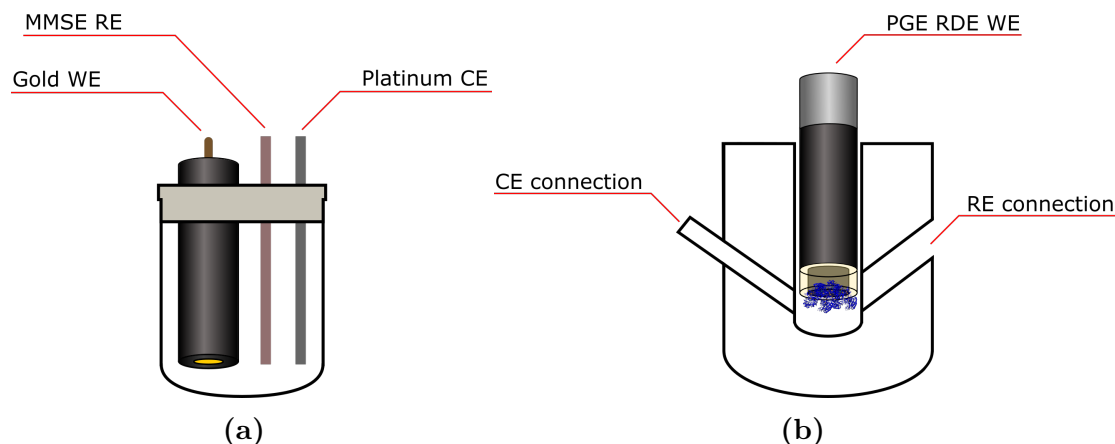


Figure 2.6: Two disk electrode cells utilised in this Thesis. **(a)** The undivided cell used for impedimetric measurements on molecular monolayers immobilised on gold electrodes. MMSE: mercury-mercurous sulfate electrode. **(b)** The rotating disk electrode, RDE, cell used for RDE electroenzymatic experiments performed in this Thesis.

rotating the disk electrode to induce convection currents, thereby increasing mass transport of the enzyme substrates to the electrode surface.

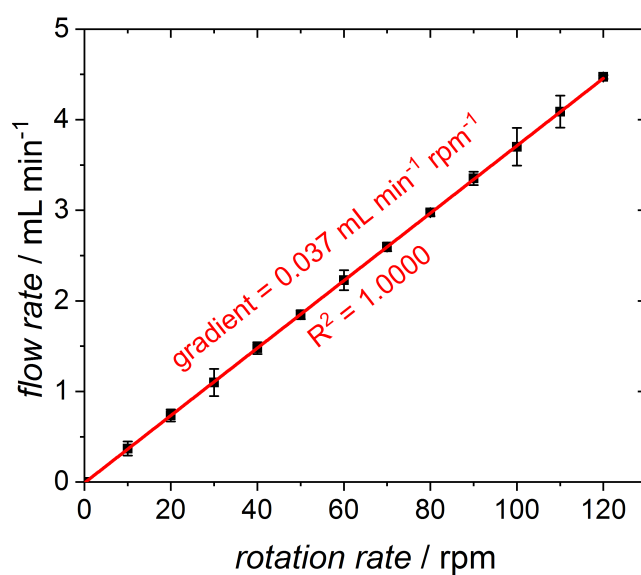
Figure 2.6b shows the diagram for the RDE used in this Thesis. The cell (maximum volume 10 mL) was cleaned with a copious amount of ultrapure water and clamped before use. Once filled with electrolyte, the CE and RE were washed and inserted into each arm of the cell. The WE was prepared, as described where relevant in Chapter 4 and screwed onto the RDE rotor, before inserting into the RDE cell to a depth of approximately 1 cm below the surface of the electrolyte.

2.4.4.2 Electrochemical flow cells

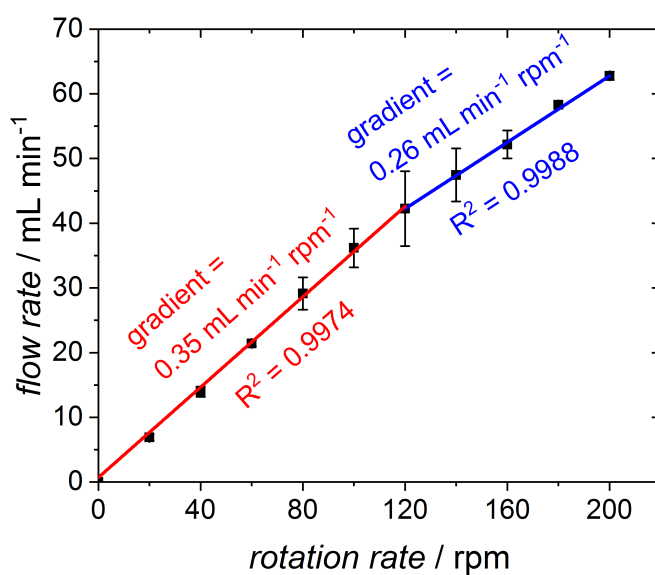
Chapters 5 and 6 describe the development of the electrocatalyst for use within an electrochemical flow cell. Two cells are used in this Thesis, one described as a ‘mushroom’ and the other a ‘star’ electrochemical flow cell, based on the shape of the cell when in operation. The details and preparation of these cells are described in Section 5.1 on page 139.

2.4.4.3 Peristaltic pump calibration

For flow electrochemistry experiments, a peristaltic pump (Watson-Marlow 120U/D1) was used to induce a flow through the system. Two different sizes of peristaltic



(a)



(b)

Figure 2.7: Calibration of peristaltic pumps (a) using peristaltic tubing of inner diameter 1.6 mm, and (b) using peristaltic tubing of inner diameter 3.2 mm.

tubing was used, one with an inner diameter of 1.6 mm and another with an inner diameter of 3.2 mm. The larger diameter tubing has a higher volume flow capacity. In order to convert rotation rate of the pump rotor to a flow rate, for both sizes of tubing, the mass of water passed through the pump after 60 s was measured using a balance. This was repeated three times and the standard deviation was used to calculate the errors of each value.

2.5 ATR-FTIR for probing gold interfaces

Fourier-transform infrared spectroscopy, fitted with an *attenuated total reflectance* accessory, ATR-FTIR, was used to probe the C–H stretches of the molecular alkanethiol monolayers immobilised on gold surfaces. Gold substrates were prepared by vapour deposition of 10 nm of chromium followed by 100 nm of gold onto Si(100) substrates (Silicon Materials). These were cut to size and then immersed in hot piranha solution (concentrated $\text{H}_2\text{SO}_4 : \text{H}_2\text{O}_2$, v/v 3 : 1) for 5 s then washed with ultrapure water and ethanol before immediate immersion into 1 mM thiol/ethanol solutions for 70 h at room temperature in the dark. After the incubation, the substrates were washed with ultrapure water and dried with N_2 gas.

ATR-FTIR spectra were recorded using a Shimadzu IRTracer-100 fitted with a Quest[®] ATR accessory (Specac). Atmospheric background scans were recorded taking an average of 65 scans with Happ-Genzel apodization. Data was collected between the wavelengths 600 cm^{-1} and 4500 cm^{-1} and averaged over 65 scans using a resolution of 4 cm^{-1} for each measurement.

2.6 Ultraviolet-visible spectroscopy

2.6.1 Ultraviolet-visible spectroscopy theory

For measurement of the concentration of NAD^+ or NADH , *Ultraviolet-visible spectroscopy*, UV-Vis, was used. This type of spectroscopy measures the absorbance of light in the UV-Vis spectrum, between the wavelengths 200 nm and 800 nm. The absorption of energy in this region relates to an electronic transition of electrons

from the HOMO to LUMO bonding orbitals in the sample. The possible transitions in this energy range, in order of decreasing transition energy, are as follows.

$$\sigma \rightarrow \sigma^* > n \rightarrow \sigma^* > \pi \rightarrow \pi^* > n \rightarrow \pi^* \quad (2.34)$$

$\sigma \rightarrow \sigma^*$ transitions tend be very rare in the UV-Vis region at least, as the σ -bonding orbitals are strongly bonding, and if these transitions are possible, they would occur at the higher energy end of the spectrum. UV-Vis is a useful technique for measuring the energy of transitions including π orbitals, in particular $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions in conjugated systems. These transitions are lower in energy and so occur at the lower energy end of the spectrum.

The UV-Vis electronic transitions for the NAD^+ or 1,4-NADH cofactor are summarised in Figure 2.8. The structure of NAD^+ /1,4-NADH (Figure 2.8a) contains two rings, the nicotinamide ring and the adenine ring, connected together by two phosphate groups. Both the nicotinamide and adenine rings contain aromatic or conjugated rings and exhibit transitions into the π^* orbital. In NAD^+ , each of the nicotinamide and adenine ring are conjugated systems and so exhibit $\pi \rightarrow \pi^*$ excitations at 260 nm. Upon the gain of a hydride onto the nicotinamide to form 1,4-NADH, the aromaticity is disrupted as the hydride adds on to the 4-position of the ring. The electronic transition for the nicotinamide ring, observed during UV-Vis spectrometry is, now a $n \rightarrow \pi^*$ excitation at 340 nm. An example spectrum for NAD^+ (white trace) and 1,4-NADH (black trace) is shown in Figure 2.8b.

The concentration of NAD^+ and 1,4-NADH can be determined from UV-Vis spectroscopy using the Beer-Lambert law, as shown below.

$$A = \epsilon cl \quad (2.35)$$

A is the absorbance, ϵ is the molar extinction coefficient, c the concentration of the species in the sample, and l the path length, which in all cases in this Thesis has a value of 1 cm. The use of the Beer-Lambert law and UV-Vis spectroscopy to measure the conversion of NAD^+ is described further in Section 5.1.3 on page 144. Calibration of the UV-Vis measurements are described in Section 2.6.2 on page 62.

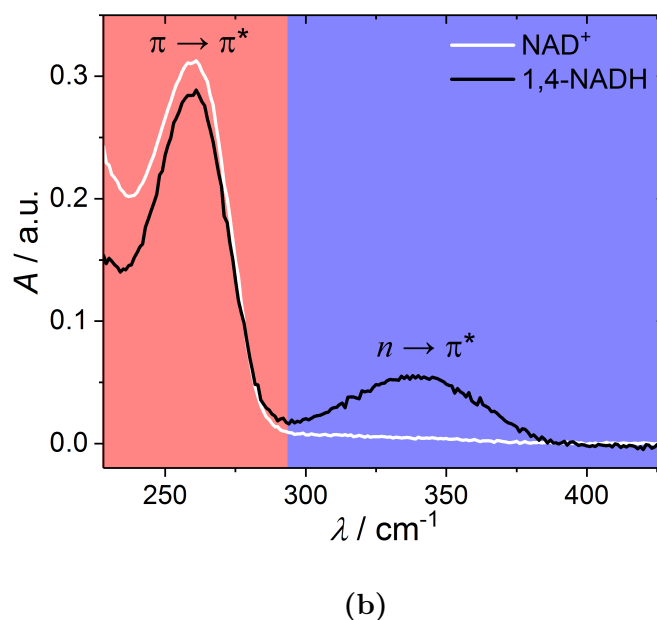
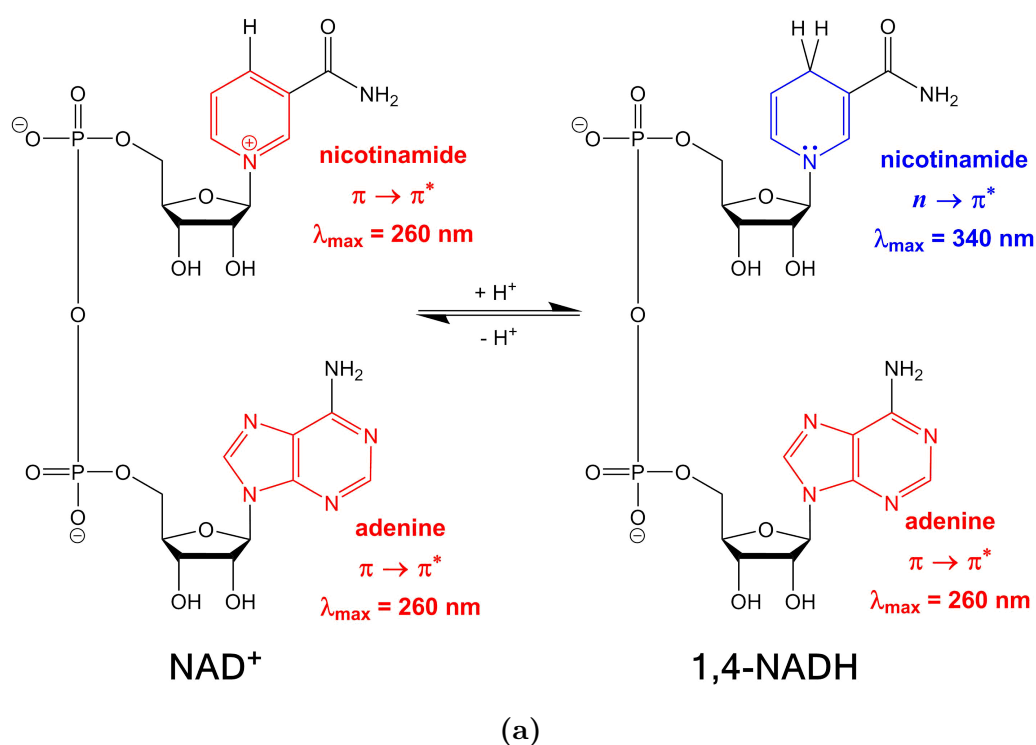


Figure 2.8: The UV-Vis spectroscopy of NAD⁺ and 1,4-NADH. (a) Shows the structures of NAD⁺ and 1,4-NADH, with the nicotinamide and adenine aromatic or conjugated rings highlighted being active in the UV-Vis region. Red indicates a $\pi \rightarrow \pi^*$ electronic transition with $\lambda_{\text{max}} = 260 \text{ nm}$. Blue indicates an $n \rightarrow \pi^*$ electronic transition with $\lambda_{\text{max}} = 340 \text{ nm}$. (b) shows an example UV-Vis spectrum for both NAD⁺ (white trace) and 1,4-NADH (black trace). The colour regions relate to the electronic transitions at the corresponding conjugated structure in (a).

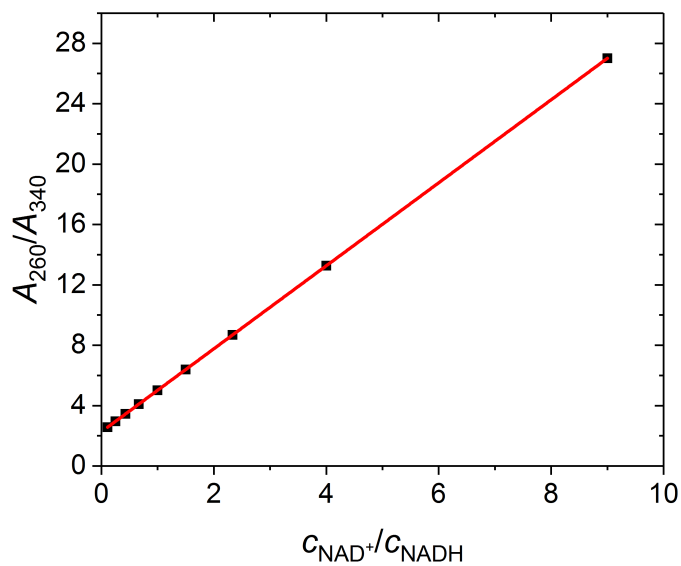
The buffers used in the reactions performed generally have strong absorbance below approximately 220 nm. Therefore a baseline spectrum is measured using buffer only and subtracted from the sample measurements.

The UV-Vis spectrometer used for all experiments presented in this Thesis is a Cary 60 (Agilent), with a holder (Agilent) for a UV-Vis cuvette (Hellma) of volume 1 mL with a defined path length of 1 cm. For flow experiments, the UV-Vis cuvette was treated as a reservoir with a flow inlet and outlet passing through the modified septum cap on the head of the UV-Vis cuvette. The cell holder also has a Peltier element to maintain temperature control at 30 °C during flow experiments presented in Chapters 5 and 6. This setup was used for *in situ* measurement of the cofactor in the solution. The growth of the 340 nm absorbance peak was monitored to determine the appearance of 1,4-NADH in solution, as the peak at 260 nm absorbance is common to both NAD^+ and NADH. For the measurement of *ex situ* samples, they were diluted 20-fold and measured in the same type of UV-Vis cuvette.

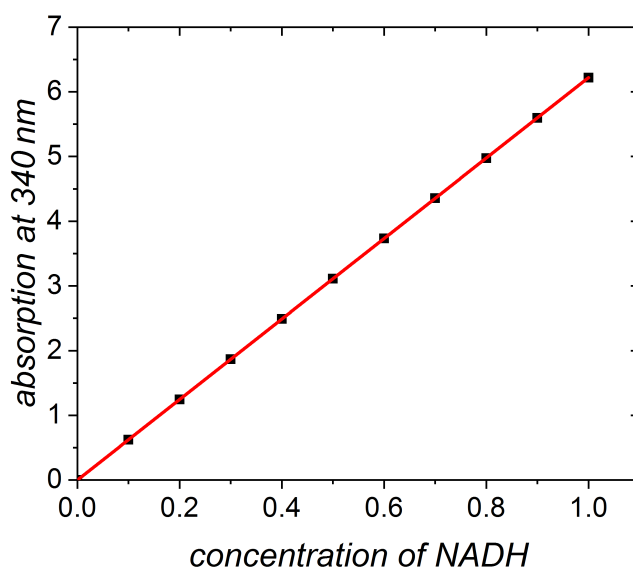
2.6.2 NAD^+ /NADH concentration calibration curves

It has previously been observed that buffered solutions make NAD^+ and NADH cofactors less absorbing in the UV-Visible spectrum. Performing calibration curves of absorbance against concentration of NAD^+ and NADH would not be accurate either, as a result of this phenomenon. Therefore the UV-Vis spectroscopy response was assumed to be linear and theoretical calibration curves were calculated from literature values (Figure 2.9).^[153]

For samples that were taken from the reaction solutions, they were diluted 20-fold before performing UV-Vis spectroscopy, in order that the absorption peak at 260 nm did not oversaturate the detector. The ratio of the two peaks were measured and knowing the total concentration of cofactor, the resulting concentrations of NAD^+ and NADH were calculated.



(a)



(b)

Figure 2.9: (a) Plot of the ratio of absorbances at 260 nm and 340 nm can be used to calculate the ratio of concentrations of NAD^+ and NADH. (b) Plot of the Beer-Lambert law to calculate the concentration of NADH, for in-line UV-Vis measurements.

$$A_{260} = \epsilon_{260,+}c_+l_+ + \epsilon_{260,H}c_Hl_H \quad (2.36)$$

$$A_{340} = \epsilon_{340,H}c_Hl_H \quad (2.37)$$

$$\frac{A_{260}}{A_{340}} = \frac{\epsilon_{260,+}c_+l_+ + \epsilon_{260,H}c_Hl_H}{\epsilon_{340,H}c_Hl_H} \quad (2.38)$$

$$\frac{A_{260}}{A_{340}} = \frac{\epsilon_{260,+}c_+ + \epsilon_{260,H}c_H}{\epsilon_{340,H}c_H} \quad (2.39)$$

$$\frac{A_{260}}{A_{340}} = \frac{\epsilon_{260,+}}{\epsilon_{340,H}} \cdot \frac{c_+}{c_H} + \frac{\epsilon_{260,H}}{\epsilon_{340,H}} \quad (2.40)$$

On the other hand, for in-line UV-Vis measurements, the samples cannot be diluted, meaning that the detector is saturated at 260 nm. Therefore only the peak at 340 nm is monitored for linear calculation of NADH concentration.

2.7 High performance liquid chromatography

High performance liquid chromatography, HPLC, is an analytical technique used to determine the structures of cofactor reduction products. This is a useful technique to separate the different molecules in the sample solution, in particular the similar structures of NAD⁺ and 1,4-NADH and therefore determine the reduction products of biological electrochemical reduction of NAD⁺.

The sample was removed from the reaction and placed in an Eppendorf centrifuge tube with Acetonitrile (20%vol). The sample was centrifuged (Sigma 1–4, 14 000 × *g*, 2 min. An 100 µL aliquot was analysed with HPLC at two wavelengths, 260 nm and 340 nm.

The equipment used was a Shimadzu Prominence HPLC equipped with a CBM-20 system controller, LC-20AD superior solvent delivery system, SIL-20A high-throughput autosampler, CTO-10AS column oven and SPD-20A UV-Vis absorbance detector, 150455 SeQuant[®] ZIC[®]-HILIC 5 µm, 200 Å HPLC column (150 × 4.6 mm) with a 150535 SeQuant[®] ZIC[®]-HILIC Guard (20 × 2.1 mm), oven temperature 40 °C, Ammonium Acetate (20 mM, pH 7.5) in Milli-Q/Acetonitrile gradient, flow rate

1 mL min⁻¹, 10 μ L injections, LabSolutions analysis software. The following method was performed: ACN (20 mM ammonium acetate pH 7.5)/H₂O (20 mM ammonium acetate pH 7.5) 100 : 0 hold for 1 min; then up to 60 : 40 over 29 min; then up to 40 : 60 over 20 min and then hold for 7 min: $t_R(1,4\text{-NADH}) = 19.0$ min, $t_R(\text{NAD}^+) = 20.5$ min, $t_R(\text{inactive NADH}) = 24.0$ min. Standards of pure NAD⁺ and 1,4-NADH were ran to determine the residence time of these two cofactors.

2.8 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance spectroscopy, NMR, was used to determine the conversions of the C=X substrate used for bioelectrochemical reduction with NADH-dependent oxidoreductases, described in Chapters 4 to 6. Additionally in Chapter 6, NMR was used to determine the structures of the NAD⁺/NADH cofactors.

Samples were prepared by injecting 500 μ L of the sample directly into clean NMR tubes (Norell) with 100 μ L of D₂O and shaken. The equipment used was a Bruker AVIIIHD 400 nanobay instrument, equipped with 5 mm *z*-gradient broadband multinuclear probe, 60-position SampleExpress robotic sample changer and controlled with TOPSPIN 3 software. Reaction samples were all aqueous, therefore in order to suppress the dominant H₂O signal, a standard presaturation technique was employed using the Bruker noesygprr1d pulse sequence.

2.9 Alkanethiol monolayer film preparation

Chapter 3 investigates the electrochemical properties of *self-assembled monolayer films*, SAMs, on polycrystalline gold electrodes. These were prepared by mechanical polishing with diamond spray (0.1 μ m, Kemet) on soft cotton wool (Boots). The electrodes were sonicated in ultrapure water for 5 min and then placed in hot piranha solution (concentrated H₂SO₄ : H₂O₂, v/v 3 : 1) for 5 min before sonication in ultrapure water again for 5 min. Electrodes were then electrochemically polished in 0.5 M KOH between -0.5 V and -1.8 V (vs pseudo-Ag reference electrode) overnight, followed by electrochemically polishing in 0.1 M H₂SO₄ between 0 V

and 1.25 V until a stable gold/gold-oxide reduction peak is observed, the area of which can be used to determine the electrochemically active surface area of the gold disk electrode.^[154]

Alkanethiol films were formed from 1-octanethiol (C8), 1-decanethiol (C10), 1-dodecanethiol (C12) and 1-hexadecanethiol (C16) (all Sigma-Aldrich) and were prepared by immersing the freshly polished polycrystalline gold electrodes in 1 mM thiol/ethanol solutions for 70 h at room temperature in the dark.

2.10 Enzyme handling

2.10.1 NAD⁺-reducing enzymes

The *NAD⁺-reductase*, NAD⁺-red, used in this Thesis for bioelectrochemical reduction of NAD⁺ was prepared and purified by Holly Reeve and Miguel Ramirez from the Vincent Group, according to a published protocol.^[155] The NAD⁺-red is a construct of the NAD⁺ reducing soluble hydrogenase, SH, from *Ralstonia eutropha* (*R. eutropha*) with inactive hydrogenase following a single amino acid substitution (I64A) in the hydrogenase large subunit,^[155] so that it only retains NAD⁺-reductase activity. It has been observed that this enzyme also undergoes O₂ reduction at the flavin at similar potentials to NAD⁺ reduction.^[54] Therefore all biocatalytic electrosynthesis experiments were performed in a glovebox (Glove Box Technology) under a N₂ atmosphere.

NAD⁺-red is represented in figures as described in Section 4.1 on page 92.

2.10.2 NADH-dependent C=X reducing enzymes

Two NADH-dependent C=X reducing enzymes were used for the bioelectrochemical reductions of C=X substrates, an *L-lactate dehydrogenase*, LDH, from rabbit muscle (Sigma-Aldrich), and an *L-alanine dehydrogenase*, alaDH, recombinant, expressed in *Escherichia coli* (Sigma-Aldrich).

2.10.3 Immobilising enzymes onto carbon nanoparticles

The NAD⁺-red/BP particles were prepared, first by sonicating 20 mg mL⁻¹ of BP2000 nanoparticles in purified and degassed water for at least 60 min, to give an evenly distributed suspension of dispersed carbon black nanoparticles. Meanwhile 30 μ L of purified NAD⁺-red enzyme was allowed to thaw slowly to 4 °C in a glovebox with an N₂ atmosphere. Once thawed, 10 μ L of the BP slurry was injected into 30 μ L of NAD⁺-red and incubated for 120 min at 4 °C. The particles were allowed to settle and samples were taken from the sedimented suspension and used immediately for electrochemical experiments.

The NAD⁺-red/alaDH/BP and NAD⁺-red/LDH/BP particles for use in Chapter 4 experiments were prepared, first by sonicating 20 mg mL⁻¹ of BP2000 nanoparticles in purified and degassed water for at least 60 min to obtain an evenly distributed suspension of dispersed carbon black nanoparticles. Meanwhile 10 μ L of purified NAD⁺-red enzyme was allowed to thaw slowly to 4 °C in a glovebox with an N₂ atmosphere. Once thawed, 0.2 mg of alaDH or LDH (20 μ L) was added to the NAD⁺-red in addition to 2 μ L of the BP slurry, mixed and incubated for 120 min at 4 °C. They were then used immediately for preparation of a high surface area electrode. The particles were allowed to settle and samples were taken from the sedimented suspension and used immediately for electrochemical experiments.

2.11 Chemicals and reagents

2.11.1 Buffers and electrolytes

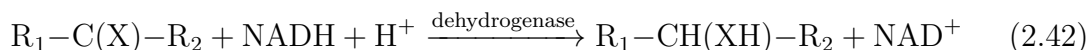
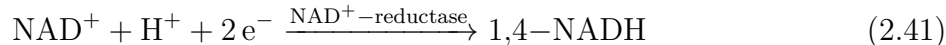
2.11.1.1 Perchlorate electrolyte for impedance measurements

For electrochemical experiments on self-assembled monolayers performed in Chapter 3, a solution of 0.15 M NaClO₄ was chosen as the electrolyte. It was necessary to choose a salt with an anion with a large diameter in order to avoid excessive anionic ingress into the prepared alkanethiol monolayer film. Therefore sodium perchlorate was chosen as the electrolyte salt, as used in previous studies in the Davis Group.^[154]

0.15 M NaClO₄ solution was made by dissolving 5.27 g of NaClO₄ (99.99% purity, Fischer) into ultrapure water (MilliQ filtered, 18.2 MΩ cm) in a volumetric flask of volume 250 mL.

2.11.1.2 Buffers for electroenzymatic C=X reductions

For the enzyme-based electrochemistry performed in Chapters 4 to 6, a buffered-electrolyte was used for electrochemistry experiments. Buffered solutions maintain the pH of a solution as a result of a change in the concentration of protons due to a redox reaction occurring in the solution. For example, the electrocatalytic reductions of C=X substrates are of interest, and these are reduced via the addition of a proton from solution and a hydride from the reduced nicotinamide cofactor, NADH, as shown in equations (2.41) to (2.42).



In these electroenzymatic reductions, there is a continuous removal of protons from solution, leading to an increase in the pH of the solution. Without a buffer solution, there would be a shift in the reduction potential for the reduction of NAD⁺. According to the Nernst equation for the electrochemical reduction of NAD⁺,

$$E_{\text{red}} = E_{\text{red}}^{\ominus} - \frac{RT}{nF} \ln \frac{[\text{NADH}]}{[\text{NAD}^+][\text{H}^+]} \quad (2.43)$$

as the concentration of H⁺ in the solution decreases in an unbuffered electrolyte, the reduction potential for the NAD⁺/NADH couple would become more negative. This means that an increasing overpotential would have to be applied to the electrode for the same catalytic current. In addition to this, enzymes generally require specific pH values near neutrality in order to operate successfully. These two reasons highlight the importance of buffered electrolytes in electroenzymatic chemistry, at least at the development stage.

Buffer systems often operate in a range ± 1 of the pK_a values. In this Thesis, two organic buffers are used, Tris-HCl for experiments at pH 8 for most experiments, and one experiment using BIS-TRIS-HCl for pH 6. Table 2.1 shows the buffer ranges for each of these two buffers.

Buffers were prepared by dissolving the Trizma[®] (6.057 g) or BIS-TRIS (10.462 g) base (both Sigma-Aldrich) in ultrapure water (MilliQ filtered, 18.2 M Ω cm) in a 500 mL volumetric flask, with a resulting concentration of 100 mM. The buffer solution was then titrated to the correct pH using concentrated HCl to form Tris-HCl buffer at pH 8 and BIS-TRIS-HCl buffer at pH 6. The pH was corrected whilst stirring the buffer solution and monitoring the pH using a Ag/AgCl pH electrode (Fisher) at 25 °C.

2.11.2 Cofactors

The cofactors used in this work were NAD⁺ and NADH (Melford) and were freshly made into 100 mM stocks in ultrapure water for each day of use. It has been observed previously in the Vincent Group that any impurities in the provided cofactors only affect the activity of the NAD⁺-reductase enzyme above ~ 1 mM, however this would not significantly affect the results displayed in this Thesis as all experiments were performed with a cofactor concentration of 1 mM. It has also been reported previously in the Vincent Group that the NADH contains small amounts of the oxidised form of the cofactor, NAD⁺. However this was of no importance for the experiments presented in this Thesis as NADH was only used for reference purposes.

2.11.3 Reagents

The substrates used for electrocatalytic reduction experiments, pyruvate and ammonium chloride, as well with all the C=X substrates used for blank carbon elec-

Table 2.1: Buffer pK_a values and buffer ranges provided by Sigma-Aldrich.^[156]

pH required	Buffer salt	pK_a at 25 °C	Buffer range
8	Tris (Trizma [®])	8.06	7.0–9.0
6	BIS-TRIS	6.50	5.8–7.2

trode screening, acetophenone, phenylethanol, pyruvate, lactate, D-alanine, 2,3-butanedione, acetoin, 3-methyl-2-cyclohexanone, 3-methylcyclohexanone and DMSO, were all purchased from Sigma-Aldrich and used directly as provided. 100 mM stock solutions of these substrates were made freshly each day in ultrapure water.

3

Defining the ionic characteristics of self-assembling monolayers

Contents

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The application of *self-assembled monolayers*, SAMs, within organic electrochemical devices has increased rapidly in recent years, enhancing the stability and response of organic transistors, electronic junctions and in particular, in the field of label-free ultra-sensitive biosensors.^[33,157,158] Understanding and characterising the electrochemical and dielectric properties of the SAM in such devices is important to exploit the full stability and sensitivity of electrochemical biosensors. This chapter describes the experimental characterisation of the dielectric properties of

simple alkanethiol SAMs on semiconducting gold substrates, using a pre-developed mathematical model.^a With this information, it is hoped that non-Faradaic label free, antibody proximal biosensors, supported by alkanethiol monolayer films, can be developed to be accurate, sensitive and reproducible.

3.1 Mathematical overview of ionic self-assembled monolayers

Bueno and coworkers^[154,159–164] have developed more general mathematical models to describe the electrochemical characteristics of self-assembled monolayers on surfaces. Of particular focus is the development of a general model of capacitance for molecular films on conductive surfaces, that takes into consideration both classical and quantic contributions. When an organic monolayer forms on the surface of a conductive electrode surface, the resulting electrode-monolayer-electrolyte interface perturbs the general capacitance descriptors for plate capacitors. This section briefly describes the equations that were derived and the development of more descriptive *equivalent circuit models* for monolayer-modified capacitive plates.

3.1.1 Interface capacitance as described by Gouy-Chapman and Lindhard models

For flat surfaces between a conducting plate and solution, a typical interpretation of the capacitance is that it is closely related to only the distance from the surface of the capacitor. This is described by the Gouy-Chapman (or double-layer) model, the capacitance value of which depends on the thermodynamics of ions adsorbed on the surface:

$$C_i = \epsilon_r \epsilon_0 \kappa \quad (3.1)$$

where C_i is the associated interfacial ionic capacitance per unit area, ϵ_r the relative permittivity of the dielectric environment, ϵ_0 the dielectric permittivity of the

^aWork presented in this Chapter was completed under the supervision of Professor Jason Davis, the results of which were published in 2017.^[124]

3. Defining the ionic characteristics of self-assembling monolayers

vacuum, and κ is described as the reciprocal of the Debye length, $\kappa = 1/L_D$, as described below.

$$\kappa^2 = \frac{2(ze)^2 N_0}{\epsilon_r \epsilon_0 k_B T} \quad (3.2)$$

where z is the valence number of ions, e the charge of an electron, N_0 the ionic concentration in the bulk solution, k_B the Boltzmann constant and T the absolute temperature. Equation (3.1) is the Debye-Huckel approximation and is especially valid at low-charged interfaces. However a more universal model arises from the consideration of both the quantic, C_q , and the classical, C_e contributions to the electrochemical capacitance at a charged surface:

$$\frac{1}{C_\mu} = \frac{1}{C_e} + \frac{1}{C_q} \quad (3.3)$$

$1/C_e$ represents the classical electrostatic contributions to capacitance, rather it depends solely on the environmental and geometric properties of the capacitor. For a simple plate capacitor,

$$C_e = \epsilon_r \epsilon_0 \delta \quad (3.4)$$

with δ , the inverse of the inter-plate distance $\delta = 1/L$, not dependent on the ionic strength of the solution between the plates. This is opposed to κ defined in Equation (3.1), as the ionic strength is now considered in the quantic contribution to the total *electrochemical capacitance*, $C_\mu = e \, dQ/d\mu$.

The electrochemical capacitance relates the charge built up on the capacitor to a chemical potential difference between the two plates. There is a density of states of potential energy of each of the capacitor plates, designated $g_1(\mu) = dN_1/d\mu$ and $g_2(\mu) = dN_2/d\mu$. This contributes to the total capacitance C_μ in series with the classical electrostatic contribution, C_e , as shown below.

$$\frac{1}{C_\mu} = \frac{1}{e} \cdot \frac{d\mu}{dQ} = \frac{1}{C_e} + \frac{q}{e^2} \left[\frac{1}{g_1(\mu)} + \frac{1}{g_2(\mu)} \right] \quad (3.5)$$

$$= \frac{1}{C_e} + \frac{q}{e^2} \left[\frac{1}{dN_1/d\mu} + \frac{1}{dN_2/d\mu} \right] \quad (3.6)$$

It should be noted in the case of the ideal metallic plate capacitor, with an infinite density of states, $g_1(\mu), g_2(\mu) \gg 1$ and C_q tends to 0. This leaves only the geometrical electrostatic contributions to capacitance and so $C_\mu = C_e$.

It can be shown that for the specific case of electro-active monolayers on metal surfaces, the capacitance is resolved as $C_\mu = e^2 g_{\text{layer}}(\mu) = e^2 dN_{\text{layer}}/d\mu$, a term dominated by quantum effects described by C_q . Therefore, C_e and C_i alone are poor approximations for C_μ in the presence of a redox system.

Non-Faradaic phenomena between an electrode and an electrolyte is dependent on the ionic strength of the solution and therefore is Debye length dependent, therefore Equation (3.3) becomes:

$$\frac{1}{C_\mu} = \frac{1}{C_i} + \frac{1}{C_q} \quad (3.7)$$

However for non-Faradaic behaviour in the film, the quantic term, C_q , disappears leaving C_i as a reasonable physical approximation of non-Faradaic relaxation only. The Thomas-Fermi model, an approximation of the Lindhard descriptions of the electric field screening reveals the electrochemical capacitance per unit area.

$$C_\mu = \epsilon_r \epsilon_0 \kappa^2 \quad (3.8)$$

The Debye length here is given by the more general term for a capacitor,

$$\kappa^2 = \frac{(ze)^2 (dN_1/d\mu)}{\epsilon_r \epsilon_0} \quad (3.9)$$

which in fact contains a quantic contribution. For non-Faradaic activity, the quantic term can be approximated so that Equation (3.9) resolves to the classical Equation (3.2). In any case, this indicates that for alkanthiol film modified electrodes, with no opportunities for redox processes, the non-Faradaic, or ionic, charging process is dominant but contains markedly more information than typically assumed within the double-layer model.

3.1.2 Equivalent circuit models of organic monolayers

The electrochemical characteristics of an electrode-electrolyte interface can be represented using an *equivalent circuit model*. In the simple case of an unmodified working electrode, the double-layer phenomenon of ions from the electrolyte adsorbing onto the surface of the electrode generates a capacity to store electrical charge at the interface, described as double layer capacitor C_{DL} (Figure 3.1a). The solution resistance, R_s , is in series to the capacitive term.

On development of a SAM on the surface of the electrode, the double layer capacitance, C_{DL} , term is perturbed and the more general term, C_i , is designated for the SAM contribution to the circuit impedance (Figure 3.1b). However it has been observed that at high frequencies ($\nu > 10$ kHz) there is a ‘straying’ deviation from the impedance^[151] originating from the translational permeation of ions into the bulk SAM through collapsed sites and pinholes within the film structure.^[151,165] A parallel resistance element to C_i , depicted in Figure 3.1c as R_m , is attributed to these effects.^[150,165,166]

A further equivalent circuit has been derived by Góes et al.,^[154] where the capacitance characteristics of the inherent or field-induced dipoles and the ionic field to the electrode potential are considered. The complex capacitive response

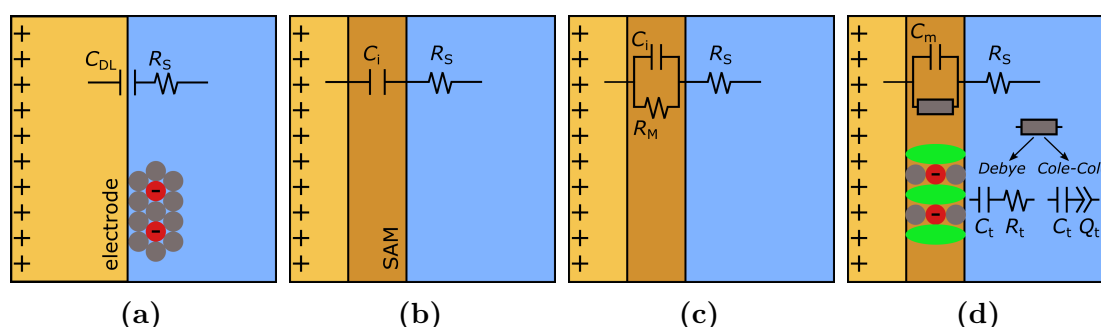


Figure 3.1: The development of four ‘equivalent circuit models’ for SAMs on an electrode surface in contact with an electrochemically inert electrolyte. **(a)** Unmodified electrode exposed to inert electrolyte. **(b)** Electrode modified with SAM perturbs the capacitance term. **(c)** Inclusion of a resistance term contribution from the SAM.^[165] **(d)** A more realistic model introduced in this chapter; the ingress of ions or solvent molecules can be described with the Debye (ideal) or Cole-Cole circuit elements.^[154] Grey: solvent molecules; red: anions from solution; green: SAM molecule (thiol).

for the dynamic SAM relaxations, assuming homogeneity (ideal SAM), is described using the Debye model in Equation (3.10).

$$C^*(\omega) = C_m + \frac{C_t - C_m}{1 + j\omega\tau_c} \quad (3.10)$$

$C^*(\omega)$ is defined as the complex capacitance of the film, ω is the electronic perturbation frequency and $j = \sqrt{-1}$. Three electrochemical circuit terms are introduced here:

- C_m : the combined bulk capacitance of the SAM and the double-layer capacitance, with the relation $1/C_m = 1/C_b + 1/C_{DL}$;
- C_t : the dynamic ionic and dipolar contribution to the SAM capacitance;
- τ_c : the angular time constant of the resolved characteristic frequency of the film, defined as $\tau_c = 1/\omega_c$.

The relaxation time constant, τ_c , couples the C_t term to an inherent resistance term in series, R_t , through the relation,

$$\tau_c = (R_s + R_t)C_t \quad (3.11)$$

and so combining all the electrochemical circuit terms from equations (3.10) and (3.11) generates the equivalent circuit model in Figure 3.1d.

For non-ideal SAMs, whose contribution to impedance is phase-dependent, we can apply the more accurate Cole-Cole description of ionic relaxation times and orientation energies in the film,^[167] and Equation (3.10) transforms into Equation (3.12).

$$C^*(\omega) = C_m + \frac{C_t - C_m}{1 + (j\omega\tau_c)^{1-\alpha}} \quad (3.12)$$

In terms of the equivalent circuit description, the R_t term is replaced by an imperfect *constant phase element*, Q_t , as shown in Figure 3.1d. This model provides a more accurate and detailed description of SAM electrochemical properties than what is usually considered because it takes into consideration the perturbed, solution

phase, ionic distribution and the ionic relaxation response associated with entrapped ions within the SAM.

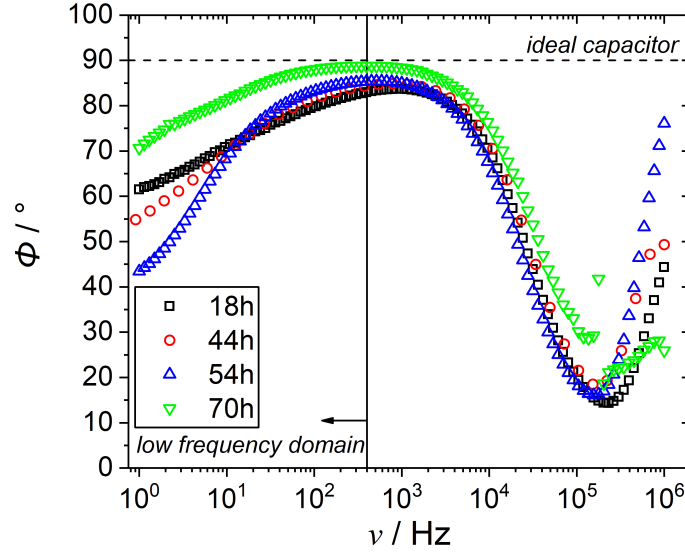
The work described in this chapter investigates the empirical application of the model shown in Figure 3.1d, in particular the effect of SAM crystallinity on the resolved ionic film charging fingerprints, contained within the term C_t . *Capacitance spectroscopy* (CS), an *electrochemical impedance spectroscopy*, EIS, based technique is used to perturb and probe the ionic characteristics of the film different lengths of alkanethiol SAMs (film thickness).

3.2 Maximising the crystallinity of self-assembled monolayer films

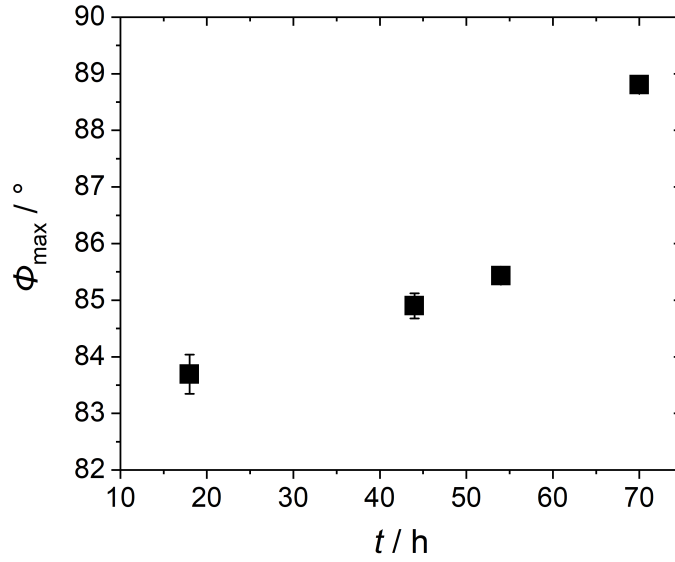
Crystallinity is a measure of structural order in a solid. In terms of a SAM adsorbed onto a metal substrate, the ideality of the film as an electrochemical capacitor is increased as the crystallinity of the film increases (maximising order and reducing defects). In order to more easily apply the Debye model description of the non-Faradaic electrochemical processes of SAM films, as opposed to the Cole-Cole model which is difficult to interpret and resolve physically, it is desirable to understand how to prepare self-assembled ‘ideal’ molecular films, of phase, $-\phi \geq 88^\circ$, onto gold substrates. This section describes how this is done by two different methods: by observing the phase of the molecular film and by measuring the stretching frequencies of particular C–H bonds.

3.2.1 The phase of self-assembled monolayer films

Self-assembled monolayer (SAM) films of 1-dodecanethiol on gold electrodes were prepared as described in Section 2.9 on page 65. Figure 3.2 shows the effect on the phase angle, ϕ , of 1-dodecanethiol SAMs as the incubation time of gold substrates in ethanolic thiol solutions increases. It can be seen from the phase-resolved data in Figure 3.2a that the measured phase of the SAM increases to a plateau at intermediate frequencies. The vertical line indicates the transition into



(a)



(b)

Figure 3.2: The phase of four different thiol monolayers after 18 h, 44 h, 54 h and 70 h incubation. **(a)** Phase plot at different applications of a.c. frequencies. **(b)** Maximum phase of the plateau observed at moderate frequencies. Note that analysis of lower frequencies is not applicable using the current model. Pre-cleaned electrodes were incubated in 1 mM 1-dodecanethiol ethanolic solution for varying incubation times, at room temperature in the dark. EIS was performed on each electrode at open-circuit potential (OCP). Electrolyte: 150 mM NaClO₄.

3. Defining the ionic characteristics of self-assembling monolayers

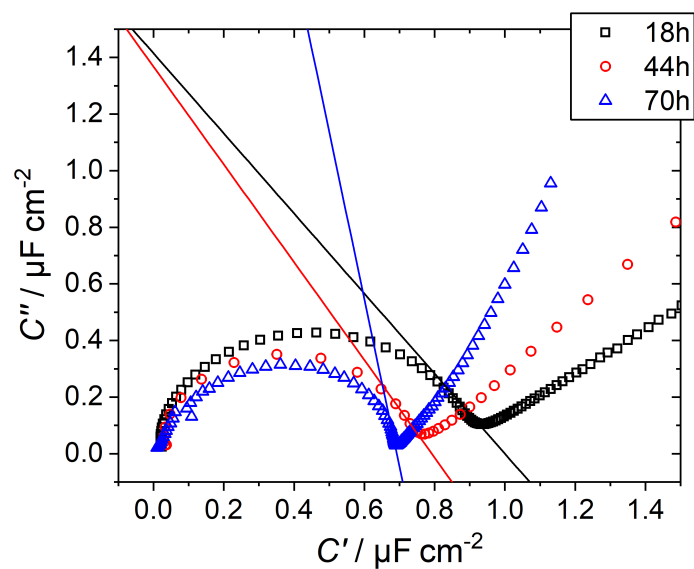
a low frequency domain, where the film behaves predominantly as capacitor C_m and therefore phase is not resolved here.

The maximum phase reached in the plateau region is plotted as a function of incubation time in Figure 3.2b and it is clear to see that as the incubation time for SAM generation increases, so does the value of $-\phi_{\max}$. In fact, at incubation times of 70 h the phase reaches 89° , very close to the value for a perfectly behaved capacitor, of 90° .^[149] Therefore in order to prepare SAMs for C_t measurement and resolution, the gold substrates were incubated in the thiolic solution for at least 70 h. Experiments at longer incubation times were performed, but there was no improvement to film ideality of 90° .

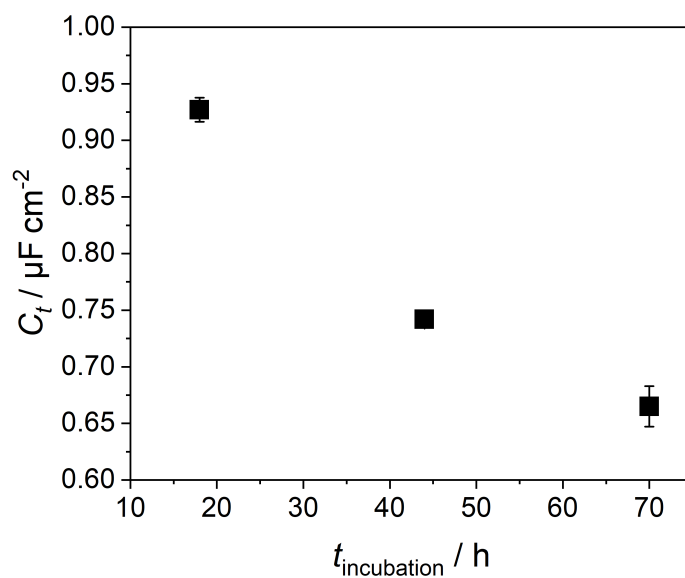
Although an incubation time of 70 h generates a maximum achievable phase of SAM film of 89° at around a frequency of 1 kHz, these films are still susceptible to ionic penetration and therefore a resolved ionic and dipolar relaxation in the C_t term, highlighted by Figure 3.3. These impedance measurements were performed in the frequency range, $1 \text{ MHz} \geq \nu \geq 10 \text{ Hz}$ applying the open circuit potential, V_{OCP} , to ensure the electrochemical response is entirely non-Faradaic in nature. The Z' and Z'' values determined with EIS were converted to C''' and C'' respectively using the relations described in Equation (2.18) (also included in the caption for Figure 3.6). With this method, and applying the Debye model from Figure 3.1d, it is therefore possible to measure the contribution of ionic ingress to the capacitance term, C_t .

The diameter of the semicircular region of the capacitance (CC) plot in Figure 3.3a is taken as the value of C_t . It is noticeable that as the incubation time increases, the diameter of the semicircular region of the CC plot decreases, therefore the value of C_t decreases (Figure 3.3b). This is an indication that, because of the increase of crystallinity, that the extent of ionic ingress of the sodium cations decreases, reducing the ionic contribution to C_t .

The deviation from ideality can also be observed by measuring the tangent of the semicircle at the point where the capacitive semicircle and the diffusion tail meet (Figure 3.3a), as an ideal capacitor, obeying Debye over Cole-Cole characteristics, should be a perfect semicircle.



(a)



(b)

Figure 3.3: The capacitance plots of three different thiol monolayers after 18 h, 44 h, and 70 h incubation. (a) The capacitance plot as a function of frequencies, with solid tangent lines at the intersection of the capacitive semicircle and the diffusion tail. (b) Resolution of the C_t term from Figure 3.3a. EIS was performed on each electrode applying V_{OCP} . Electrolyte: 150 mM NaClO_4 .

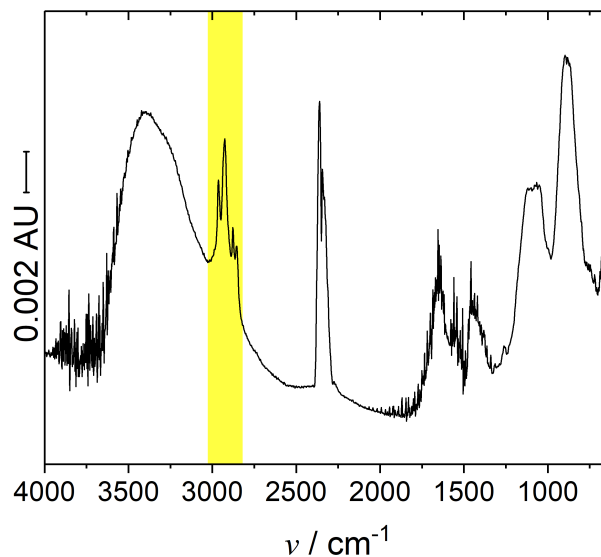
3.2.2 Measuring the energies of C–H stretches

The extent of crystallisation as a function of chain length can also be observed by measuring the vibrations of the symmetric and antisymmetric stretches of the CH₃ and CH₂ in the alkyl chains of the thiol monolayer films. Fourier Transform IR spectroscopy (FTIR) was performed on a variety of samples, with differing incubation times (18 h, 44 h and 70 h) of clean gold substrates in 1-dodecanethiol ethanolic solutions. Samples were clamped onto an attenuated total reflectance (ATR) accessory that was fitted to the spectrometer, in order to probe the non-transparent surface (see Section 2.5 on page 59).

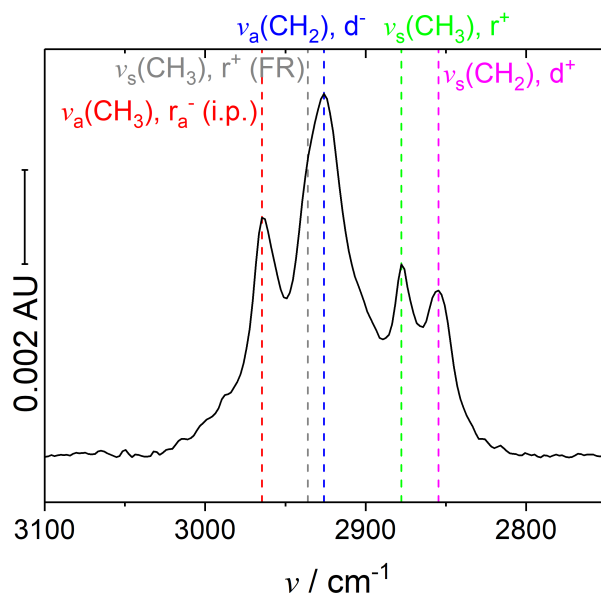
The spectrum of SAM-modified gold surfaces measured by ATR-FTIR is shown in Figure 3.4a and is background corrected by subtracting the atmospheric signals. The region of particular interest for alkanethiol SAMs is the C–H stretching in the 3000 cm⁻¹ to 2800 cm⁻¹ region (yellow, expanded and baseline corrected in Figure 3.4b). Although these peaks overlap with the broad water stretching region, they are resolved enough for quantification of the different energies in vibrations.

There are five observable stretching energies shown in Table 3.1: the symmetric and antisymmetric frequencies of each the CH₃ and CH₂ groups, and the Fermi resonance (FR) of the symmetric methyl stretching mode.^[168–170] The vibration at around 2875 cm⁻¹ is particularly susceptible to a variation in energy as a function of incubation times. Here the energy of the vibration is observed to decrease as the incubation time of the 1-dodecanethiol increases (Figure 3.5). As the incubation time is increased, the time the SAM molecules have to rearrange on the surface of the substrate also increases. This enables the SAM to form a more homogenous layer with the 1-dodecanethiol molecules orientated in the same direction, which leads to a higher proportion of dipole orientation, associated with greater crystallinity.

To investigate the effect of chain length of the thiol on the value of C_t , it is practically necessary to apply the Debye equivalent circuit model. Therefore to maximise crystallinity to reach values of $-\phi \geq 88^\circ$, the SAMs in the next section were prepared with incubation times of at least 70 h.



(a)



(b)

Figure 3.4: FTIR-ATR spectra recorded at 1-decanethiol SAM modified gold surfaces, incubated for 70 h. (a) Whole recorded spectrum, with the C–H stretching region highlighted in yellow, and (b) the C–H stretching region between 3100 cm^{-1} and 2750 cm^{-1} and baseline corrected.

Table 3.1: Vibrational energies for 1-dodecanethiol SAM after 50 h incubation.

Energy / cm^{-1}	Functional group	Mode
2965	CH_3	a, r_a^-
2936	CH_3	s, r^+ (FR)
2926	CH_2	a, d^-
2875	CH_3	s, r^+
2855	CH_2	s, d^+

s: symmetric; a: antisymmetric; r^+ : symmetrical CH_3 stretch; r_a^- : antisymmetric CH_3 stretch in skeletal plane; d^+ : symmetrical CH_2 stretch; d^- : symmetrical CH_2 stretch; FR: Fermi resonance.^[168–170]

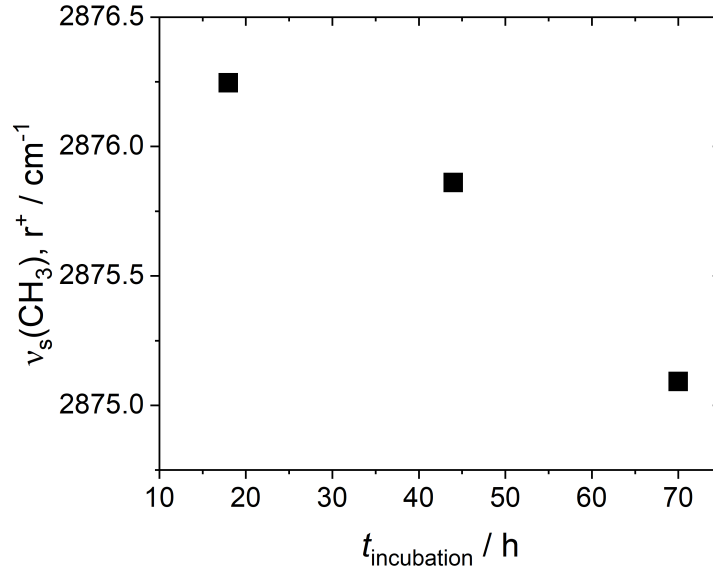


Figure 3.5: $\nu_s(\text{CH}_3), r^+$ as a function of incubation times of 1-dodecanethiol. Same preparation as in Figure 3.4, but with incubation times of 18 h, 44 h and 70 h.

3.3 Investigating the origin of C_t

Alkanethiol SAMs were prepared on freshly cleaned and smooth gold disk electrodes diameter = 1.6 mm by incubating the electrodes in 1 mM ethanolic alkanethiol solution for at least 70 h in the dark at room temperature. Non-Faradaic electrochemical impedance spectroscopy (EIS) was performed at open-circuit potential, V_{OCP} , at frequency range 10^0 Hz to 10^6 Hz in 0.15 M NaClO_4 electrolyte. The Bode plot in Figure 3.6a shows the impedance magnitude response for different thiol chain

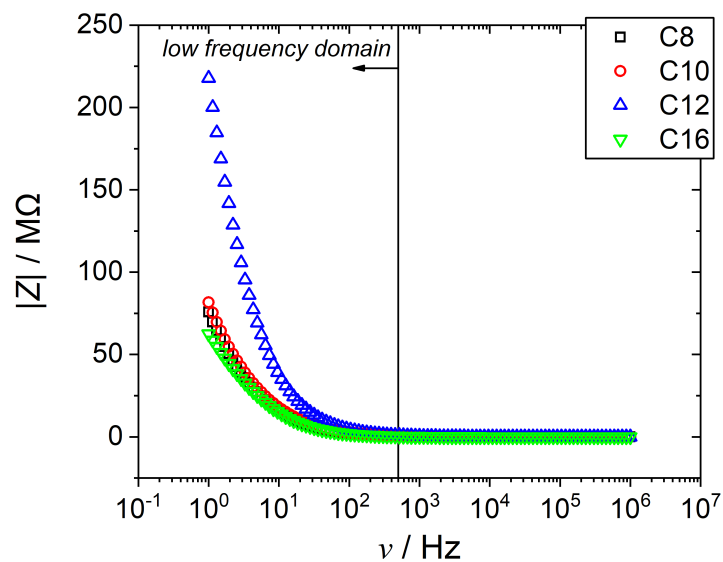
lengths. Noticeably, below a frequency value of 500 Hz, the dramatic increase in the impedance magnitude is indicative of ionic leakage within the low frequency domain, for which the Debye or Cole-Cole equivalent model for resolving C_t is invalid.

The Bode plot for the phase angle (ϕ) dependency of the SAM film is also indicative of the deviation from a perfect capacitor within the low frequency domain. The plateau region at intermediate frequencies shows the maximum phase angle, $\phi_{\max}$ achieved for each film. It can be seen that the values of $\phi_{\max}$ for each film are consistently very high, $\phi_{\max} \geq 85^\circ$, and so the Debye equivalent circuit model can approximately be applied here see Equation (3.10) and Figure 3.1.^[149] For very high frequencies, $\phi = 10^6$ Hz, the bulk and double layer capacitance, C_m , of the film dominates and C_t cannot be resolved. At mid-range frequencies, 10^2 Hz to 10^5 Hz, the C_t term contributes to the impedimetric and phase response of the film and is the region of interest for fitting of the C_t model.

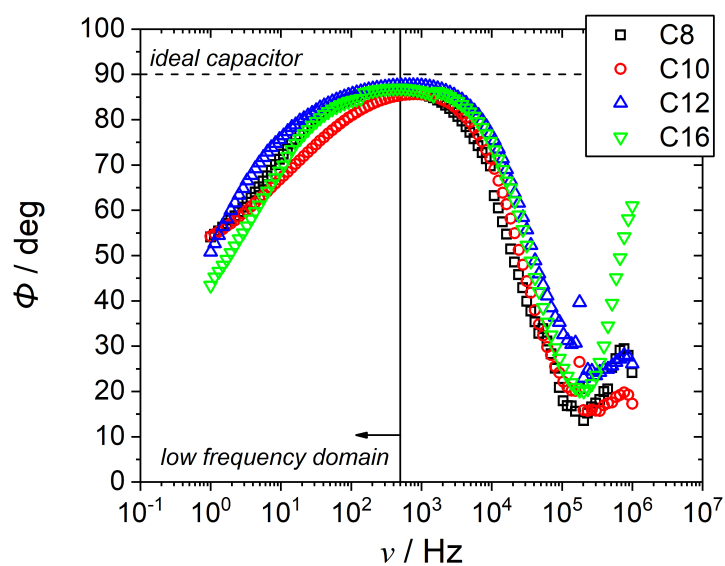
The impedance data retrieved from EIS are shown in Figure 3.6c. It can be seen that for the frequency range of the experiment, the data are not easily resolvable for capacitance contributions - indeed the plot is very much vertical within the mid-range frequencies (insert), indicating a single near-perfect capacitor in series with the solution (electrolyte) resistance.^[171] Within the lower frequency domain (higher impedance values), the deviation from the near-vertical line is again an indication of ionic leakage, for which we cannot apply the Debye or Cole-Cole model.

Using equations (2.18), also shown in the caption for Figure 3.6, the impedance plot is converted to a capacitance plot, as shown in Figure 3.6d, and C_t is easily resolved as the semicircle diameter of the plot. C_m dominates at high frequencies (x -origin of the semicircle), until it reaches $\phi_{\max}$ at the maximum diameter of the semicircle, before a collapse of the ionic integrity of the film leading to the ‘diffusion tail’, where the equivalent circuit model cannot be applied.

It can be seen that as the diameter of the film, or the length of the alkanethiol used for SAM generation, decreases, the diameter of the capacitance semicircle, or the value of C_t , decreases. The variation of the C_t term as a function of alkanethiol length can be more easily seen when looking at the real part of the capacitance as a

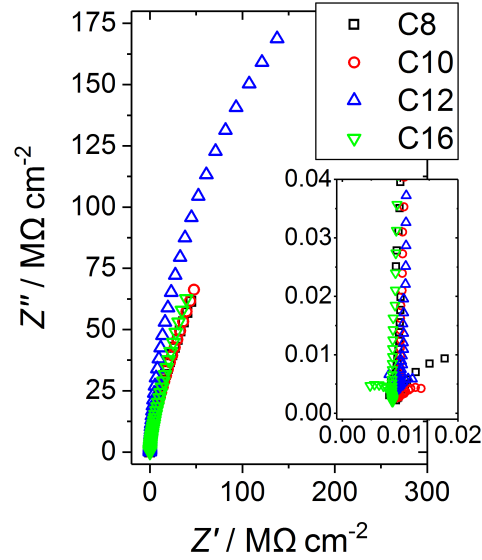


(a)

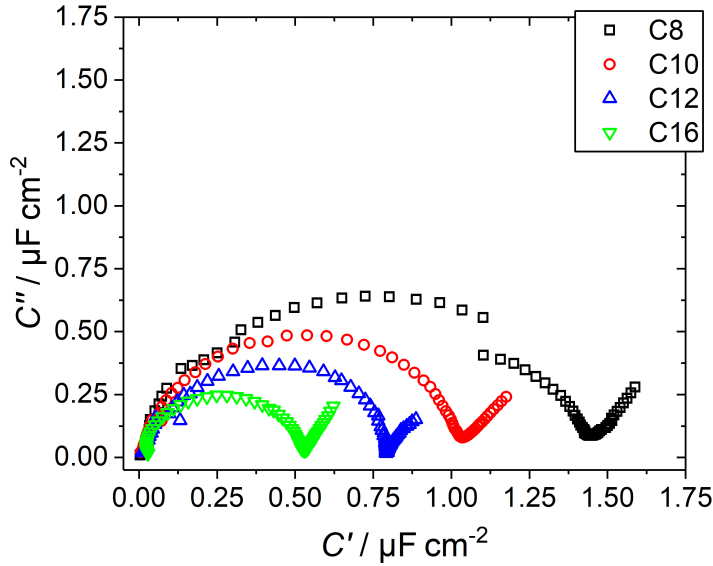


(b)

Figure 3.6: Bode and Nyquist plots for 1-octanethiol (C8), 1-decanethiol (C10), 1-dodecanethiol (C12) and 1-hexadecanethiol (C16). (a) Bode modulus plot. (b) Bode phase angle plot. (cont.)



(c)



(d)

Figure 3.6: (cont.) Bode and Nyquist plots for 1-octanethiol (C8), 1-decanethiol (C10), 1-dodecanethiol (C12) and 1-hexadecanethiol (C16). **(c)** Impedance Nyquist plot. **(d)** Capacitance Nyquist plot. Conversion from Figure 3.6c using the relations $C'(\omega) = Z''/\omega|Z|^2$ and $C''(\omega) = Z'/\omega|Z|^2$ as in Equations 2.18 on page 44. Gold substrates were incubated in 1 mM ethanolic thiol solutions for 70 h. Impedimetric measurements were taken at V_{OCP} at frequency range 10^0 Hz to 10^6 Hz in 0.15 M NaClO_4 electrolyte.

function of applied frequency, as shown in Figure 3.7a. Subtracting the capacitance value at the high frequency (10^6 Hz) from the plateau region retrieves the value of C_t . Plotting this value against the film diameter, L_D , in Figure 3.8a shows a negative and almost linear relationship in the effect of diameter on the value of C_t . This is in line with the prediction of a decreased contribution of ionic ingress of the solvent cations into the film with longer thiol-base SAMs, with higher crystallinity.

The peak in the imaginary capacitance Bode plot in Figure 3.7b relates to the time constant $\tau_c^{-1} = [(R_s + R_t)C_t]^{-1}$.^[124,154] Using this relation, we can retrieve a value for the total resistance of the electrochemical circuit, $(R_s + R_t)$. Figure 3.8b shows the plot for the combined resistance and it can be seen that as the diameter of the film increases, the resistance increases almost linearly. As the solution resistance, R_s , the resistance of the monolayer dipoles and ionic field to further field-induced perturbation, can reasonably be assumed to be consistent between experiments, the variation in resistance in the SAM increases linearly as the diameter increases. This is in line with an increase in the peak centre of τ_c^{-1} in Figure 3.7b (increase in frequency/energy) as the diameter of the film increases, for an increase of energy required for film relaxation processes.

The decrease of the τ_c^{-1} peak intensity is an indication that, although aiming for ideal Debye-behaving monolayer films in their preparation, that the R_t term is not ideal, and has a spread of values better represented by the constant phase element, $Z(\omega)_Q = [Y_0(j\omega)^\alpha]^{-1}$.^[154]

Finally the symmetric and antisymmetric methylene stretching frequencies were collected from alkanethiol films of various length, after incubation of clean evaporated gold on glass substrates in 1 mM ethanolic thiol solutions for at least 70 h. Blanks were taken in atmospheric air before samples were clamped onto an Si prism of a connected ATR accessory. The frequencies of the symmetric and antisymmetric stretches^[168,169] were then plotted as a function of SAM chain length (Figure 3.9). It can be seen that as the length of the thiols in the SAM increases, the frequencies of the methylene stretches decreases. This is further evidence for higher crystallinity (leading to reduced ionic ingress and therefore lower values of C_t) as

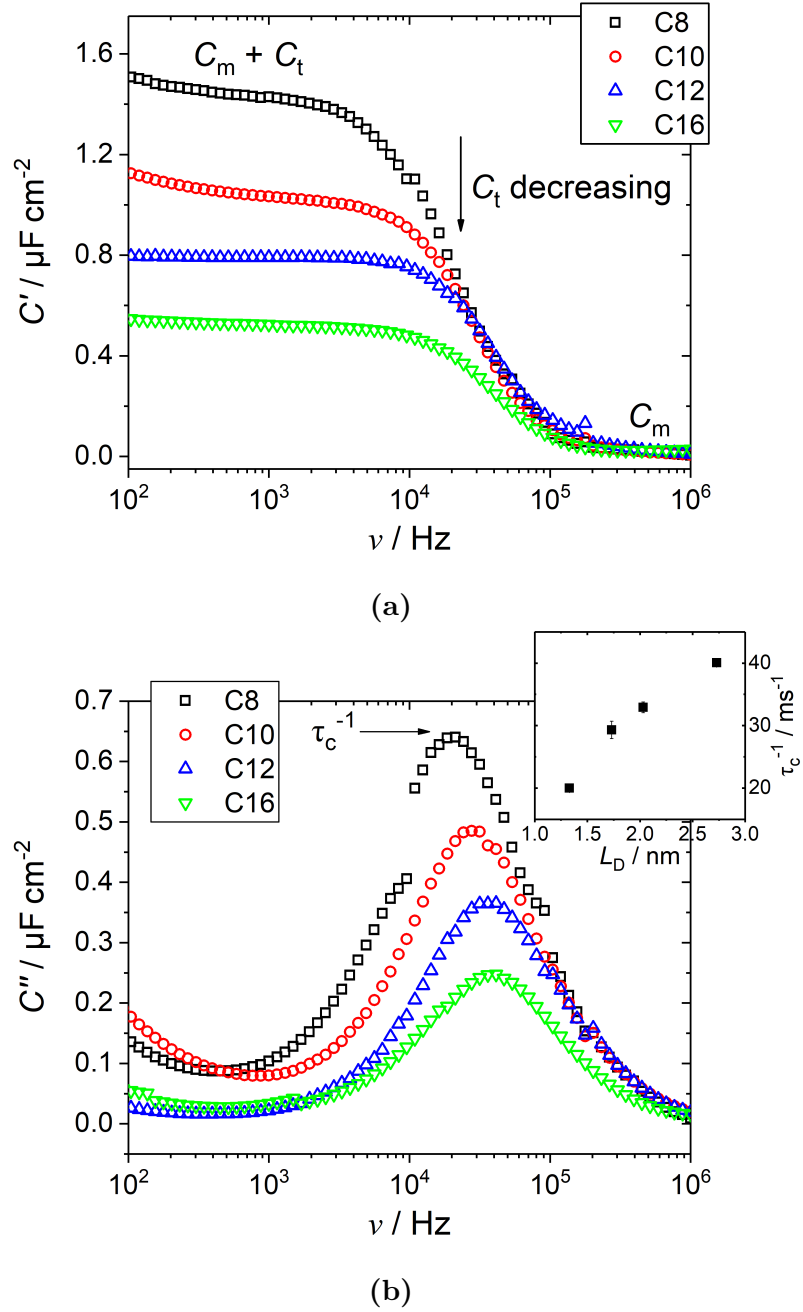
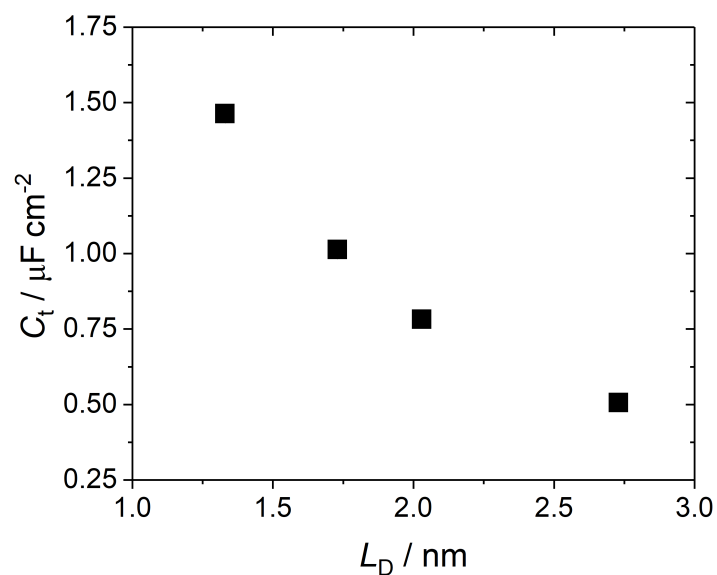
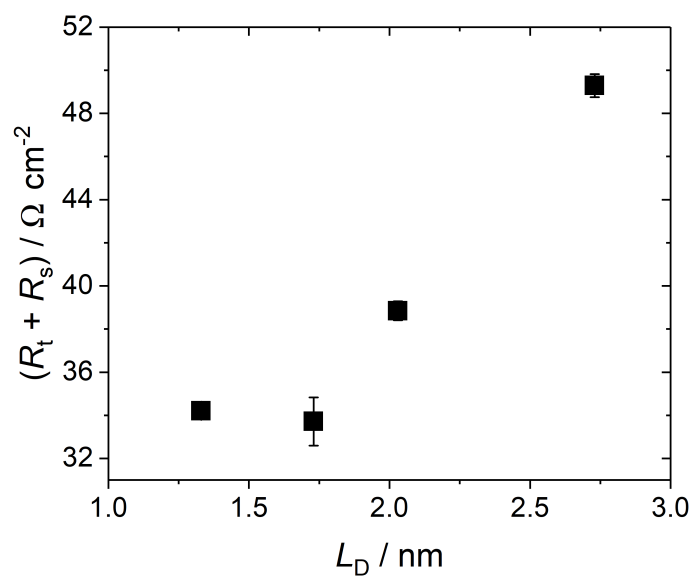


Figure 3.7: Bode plots of (a) the real, and (b) the imaginary parts of the complex resistance. The insert plots the function $\tau_c^{-1} = [(R_s + R_t)C_t]^{-1}$. This data is extracted from the capacitance Nyquist plot in Figure 3.6d on page 86.



(a)



(b)

Figure 3.8: (a) C_t , and (b) $(R_t + R_s)$ as a function of SAM thickness. These data are extracted from the capacitance Bode plots in Figure 3.7 on page 88.

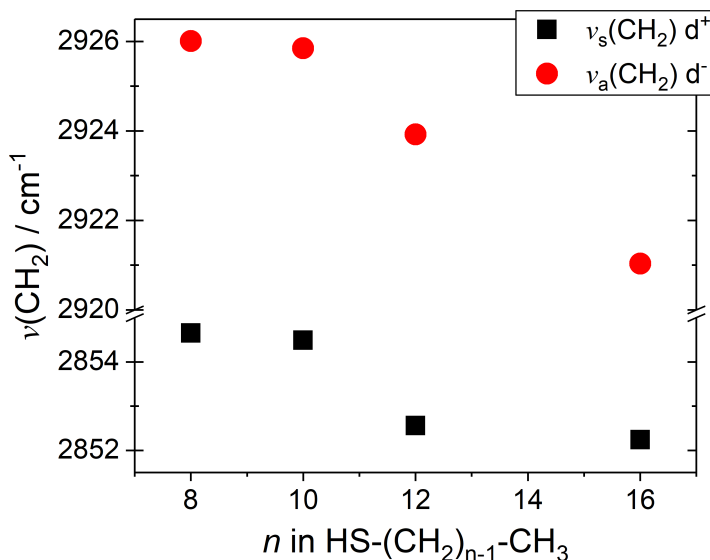


Figure 3.9: Methylene $\nu_s(\text{CH}_2)$, d^+ and $\nu_a(\text{CH}_2)$, d^- stretches as a function of alkanethiol chain length. Monolayers were prepared similarly to those in Figure 3.6.

a result of an increasing proportion of dipole orientation, which in turn increases the energy of the stretching vibrations, as the thiol length increases.

3.4 Summary

The double layer capacitance model is acceptable for a simple electrode-electrolyte interface. However, when an organic SAM is formed on the surface of the electrode, this generates an electrode-SAM-electrolyte interface which cannot be accurately described with the simple double layer capacitance model. In addition to lowering the interfacial capacitance, further electrochemical terms are resolvable: the film ionic capacitance, C_t , and associated rotational resistance $R_t = \tau_c C_t^{-1} - R_s$. The explanation for the origin of these terms and the intermediate frequency of the ionic relaxation, τ_c^{-1} , is the entrapment and ingress of cations into the SAM film. In this Chapter, I have demonstrated the empirical application of a more detailed electrochemical model for a SAM film than has been described before. The resolution of C_t enables SAM-based electrochemical devices, such as label-free biosensors, to become more accurate, sensitive and reproducible than before.

4

Development of an electroenzymatic NADH recycling method for selective biocatalytic reductions

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4.1 The NAD^+ -reducing enzyme

The essence of this Thesis is the utilisation of an enzyme that reduces the oxidised cofactor, NAD^+ , to the biologically active 1,4- NADH cofactor, powered by direct electron transfer from a carbon working electrode. The 1,4- NADH generated by this electrocatalyst goes on to be used as a two-electron (hydride) donor for specific reductions of $\text{C}=\text{X}$ substrates by oxidoreductases. This section describes the enzyme used for electroenzymatic regeneration of the NADH cofactor.

4.1.1 The electrochemistry of the soluble hydrogenase

Fundamental investigation into the electrochemical activity of the NAD^+ -reducing soluble hydrogenase (SH) from *Ralstonia eutropha* has characterised the activity of the diaphorase sub-complex, which reversibly reduces NAD^+ to 1,4- NADH .^[54] The proposed modular structure is shown in Figure 4.1.

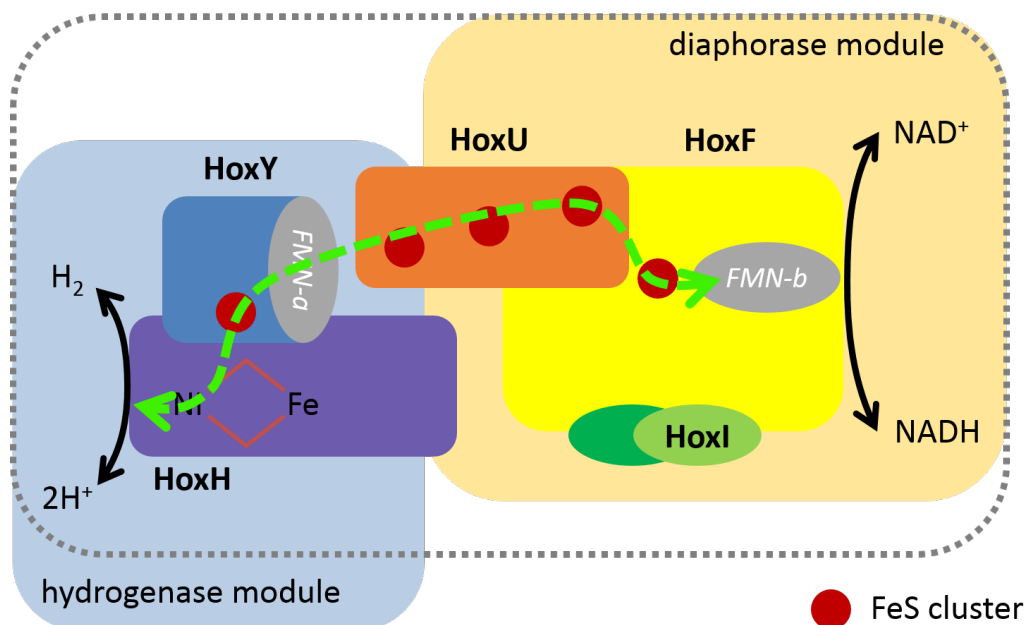


Figure 4.1: Cartoon structure of the [NiFe]-soluble hydrogenase from *Ralstonia eutropha*. Green dashed line indicates the suggested route of electron transfer between the hydrogenase and the diaphorase modules.

The enzyme is composed of two distinct modules:

- the hydrogenase module, HoxHY, which reversibly extracts electrons from the oxidation of H_2 , and
- the diaphorase module, HoxFU(I_2), which uses the two electrons from H_2 to selectively and reversibly reduce NAD^+ to 1,4-NADH.

The reversible electron transfer of two electrons from the NiFe active site in HoxH to the FMN catalytic centre of HoxF is mediated by an electron relay chain of FeS clusters, shown in red in Figure 4.1, and possibly also by the additional FMN in HoxY.^[172–176]

Lauterbach *et al.*^[54] managed to separate HoxHY from the HoxFU modules, the Hox I_2 being labile at high ionic strength and alkaline pH.^[174] The HoxFU diaphorase module has been isolated and characterised independently using various biochemical, electrochemical and spectroscopic methods.^[54,176,177]

Although there is no high resolution X-ray crystallographic structure of the SH from *R. eutropha*, there are accepted analogous enzymes for which the structure has been solved using X-ray crystallography. Shomura *et al.*^[178] successfully solved the structure of the SH isolated from *Hydrogenophilus thermoluteolus* to 2.58 Å. This structure is likely to be most similar to that of the SH from *R. eutropha*, as utilised in this project. Another source of comparison is the related peripheral arm of Complex I from *Thermus thermophilus*, as described by Sazanov and Hinchliffe^[179] and Berrisford and Sazanov.^[180]

The NAD^+ -reductase used for bioelectrochemical NAD^+ reduction in experiments described in this Thesis is the NAD^+ reducing soluble hydrogenase from *R. eutropha* with a deactivated hydrogenase module via a single amino acid substitution (I64A) in the large hydrogenase subunit, as described in Section 2.10.1 on page 66.

4.1.2 ‘ NAD^+ -red’ and its electrochemistry

Figure 4.2a shows the crystal structure for soluble hydrogenase from *H. thermoluteolus*.^[178] There are four [4 Fe–4 S] clusters and one [2 Fe–2 S] cluster in the protein, each able to transfer an electron one at a time through the protein structure, through

to the flavin in the diaphorase module, FMN, at the top of the image. The inter-cluster distance and the distance between the cluster chain and the outside of the protein is sufficiently small enough for electrons to tunnel between each cluster.

The electrochemistry of the NAD^+ -reductase enzyme used in this study, denoted ‘ NAD^+ -red’, is shown in Figure 4.2b and the onset potential for NAD^+ reduction with an NAD^+ -red-modified PGE working electrode, was determined and the catalytic current normalised). It was observed that $V_{\text{onset, NAD}^+\text{-red}} = -0.35 \text{ V vs SHE}$, so very little overpotential is required to drive the reduction of NAD^+ and very little energy is wasted in driving the reduction of NAD^+ , or conversely the oxidation of NADH.

The flavin, FMN, is the site where electrons are transferred to the cofactor NAD^+ . This is looked at in detail in Figure 4.3a, which shows the crystal structure of the active site from the peripheral arm of Complex I from *T. thermophilus*.^[180] The closest [Fe–S] cluster to the FMN, which is capable of accepting and donating two electrons (Figure 4.3b), a [4 Fe–4 S] cluster, is in the direct field of view at a distance of 7.8 Å. This distance is small enough for two electrons to be able to transfer to the FMN one by one, before the two electrons transfer again to the substrate NAD^+ .^[181] The NAD^+ is observed to coordinate on top of the FMN in the configuration shown. The geometry of the active site, dictated by the shape of the folded protein around the FMN, a β -sheet motif called the *Rossmann fold*,^[182,183] allows for the NAD^+ to bind to the FMN only in this position. This enables for the electron transfer over a distance of 3.2 Å. The current consensus is that this is a concerted hydride transfer rather than a separate electron transfer:^[54,180] the ‘hydride’ binds only to the 4-position of the nicotinamide ring. Therefore the only reduction product is the 1,4–NADH, the only biologically active structure of NADH cofactor.

The phenomenon that the NAD^+ -red enzyme specifically reduces NAD^+ to the 1,4–NADH lays the groundwork for the development of an electrochemical reduction catalyst. The next section discusses the different carbon electrode materials used in this study for the direct electron transfer from the electrode to the NAD^+ -red.

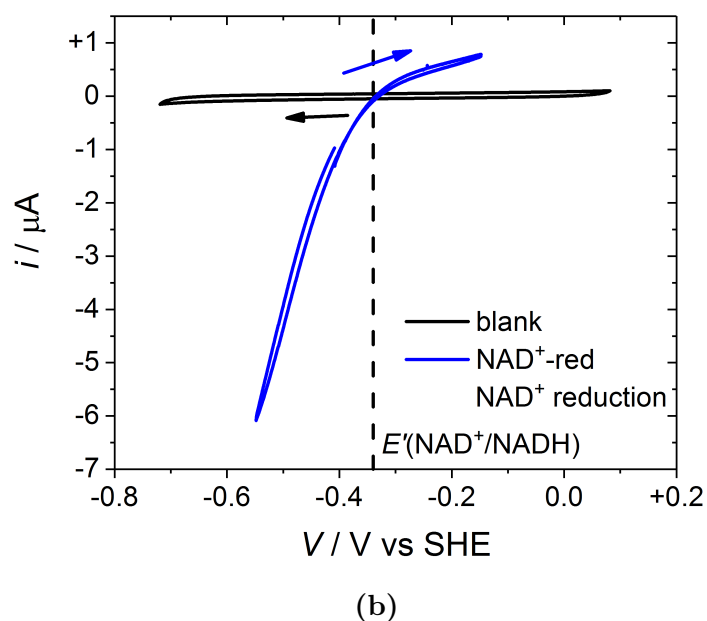
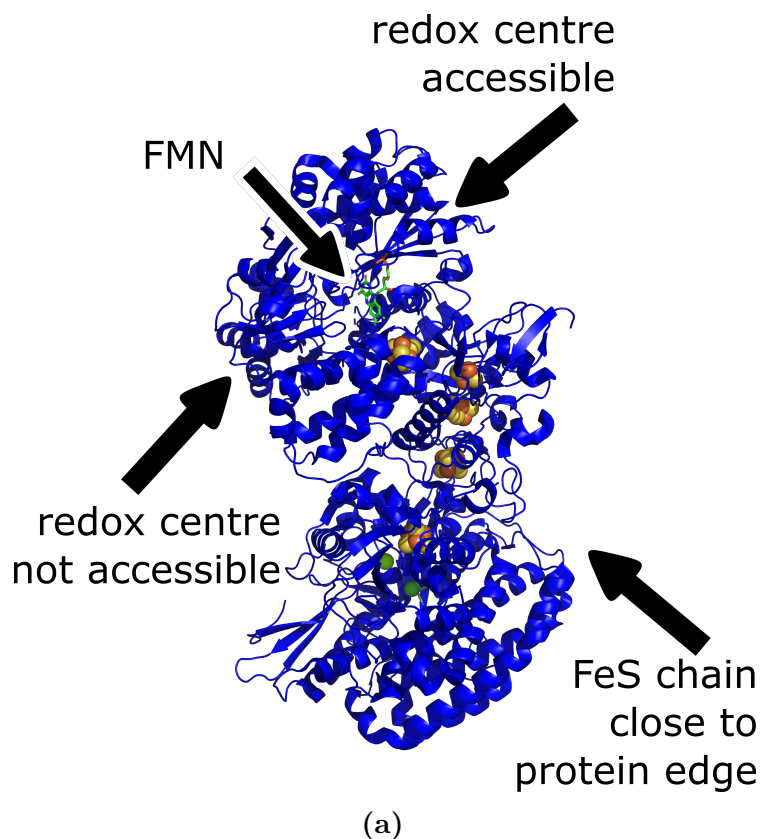
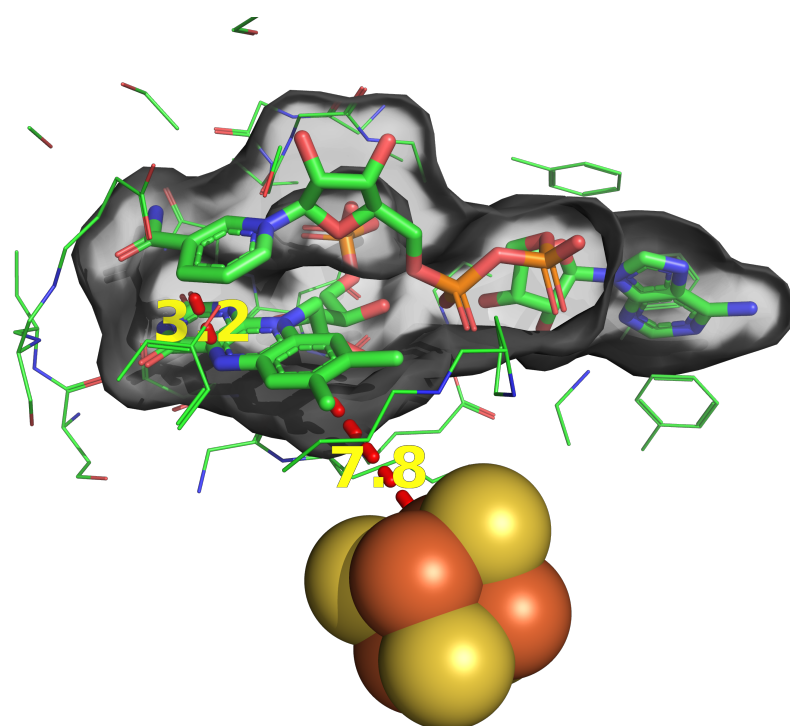
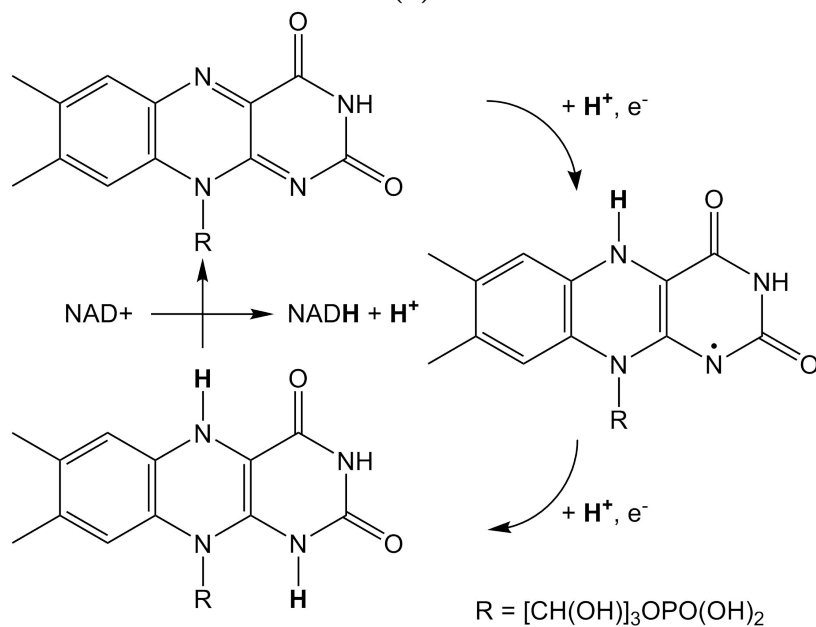


Figure 4.2: (a) Crystal structure for the soluble hydrogenase from *Hydrogenophilus thermoluteolus*,^[178] PDB code: 5XF9 (b) Cyclic voltammogram for NAD^+ reduction recorded at an NAD^+ -red-modified pyrolytic graphite, PGE, electrode (blue). A blank cyclic voltammogram recorded at an unmodified PGE electrode with no NAD^+ in solution is provided (black). Substrates: 1 mM each NAD^+ and NADH ; scan rate = 10 mV s^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.



(a)



(b)

Figure 4.3: (a) Active site of the peripheral arm of Complex I from *Thermus thermophilus*,^[180] co-crystallised with 1,4-NADH and showing the proximity of the FeS chain to the FMN, with distances shown in Ångstrom, PDB code: 3IAM. (b) The flavin, FMN, in the diaphorase module, undergoing two-electron transfer between the reduced and oxidised state.^[181]

4.2 Carbon as an electrode material

The choice of electrode material for enzymatic electrochemistry studies is a non-trivial one to make. They can range in material, electrochemical properties, bonding capabilities, topology and scalability, to list a few characteristics. Within studies described here in this Thesis, a range of carbon materials are used as a working electrode material for the exchange of electrons to directly adsorbed NAD^+ -red. With carbon's various different allotropes, high conductivity and ability for proteins to adsorb to its surface, it makes an ideal material from which to manufacture different types of electrodes for different purposes.

For analytical electrochemistry experiments using a stationary or rotating RDE, *pyrolytic graphite edge*, PGE, is chosen in this Thesis to be the working electrode material. Such PGE electrodes are a popular choice of working electrode material for metalloenzyme immobilisation, due to its roughness at the edge plane scale^[184–187] and perceived electrochemical ‘silence’ towards non-enzymatic redox reactions on typical organic reagents in water-based electrolytes,^[188] at least within the potential range of interest around enzymatic redox processes.

Another working electrode material used is the *carbon paper*, CP, electrode introduced here, but mainly discussed in the application to an electrochemical flow cell in Chapter 5 on page 137. An advantage to this material as compared to PGE is that it provides readily scalable electrodes. Supplied as large sheets, electrodes can be cut to any size and shape. Therefore any size of electrode can be manufactured using this material and is only limited by the size of the carbon paper sheet supplied.

A common technique used within the *Vincent Group* is to use carbon nanoparticles, specifically BP2000, to immobilise enzymes for heterogeneous hydrogenation catalysts. These nanoparticles can be used to build up other carbon surfaces, like the PGE or CP electrodes, to generate high surface area electrodes, whilst maintaining a small geometrical surface area of the working electrodes.

4.2.1 Analytical electrodes

To perform analytical electrochemistry, electrodes must be used that have certain characteristics:

- Low surface area — in order one can assume even immobilisation across the surface of the electrode. Also so that the solution flow is laminar and evenly spread across the surface of the electrode.
- Low capacitive current — in order to maximise the Faradaic against the non-Faradaic contributions.
- High reproducibility — so that direct comparisons in experiments can be made more faithfully.

Therefore a PGE disc electrode is chosen for the more analytical electrochemistry experiments described in this chapter. There is also a large literature base of protein film electrochemistry using a PGE electrode with a small diameter. This makes comparisons to previous studies more relevant.

Figure 4.4 shows the cyclic voltammogram recorded at a freshly polished PGE disc electrode, of diameter 2.0 mm. The electrolyte was chosen to be Trizma[®] base with a total concentration of 100 mM and corrected to pH 8 with concentrated HCl, to make Tris-HCl buffer. A fritted Pt counter electrode was used, together with a saturated calomel electrode, SCE, as a reference electrode, with potentials corrected to the standard hydrogen electrode, SHE. The scan started at the equilibrium potential for the NAD^+/NADH couple at pH 8, at $V_i = -0.32 \text{ V vs SHE}^{[189-192]}$ as shown by the arrow. The potential was then cycled $\pm 400 \text{ mV}$ around the equilibrium reduction potential, the maximum range expected for electrochemical experiments with NAD^+ -red-modified electrodes.

Within the potential range of interest, $(-0.32 \pm 0.40) \text{ V}$, the cyclic voltammetry shows that under these conditions, there are no redox events occurring, which would otherwise be shown by peaks in the forward and/or backwards direction. This indicates that there are no redox active species in or on the surface of the

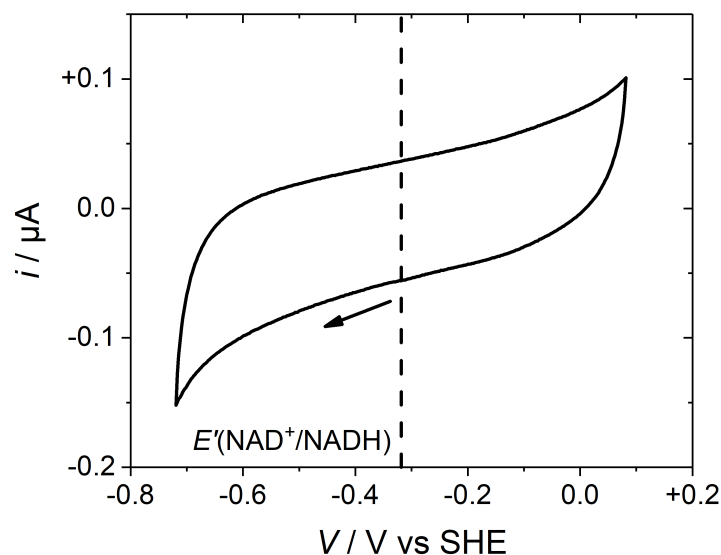


Figure 4.4: Cyclic voltammogram of freshly polished PGE working electrode in buffer containing no redox active species. Dashed line indicates the approximate standard reduction potential for the $\text{NAD}^+/\text{1,4-NADH}$ couple corrected for pH 8, although neither NAD^+ or 1,4-NADH are present in the bulk solution in this experiment. Scan rate = 10 mV s^{-1} ; rotation rate = 0 rpm; electrolyte: 100 mM Tris-HCl buffer, pH 8.

PGE electrode and none in the bulk electrolyte (purely Tris-HCl buffer). Therefore the use of this PGE electrode setup will allow for easy observation of any redox activity in further experiments in this chapter.

The next experiment to perform was a more rigorous testing of the electrochemical activity of common substrates that may be used with NADH-dependent oxidoreductase enzymes, which will be implemented later in this project for selective reductions of $\text{C}=\text{X}$ bonds. These experiments aim to see the scope of substrates that are not electroactive at a PGE electrode. A similar experiment was set up as described in Figure 4.4, this time with the $\text{C}=\text{X}$ substrate of interest, or the product of its reduction, collectively termed ‘O/R $\text{C}=\text{X}$ substrates’, in solution and at 1 mM concentration.

Figure 4.5a shows the cyclic voltammetry of a freshly polished PGE electrode in pH 8 Tris-HCl buffer with different colour traces representing each experiment with a different oxidoreductase O/R $\text{C}=\text{X}$ substrate at 1 mM in solution. The

black trace of no substrate in solution is provided as a ‘blank’. No electrochemical activity was observed with any of the enzyme substrates in solution within this potential window. This indicates that the use of PGE as an electrode material would be appropriate for analytical experiments towards developing a system for electrosynthesis, as these common O/R C=X substrates are electrochemically silent around the equilibrium potential for the NAD^+/NADH couple. Therefore any signal observed within this potential window will solely be enzymatic transformations occurring at NAD^+ -red-modified carbon electrodes. These O/R C=X substrates may be reduced or oxidised outside of this range, but this is likely to be unspecific, particularly for prochiral molecules.

Many organic molecules show low solubility in aqueous solution and DMSO is often used to aid solubility of enzymatic substrates in water. Therefore DMSO was also tested for electrochemical activity (teal trace). A reductive peak could be interpreted at about -0.68 V vs SHE, which suggests some reductive process occurring. However the current of the peak appears to be relatively small compared to the discharge capacitance of the electrode. In addition, this should not affect future experiments as the potential at which this reductive process is occurring is at a more negative potential than non-specific reduction of NAD^+ by the unfunctionalised PGE electrode, as shown by thick red trace in Figure 4.5b. Any useful electroenzymatic reductions should not occur at such negative potentials anyway, as this would run the risk of the carbon electrode additionally producing non-specific reduction products.

These results indicate that the use of carbon as a working electrode material does not interfere electrochemically with these O/R C=X enzyme substrates in solution. Therefore this avoids unspecific reduction via direct electron transfer from the PGE working electrode.

The oxidised cofactor, NAD^+ , is known to undergo direct electron transfer at carbon electrodes at high overpotential.^[54,193,194] The onset potential at which the two-electron reduction occurs at a PGE working electrode, was determined to be -0.58 V (Figure 4.5b, red trace). Therefore, there is a window for selective

4. Development of an electroenzymatic NADH recycling method for selective biocatalytic reductions

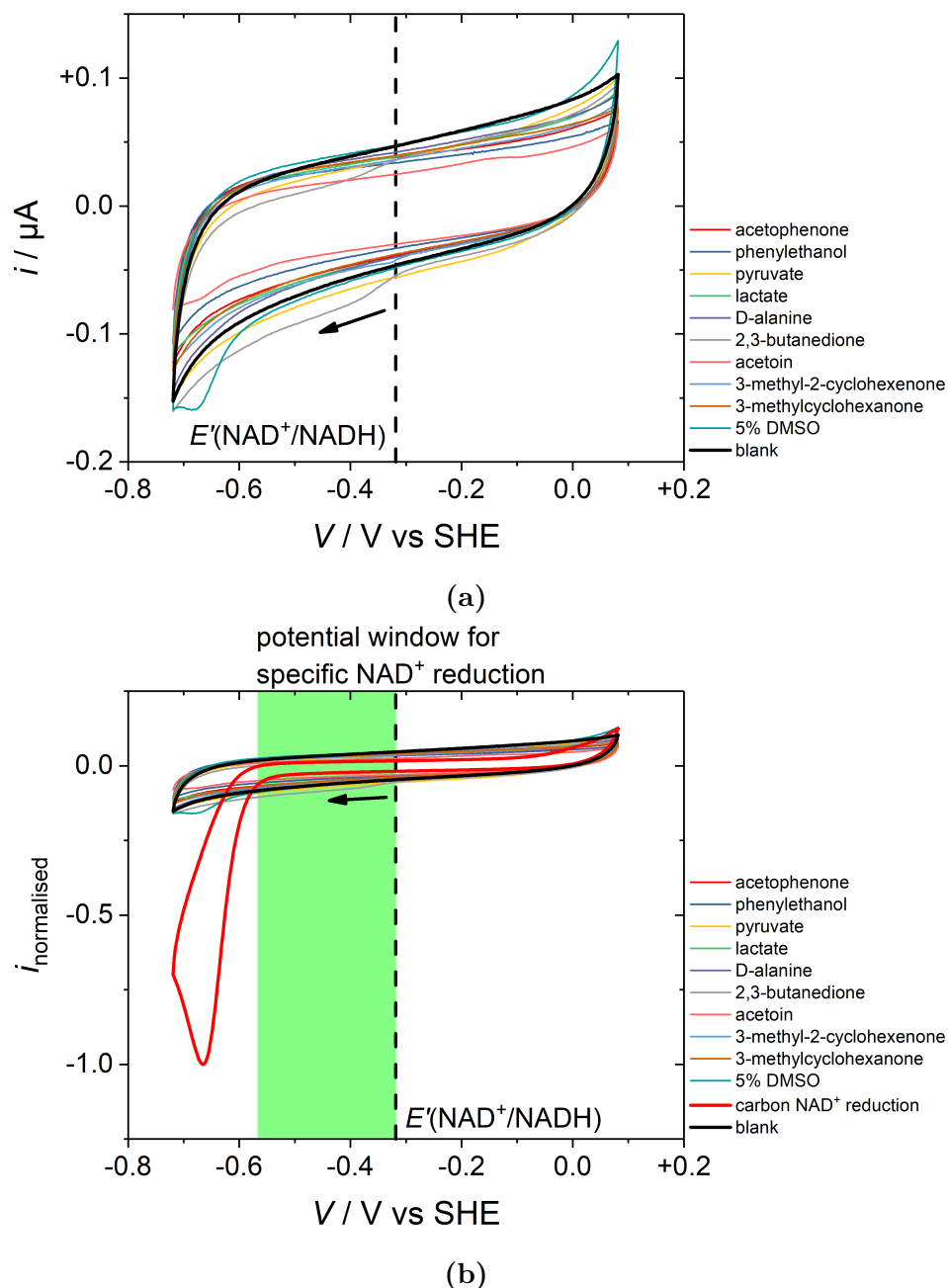


Figure 4.5: Cyclic voltammograms recorded at a PGE working electrode in the presence of O/R C=X substrates for some readily available oxidoreductases. **(a)** Detailed view of current response compared to a blank. Thin colour traces are voltammograms of a PGE working electrode in the presence of 1 mM ‘substrates’: acetophenone (red), phenylethanol (blue), pyruvate (yellow), lactate (green), D-alanine (purple), 2,3-butanedione (grey), acetoin (pale red), 3-methyl-2-cyclohexenone (pale blue), 3-methylcyclohexanone (brown), 5% DMSO (teal). **(b)** Scans from **(a)** plus scan in the presence of 1 mM NAD^+ with an unmodified PGE working electrode (thick red trace), normalised with respect to i_{max} . The thick black trace is a blank voltammogram without any substrates, as in Figure 4.4. Scan rate = 10 mV s^{-1} ; rotation rate = 0 rpm; electrolyte: 100 mM Tris-HCl buffer, pH 8.

catalytic reduction of NAD^+ to 1,4-NADH between the thermodynamic potential of the NAD^+/NADH couple at pH 8 and the onset potential for non-catalytic NAD^+ reduction at the unmodified PGE working electrode, as shown by the green box in Figure 4.5b and numerically in Equation (4.1).

$$-0.58 \text{ V} < V_{\text{onset, PGE}} < -0.32 \text{ V vs SHE} = E'_{\text{NAD}^+/\text{NADH}} \quad (4.1)$$

4.2.2 Scalable high surface area electrodes

Similar experiments, to test the electrochemical (in)activity of other types of carbon working electrodes towards various O/R $\text{C}=\text{X}$ substrates were performed. These are carbon electrodes which are more easily scalable than the PGE electrode, though less useful for analytical measurements. Experiments were performed using:

- carbon paper, CP, electrodes (Figure 4.6a), and
- BP2000 carbon nanoparticles on PGE, BP/PGE, electrodes (Figure 4.6b).

4.2.2.1 Carbon paper electrode

First, the selected common oxidoreductase O/R $\text{C}=\text{X}$ substrates were tested for electrochemical activity at unmodified carbon paper electrodes. These were prepared by cutting a small piece of carbon paper and placing it in between the two layers of lamination plastic. Between the carbon paper and the bottom layer of the laminating plastic was placed the end of a length of carbon fibre. The top side of the laminating plastic was cut using a punch, creating a circular hole of diameter 0.8 cm, exposing an area of 0.5 cm^2 of the CP electrode. The sandwich of the carbon paper, carbon ribbon and the laminating plastic were laminated together using a soldering iron.

Figure 4.6a shows the cyclic voltammetry experiments performed at unmodified CP electrodes in 100 mM Tris-HCl buffer at pH 8, with no enzyme substrate (black trace) and a selection of various enzyme substrates. The discharge capacitance, as read by half the width of the voltammogram, is much higher in value than observed from the measurements made at PGE electrodes, with a value of $6 \text{ }\mu\text{F}$ compared to $0.05 \text{ }\mu\text{F}$ (see Figure 4.5a on page 101). This is roughly in line with the scaled increase

4. Development of an electroenzymatic NADH recycling method for selective biocatalytic reductions

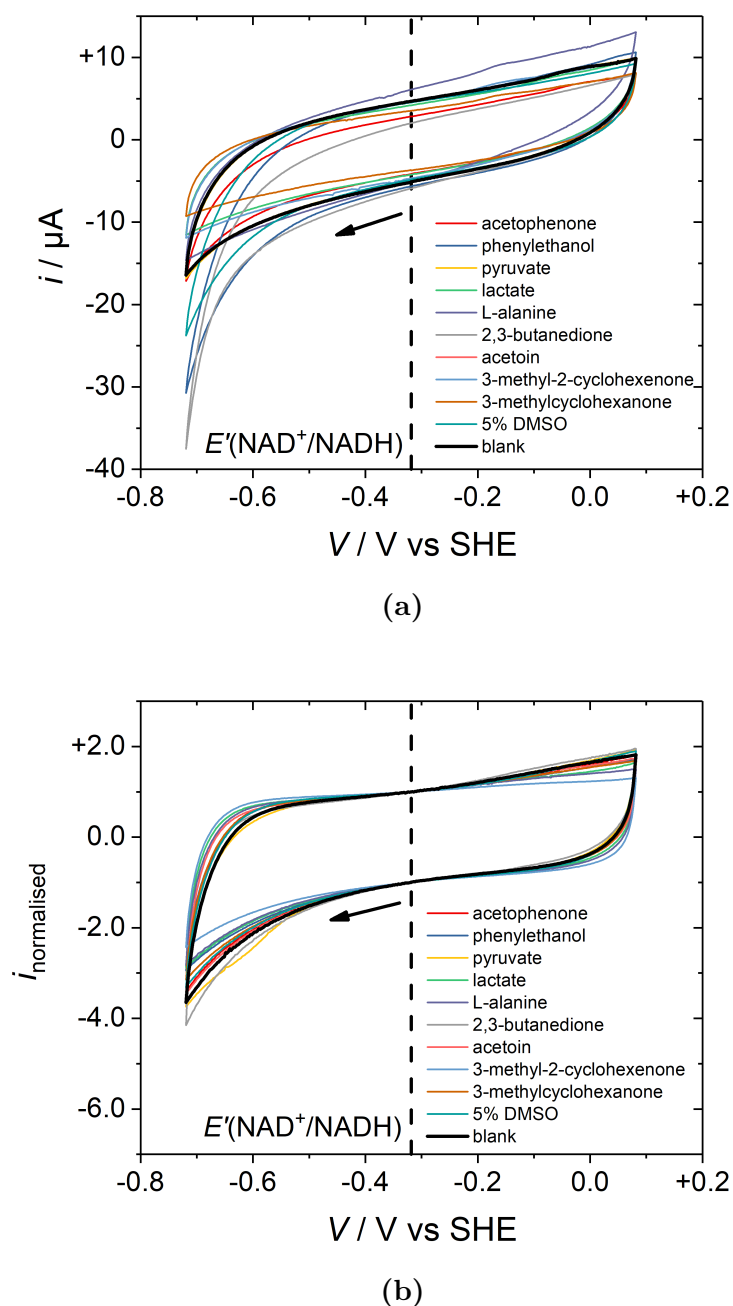


Figure 4.6: Cyclic voltammograms of (a) carbon paper (CP) working electrodes, and (b) high surface area BP2000 nanoparticles on PGE carbon electrodes (BP/PGE), in the presence of some commonly used oxidoreductase O/R C=X substrates. Trace colours are the same as on Figure 4.5 on page 101. Scan rate = 10 mV s^{-1} ; rotation rate = 0 rpm; electrolyte: 100 mM Tris-HCl buffer, pH 8.

in geometrical surface area of the working electrode surface, moving from 0.03 cm^2 for the PGE electrode to 0.5 cm^2 for the CP electrodes, a factor of 12 times larger.

Also, there is no observation of any defined redox phenomena occurring with any of the O/R C=X substrates that are tested in solution. This means, much like the PGE electrode, this working electrode material will not interfere with any of the O/R C=X substrates and that any redox transformation of the O/R C=X substrates is not occurring through a direct electron transfer with the a CP electrode. Therefore this scaleable electrode is a suitable material for use in electrosynthesis experiments, and as a scale-up electrode material.

4.2.2.2 Carbon nanoparticle electrode blank cyclic voltammetry

Next, the selected common oxidoreductase substrates were tested for electrochemical activity with BP2000 nanoparticles. Carbon nanoparticles are a useful material for increasing the surface area of electrodes, whilst maintaining a relatively small geometrical surface area. It has been calculated that these particles have a very high surface area of $(1332 \pm 22)\text{ m}^2\text{ g}^{-1}$.^[76] In particular, BP2000 carbon nanoparticles are particularly good for non-covalent immobilisation of the NAD^+ -red enzyme and this is discussed in more detail in Section 4.4.1 on page 108.

Figure 4.6b shows cyclic voltammetry experiments performed at unmodified BP/PGE electrodes in 100 mM Tris-HCl buffer at pH 8, with no enzyme O/R C=X substrate (black trace) and a selection of various enzyme O/R C=X substrates. Firstly a particle slurry was made by sonicating 20 mg mL^{-1} of BP2000 in ultrapure water for 60 min. Then, $0.5\text{ }\mu\text{L}$ of this particle slurry was drop cast on a stationary and freshly polished PGE electrode, before placing in an electrochemical cell. Cyclic voltammetry experiments were performed immediately, with a newly prepared BP/PGE electrode made for each substrate investigated.

The magnitude of the discharge capacitance was quite sensitive to the surface area of the particle film drop cast onto the electrode and although the mass of the particles was roughly the same in each case, the duration and power of sonication would affect the size of the nanoparticles aggregates created. Also each substrate

investigated could possibly adsorb to the BP particles at different rates, creating a different insulating effect to the surface of the particles. Therefore for ease of reading, the cyclic voltammogram traces were normalised to the discharge capacitance, C_d the width of the voltammogram at a potential where no Faradaic current was observed, of the black trace with no O/R C=X substrates in solution.

In all experiments with different O/R C=X substrates in solution, no redox activity was observed. This again confirms the suitability of the use of BP2000 particles as an electrode material, being electrochemically silent in the presence of selected O/R C=X substrates in solution.

The results in this section indicate that each carbon material tested, PGE, CP and BP2000, show no electrochemical activity in the Tris-HCl buffer at pH 8 and also with all of the selected C=X substrates. The next section discusses the NAD^+ -reductase enzyme utilised in this study for NAD^+ reduction. The enzyme reduces NAD^+ to the biologically active 1,4-NADH cofactor, which can be used as the two electron carrier for two-electron C=X reductions by NADH-dependent oxidoreductases.

4.3 Confirmation of the NAD^+ -red electrochemical activity

Understanding the structure of the NAD^+ -red enzyme and the chemical ‘silentness’ of carbon electrode materials, experiments were performed at the analytical PGE electrode to confirm its electrochemical activity towards NAD^+ reduction with previous work.^[54,195]

The electrochemistry of the NAD^+ -red was first tested using a PGE RDE, as shown in Figure 4.7a. NAD^+ -red (0.5 μL , 0.7 μg) was drop cast onto the surface of a freshly cleaned and polished PGE electrode. The protein film was allowed to partially dry for approximately 60 s. The PGE electrode was then screwed onto the RDE rotor and placed inside an electrochemical cell containing 100 mM Tris-HCl buffer at pH 8.

The results from the cyclic voltammetry experiments performed at this electrode are shown in Figure 4.7. The grey trace in Figure 4.7b shows the response in

absence of NAD^+ cofactor with an unmodified PGE electrode. This matches the electrochemistry discussed in Section 4.2 on page 97. Once the PGE electrode is modified with NAD^+ -red, another blank was measured in the absence of cofactor, with this result shown in black. The discharge capacitance decreases slightly from $10\text{ }\mu\text{F}$ to $9\text{ }\mu\text{F}$, which can be explained by the NAD^+ -red enzyme being slightly insulating to the PGE electrode.

The red trace in Figure 4.7b shows the cyclic voltammetry recorded at a NAD^+ -red-modified PGE working electrode. Upon addition of 1 mM NAD^+ to the bulk solution, a catalytic wave of magnitude $-0.5\text{ }\mu\text{A}$ at -0.51 V appears with an onset potential of -0.35 V . This shows that the NAD^+ -red-modified PGE is reducing the NAD^+ at this potential, as electrons are transferred from the surface of the PGE electrode, through the four FeS clusters for a two-electron reduction of FMN. The two-electrons then reduce NAD^+ . The potential window was not widened beyond -0.56 V below this potential, as it has been previously observed that the enzyme is not stable and the catalytic activity decreases.^[195] Also, the PGE will directly and unspecifically reduce NAD^+ at the surface of the electrode at potentials more negative than -0.58 V .

The change in the slope of the red trace in Figure 4.7b indicates that the enzyme is substrate-limited. Upon rotation of the electrode at 1000 rpm , the catalytic current only increases slightly to $-0.6\text{ }\mu\text{A}$ at -0.51 V . Upon faster rotation speeds, the current decreases dramatically, as a result of the enzyme film instability.

The results shown in Figure 4.7c are for a similar experiment performed at a freshly prepared electrode, with 1 mM of NAD^+ already in solution. The red trace again shows a catalytic wave, with the onset potential of -0.35 V . The magnitude of the reduction current at -0.51 V , a potential close to the value chosen for electrosynthesis later in this chapter (Section 4.5.2), is roughly the same for a stationary electrode at $-0.45\text{ }\mu\text{A}$. This is explained by the observation made in earlier work that the FMN itself becomes dissociated from the protein upon reduction if there is no substrate in solution.^[54,175,196] This means that part of

4. Development of an electroenzymatic NADH recycling method for selective biocatalytic reductions

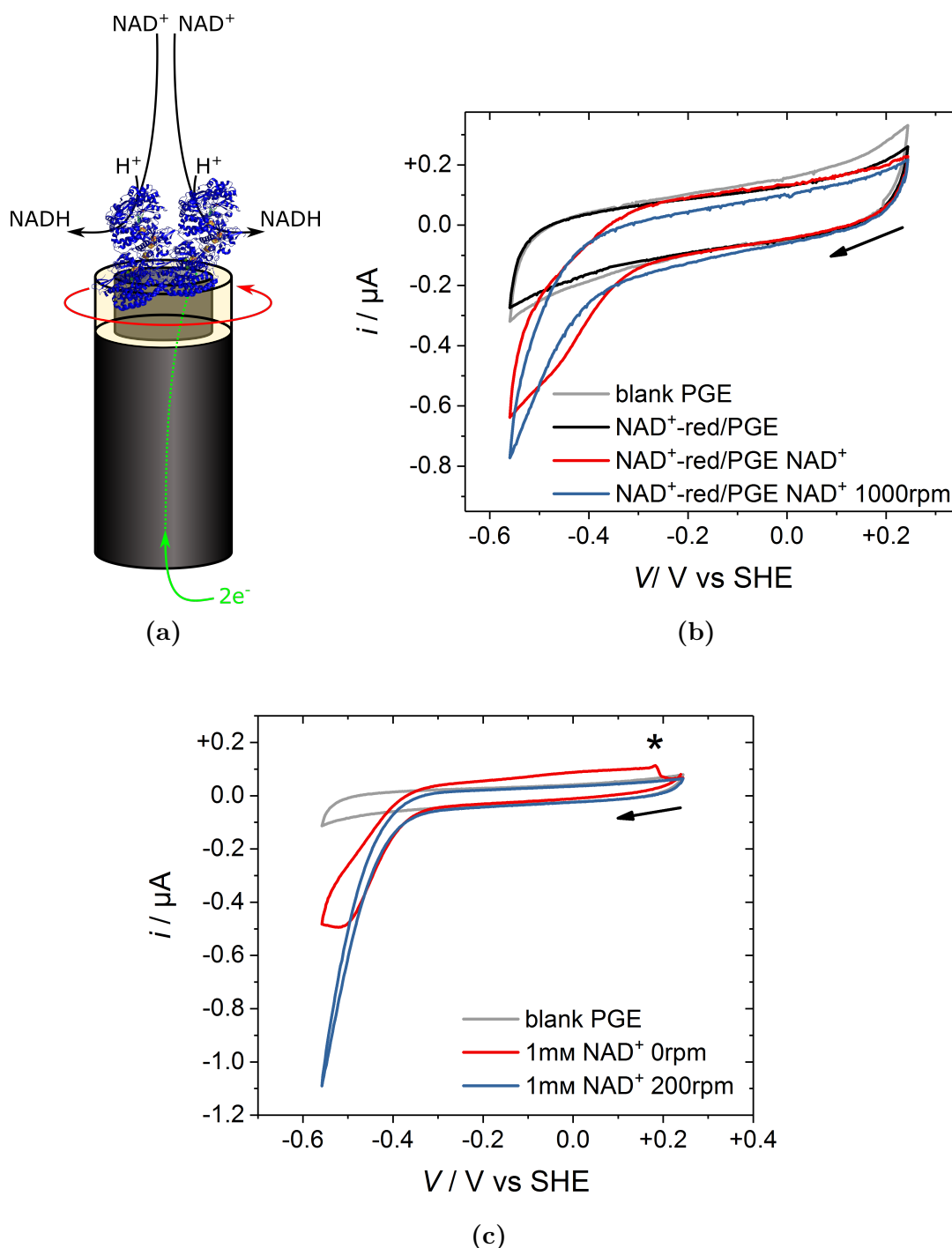


Figure 4.7: Cyclic voltammograms recorded at an NAD⁺-red-modified PGE electrode. (a) Diagram showing the immobilisation of NAD⁺-red enzyme onto a PGE. (b) Dashed grey: freshly polished, unmodified stationary PGE electrode; solid grey: NAD⁺-red-modified PGE electrode (NAD⁺-red/PGE) blank; red: same NAD⁺-red/PGE electrode with 1 mM NAD⁺ in the electrolyte. (c) Red: as red trace in (b); blue: with rotation rate = 200 rpm applied at asterisk. Scan rate = 10 mV s⁻¹; electrolyte: 100 mM Tris-HCl buffer, pH 8.

the protein sample was likely to have lost its FMN in the experiment shown in Figure 4.7b, therefore this situation was avoided in all future experiments.

When the electrode is rotated at 200 rpm (blue trace), convectional flow is induced within the bulk solution, therefore increasing mass transport of substrate, NAD^+ , into the NAD^+ -red catalytic layer. Here, the onset potential remains the same, however the magnitude of the catalytic current at -0.51 V increases to $-1.05\text{ }\mu\text{A}$. There is also a change in the shape of the reductive wave, from a trace that appears to be limiting to one that appears straight and not reaching a limiting reduction current. This change indicates that this catalytic process was mass transfer limited and this is partially corrected by rotating the electrode.

This section has described how direct electron transfer is observed for non-covalently bound NAD^+ -red on PGE electrodes and the reduction of NAD^+ is possible at small overpotentials when the electrode is modified with NAD^+ -red. The next section describes the development and electrochemistry of NAD^+ -red/BP particles for high surface area electrodes.

4.4 Development of NAD^+ -reducing enzyme modified particle electrodes

The currents achieved with NAD^+ -red-modified PGE electrodes was modest, and in line with previous observations in the research group. These analytical electrodes are useful for characterisation, but for electrosynthesis, a high amount of catalyst per geometrical area is desirable. This section describes the use of carbon black nanoparticles as an enzyme support and as a high surface electrode to improve catalytic current outputs.

4.4.1 Carbon particles as an enzyme support

Reeve *et al.*^[52] have shown that carbon black, in particular BP2000, is a suitable material for immobilisation of the NAD^+ -red enzyme, for work in heterogeneous catalysis. Figure 4.8a shows the immobilisation of the NAD^+ -red onto the BP carbon black nanoparticle and Figure 4.8b these NAD^+ -red particles drop cast on a

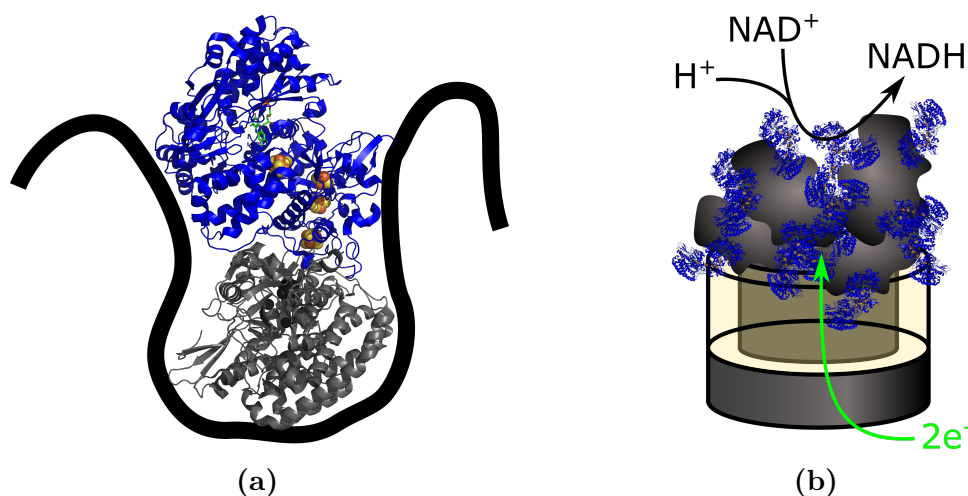


Figure 4.8: (a) Ribbon representation of the [NiFe]-soluble hydrogenase from *R. eutropha* immobilised within a pore of a BP carbon nanoparticle. The knocked-out (inactive) hydrogenase module is shown in grey. (b) Diagram of NAD⁺-red immobilised onto BP nanoparticles, drop cast on the surface of a PGE electrode.

PGE electrode, NAD⁺-red/BP/PGE. The non-covalent attachment of the enzyme to the carbon surface is spontaneous, similar to the PGE, and kinetically stable under aqueous conditions. Serendipitously, the average pore size of BP is roughly the same for that of the NAD⁺-red, creating a three-dimensional environment of intermolecular interactions between the BP and the amino acids in the enzyme protein chain. The shorthand for describing immobilised NAD⁺-red on BP carbon black nanoparticles is ‘NAD⁺-red/BP’.

In the cases described in this Thesis, these NAD⁺-red/BP nanoparticles can be supported on carbon electrodes to increase electrode surface area without increasing the geometrical surface area. This provides a useful platform to ensure as much of the NAD⁺-red immobilises onto a conductive carbon surface as possible, in order to maximise the catalytic current for NAD⁺ reduction.

4.4.2 Electrochemistry of enzyme-modified carbon particles

Particles prepared by following the protocol in Section 2.10.3 on page 67 were then tested for their electrochemical activity. A 0.5 μ L aliquot of the NAD⁺-red/BP suspension was drop cast onto an PGE RDE. The particles on the electrode were

allowed to dry partially. The PGE RDE was then immediately put on an RDE rotor and inserted into an electrochemical cell containing 100 mM Tris-HCl buffer at pH 8.

The traces in Figure 4.9a shows cyclic voltammograms recorded at a PGE working electrode modified with NAD^+ -red (dashed) or NAD^+ -red/BP particles (solid). The grey traces indicate a blank experiment recorded at a these electrodes with no cofactor in solution. Immediately obvious is the vast increase in the discharge capacitance of the electrode as compared to the unmodified PGE electrode in Figure 4.7 on page 107, from roughly 10 μF to 200 μF , a 20-fold increase. Assuming the PGE electrode has similar electrostatic properties to the BP particles, this indicates the electrochemically active surface area of the electrode has increased 20-fold.

The solid red trace in Figure 4.9a indicates a modest catalytic current of $-4 \mu\text{A}$ at a stationary electrode. Figure 4.9b shows the effect of increasing rotation rate of the same NAD^+ -red/BP/PGE electrode. The red trace shows the catalytic wave for NAD^+ reduction being very much limited by mass transport. Rotating the electrode increases the size of the current response at the NAD^+ -red/BP/PGE electrode. Increasing the rotation rate up to about 3000 rpm maximises the catalytic activity of the electrode to $-15.0 \mu\text{A}$, a 275% enhancement of the reductive current relative to the stationary electrode. This shows that the NAD^+ -red/BP/PGE electrode is highly dependent on having good mass transport through the BP particle network. This is a typical current-rotation rate response observed at similarly manufactured electrodes, with reduction currents at -0.51 V within $2 \mu\text{A}$ of each other.

Compared to the NAD^+ -red/PGE electrode, it is observed that at the maximum current achieved with rotating the NAD^+ -red/BP/PGE electrode has a 14-fold catalytic enhancement at -0.51 V to $-15.0 \mu\text{A}$ from $-1.05 \mu\text{A}$ at the NAD^+ -red/PGE electrode. This is mainly in part due to being able to pack more enzyme onto the surface of the electrode as a result of the BP particles. Table 5.4 compares the electrochemical characteristics of the two PGE electrodes, with and without BP particles as a NAD^+ -red support.

It is observed that the BP particle electrodes are more limited by mass transport than the ideal PGE electrodes (the NAD^+ -red/PGE electrode, Figure 4.7c, only

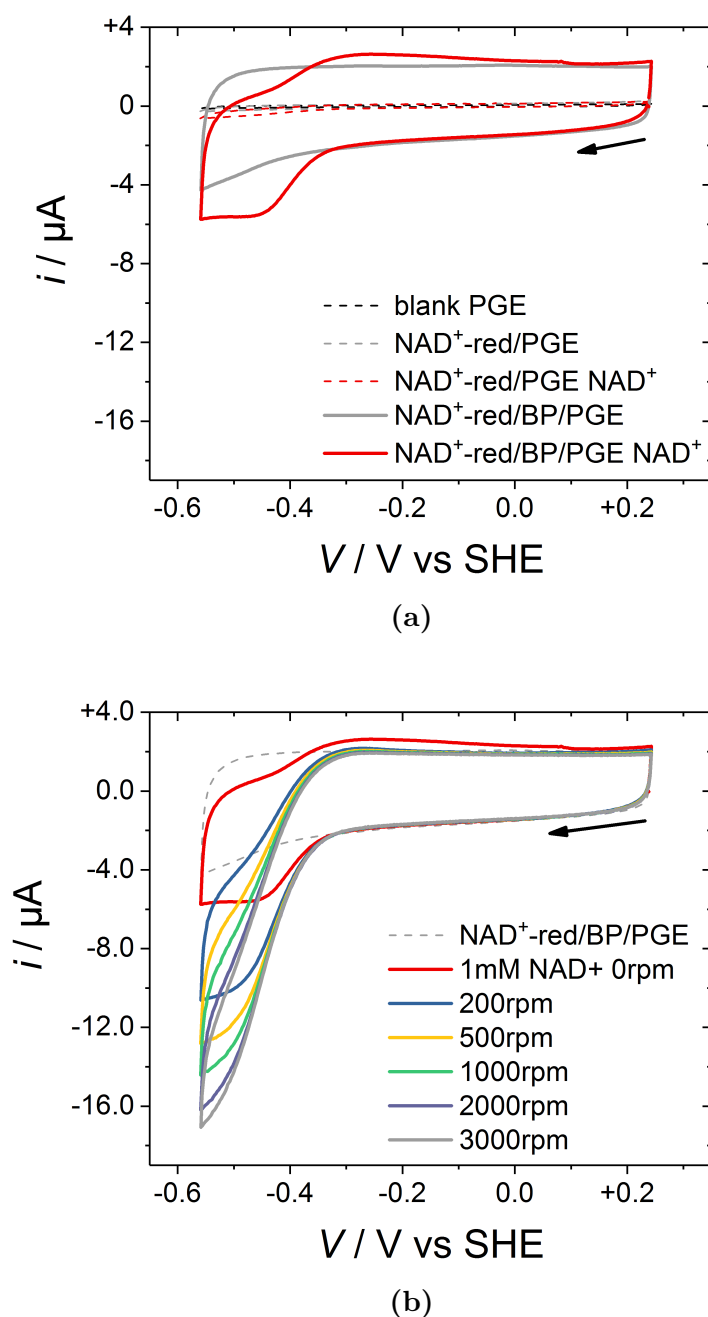


Figure 4.9: Cyclic voltammograms recorded at an $\text{NAD}^+\text{-red/BP/PGE}$ electrode. **(a)** Compares the stationary electrode cyclic voltammetry response to that of an electrode without BP particles, $\text{NAD}^+\text{-red/PGE}$. **(b)** The same $\text{NAD}^+\text{-red/BP/PGE}$ electrode as used in **(a)** but varying the rotation rate of the electrode. Similar responses are observed at similarly manufactured electrodes, variation in current responses at 0.51 $\text{V} = \pm 2 \mu\text{A}$. Scan rate = 10 mV s^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

4.4. Development of NAD^+ -reducing enzyme modified particle electrodes

Table 4.1: Table comparing the characteristics of NAD^+ -red/PGE electrodes.

	NAD^+ -red/PGE	NAD^+ -red/BP/PGE
$A_{\text{geometrical}} / \text{cm}^2{}^a$	0.03	0.03
$A_{\text{total}} / \text{cm}^2{}^b$	0.03	88.8
$C_d / \mu\text{F}^c$	10	200
$m_{\text{NAD}^+\text{-red}} / 10^{-3} \text{ mg}^d$	0.7	4.7
$i_{\text{max}} / \mu\text{A}^e$	-1.05	-15.0
$i_{\text{max}}/m_{\text{NAD}^+\text{-red}} / \mu\text{A mg}^{-1}$	-1500.0	-3191.5
$i_{\text{max}}/A_{\text{geometrical}} / \mu\text{A cm}^{-2}$	-35.0	-500.0
$i_{\text{max}}/A_{\text{total}} / \mu\text{A cm}^{-2}$	-35.0	-0.169

^aThe geometrical surface area of the working electrode.

^bThe total surface area of the working electrode assuming the surface area of the BP2000 nanoparticles is $(1332 \pm 22) \text{ m}^2 \text{ g}^{-1}$, from BET analysis.^[76]

^cThe discharge capacitance of the electrode calculated from the blank cyclic voltammogram.

^dMass of NAD^+ -red on the electrode, assuming all the applied enzyme is absorbed onto the carbon surface. For NAD^+ -red/BP/PGE electrodes roughly $\frac{1}{3}$ of the particles from the preparation are used each time.

^eMaximum catalytic current achieved at no mass transport limitation.

needs to be rotated at 200 rpm for the current to become independent of mass transport as compared to 3000 rpm for the NAD^+ /BP/PGE electrode, Figure 4.9b). Further mass transport limitations are likely to occur inside the BP nanoparticle network giving rise to a smaller proportion of immobilised electroactive NAD^+ -red on the BP particles. The values given in Table 5.4 are therefore lower-bound estimates.

The data shown in each column in Table 5.4 are recorded at the same PGE working electrode, therefore the value of $A_{\text{geometrical}}$ is the same for each setup. However when you compare this to the value of the discharge capacitance, C_d , for each electrode, there is a 20-fold increase in C_d for the NAD^+ -red/BP/PGE electrode as compared to the NAD^+ -red/PGE electrode. Assuming C_d is proportional to the electrochemically active surface area, this is an indication of a 20-fold increase in the electrochemically active surface area of the electrode, i.e. A_{total} increases from 0.03 cm^2 for the NAD^+ -red/PGE electrode, to 88.8 cm^2 for the NAD^+ -red/BP/PGE electrode.

Ratios of $i_{\text{max}}/m_{\text{NAD}^+\text{-red}}$ for the NAD^+ -red/PGE electrode are $-1500.0 \mu\text{A mg}^{-1}$ (NAD^+ -red) and for the NAD^+ -red/BP/PGE electrode, $-3191.5 \mu\text{A mg}^{-1}$ (NAD^+ -red). This indicates that twice as much of the enzyme immobilised on the particles is

electrochemically active as the enzyme immobilised on the PGE electrode. Therefore for the same geometrical surface area, more NAD⁺-red enzyme can be immobilised on to the BP/PGE particle electrode as compared to the PGE electrode, generating more current overall when comparing the $i_{\text{max}}/A_{\text{geometrical}}$ values in the table. This means that a greater proportion of the NAD⁺-red is electrochemically active on the NAD⁺-red/BP/PGE electrode, which is indicative of the structure of the BP nanoparticles driving some kind of order of the NAD⁺-red, possibly due to the similar pore sizes to the enzyme itself (see Figure 4.8a). Comparing the values of $i_{\text{max}}/A_{\text{total}}$ for the NAD⁺-red/PGE ($-35.0 \mu\text{A cm}^{-2}$) and the NAD⁺-red/BP/PGE ($-0.169 \mu\text{A cm}^{-2}$) implies that there is much higher proportion of carbon covered by NAD⁺-red on the PGE as compared to the NAD⁺-red/BP/PGE particle electrode.

This section has described the electrochemical response of directly immobilised NAD⁺-red modified electrodes and NAD⁺-red modified carbon black nanoparticles on PGE electrodes. It has highlighted the usefulness of carbon black nanoparticles as a method of increasing the surface area of a carbon electrode, increasing enzyme immobilisation and therefore catalytic activity. It has also highlighted the importance of good mass transport through the catalytic layer of the electrode. The next section discusses using an NADH-dependent oxidoreductase to use the electroenzymatically generated 1,4-NADH for specific C=X reductions.

4.5 Coimmobilising an NADH-dependent enzyme with the NAD⁺-red

There are hundreds of naturally occurring 1,4-NADH dependent enzymes, each each with a unique active site for specific molecular reductions. The cofactor 1,4-NADH supplies the two electrons as a hydride. The coenzyme, 1,4-NADH, associates with with the C=X substrate within the active site of the oxidoreductase. The mechanism of the two electron transfer from the cofactor to the substrate is uncertain, however it is suggested by Scrutton and coworkers that it is possibly a concerted hydride transfer.^[197] Within the active site, the hydride will always add to the substrate in the same orientation, ensuring 100% enantiomeric specificity of the reduced product.

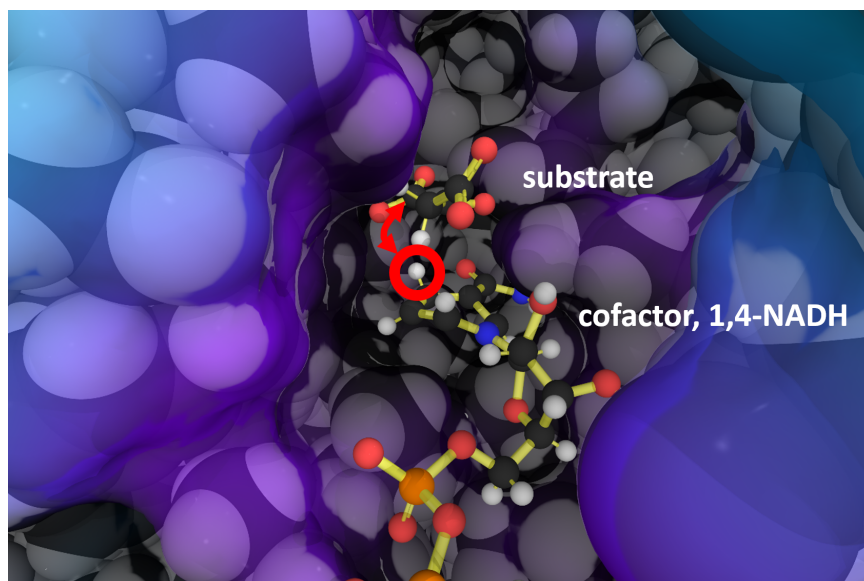


Figure 4.10: Crystal structure of an active site of a 1,4-NADH-dependent L-lactate dehydrogenase, LDH. The molecule underneath is 1,4-NADH and above is the pyruvate substrate. The red circle indicates the labile hydride at the 4-position on the nicotinamide ring. Rabbit muscle LDH in complex with NADH and oxaloacetate crystal structure resolved by Alam *et al.*,^[198] PDB code: 5NQQ.

For example, the crystal structure of the active site of a 1,4-NADH-dependent enzyme, an L-lactate dehydrogenase, is shown in Figure 4.10. The shape of the active site is the main dictator for the orientation of both the substrate (above) and the NADH cofactor (below) associating in the protein. The hydride highlighted is the hydride in the 4-position of the nicotinamide ring, which is orientated towards the co-associated substrate. The two electrons and associated proton add to the underside of the substrate. The second proton then follows, most likely taken from aqueous solution.

The use of these 1,4-NADH-dependent enzymes is fairly desirable for their specificity, in that, for the case of naturally occurring enzymes in nature, they have evolved to be specific in reducing the $\text{C}=\text{X}$ substrate whilst within the active site, controlled by the stereochemistry of the active site. This ensures that only one enantiomeric product emerges from the reduction. These enzymes commonly have very high enantiomeric excesses of $> 99\%$, and therefore can be very useful in stereoselective syntheses.

4.5.1 NADH-dependent enzymes used in this study

Fortunately, there is a large selection of readily available NADH-dependent oxidoreductases, that can be bought from major chemical suppliers, any of which would be suitable as model systems for this electroenzymatic catalyst. The main NADH-dependent oxidoreductases used in the experiments described in this Thesis are:

- L-alanine dehydrogenase, alaDH, and
- L-lactate dehydrogenase, LDH.

These are used as they are known to have high activities and will not be a limiting factor in this electroenzymatic catalytic system. This will also ensure that oxidised cofactor (NAD^+) pool is kept high and keep the NAD^+ -red more active still. Realistically, once there is a supply of biologically active 1,4-NADH, any enzyme that uses the NADH cofactor as a hydride source can be utilised, opening up a wide range of possible molecular substrates this catalyst could work with.

4.5.2 Driving NADH-dependent biocatalysis

In Sections 4.1.2 and 4.4.2 on pages 93 and 109, it has been shown that carbon working electrodes with NAD^+ -red immobilised onto the surface can reduce NAD^+ to NADH at a mild overpotential. This section describes the coupling of the electroenzymatic cofactor regeneration system developed thus far with oxidoreductase enzymes, to drive the 1,4-NADH-dependent reduction of $\text{C}=\text{X}$ containing substrates.

Figure 4.11a shows a diagram of the electrochemical setup for the next experiments described in this section. The NAD^+ -red/alaDH/BP particles are prepared as described in Section 2.10.3 on page 67 and then 0.5 μL of the suspension is drop cast on to the surface of a PGE electrode. With all substrates present in solution, 1 mM NAD^+ , 10 mM of both pyruvate and NH_4Cl , the NAD^+ close to the surface of the electrode is reduced by the NAD^+ -red. This is controlled by convection currents, which in turn are controlled by the rotation rate of the electrode. The reduced cofactor, NADH, at the surface of the electrode can be passed to the alaDH. In the active site of the alaDH, the NADH provides the 2e^- and returns to the surface of

the electrode again as NAD⁺, while pyruvate transforms to L-alanine. The NAD⁺ re-enters the cycle to be reduced back to NADH by the NAD⁺-red.

The next two experiments aim to investigate the effect of both mass transport, adjusted by the rotation rate of the electrode, and also to investigate the current response of the electrode when the alaDH is with and without substrate. The experimental schemes are shown in the subsequent two Figures, 4.11b and 4.11c.

4.5.2.1 Investing the current response at an NAD⁺-red/alaDH/BP/-PGE electrode: rotation rate jumps then pyruvate injections

Figure 4.12 shows the chronoamperometry experiment of a PGE electrode modified with NAD⁺-red and alaDH co-adsorbed onto BP nanoparticles. After being drop cast on the PGE RDE, the particles were dried partially for approximately 60 s and the electrode was immediately put on an RDE rotor and inserted into an electrochemical cell with 100 mM Tris-HCl buffer at pH 8, with 1 mM NAD⁺.

The enzyme alaDH, driven by the hydride carrier 1,4-NADH, reduces pyruvate to L-alanine, a reductive amination, which requires a nitrogen source. Therefore, a stoichiometric amount of 10 mM NH₄Cl was included in the solution. However 0 mM of pyruvate was in the solution at initial conditions.

Figure 4.12a shows the current response of a NAD⁺-red/alaDH/BP/PGE electrode, with the alaDH under non-turnover conditions as no pyruvate substrate is included in the electrolyte solution. The effect of rotation rate of the working electrode on the current response is observed, with 1 mM NAD⁺ in solution. The initial rotation rate of the electrode is 0 rpm giving an initial current of $-2.2 \mu\text{A}$, a similar current compared to the same electrode but without co-immobilised alaDH (Figure 4.9). Here the mass transport is dependent solely on the native diffusion dynamics in the solution. As the rotation of the working electrode is increased to 200 rpm, there is induced convection contributing to the mass transfer of substrates to the electrode from the bulk solution, aiding the supply of substrate to the NAD⁺-red on the electrode and also aiding removal of the products from the catalytic layer of the electrode. Therefore with higher mass transport increasing the effective concentration of NAD⁺ at the surface of the electrode, the NAD⁺-red are more

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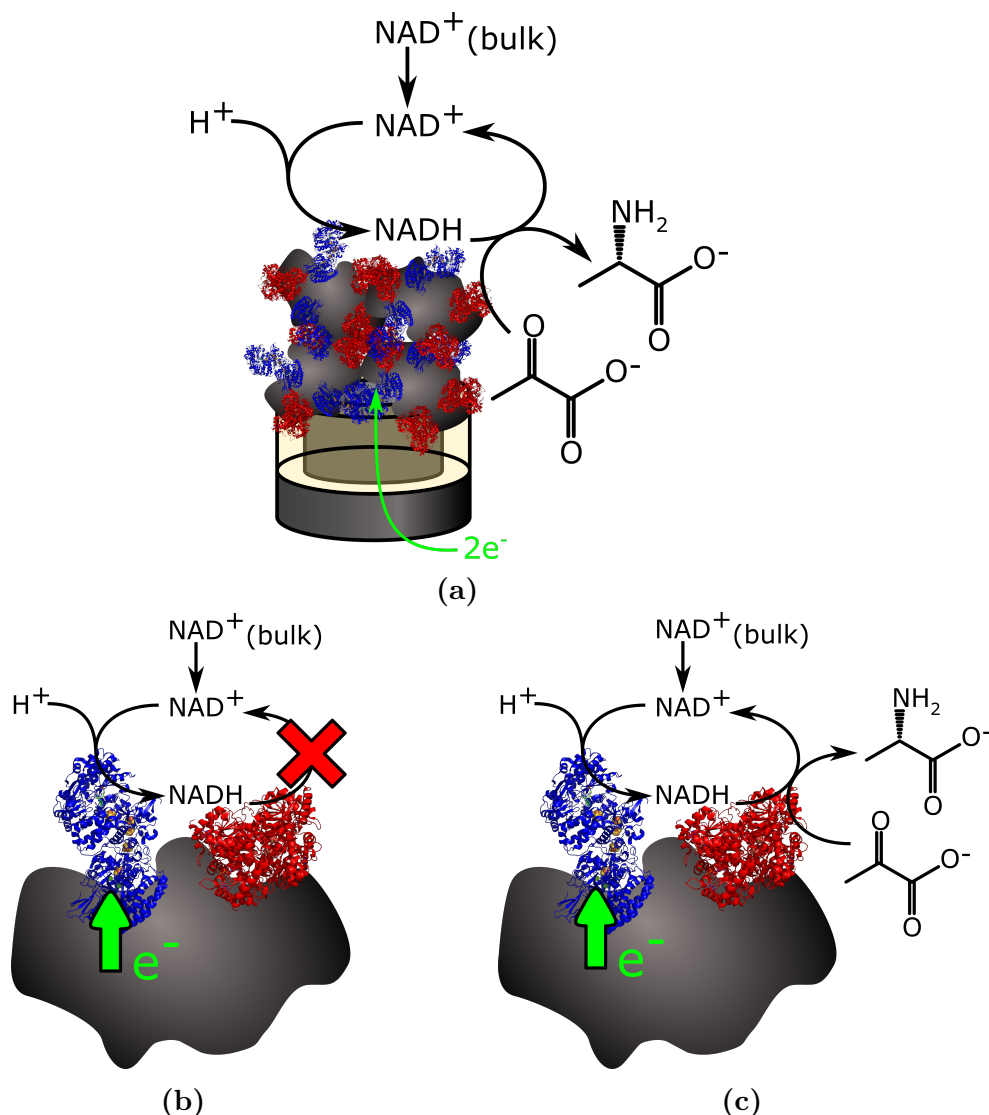


Figure 4.11: Figures of the reactions occurring on a BP electrode modified with NAD⁺-red and alaDH. **(a)** A PGE electrode immobilised with NAD⁺-red/alaDH/BP particles. **(b)** With no alaDH substrate in the bulk solution, there is no oxidation of NADH back to NAD⁺. **(c)** When pyruvate is injected as a substrate for alaDH, the alaDH reduces the pyruvate to L-alanine and oxidises the NADH back to NAD⁺ near the surface of the carbon.

electrochemically active, which contributes to a higher reductive current response. This is observed in Figure 4.12a. With each subsequent step of increasing rotation rate, the current increases to a limiting current near 3000 rpm of $-6.5 \mu\text{A}$.

At the highest rotation rate (maximum mass transport), pyruvate injections, each adding $625 \mu\text{M}$, were added sequentially. At the first injection of pyruvate, the reductive current increases from $-6.3 \mu\text{A}$ to $-8.0 \mu\text{A}$. This can be explained by

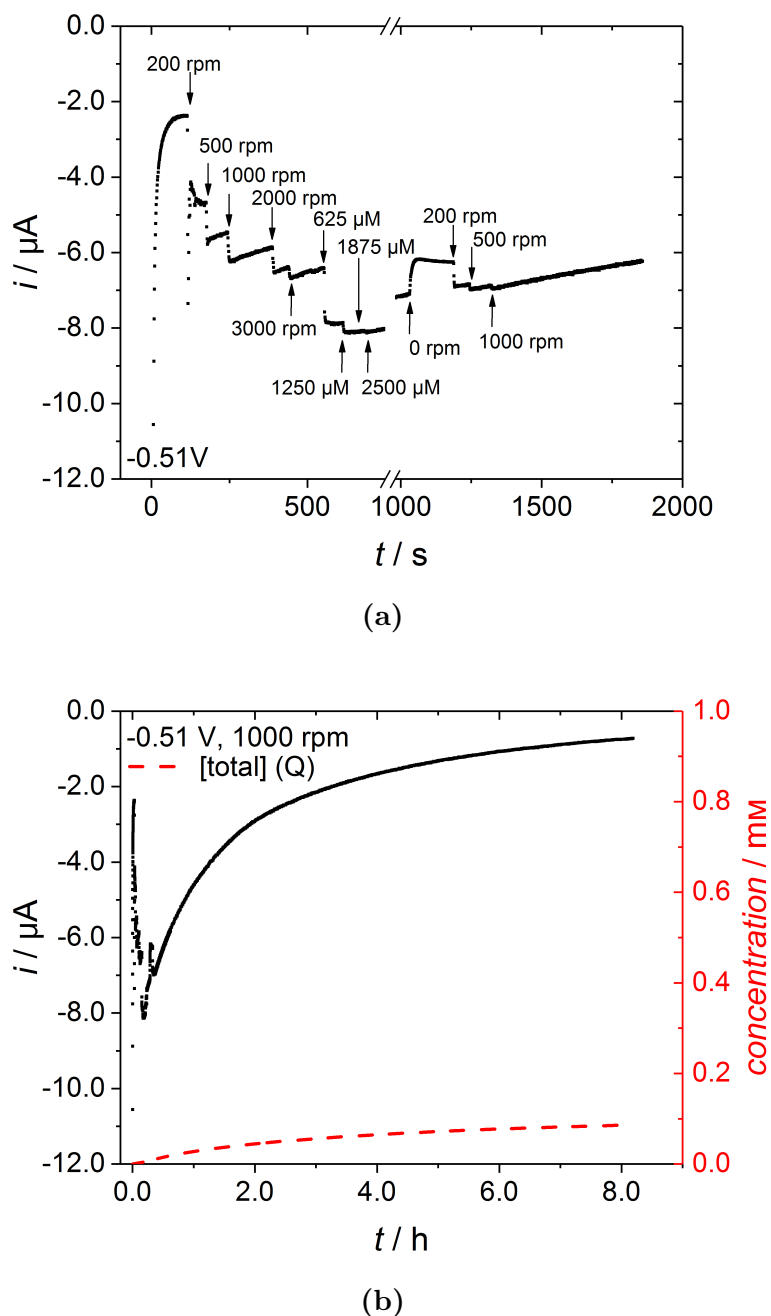


Figure 4.12: Electrochemistry experiments performed at a PGE electrode modified with NAD⁺-red/alaDH/BP particles. **(a)** Chronoamperometry experiment measuring the current response with injection of the pyruvate substrate and with different rotation rates of the electrode. **(b)** Extended-time chronoamperometry experiment with the same electrode, measuring the current response of the electrode over 8 h at 1000 rpm. Red dashed trace shows the predicted concentration of all reduction products generated as a function of the charge passed. WE potential: -0.51 V vs SHE; initial concentrations: NAD⁺ = 1 mM, pyruvate = 0 mM, NH₄Cl = 10 mM; 10 mM of total injected pyruvate is achieved after 1000 s; electrolyte: 100 mM Tris-HCl buffer, pH 8.

an enhanced NAD^+ concentration as a result of the alaDH undergoing catalytic reduction of pyruvate. The 1,4-NADH is used as the cofactor for the alaDH reduction. It is then released from the enzyme in the oxidised state (NAD^+) and should remain near the surface of the carbon. Meanwhile, the maximised mass transport still provides the same concentration of NAD^+ to the surface of the electrode from the bulk solution. Therefore the amount of NAD^+ at the surface of the electrode is provided from two sources: the bulk solution and also from the alaDH. This enhanced concentration of NAD^+ increases the activity of the NAD^+ -red enzyme which is shown by the increase in the magnitude of the reduction current.

This is observed also when the rotation of the electrode is returned to 0 rpm. Comparing the reduction currents for the stationary electrode without pyruvate, at $t = 0$ s, and with 10 mM pyruvate, at 1100 s, the reduction current increases from $-2.2 \mu\text{A}$ to $-6.0 \mu\text{A}$. This increase in the reduction current when the alaDH is operating must be due to the enhanced NAD^+ concentration effect as the alaDH supplies NAD^+ back to the surface of the electrode, increasing the NAD^+ -red activity.

Another significant difference after the injection of pyruvate is that the current is more stable over time. This could be due to an increase in stability of the NAD^+ -red as there is not a build up of NADH now that the NADH-dependent alaDH is operating under turnover conditions with two substrates. Alternatively it could be that any loosely bound enzyme has fallen off the carbon particles by this point in the experiment.

At 1300 s the electrode is rotated again at 1000 rpm, a compromise rotation rate between maximum mass transport of substrates and film stability. The chronoamperometric response is recorded over 8 h and Figure 4.12b shows the continued and complete experiment. The current dissipation over time is attributed to a loss of enzyme from the electrode into the bulk solution.

The red dashed trace in Figure 4.12b shows the predicted concentration of NADH generated over the 8 h experiment, as calculated from the charge passed through the catalyst. It can be seen that 0.1 mM is predicted to have been generated. If it is assumed that all the NADH generated is used by the alaDH

immediately, then this would mean that 0.1 mM of L-alanine has been generated too. More analytical experiments with long time electrosynthesis experiments to quantify and confirm the concentrations of products are performed with more robust systems in Chapter 5 on page 137.

4.5.2.2 Investing the current response at an NAD⁺-red/alaDH/BP/-PGE electrode: pyruvate injections then rotation rate jumps

A second chronoamperometry experiment was performed with a similar electrode, this time first investigating the effect of substrate injections under a mass transport limiting condition, then secondly increasing the rotation rate. This is shown in Figure 4.13. The electrode starts with a rotation of 0 rpm and 0 mM pyruvate, giving rise to a current response of $-1.8 \mu\text{A}$ due to NAD⁺ reduction, a similar value as observed at the start of the chronoamperometry experiment shown in Figure 4.12a. When convection is induced by increasing the rotation rate to 300 rpm, the current jumps to $-6.0 \mu\text{A}$ consistent with improved mass transport to the catalytic layer. After applying 300 rpm, there is a slow increase of reductive current, which may be due to increased ‘wetting’ of the catalytic layer, allowing the NAD⁺ to reach more NAD⁺-red enzymes further inside the catalytic layer.

The current is stabilised with subsequent injections of the alaDH substrate, pyruvate. Upon these injections, each adding $635 \mu\text{M}$, there are only small increases in the reductive current. It has been shown that this electrode does respond to injections of pyruvate in Figure 4.12. Therefore during rotation at 300 rpm, it appears that pyruvate supply, or alanine removal is strongly mass transport limited.

The rotation rate of the electrode is then increased stepwise from 300 rpm at $t = 310 \text{ s}$ to 500 rpm, from which the current increases from $-6.5 \mu\text{A}$ to $-7.8 \mu\text{A}$. The subsequent increases in the electrode rotation rate produce similarly sized increases in the magnitude of the reductive current. The response in current to the rotation rate is larger than what is observed in Figure 4.12a. This could be because better mass transport of NAD⁺ and pyruvate from the bulk solution to the surface become critical when pyruvate is available at higher concentrations. The increase in NAD⁺ at the surface increases the catalytic rate of the NAD⁺-red and

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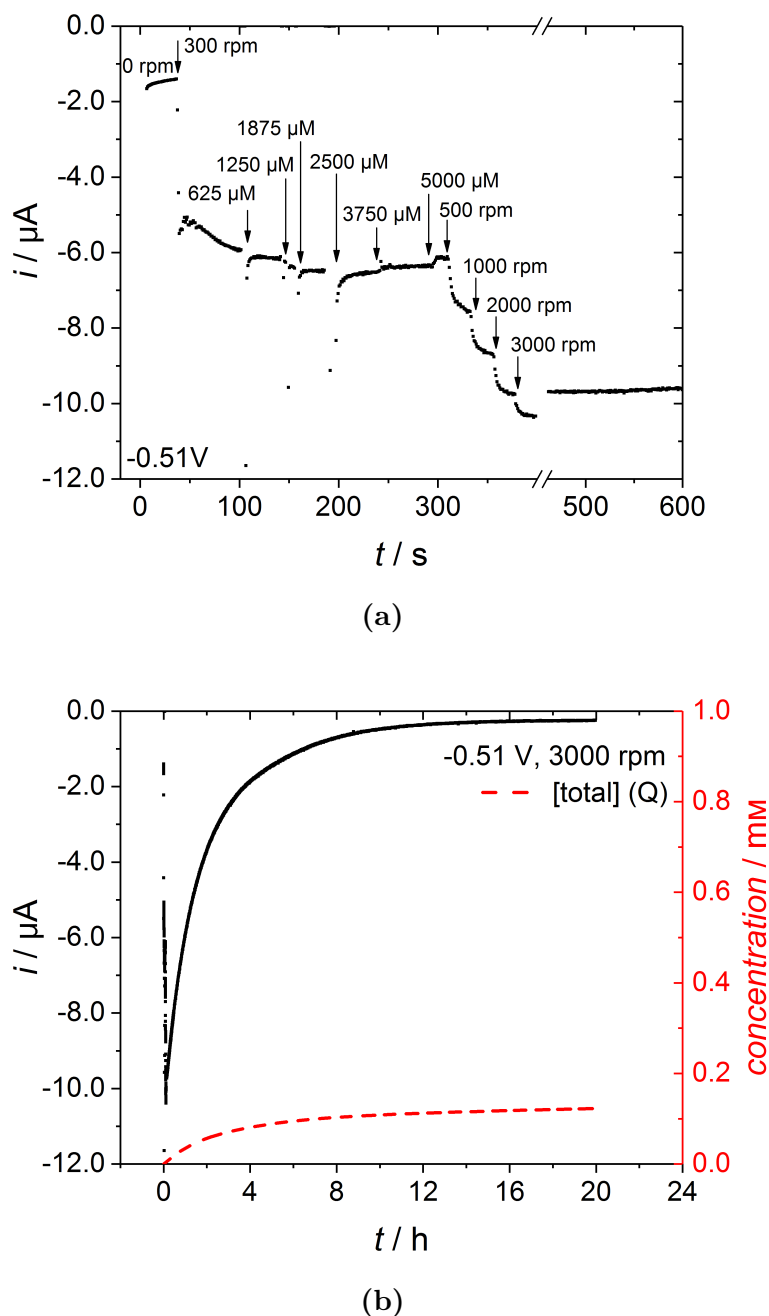


Figure 4.13: Electrochemistry experiments performed at a PGE modified with NAD⁺-red/alaDH/BP particles. **(a)** Chronoamperometry experiment measuring the current response with different rotation rates of the electrode and with injection of the pyruvate substrate. **(b)** Extended-time chronoamperometry experiment with the same electrode, measuring the current response of the electrode over 20 h. Red dashed trace shows the predicted concentration of all reduction products generated as a function of the charge passed. WE potential: -0.51 V vs SHE; initial concentrations: NAD⁺ = 1 mM, pyruvate = 0 mM, NH₄Cl = 10 mM; 10 mM of pyruvate is achieved after 1000 s; electrolyte: 100 mM Tris-HCl buffer, pH 8.

the increase of pyruvate to the surface of the electrode would increase the rate at which alaDH would reoxidise NADH to NAD⁺ at the surface of the electrode, increasing the effective concentration further still. Additionally, with increase in the rotation rate, there is improvement in the removal of the product, L-alanine, driving the alaDH reaction equilibrium further.

The maximum current reached at 1 mM NAD⁺, 10 mM pyruvate and NH₄Cl and at 3000 rpm for the working electrode is $-10\ \mu\text{A}$. The current response appears to be stabilised and not significantly increasing or decreasing in magnitude over a timescale of 100 s. This is indicative of the catalytic layer reaching an equilibrium saturation for the catalytic layer for both the NAD⁺ and pyruvate (and NH₄Cl) substrates.

Chronoamperometry was performed over 24 h using this electrode at a potential of $-0.51\ \text{V}$, with the current response over this time period shown in Figure 4.13b. The red dashed trace shows the total predicted conversion of NAD⁺ to NADH and pyruvate to alanine, as calculated from the charge transferred through the working electrode. Over 24 h, it is predicted that 0.15 mM of NADH and alanine has been generated by the electrode. If it is assumed that all the generated NADH is immediately used by the alaDH for pyruvate reduction, it can be calculated that 0.15 mM of pyruvate has been converted to alanine. Although the length of this 24 h experiment is 3-times the length of the similar experiment in Figure 4.12b, the total concentration of NADH and alanine to have been produced is only 0.5-times that in Figure 4.12b. This indicates that after about 10 h of electrosynthesis with this electrode, the current dissipates so much that there is limited conversion of NAD⁺ and pyruvate after this time. This result indicates that the catalytic film on the electrode in this experiment is in fact less stable than the previous one. This is indicative of the different stabilities of different electrode films and the difficulty in direct comparisons between electrodes.

In this section, it has been shown how a NAD⁺-red/alaDH/BP/PGE electrode responds to injections of pyruvate and with increasing the rotation rate of the electrode. Current increases with increased pyruvate concentration as a result of the surface NAD⁺ concentration enhancement effect after oxidation within the alaDH. There

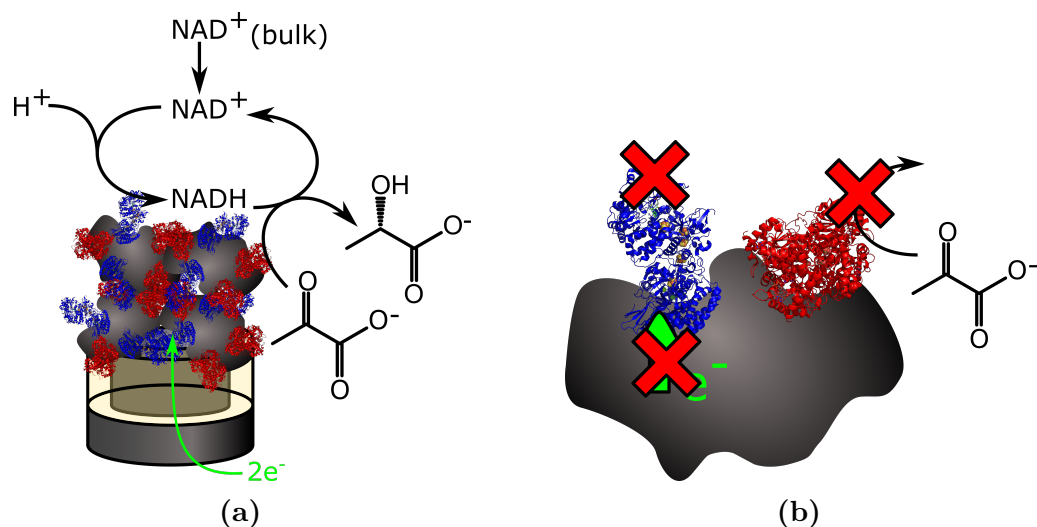


Figure 4.14: Figures of the reactions occurring on a BP electrode modified with NAD⁺-red and LDH. **(a)** A PGE electrode immobilised with NAD⁺-red/BP particles. **(b)** The NAD-red immobilised on BP nanoparticles reduces NAD⁺-red to NADH. With no NAD⁺ substrate in the bulk solution, there is no NADH of pyruvate to L-lactate.

is not much of an increase in the reductive current as the concentration of pyruvate increases beyond the concentration of the reduced cofactor NAD⁺. A high rotation rate of about 3000 rpm is required to maximise mass transport of the substrates to the catalytic layer. The next subsection discusses the reductive current response with increasing concentration of NAD⁺ for a composite NAD⁺-red/LDH/BP/PGE electrode, along with some kinetic analysis of the NAD⁺-red enzyme.

4.5.2.3 Investigating the NAD⁺-red enzyme kinetics when coimmobilised and cooperational with an oxidoreductase

Next, an experiment was designed to measure an ‘apparent’ or ‘operational’ K_M for the NAD⁺-red enzyme on BP particles, whilst in an environment of an LDH enzyme maintaining a high concentration of NAD⁺ at the surface of the electrode. Figure 4.14 shows the experiment performed with a NAD⁺-red/LDH/BP/PGE electrode. The setup is similar than that shown in Figure 4.11, except an LDH, with a higher k_{cat} than the alaDH, is used.

In this experiment, a NAD⁺-red/LDH/BP/PGE electrode (LDH = L-lactate dehydrogenase) was prepared in a similar fashion to the NAD⁺-red/alaDH/BP/PGE

electrode, as described in Section 2.10.3 on page 67. An experiment was performed to investigate the reductive current response when limiting and then increasing the concentration of NAD⁺ solution. LDH was chosen here as it has very fast turnover frequency as compared to the NAD⁺-red, so it should not limit the current itself. The substrate for LDH, pyruvate, was at 0 mM as part of the initial conditions. No NH₄Cl was required as it is not a reductive amination enzyme, rather pyruvate is reduced to lactate and no nitrogen source is required.

Figure 4.15a shows the reductive current response with injections of 125 μ M NAD⁺. The potential of the NAD⁺-red/LDH/BP/PGE electrode was held at -0.51 V and the electrode is rotating at 300 rpm. This rotation rate was a balance between good mass transport and good stability. A small amount of NAD⁺ cofactor, 25 μ M, is added immediately to protect the flavin in NAD⁺-red, to avoid dissociation of the FMN from the NAD⁺-red.^[54,175,196] The increase in reductive current is due to the NAD⁺-red beginning the turnover of NAD⁺ to NADH. 10 mM of the LDH substrate, pyruvate, is then injected into the solution. The reductive current enhancement here is due to the enhanced NAD⁺ concentration effect and the effect of driving the NAD⁺/NADH equilibrium forward, as observed in the previous two sections.

As significant amounts of NAD⁺ are added to the electrolyte solution, 125 μ M of NAD⁺ each injection, the reductive current increases in magnitude. This is because the NAD⁺-red enzyme is more active with each increase in concentration of NAD⁺. Therefore the number of moles of electrons passing from the carbon electrode to the NAD⁺-red enzymes is increasing, which increases the reductive current of the electrode.

Following an NAD⁺ injection to take the concentration to 1025 μ M, the current reaches a maximum limit. However, this may not indicate that all of the electroactive NAD⁺-red enzyme is operating at maximum turnover conditions, as there are possibly mass transport limitations inside the nanoparticle network on the electrode. However, the rotation rate was not increased to test this in order to avoid significant instability and loss of reduction current.

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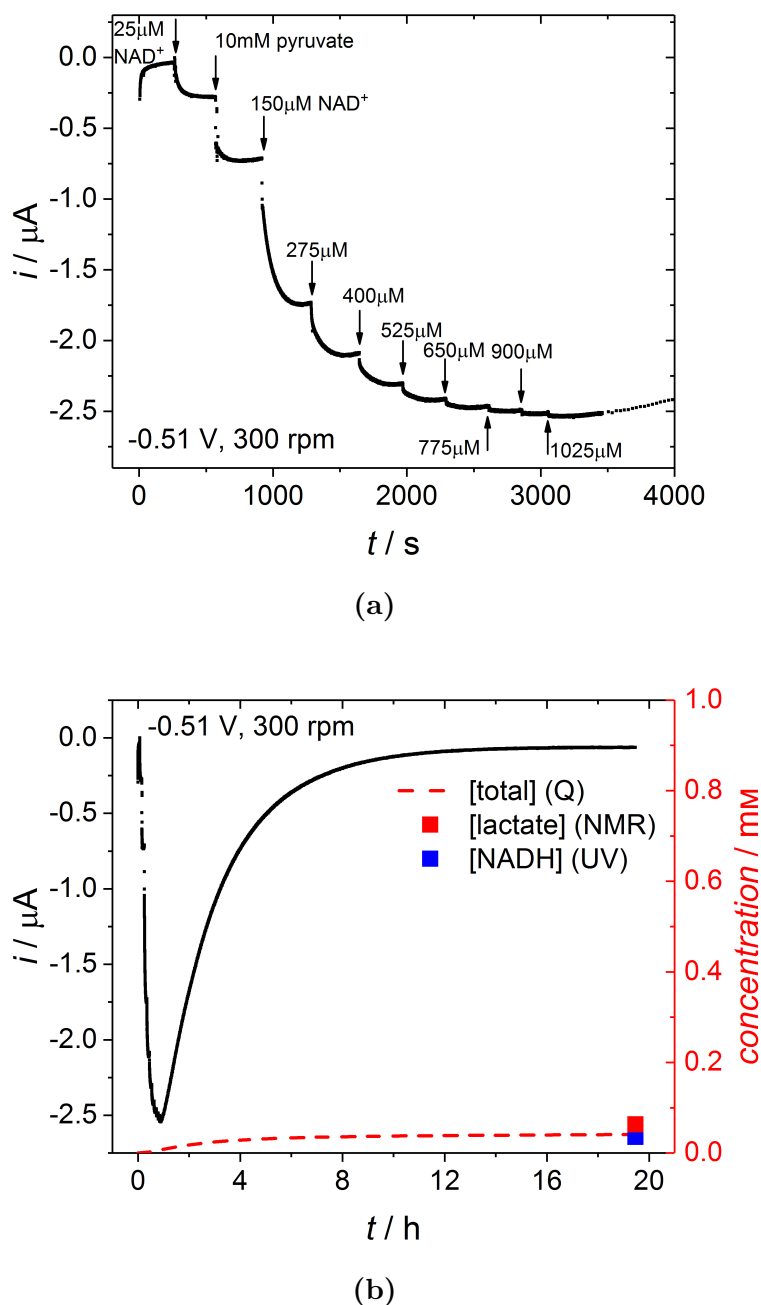
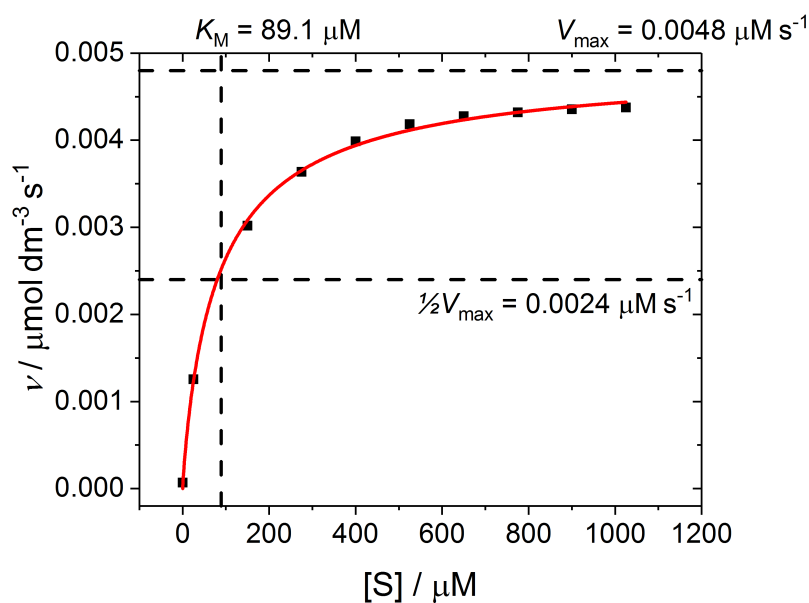
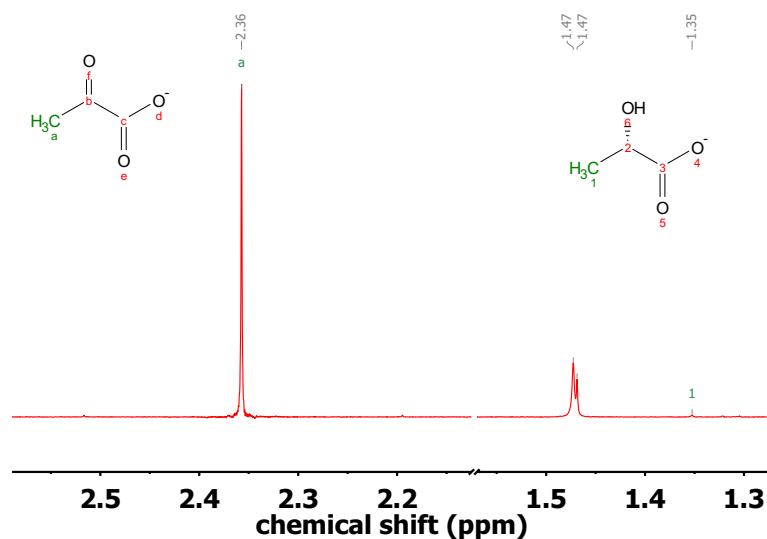


Figure 4.15: Concentration assay and related chronoamperometry for a NAD⁺-red/LDH/BP/PGE electrode. **(a)** NAD⁺ concentration injections performed in the presence of 10 mM and rotating at 300 rpm. **(b)** Chronoamperometry (electrosynthesis) at the same electrode. Red dashed trace shows the predicted concentration of all reduction products generated by the electrocatalyst by charge passed. Blue dot shows final concentration of NADH in the solution determined by UV-Vis spectroscopy, the red dot shows the final concentration of lactate in the solution determined by ¹H NMR. (cont.)



(c)



(d)

Figure 4.15: (cont.) Concentration assay and related chronoamperometry for a NAD^+ -red/LDH/BP/PGE electrode. (c) Minchaelis-Menten reactivity plot for the electrode. (d) ^1H NMR spectrum in the product region measured from sample taken at the end of the chronoamperometry experiment in Figure 4.15b. WE potential: -0.51 V vs SHE; substrate: NAD^+ injections to 1 mM , 10 mM pyruvate; electrolyte: 100 mM Tris-HCl buffer, $\text{pH } 8$; 300 rpm rotation rate.

The electrode was used to perform long term electrosynthesis over 20 h. The red dashed trace shows the predicted total concentration of reduced species, i.e. NADH and lactate, generated at the NAD⁺-red/LDH/BP/PGE electrode, which is calculated from the charge passed, Q , of about 0.04 mM. The blue point shows the actual amount of NADH detected after 20 h, measured using UV-Vis spectroscopy to be about 0.04 mM. The presence of any of the reduced product, lactate was also measured using ¹H NMR spectroscopy (a more in depth discussion on the use of ¹H NMR spectroscopy for product detection is later in Section 5.1.4 on page 146). A lactate peak (Figure 4.15d, peak ‘1’), of chemical shift 1.35 indicates that there is some lactate present and that the bioelectrocatalyst has been successful at reducing pyruvate to lactate, albeit at very small quantities (0.06 mM). The development of systems with higher final product concentrations in Chapter 5 on page 137 have more reliable product analyses.

A good kinetic interpretation of the NAD⁺-red enzyme was possible from the NAD⁺ injection chronoamperometry experiment in Figure 4.15a. Figures 4.15c is a visual interpretation of *Michaelis-Menten* kinetics, a model for enzyme kinetics. The Michaelis-Menten relation between reaction rate, ν , at an ideal electrode, with substrate concentration of the NAD⁺-red enzyme, $[S]$, the concentration of NAD⁺, is shown in Equation (4.2).

$$\nu = \frac{d[P]}{dt} = \frac{V_{\max}[S]}{K_M + [S]} \quad (4.2)$$

The rate of reaction against substrate concentration is plotted in Figure 4.15c, where the rate and therefore activity is proportional to half the current of the electrode at each NAD⁺ concentration. The fit of a hyperbolic function to this plot has an R^2 value of 0.999, so there is good fitting to the Michaelis-Menten model. The upper asymptote is taken as the maximum possible ‘operational rate’ of the enzyme, $V_{\max}$. The x -value at which this plot crosses the y -value of $1/2V_{\max}$ is equal to the Michaelis-Menten equilibrium constant, K_M . The kinetic data for this electrode setup is shown in Table 4.2

Table 4.2: Operational Michaelis-Menten kinetic data for NAD⁺-red in the NAD⁺-red/LDH/BP/PGE electrode.

Catalytic variable	Value
‘Operational’ $V_{\max}$	$4.8 \times 10^{-3} \mu\text{M s}^{-1}$
‘Operational’ K_M	$89.1 \mu\text{M NAD}^+$
‘Operational’ k_{cat}	0.24 s^{-1}

Table 4.3: K_M values for the NAD⁺-red enzyme with NAD⁺ substrate.

Source of K_M	$K_M / \mu\text{M}$
True value ^a	197 ± 28
‘Operational’ value ^b	89.1

^aFrom Lauterbach *et al.*^[54]

^bAs a part of the NAD⁺-red/LDH/BP/PGE electrode in this study.

The value of K_M is not a true value of the Michaelis-Menten constant for the NAD⁺-red enzyme, rather it is the Michaelis-Menten constant for the NAD⁺-red enzyme when a part of the NAD⁺-red/LDH/BP/PGE electrode and in the presence of the C=X substrate. Therefore it is more appropriate to call this an ‘operational’ K_M . Table 4.3 compares this determined ‘operational’ K_M to the true value determined in previous studies,^[54] the value determined here is smaller in magnitude at $89.1 \mu\text{M}$ rather than the true value of $(197 \pm 28) \mu\text{M}$.

This experiment has shown that when the NAD⁺-red is immobilised as part of a NAD⁺-red/LDH/BP/PGE electrode, the ‘apparent’ K_M of 89.1 is roughly half that for the true value. This is indicative of a larger binding affinity of NAD⁺ for the NAD⁺-red enzyme in this electrode setup. This may be due to the complex NAD⁺ concentration enhancement effect where the LDH is constantly removing NADH and converting it back to NAD⁺ to provide an enhanced concentration at the surface of the electrode between the enzymes. Therefore less NAD⁺ is needed to be injected to the bulk solution to have the same activity as the NAD⁺-red without the LDH.

The value of an ‘operational’ k_{cat} can also be determined with this experiment, if the amount of initial enzyme is assumed to be equal to the amount immobilised on the carbon nanoparticles. The value of ‘operational’ k_{cat} is calculated as shown

in Equation (4.3).

$$k_{\text{cat}} = \frac{V_{\text{max}}}{[\text{E}]_0} \quad (4.3)$$

The value of this ‘operational’ k_{cat} , a turnover frequency, calculated from the Michaelis-Menten model is calculated to be 0.24 s^{-1} . This is probably a lower-bound estimate however because of the assumptions made. The amount of enzyme exposed to the BP particles for the immobilisation step is known, but all the enzyme may not be immobilising onto the electrode in a way that is electroactive. In addition to this, not all the assumed immobilised will have sufficient mass transport. However, this is a reasonable gauge on the operational rate constant for the reduction of pyruvate to lactate at this electrode under these conditions.

This section has discussed the electrochemistry of two different BP particle electrodes, one with alaDH co-immobilised with NAD^+ -red, the NAD^+ -red/alaDH/BP/-PGE electrode, and the other with LDH co-immobilised with NAD^+ -red, the NAD^+ -red/LDH/BP/PGE electrode. Chronoamperometry experiments were performed to measure the impact of substrate injections with the catalytic reductive current response, and also the rotation rate in the case of the NAD^+ -red/alaDH/BP/PGE electrode. Preliminary results indicate that there is NADH being produced by the NAD^+ -red enzyme and that it is being used by the alaDH and the LDH. The next stages in electrocatalyst development require an improvement in the catalytic current to produce reasonable quantities for product analyses. The next two sections show the effect of pH (Section 4.6.1) and covalent immobilisation (Section 4.6.2) on the catalytic activity of the NAD^+ -red enzyme on BP nanoparticles.

4.6 Conditions affecting the magnitude of catalytic activity of the NAD^+ -red enzyme

4.6.1 Comparing the effect of pH on NAD^+ -red on BP particle electrodes

In this section, NAD^+ -red immobilised on BP carbon black nanoparticles are produced and electrochemistry experiments are performed with an electrolyte solution

of pH 6 BIS-TRIS-HCl buffer. The availability of more protons in the bulk solution should change the redox potential of the NAD^+/NADH couple, as described by the Nernst equation (see Section 2.1.3 on page 41).

Figure 4.16 shows the electrochemistry experiments performed at a NAD^+ -red/BP/PGE electrode with a bulk electrolyte BIS-TRIS-HCl buffer at pH 6. First, cyclic voltammetry experiments were performed at different rotation rates (Figure 4.16a). The red trace shows the voltammetric response with 1 mM of NAD^+ in solution. As before with the experiments performed at pH 8 (Section 4.4.2 on 109), the onset potential is -0.35 V vs SHE. At 0 rpm, the current reached at pH 6 is roughly $-7.0\text{ }\mu\text{A}$ at -0.51 V , compared to the $-4.0\text{ }\mu\text{A}$ achieved at a similar NAD^+ -red/BP/PGE electrode at pH 8 at a similar potential. Although it might seem at first like an improvement in the reduction current, it is difficult to compare particle electrodes between different experiments, as the mass of particles, the diameter of particles and the exact amount of enzyme immobilised in an active state on the surface of the nanoparticles cannot necessarily be assumed to be the same.

When the electrode rotation is increased stepwise from 0 rpm to 3000 rpm, the current increases from $-7.0\text{ }\mu\text{A}$ to $-13.0\text{ }\mu\text{A}$, a current enhancement of 86%. However at this rotation rate with the similar NAD^+ -red/BP/PGE electrode at pH 8, the current enhancement due to rotation is 275%, from $-4.0\text{ }\mu\text{A}$ to $-15.0\text{ }\mu\text{A}$. Therefore because of the large enhancement in the current and larger magnitude of i_{max} achieved with high mass transport at pH 8, it seems that working at pH 8 conditions is more preferable than working at pH 6.

The effect of pH on the discharge capacitance, C_d , of the NAD^+ -red/BP/PGE electrode has little effect. At pH 6, the electrode appears to have a value of $200\text{ }\mu\text{F}$, which is the same value that is observed with the similar experiment performed at pH 8.

This electrode was then used immediately for a chronoamperometry experiment. The red dashed trace shows the predicted concentration of NADH being generated as a function of time and calculated from the charge passed through the working electrode. After 24 h, it is predicted that 0.15 mM of NADH has been generated by

4. Development of an electroenzymatic NADH recycling method for selective biocatalytic reductions

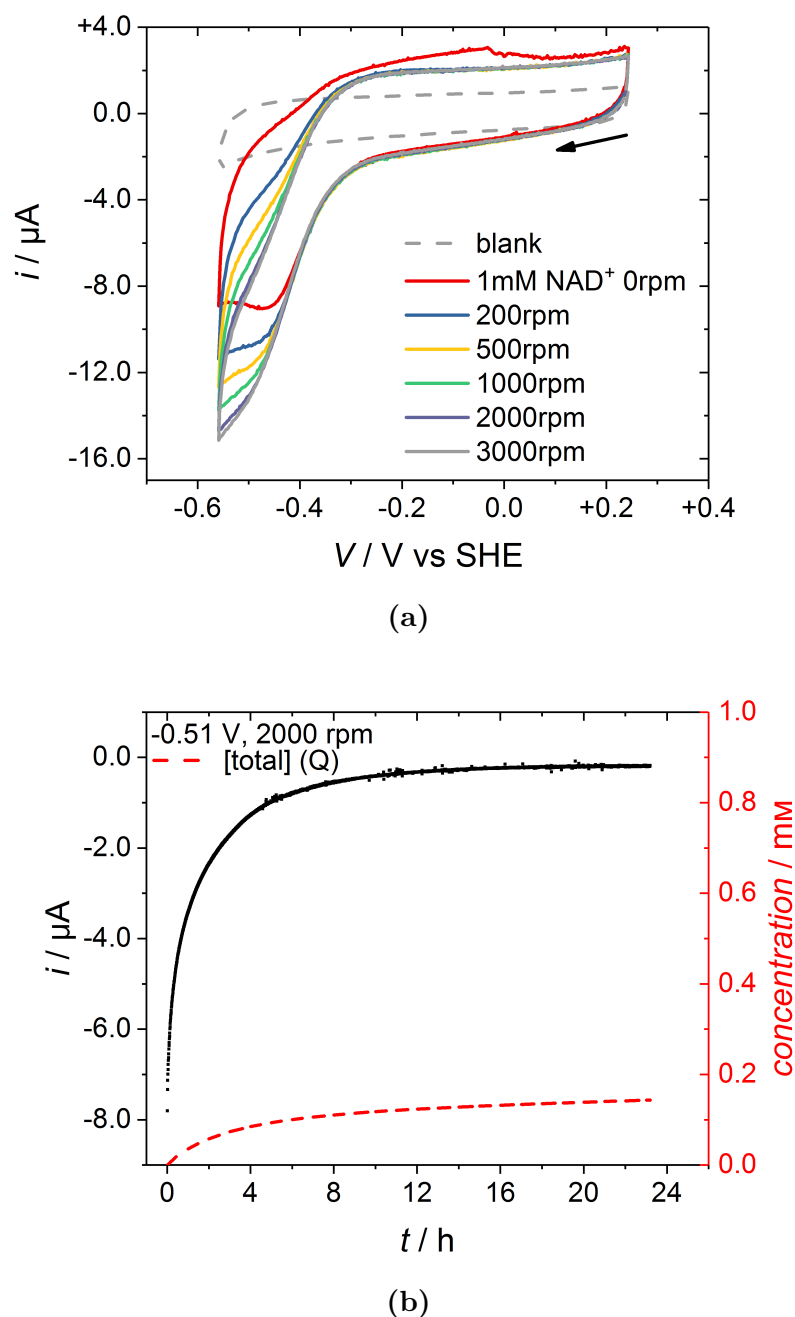


Figure 4.16: Electrochemistry experiments recorded at an NAD^+ -red/BP/PGE electrode at pH 6. **(a)** Cyclic voltammograms showing the effect of WE rotation rate, compared to a blank BP/PGE electrode (grey); scan rate: 0.01 V s^{-1} . **(b)** Extended time chronoamperometry recorded at the same electrode at a WE potential of -0.51 V over 24 h. Red dashed line: total reduction products as calculated from the total charge passed. Substrate: 1 mM NAD^+ ; electrolyte: 100 mM BIS-TRIS-HCl, pH 6.

the electrode under pH 6 conditions. This is a similar value to the concentration predicted by charge passed through NAD^+ -based electrodes at pH 8, if not a little higher. However, not all electrodes are directly comparable and the difference in concentration values are so small, this could be due to a small or random difference.

A similar chronoamperometry experiment was performed with an NAD^+ -red/-alaDH/BP/PGE electrode. Figure 4.17 shows the current response when this electrode is held at a potential of -0.51 V vs SHE. The red dashed trace shows the predicted total concentration of reduction products (NADH and alanine) being generated, calculated from the charge passed through the electrode. After 21 h, the concentration of the reduction products is predicted to be 0.2 mM . This is confirmed with ^1H NMR spectroscopy, which indicates a concentration of lactate of 0.18 mM (red point), which indicates a 90% Faradaic efficiency in this system. The concentration of NADH is measured at 0.02 mM (blue point), as determined

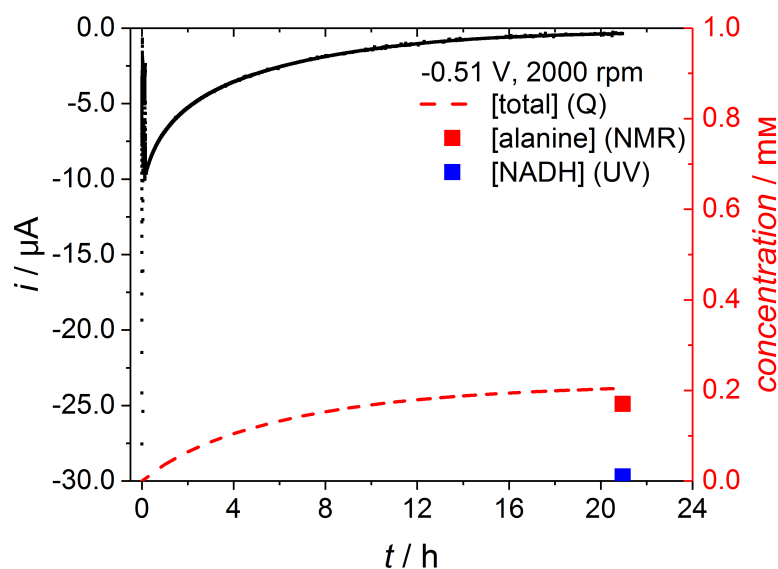


Figure 4.17: Extended time chronoamperometry recorded at an NAD^+ -red/alaDH/BP/PGE electrode at a WE potential of -0.51 V over 24 h. Red dashed line: total reduction products as calculated from the total charge passed. Red point: concentration of alanine measured by ^1H NMR spectroscopy; blue point: concentration of NADH measured by UV-Vis spectroscopy. Substrates: 1 mM NAD^+ , 10 mM pyruvate, 10 mM NH_4Cl ; electrolyte: 100 mM BIS-TRIS-HCl, pH 6.

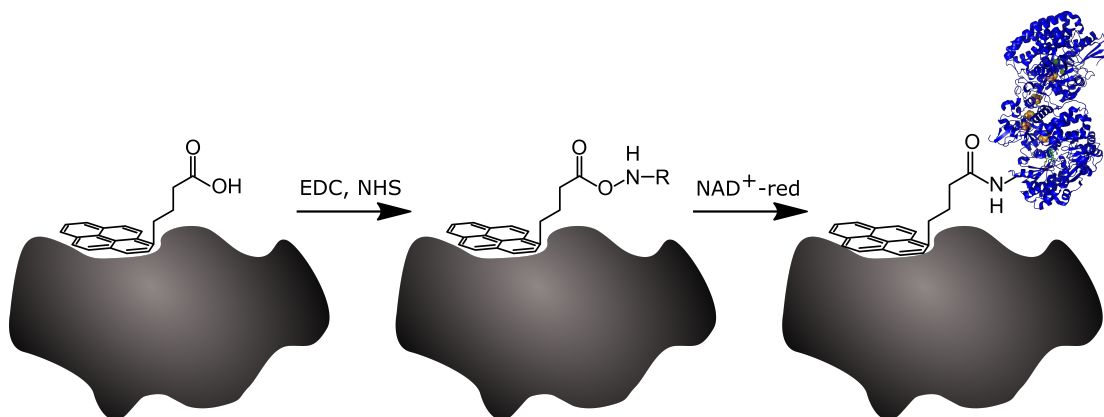
by UV-Vis spectroscopy, indicating that there was no intermediate NADH in the bulk solution and it was used by the alaDH for the reduction of pyruvate to lactate, resulting in the reoxidation of the cofactor. Overall, this is a slight improvement in the production rate of alanine, but this could be due to the small variations between electrode preparations.

This section has shown that electrochemical experiments performed with these enzyme modified particles perform just as well at pH 6 as they did at pH 8. This indicates that this system has the ability to work with a wide range of NADH-dependent oxidoreductases on either side of neutral pH. The next section discusses a method for covalent attachment of the NAD⁺-red enzyme onto the carbon black BP nanoparticles.

4.6.2 Effect of a covalent bonding method of proteins to carbon surfaces

A method for covalent attachment of proteins to a graphite surface was investigated in relation to enzyme reductive activity. A common method is by using 1-Pyrenebutyric acid (py), where the π - π stacking phenomenon generates a strong association with the carbon substrate,^[73,199–202] see Scheme 4.1. The protein is then covalently attached to the electrode by esterification of the CO₂H group by EDC-NHS coupling-Chemistry.^[74,203–206] Therefore, immobilisation of NAD⁺-red covalently onto BP carbon black nanoparticles were prepared, as shown in the reaction Scheme 4.1.

To prepare carbon nanoparticles for covalent coupling of the NAD⁺-red enzyme, 20 mg mL⁻¹ of BP2000 carbon black nanoparticles were suspended in DMF, then shaken and sonicated for 60 min. Then, py was added to the suspension to make up 20 mM, before the suspension was sonicated again for 15 min. The suspension was left overnight at 4 °C, shaking periodically. The particles were then centrifuged and washed four times with DMF, then six times with ultrapure water. The particles were then mixed with 0.4 M EDC and 0.1 M NHS-Na and left at 4 °C for 120 min. The resulting BP-py-NHS particles were then washed with ultrapure water four



Scheme 4.1: Reaction scheme for covalent NAD^+ -red bonding to BP nanoparticles.

more times. NAD^+ -red was thawed to 4°C and the BP-py-NHS particles were added to the NAD^+ -red enzyme at a ratio of 1:3 by volume and incubated at 4°C overnight to generate the BP-py-NHS- NAD^+ -red particles, shown in Scheme 4.1.

For electrochemical measurements, $0.5\ \mu\text{L}$ of the BP-py-NHS- NAD^+ -red particles were drop cast onto the surface of a freshly cleaned and polished PGE electrode. The BP-py-NHS- NAD^+ -red/PGE electrode was then immediately placed in $100\ \text{mM}$ Tris-HCl buffer at pH 8 containing $1\ \text{mM}$ NAD^+ . Cyclic voltammetry experiments were then performed at this electrode.

Figure 4.18 shows the cyclic voltammetry performed at the BP-py-NHS- NAD^+ -red/PGE electrode with $1\ \text{mM}$ NAD^+ in solution. An onset potential of $-0.35\ \text{V}$ vs SHE is observed, similarly as before with the non-covalent NAD^+ -red/BP/PGE electrode (Figure 4.9 on page 111). However, the discharge capacitance, C_d has decreased from $200\ \mu\text{F}$ to $50\ \mu\text{F}$, a four-fold decrease. This could be indicative of a decrease in the amount of enzyme associated with the BP nanoparticles and therefore this particle electrode behaving less like an insulator.

The maximum reduction current has also decreased using the BP-py-NHS- NAD^+ -red/PGE electrode as compared to the NAD^+ -red/BP/py/PGE electrode. At $-0.8\ \text{V}$, the i_{max} with the BP-py-NHS- NAD^+ -red/PGE is approximately $-6.0\ \mu\text{A}$ as compared to a value of at least $-14.0\ \mu\text{A}$ with the non-covalent particle electrode (however the range of values between repeats varied considerably between $-2\ \mu\text{A}$ and

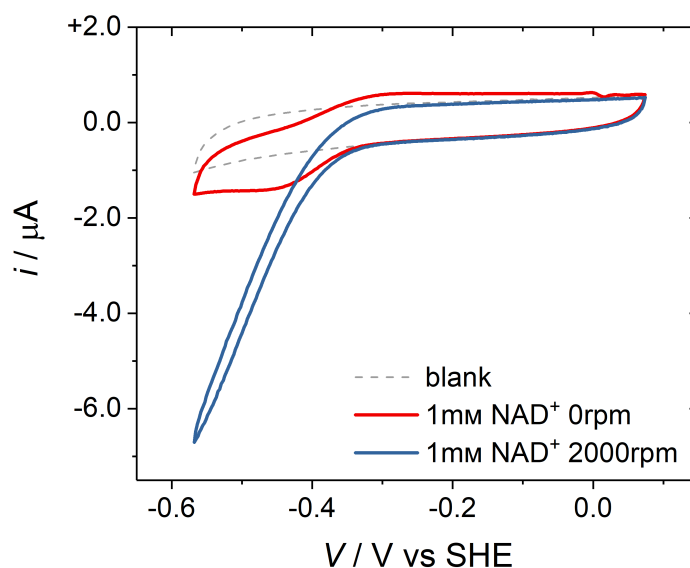


Figure 4.18: Cyclic voltammograms recorded at an NAD⁺-red/BP/py/PGE electrode with 1 mM in solution at a stationary (red) and rotating (blue) electrode. A blank response (grey dashed trace) is present for comparison. Scan rate = 10 mV s⁻¹; electrolyte: 100 mM Tris-HCl buffer, pH 8.

−12 μA). This is further indicative of the BP-py-NHS-NAD⁺-red/PGE electrode having fewer NAD⁺-red enzymes on the electrode. This could be explained by the fact that NAD⁺-red can only be attached to the surface of the BP particles covalently through EDC/NHS coupling-Chemistry, where there are fewer binding positions as compared to non-covalent immobilisations.

Another possibility is that the covalent attachment of the enzyme onto the py-NHS ester orientates the enzyme so that direct electron transfer from the carbon electrode to the FeS clusters in the NAD⁺-red is not possible. A covalent bonding process could position the NAD⁺ in a discrete number of orientations, which is often not the case with non-covalent attachments. However, the protein structure for the NAD⁺-red is not well known enough to try and model this behaviour.

The covalent BP-py-NHS-NAD⁺-red/PGE electrode has been shown to have lower reduction currents than with the particle electrodes prepared non-covalently. Therefore the non-covalent particle preparation is a better method for NAD⁺-red immobilisation.

4.7 Summary

This chapter has discussed the use of NAD⁺-red immobilised onto carbon working electrodes as an appropriate electrocatalyst for NAD⁺ reduction to NADH. The NADH produced can be used for fuelling NADH-dependent oxidoreductase enzymes, such as alaDH and LDH, for specific C=X reductions. The presence of the oxidoreductase enzyme creates a NAD⁺ concentration enhancement effect at the surface of the electrode, which in turn makes the NAD⁺-red more active.

The use of BP nanoparticles enables greater loading of enzyme onto the same geometrical area of the working electrode. However, these particles are more limited by mass transport than by using the PGE for direct NAD⁺ immobilisation. Carbon substrates are good materials for working electrodes as they demonstrate good non-covalent immobilisation of enzymes onto the surface and are electrochemically silent towards various C=X substrates. The wide range of conductive allotropes allow for great flexibility in working electrode design. Also, there is a sizeable potential window for the NAD⁺-red to operate in before the carbon electrode reduces NAD⁺ directly, creating unspecific reduction products that are biologically inactive.

Changing the pH of the electrolyte had no major effect on the catalytic reduction current of the NAD⁺-red/BP/PGE electrode and little change in the onset potential of the reductive wave. Covalently attaching the NAD⁺ onto BP nanoparticles saw a decrease in the catalytic current due to lower enzyme loading onto the BP particles.

The next chapter investigates the application of this electrochemical system to an electrochemical flow cell to maximise mass transport to the working electrode. Moving towards a flow system also makes this electrocatalyst more desirable to the chemical industry, who are increasingly finding flow chemical systems more appealing than batch systems.^[59] More comprehensive product analyses are also performed by UV-Vis, ¹H NMR and HPLC methods to show the specificity of the electrocatalyst.

5

Application to electrochemical flow systems

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In Chapter 4, it was shown that a bi-enzyme electrocatalyst can be developed for the reduction of various C=X substrates. The analytical PGE electrode was used for good and uniform mass transport, but is limited in enzyme loading. Larger nanoparticle-derived electrodes were developed, but were found to be more mass transport limited. Therefore in this chapter, an electrochemical flow cell is used to improve mass transport at high surface area electrodes. In addition to this, it is becoming more desirable for industrial catalytic reactions to be performed in flow systems. One advantage to flow processes over batch processes is that conditions are highly tuneable, in particular the flow rate and therefore the mass transport of chemical substrates and products;^[207–209] the greater efficiency of these flow chemical systems improves Green Chemistry credentials, such as improved heat transfer, improved impurity minimisation and increased reaction speed, to the process, as discussed in Section 1.2.8 on page 24.

The use of an electrochemical flow cell should aid in the improvement of mass transport, meaning the use of higher surface electrodes is possible without compromising substrate or product flux. This means that more enzyme can be used to gain faster C=X substrate reductions. This chapter discusses the development of

electrochemical flow cells to drive electrochemical NADH generation and specific C=X substrate reductions.

5.1 Electrochemical flow cell setups and in-line product analysis

Two different electrochemical flow cells, designed in house and modified during this project, are used in the experiments described in this chapter. For the sake of clarity, the two different electrochemical flow cells are named the ‘mushroom’ electrochemical flow cell and the ‘star’ electrochemical flow cell.

Whichever flow cell is used, the setup of the flow chemistry system is usually the same, as shown in Figure 5.1. The reagents are injected into the flow system, which is filled with Tris-HCl buffer at pH 8. The bulk solution passes through a peristaltic pump, the flow rate of which can be adjusted. The solution then passes through the bioelectrochemical flow reactor, which contains the carbon-based electrode with immobilised NAD⁺-red with or without LDH. Upon exiting the bioelectrochemical flow cell, the solution passes through a UV-Vis cuvette and spectrometer, which takes periodic UV-Vis readings in the 800 nm to 220 nm range.

After leaving the UV-Vis cuvette (reservoir), the solution returns to the peristaltic pump, to be passed through the flow system again. This type of flow chemistry is called *semi-continuous flow*, where the bulk solution repeatedly passes through

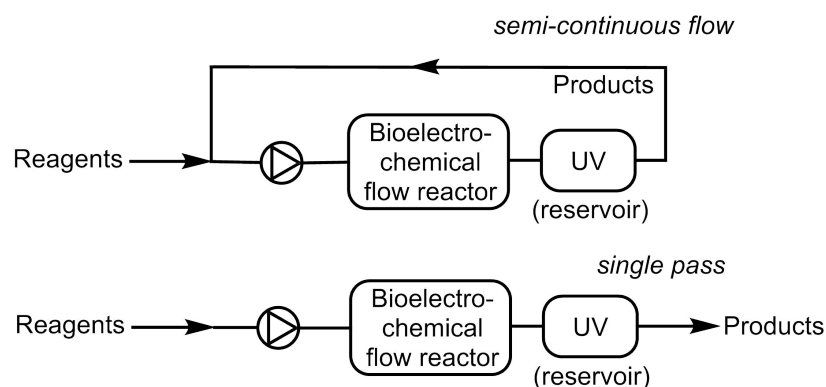


Figure 5.1: Flow diagrams for the semi-continuous flow setups for the experiments described in this chapter, as well as a single pass flow setup. Pump: circle with a right-pointing triangle in direction of flow.

the electrochemical flow reactor. This differs to *single pass* (also Figure 5.1) where the bulk solution only passes through the bioelectrochemical flow cell once and then collected after passing through the UV-Vis cuvette and spectrometer. Although single pass flow chemistry is the preferred method in industrial chemical reactions, only the semi-continuous flow is used in this study. This is because, the NAD⁺-red derived carbon electrodes are very highly mass transport limited and require relatively high flow rates to alleviate mass transport limitations at the electrode. In fact, flow rates up to 12 mL min⁻¹ are used in this chapter, for which the single pass setup would not be appropriate, where lower flow rates in the range of 0.01 mL min⁻¹ to 1 mL min⁻¹,^[210] are generally used to maintain higher conversions at the electrode.

Both electrochemical flow cells were inherited and adapted into flow electrosynthesis cells. They both comprise two parts: the head of the cell, which holds in place the fitting for the electrodes and the flow tubing, and the baseplate of the cell, onto which the carbon paper electrode is placed and the cell head is screwed into place. The cells are designed so that the reaction solution goes into the cell through the top of the cell head, creating a downwards flow onto the working electrode. Once at the surface of the working electrode, the solution exits the cell either from the side or back to the top of the cell head. This results in a flow of the reaction solution onto the working electrode.

The convectional flow effects for NAD⁺ reduction at an RDE and within the electrochemical flow cell are shown schematically in Figure 5.2a. When rotating the PGE, the convection current induces the solution up to the face of the electrode and it is then spun away, as shown by the orange arrows in the figure. The NAD⁺ is brought from the bulk solution to the surface of the PGE electrode, before it is converted to NADH by the NAD⁺-red (blue). After the reduction, the NADH is spun back into the bulk solution. Inside the electrochemical flow cell, the reaction solution flows down onto the carbon paper electrode, creating a similar convection effect to that observed at a PGE.

A similar effect to convection of the reaction solution is seen for the reduction of pyruvate at the PGE electrode and with the electrochemical flow cell, as shown by

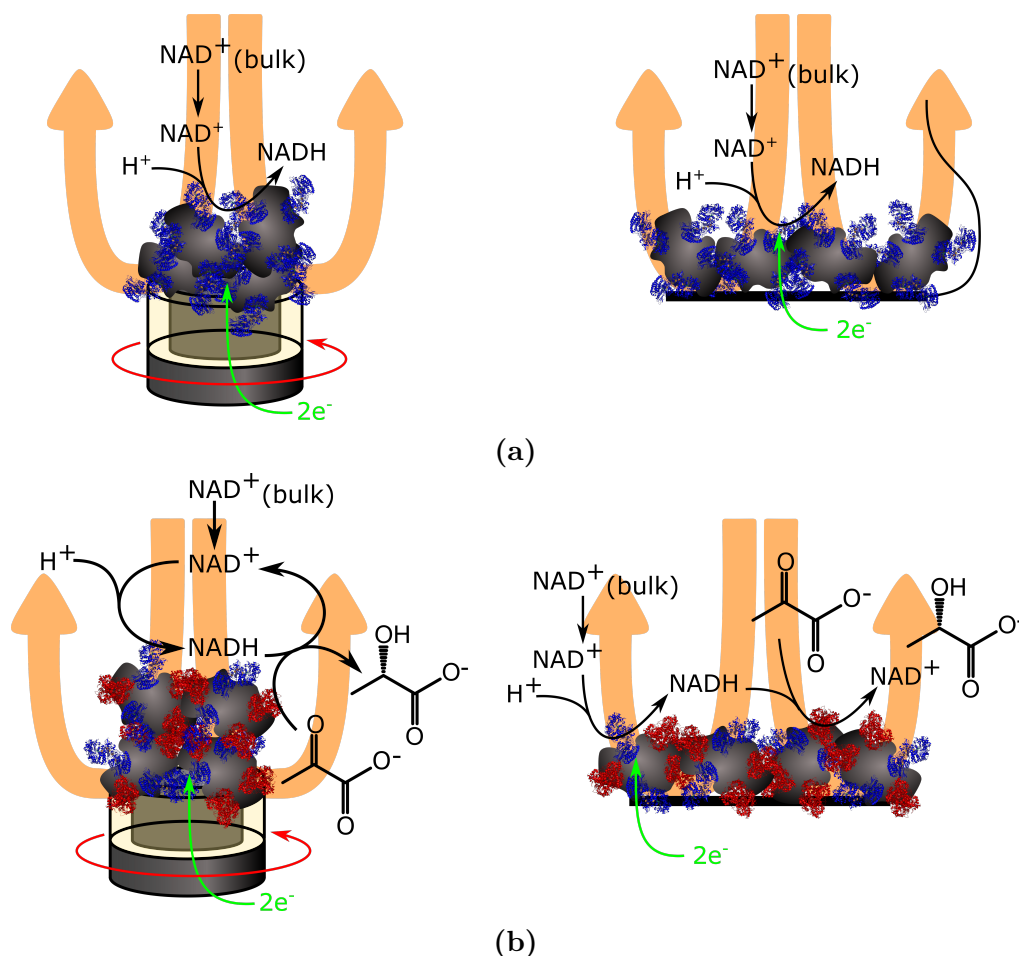


Figure 5.2: Diagram of the mass transport for (a) NAD^+ , and (b) NAD^+ and pyruvate reduction, at a rotating RDE (left) and within an electrochemical flow cell (right). The orange arrows indicate the flux of cofactor and substrate being similar for both the RDE and the electrochemical flow cell.

the orange arrows in Figure 5.2b. Based on assumptions provided by the previous chapter, at the RDE the NAD^+ is brought from the bulk solution to the surface of the PGE electrode, where it is reduced to NADH by the NAD^+ -red (blue). This remains near the catalytic surface of the electrode and is used by the LDH (red) to reduce pyruvate, which has been brought from the bulk solution as a result of the convection current, into lactate. The lactate product is spun back into the bulk solution, avoiding accumulation at the catalytic surface. Meanwhile, the NAD^+ remains near the catalytic surface for re-reduction back into NADH by the NAD^+ -red.

This is similar to the carbon paper within the electrochemical flow cell. The direct motion of the bulk solution down onto the carbon paper bring the two

substrates, NAD^+ and pyruvate to the surface of the electrode and into the catalytic layer. NAD^+ is reduced to NADH by the NAD^+ -red (blue), which remains near the catalytic layer of the electrode where it is used immediately by the LDH (red) for the reduction of pyruvate to lactate. The resulting NAD^+ remains close to the catalytic layer of the electrode and is re-reduced back to NADH by the NAD^+ -red. The generated lactate is flowed back out of the electrochemical cell with the flow of the bulk solution.

The design of the two different flow cells, the ‘mushroom’ and the ‘star’ electrochemical flow cells, are discussed in detail in the next two sections.

5.1.1 The ‘mushroom’ electrochemical flow cell

The ‘mushroom’ electrochemical flow cell is so called because the cell head is shaped like a mushroom, which holds the tubing inlet and outlet and also the electrodes (see Figure 5.3a). The cell head is pushed and clicked into place by a large circular ring on the baseplate. This ring on the baseplate has an O-ring in place to secure the inside of the cell from leaking. The carbon paper working electrode on the baseplate is connected to the outside of the cell by a thin strip of carbon fibre going through a small hole in the top of the mushroom cell head, through to the top of the carbon paper electrode. The counter and reference electrodes are connected through the screw holes on either side of the cell head. The internal dimensions of the ‘mushroom’ electrochemical flow cell are a cylinder of diameter 12 mm and depth 1.5 mm, with total volume 170 μL .

Figure 5.3b shows the ‘mushroom’ cell in operation. The inlet and outlet for the reaction mixture are seen to go into the top side of the cell head. The long electrode is the *saturated calomel electrode*, SCE, and the platinum counter electrode can be seen coming out the front of the cell head. The carbon ribbon can be seen coming out the top of the cell head, connecting to the carbon paper on the inside of the cell. The red peristaltic pump can be seen in the foreground set at 26 rpm, relating to a flow rate of 0.96 mL min^{-1} . The pump calibration is discussed in

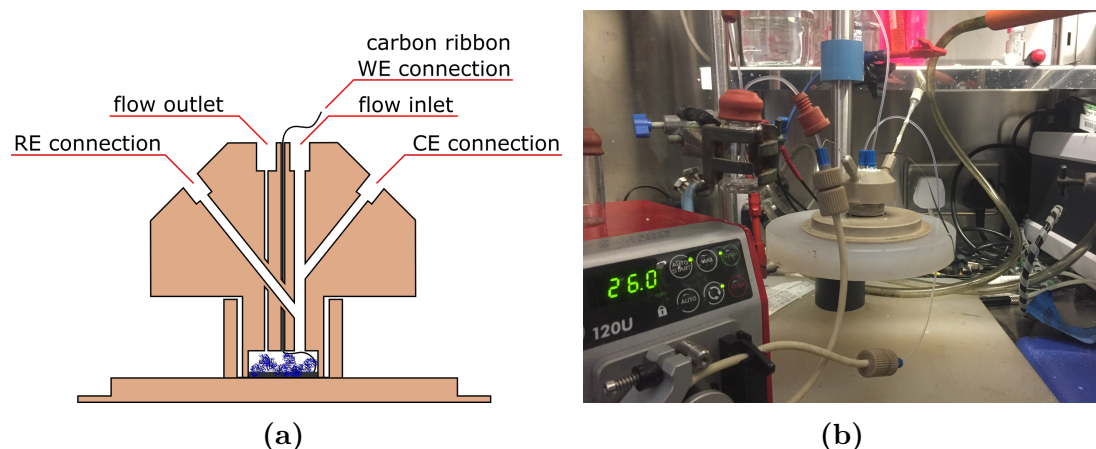


Figure 5.3: (a) Cross-section and (b) picture of the ‘mushroom’ electrochemical flow cell in operation. WE: working electrode; CE: counter electrode; RE: reference electrode.

Section 2.4.4.3 on page 57. In this example, the UV-Vis cuvette is replaced by a glass vial, but both act as a reservoir.

5.1.2 The ‘star’ electrochemical flow cell

The ‘star’ electrochemical flow cell is so called because the cell head here has the three electrodes and the flow outlet connected to the side of the cell head, creating a ‘star’-like shape when in use. In this setup there is no ring securing the cell head in place, rather the carbon paper electrode is laid on the surface of the baseplate and the cell head is screwed into position on top of the carbon paper (see Figure 5.4a). The method of connecting the carbon paper to the outside this time is by using a *glassy carbon*, GC, rod that comes out the side of the cell head. The reaction mixture flows into the top of the cell head and exits from the side of the cell head, so the fluid dynamics on the inside of the cell are inherently slightly different to the fluid dynamics inside the ‘mushroom’ electrochemical flow cell. The internal dimensions of the ‘star’ flow cell are $4.00 \times 6.00 \times 7.46$ mm, with total volume 180 μ L. This is a similar volume when compared to the ‘mushroom’ electrochemical flow cell, but it has a smaller cross section and a deeper cavity.

The ‘star’ electrochemical flow cell can be seen in use outside the glovebox in Figure 5.4b. The large electrode coming out the left side of the cell head is the

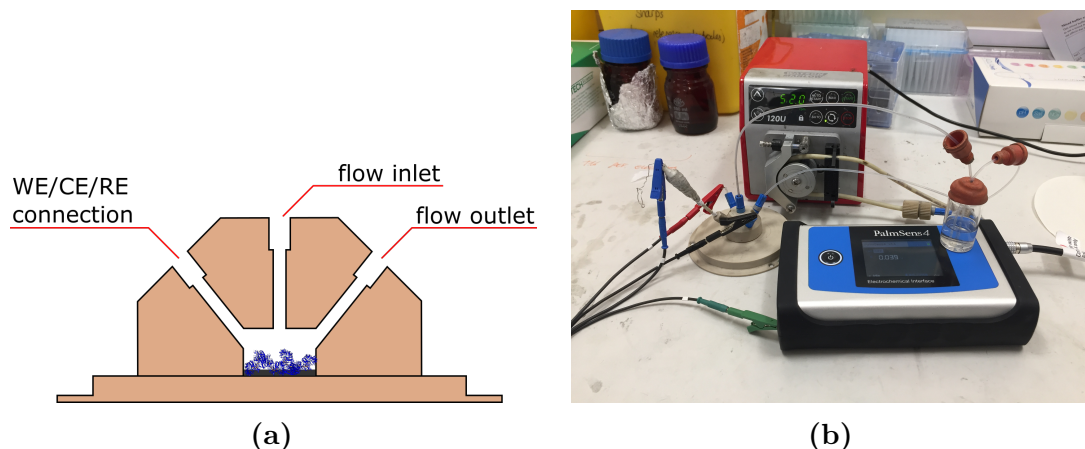


Figure 5.4: (a) Cross-section and (b) picture of the ‘star’ electrochemical flow cell in operation. WE: working electrode; CE: counter electrode; RE: reference electrode.

SCE reference electrode, with the platinum counter coming out the front of the cell head and the glassy carbon rod coming out the back. The flow inlet is through the top of the cell head, with the outlet coming out the right hand side. Again, the red peristaltic pump can be seen and the UV-Vis cuvette is replaced with a glass vial. The blue instrument is the PalmSens4 potentiostat used to control and measure the electrochemistry in most of the experiments in this chapter.

5.1.3 In-line NADH determination using UV-Vis spectrometry

An advantage of flow reactions is that in-line product analysis is possible. In most of the flow experiments presented in this chapter, the flow setup was designed so that the reaction mixture passes through a UV-Vis cuvette held in a peltier cell holder in a UV-Vis spectrometer, for in-line NADH measurement. This brings two advantages to the flow setup, as compared to a batch-type electrochemistry:

- It is possible to have a live measurement of NADH in the reaction mixture.
- The peltier heats the cuvette to 30 °C, meaning that temperature control over the reaction solution is possible.

Therefore, the in-line UV-Vis analysis for NADH provides a useful spectroscopic addition to the experiment.

Table 5.1: Literature values of molar extinction coefficients of NAD^+ and NADH .^[153]

Molecule	λ_{max} / nm	ϵ / $\text{mM}^{-1} \text{cm}^{-1}$
NADH (adenine ring)	260	14.1
NADH (nicotinamide ring)	340	6.22
NAD^+ (adenine and nicotinamide ring)	260	17.4

NADH is UV-Vis active, with two aromatic centres contributing to two different absorbance wavelengths (see Section 2.6.1 on page 59), summarised in Table 5.1. An example spectrum of an experimental sample which contains a mixture of NADH and NAD^+ is shown in Figure 5.5a. This sample was diluted 20-fold in order to be able to detect two peaks at 260 nm and 340 nm. However, at the experimental concentrations of 1 mM the NAD^+ and NADH are too absorbing at 260 nm, as shown in Figure 5.5b. Therefore only the peak at 340 nm is monitored by in-line measurement, where dilution is not possible for in-line NADH UV-Vis analysis.

As the peak at 340 nm is solely contributed by the reduced nicotinamide ring in NADH , the Beer-Lambert law can be used to calculate the concentration of NADH flowing through the UV-Vis cuvette at the time of sampling, as shown in Equation (5.1).

$$c_{\text{NADH}} = \frac{A}{\epsilon l} = \frac{A}{6.22} \quad (5.1)$$

Note that the absorbance, A , is background corrected and adjusted for a moving baseline by subtracting the absorbance value at a non-UV-Vis active value at 450 nm. Also the pathlength across the cuvette is 1 cm.

Figure 5.5b shows an example of the increase in absorbance at 340 nm, which can be used to measure the increase in NADH for an in-line measurement of NAD^+ reduction to NADH . This provides another precise method for calculating enzyme activity at a certain time, which can also be compared to the electrochemical activity of the enzyme at the electrode. The concentration of NADH determined by UV-Vis measurements is plotted as a function of time as a blue trace alongside the current or potential response during electrosynthesis experiments.

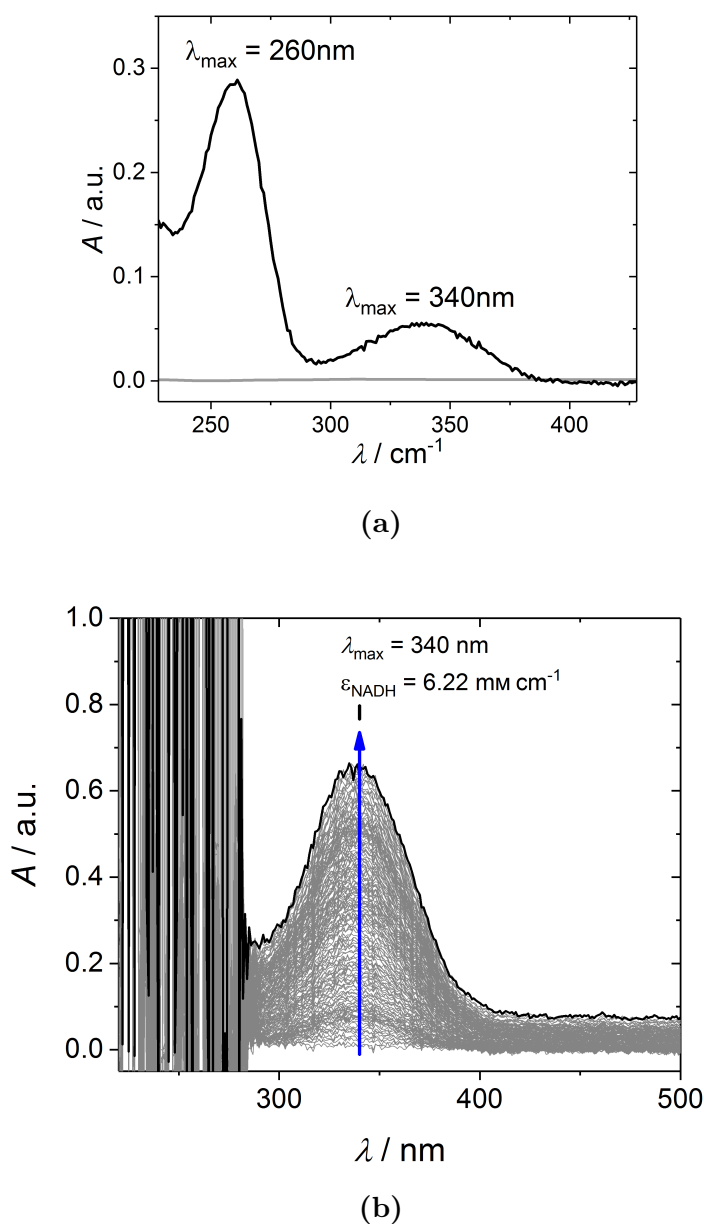


Figure 5.5: UV-Vis spectroscopy for in-line measurement of NADH . (a) UV-Vis spectrum of a roughly equimolar solution of NAD^+ and NADH (b) The increase in absorption at 340 nm can be used as a method of measuring the production of NADH .

5.1.4 Lactate product analysis using ^1H NMR spectroscopy

Conversion of pyruvate to lactate is analysed using ^1H NMR. Figure 5.6 shows an example ^1H NMR spectrum of a sample taken at the end of an electrosynthetic reduction of pyruvate to lactate. The methyl group is used as the diagnostic peak for

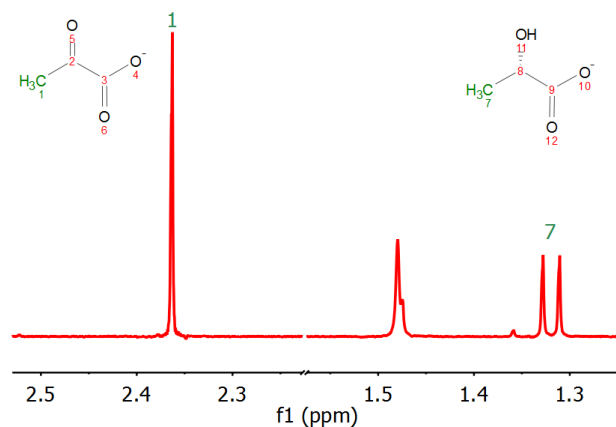
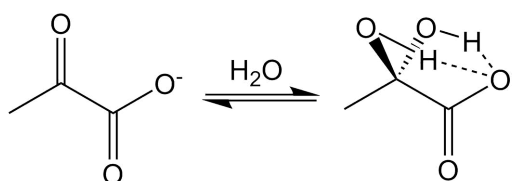


Figure 5.6: Example ^1H NMR spectrum for a sample mixture with pyruvate and lactate.

Table 5.2: Chemical shifts used for pyruvate and lactate measurements.

Molecule	δ , ^1H NMR (400 MHz, 10 v/v% D_2O)
pyruvate	2.36 (s, 3 H)
pyruvate hydrate	1.48 (s, 3 H)
lactate	1.23 (d, 3 H)

both pyruvate, $\delta 2.36$ (s, 3 H) and lactate $\delta 1.23$ (d, 3 H), as shown in Table 5.2 and these signals are used to calculate the concentration of both pyruvate and lactate in solution. There is another peak in the spectrum at $\delta 2.36$ (s, 3 H) attributed to a hydrate of pyruvate,^[211] the structure of which is shown in Scheme 5.1. Therefore



Scheme 5.1: Equilibrium between pyruvate and the hydrate.

the integral of the hydrate peak is also taken into consideration when calculating the total amount of pyruvate in the sample, as described in Equation (5.2).

$$[\text{lactate}] = [\text{total}] - [\text{pyruvate}] - [\text{hydrate}] \quad (5.2)$$

For the experiments presented in this Thesis, in-line ^1H NMR was not possible, therefore as an alternative, samples of the reaction mixture at the end of the

electrosynthesis were taken for ^1H NMR analysis. However, this does not rule out the use of in-line ^1H NMR as a future method for product analysis. In this chapter the use of UV-Vis spectroscopy as the sole in-line method for the analysis of NADH demonstrates the power of in-line product analysis.

5.2 Reduction of NAD^+ using the ‘mushroom’ electrochemical flow cell

An electrochemical flow experiment for the electrosynthetic reduction of NAD^+ to NADH was performed using the ‘mushroom’ electrochemical flow cell. First, NAD^+ -red/BP particles were manufactured by incubating 50 μL of NAD^+ -red (1.4 mg) with 10 μL of BP nanoparticle slurry (20 mg mL^{-1} in ultrapure water) for 60 min at 4 $^\circ\text{C}$. A piece of *carbon paper*, CP, was cut to fit inside the ‘mushroom’ cell (0.5 cm^2) and the prepared NAD^+ -red/BP particles were injected on top of the CP on the baseplate. The head of the cell was immediately put into place on top and 2.3 mL of the electrolyte solution with 1 mM NAD^+ was flowed into the cell as a semi-continuous flow and through a UV-Vis spectrometer. A chronoamperometry experiment was performed holding the cell at a potential of -0.51 V vs SHE, the same potential used for the chronoamperometry experiments performed in Chapter 4, for 14 h. The results are shown in Figure 5.7.

The black points show the current response of the cell when a potential of -0.51 V is applied. After the initial relaxation period, the current is approximately $-8\text{ }\mu\text{A}$, which decreases to $-3\text{ }\mu\text{A}$ after 14 h. The predicted possible amount of NADH made from the reduction of NAD^+ , based on charge passed assuming all the electrons are only being used for the two-electron reduction of NAD^+ to NADH, is shown as a blue dashed line. This gives a predicted upper limit NADH concentration of 0.58 mM. The blue point shows the concentration of NADH to be 0.49 mM as determined by UV-Vis from a sample taken from the bulk solution at 14 h. A Faradaic efficiency can be determined by using these two numbers and Equation (5.3).

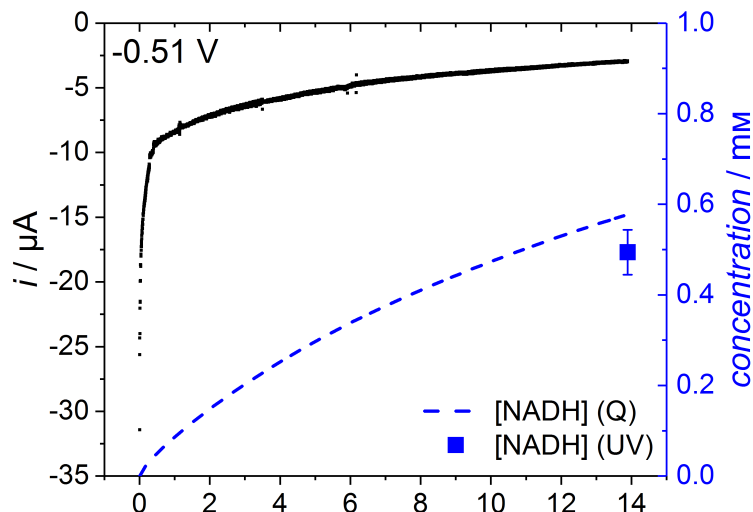


Figure 5.7: NAD⁺ reduction chronoamperometry experiment performed with a NAD⁺-red/BP/CP electrode within the ‘mushroom’ electrochemical flow cell. Applied potential: -0.51 V vs SHE. The blue dashed line shows the predicted generation of NADH calculated from the charge passed through the working electrode. The blue dot indicates the concentration of NADH present after 14 h determined by UV-Vis. Cofactor: 1 mM NAD⁺; flow rate: 0.96 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

$$\eta_{\text{Faradaic}} = \frac{[\text{NADH}]_{\text{UV}}}{[\text{NADH}]_{\text{Q}}} * 100 \quad (5.3)$$

$$= (84.5 \pm 8.4) \% \quad (5.4)$$

A moderate Faradaic efficiency of 84.5% indicates that this is an efficient process where most of the electrons are being used to reduce NAD⁺ to NADH. The loss of 15.5% of electrons could be due to some sticking of NADH at the carbon electrode, therefore not being in the bulk solution to be measured by UV-Vis.

This experiment is useful to indicate that an electrochemical flow is an appropriate method for electrosynthesis. It combines a large geometrical surface area CP with the high surface area BP to form a large total electroactive area of the working electrode. A 58% conversion was measured after performing electrosynthesis at this electrode after 14 h, providing a modest value to build from throughout this

chapter. The next section discusses the improvement of NAD^+ -red immobilisation onto BP nanoparticles, with their electrochemical activities measured within the ‘mushroom’ flow cell.

5.3 Investigating the effect of the BP nanoparticle mass ratio for NAD^+ -red immobilisation

The NAD^+ -red/BP particles are used to provide a high surface area for immobilisation of the NAD^+ -red enzyme on the working electrode and the particles were then spotted onto CP electrodes. This section discusses efforts towards optimisation of the relative mass of BP required for NAD^+ -red immobilisation, and the effect on the electrochemical response.

The following section has particle-based electrodes prepared in a similar fashion to that described in Section 5.2, with the mass of carbon used for protein immobilisation increased each time. The amount of NAD^+ -red enzyme used for each experiment is constant (30 μL of 1.4 mg mL^{-1}). The enzyme is first allowed to thaw slowly to 4 °C. In the meantime, a BP nanoparticle slurry is prepared by dispersing 20 mg mL^{-1} of BP in ultrapure water by sonication for 60 min. Then, a different volume of the particle slurry is injected into aliquots of 30 μL of NAD^+ -red and incubated overnight. The volumes of BP slurry and NAD^+ -red used for particle preparation in each section are summarised in Table 5.3. The supernatant is removed and the same amount of LDH (30 μL , 10 mg mL^{-1}) is added to the particles each time and incubated for a further 60 min at 4 °C.

After the enzyme immobilisation step, the particles were then spotted onto CP of area 0.5 cm^2 and allowed to dry partially for about 2 min. The ‘mushroom’ cell

Table 5.3: Volumes of BP slurry and NAD^+ -red used for particle immobilisation.

Section	Electrode	V_{BP} / μL	$V_{\text{NAD}^+\text{-red}}$ / μL	$m_{\text{BP}}/m_{\text{NAD}^+\text{-red}}$
5.3.1	A	5	30	2.4
5.3.2	B	10	30	4.8
5.3.3	C	20	30	9.5

head was then immediately put on top of the NAD⁺-red/BP/CP electrode and electrochemical experiments were performed immediately.

5.3.1 Investigating the NAD⁺-red/LDH/BP/CP electrode with mass ratio 2.4: electrode A

Figure 5.8 shows the electrochemistry experiments performed at a NAD⁺-red/LDH/-BP/CP electrode with a BP to NAD⁺-red mass ratio, $m_{\text{BP}}/m_{\text{NAD}^+-\text{red}}$, of 2.4. First a cyclic voltammogram was performed at the electrode, with the response shown in Figure 5.8a. The electrolyte was 100 mM Tris-HCl with 1 mM NAD⁺ in solution. The discharge capacitance of the electrode was 700 μF consistent with the expected high carbon surface area. Also important to note is the sloping voltammogram in non-redox regions. This could be attributed to an increase in the resistance of the working electrode, by not having a particularly good connection between the NAD⁺-red/BP particles and the CP electrode and between the NAD⁺-red/BP particles themselves. One reason for this could be due to the particles being ‘flooded’ out by the NAD⁺-red and becoming too insulating to allow a good electrochemical conductivity between the particles and the CP.

The onset potential for NAD⁺ reduction appears at -0.4 V , which is a more negative potential than observed for previous electrodes in Chapter 4. This can be explained by the fact that the sloping CV is hiding the true value of the onset potential. The electrode is not under mass transport limitation, as the reduction peak of the electrode does not tail off at negative potentials. With this, a maximum reduction current of $-15\text{ }\mu\text{A}$ is achieved at -0.56 V , i_{max} . Compared to the analytical NAD⁺-red/BP/PGE electrodes discussed in Chapter 4, this is not a good current achieved per milligram of NAD⁺-red. This is examined more closely, along with the other NAD⁺-red/BP/CP electrodes assessed in this section, ahead in Section 5.3.4 on page 160.

In line with normal practice in the field of organic synthesis, as discussed in Section 2.3.2, a chronopotentiometry experiment was performed at the same electrode (Figure 5.8b), after a 10 mM injection of pyruvate, and with the applied

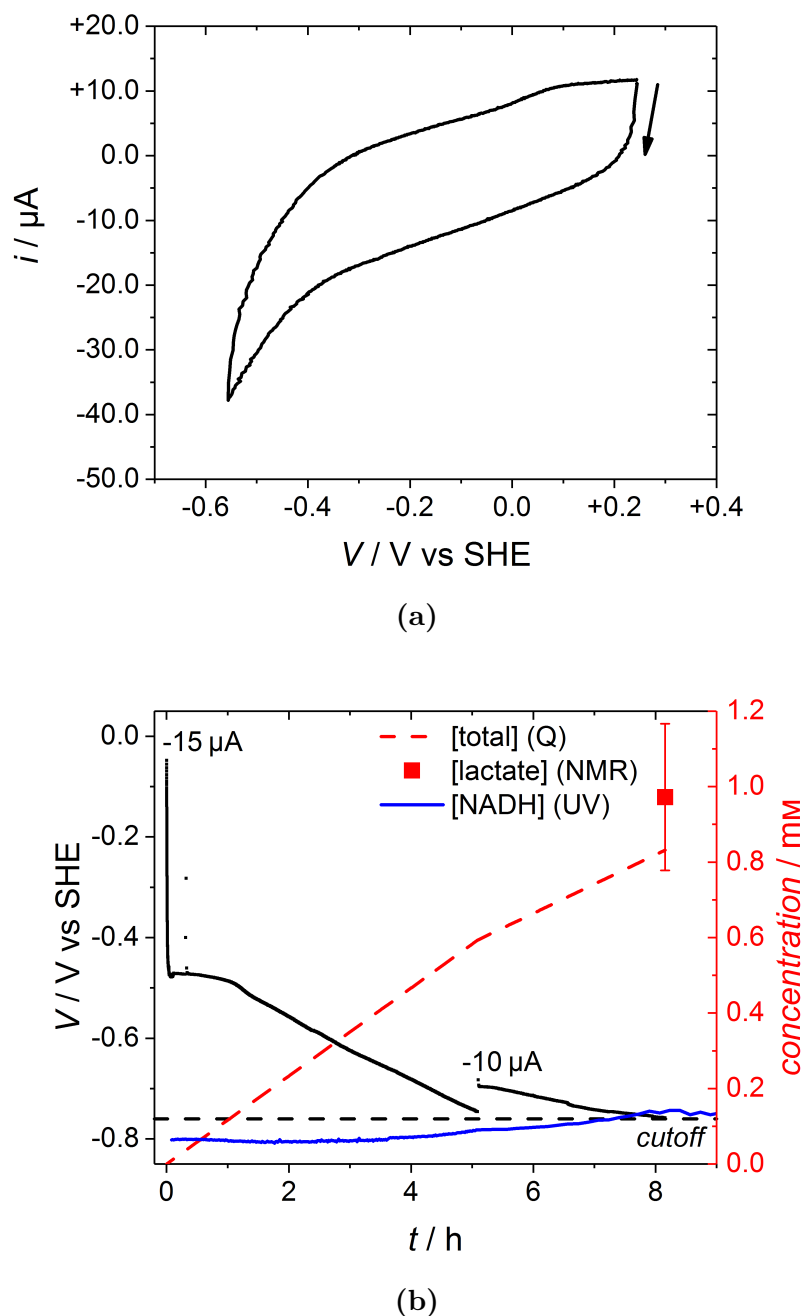


Figure 5.8: Electrochemistry experiments performed at a NAD^+ -red/LDH/BP/CP electrode ($m_{\text{BP}}/m_{\text{NAD}^+-\text{red}} = 2.4$). (a) Cyclic voltammetry at this electrode with 1 mM NAD^+ in solution. (b) Chronopotentiometry experiment performed with 1 mM NAD^+ and 10 mM pyruvate in solution; applied currents are indicated on the plots. Black points: potential of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; red point: [lactate] calculated by ^1H NMR; blue trace: NADH calculated by in-line UV-Vis; black dashed line: potential cutoff at -0.76 V vs SHE . Conditions for (a) and (b); flow rate: 2.08 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

currents indicated on the figure. After a period of stability for 1 h, the potential of the electrode becomes more negative until it reaches the set safety cutoff of -0.76 V at 5 h. The cutoff is applied for a few reasons:

- To protect the enzyme from extremely negative potentials.
- To avoid reaching a potential at which unselective NAD^+ reduction occurs at the carbon itself.
- To avoid H_2 evolution at the carbon electrode at extremely negative potentials.

The potential of the electrode shifts to more negative values due to loss of catalytic activity meaning that a greater driving force is required to achieve the same current. The amount of NAD^+ -red can be unstable and be lost into the bulk solution, or simply deactivate over time. Therefore in order to maintain the current, the potentiostat applies a greater energy (potential) to the cell in order for each NAD^+ -red enzyme to work harder.

At 5 h, the current is reset to a lower magnitude and the chronopotentiometry restarted. The enzyme film seems to be more stable at $-10\text{ }\mu\text{A}$, shown by a shallower gradient by the black points on the graph, suggesting that the NAD^+ -red is more stable at this current. However, the enzyme film may simply have stabilised over time as loosely associated enzyme washes away.

The red dashed trace shows the predicted amount of lactate and NADH generated by the electrocatalyst, derived from the charge passed through the electrode and accounting for the two-electron transfer. A sample was taken from the bulk solution at the end of the experiment and used for ^1H NMR spectroscopy to detect a quantity of lactate produced, which is shown by the red point. The total concentration of reduction products produced as predicted by the charge passed is within the margin of error of the concentration of lactate measured by ^1H NMR. The blue trace indicates the concentration of NADH as determined by in-line UV-Vis, which shows that there is a slight accumulation of NADH in solution over time (0.1 mM). This could be due to not enough LDH being immobilised onto the BP particles

and therefore an accumulation of NADH at the surface, some of which can move back into the bulk solution.

5.3.2 Investigating the NAD^+ -red/LDH/BP/CP electrode with mass ratio 4.8: electrode B

The experiment performed in Section 5.3.1 was repeated, however this time using a NAD^+ -red/LDH/BP/CP electrode with a BP to NAD^+ -red mass ratio, $m_{\text{BP}}/m_{\text{NAD}^+\text{-red}}$, of 4.8. Figure 5.9 shows the electrochemistry experiments performed at this electrode in the ‘mushroom’ electrochemical flow cell. First a cyclic voltammetry experiment was performed on the electrode with 1 mM NAD^+ in solution, with the results shown in Figure 5.9a. The periodic steps in current are attributed to the periodic pulse of the peristaltic pump. The discharge capacitance for this electrode is 1000 μF , only slightly higher than with electrode A despite having twice as much BP. This suggests that there is better coverage of enzyme on the BP nanoparticles.

The onset potential for the NAD^+ reduction at the NAD^+ -red/LDH/BP/CP electrode is roughly at -0.32 V . The reduction peak does not seem to be under mass transport limitation, as the slope is constant on the catalytic wave. The maximum reduction current achieved is $-25\text{ }\mu\text{A}$, which is a significant increase compared electrode A with half as much BP.

Figure 5.9b shows a chronopotentiometry experiment performed at this electrode. The applied current is $-8\text{ }\mu\text{A}$, roughly a quarter of the maximum reduction current achievable at this electrode at -0.56 V , defined as i_{max} . This was to attempt to avoid destabilising the enzyme film too much, as seen in Figure 5.8b, so that a longer experiment would be able to be performed. The experiment was able to be performed for 26 h (there was a gap in the experiment between 8 h and 14 h), without even reaching the safety cutoff potential of -0.56 V . Applying a current of approximately $1/4i_{\text{max}}$ seems to aide stability, as the chronopotentiometry experiment at electrode B was found to last at least three times longer than the experiment with electrode A.

The red dashed line indicates the total concentration of reduced species, calculated from charge and assuming a two-electron reduction process, i.e. NADH and

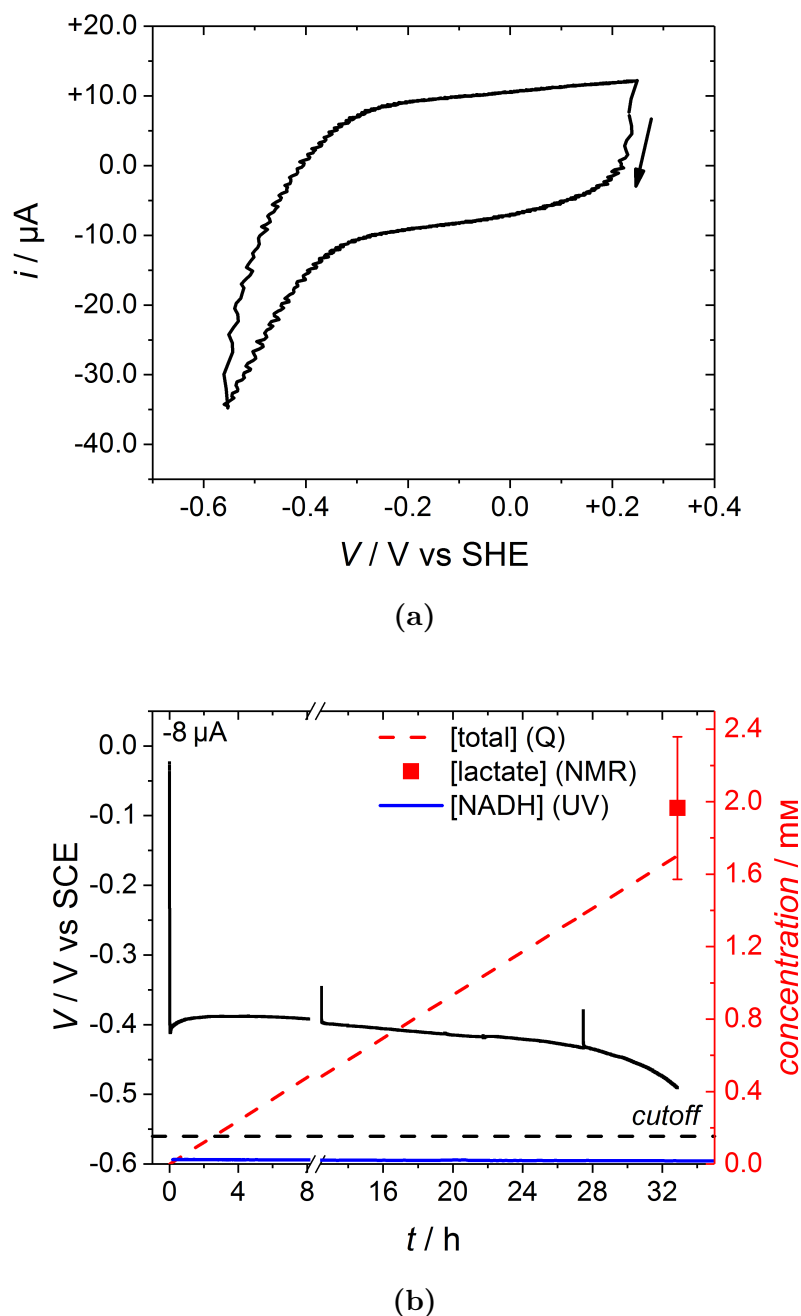


Figure 5.9: Electrochemistry experiments performed at a NAD^+ -red/LDH/BP/CP electrode ($m_{\text{BP}}/m_{\text{NAD}^+\text{-red}} = 4.8$). **(a)** Cyclic voltammetry at this electrode with 1 mM NAD^+ in solution. **(b)** Chronopotentiometry experiment performed with 1 mM NAD^+ and 10 mM pyruvate in solution; applied currents are indicated on the plots. Black points: potential of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; red point: [lactate] calculated by ^1H NMR; blue trace: NADH calculated by in-line UV-Vis; black dashed line: potential cutoff at -0.56 V vs SHE . Conditions for **(a)** and **(b)**; flow rate: 2.08 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

lactate. At 32 h a total of 1.8 mM reduced species have been generated. This is more than what was produced by the electrode A, which had less carbon and a higher applied current relative to i_{max} , which was causing greater instability in potential. This is in agreement with the concentration of lactate measured by ^1H NMR at the end of the experiment, as shown by the red point. This shows that there is nearly 100% Faradaic efficiency at this electrode, which indicates that all the electrons are going into reduction of pyruvate and that there is little energy wasted.

The concentration of NADH in the bulk solution is also measured throughout the whole experiment, shown by the blue trace. It can be seen that there is very little, accumulation of the reduced cofactor in the bulk solution. This indicates that once the NAD^+ is reduced to NADH at the electrode, it is used immediately by the LDH bound locally to the NAD^+ -red, meaning that little NADH has the chance to move back into the bulk solution. Another advantage of this observed effect is that the immediate consumption of NADH by the LDH and regenerates NAD^+ at the surface of the electrode, which contributes to an enhanced local NAD^+ concentration, making the electrocatalyst more active.

Preparing NAD^+ -red/LDH/BP particles with a relative mass ratio of 4.8 ultimately creates a more active and more stable NAD^+ -red/LDH/BP/CP electrode than using a mass ratio of 2.4. The electrocatalyst lasted longer with a set current value of approximately $1/4i_{\text{max}}$, and in the end produced more lactate than before. The next section describes a similar experiment, but with particles made with a higher $m_{\text{BP}}/m_{\text{NAD}^+\text{-red}}$ mass ratio still.

5.3.3 Investigating the NAD^+ -red/LDH/BP/CP electrode with mass ratio 9.5: electrode C

A similar experiment was performed using an NAD^+ -red/BP/CP electrode, prepared in the same way as before but this time with a BP to NAD^+ -red mass ratio, $m_{\text{BP}}/m_{\text{NAD}^+\text{-red}}$, of 9.5. The electrochemistry experiments at this electrode were performed in the ‘mushroom’ electrochemical flow cell and the results are shown in Figure 5.10. Firstly a cyclic voltammetry experiment was performed with 1 mM

of NAD^+ in solution, as shown in Figure 5.10a. The discharge capacitance of this electrode is significantly larger than observed with the previous two electrodes, at a value of $4000\ \mu\text{F}$. Whilst only doubling the mass of the BP used in particle manufacture for this electrode as compared to before, the discharge capacitance increase four-fold from $1000\ \mu\text{F}$. This indicates that there is significantly larger surface area of the particles that are unmodified, allowing for greater charging of the surface. This indicates that this mass ratio is probably higher than necessary for full enzyme immobilisation.

The onset potential for this electrode appears to be around $-0.4\ \text{V}$. However, similar to electrode A, the blank area of the cyclic voltammogram is sloping, which may be hiding the true onset potential for NAD^+ reduction at this electrode. As with each of these electrodes, the NAD^+ reduction of this enzyme does not appear to be under mass transport limitation, as observed by the constant slope of the downward wave of the reduction peak. However the unusually high discharge capacitance of the electrode may be skewing the shape of the relatively small reduction wave. The maximum reduction current achieved at this electrode is ca $-35\ \mu\text{A}$, which is slightly larger than that observed at electrode B. This could be a result of more NAD^+ -red adsorbed onto the electrode, or more LDH being absorbed onto the larger total surface area of the BP nanoparticles, meaning that there is a greater contribution to the NAD^+ concentration enhancement at the surface of the electrode, as a result of the LDH oxidation of NADH.

A chronopotentiometry experiment was performed at the electrode, with the applied potentials indicated on the graph in Figure 5.10b. The first applied current was $-10\ \mu\text{A}$, a value slightly larger than that for $1/4i_{\text{max}}$ ($-8.75\ \mu\text{A}$). It seems that this electrode is fairly unstable, with the potential of the cell reaching the initial cutoff potential of $-0.61\ \text{V}$ after just 2 h. The experiment was restarted at the lower current of $-5\ \mu\text{A}$, but still only lasting for 3 h before reaching the adjusted the potential cutoff at $-0.56\ \text{V}$. The potential cutoff was moved more positive as it was previously a little too close to the potential at which there is NAD^+ reduction at the carbon itself. The experiment was started for a third time at a

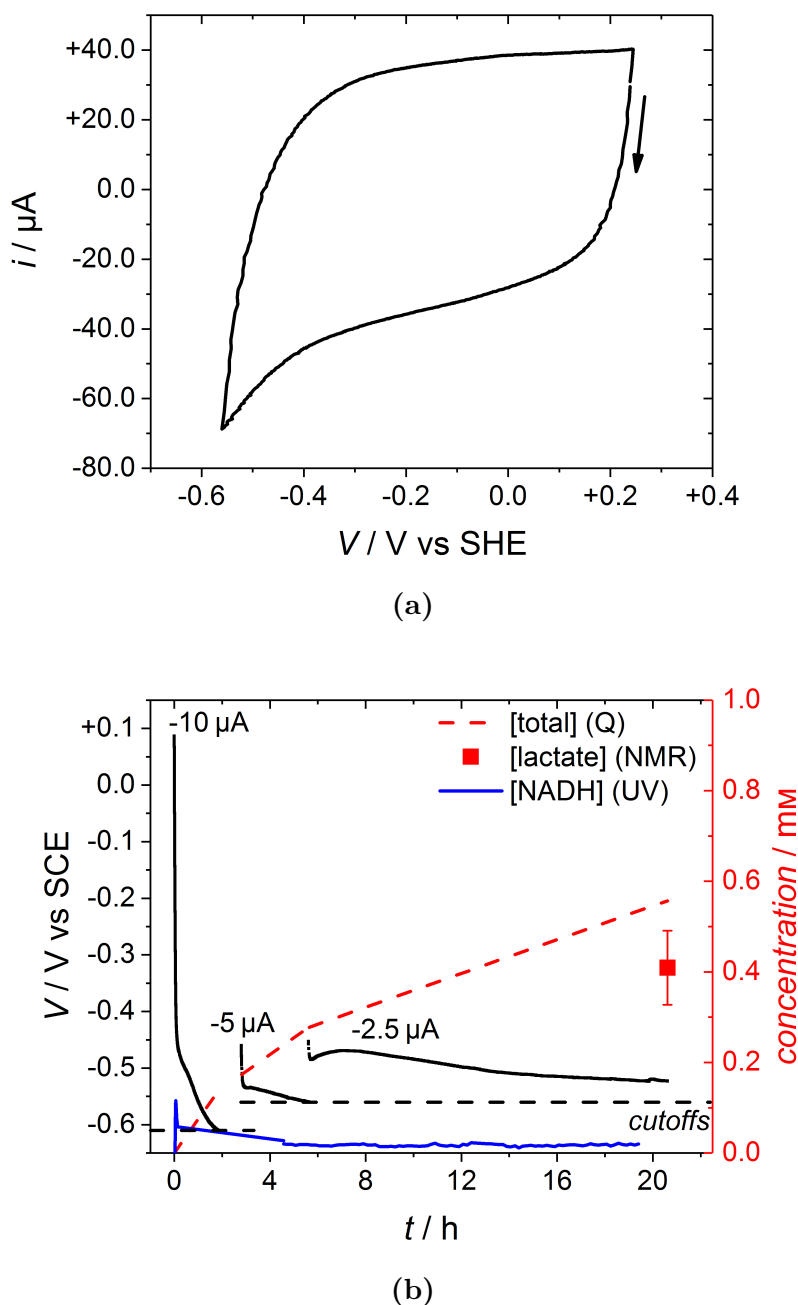


Figure 5.10: Electrochemistry experiments performed at a NAD^+ -red/LDH/BP/CP electrode ($m_{\text{BP}}/m_{\text{NAD}^+-\text{red}} = 9.5$). (a) Cyclic voltammetry at this electrode with 1 mM NAD^+ in solution. (b) Chronopotentiometry experiment performed with 1 mM NAD^+ and 10 mM pyruvate in solution; applied currents are indicated on the plots. Black points: potential of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; red point: [lactate] calculated by ^1H NMR; blue trace: NADH calculated by in-line UV-Vis; black dashed line: potential cutoff at -0.61 V until 3 h then -0.76 V vs SHE. Conditions for (a) and (b); flow rate: 2.08 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

current of $-2.5\ \mu\text{A}$, and lasted 15 h without reaching the cutoff potential. With every lower applied current, the gradient of the potential vs time trace became shallower, indicating that the electrode becomes increasingly more stable. However, it is clear that this electrode is a lot less stable than the previous two NAD^+ -red/LDH/BP/CP electrodes.

The total predicted reduced species, as calculated from the charge passed through the electrode, is shown as the red dashed line. After 21 h, a total of 0.6 mM of lactate and NADH is predicted to have been generated by the electrocatalyst, as compared to the actual concentration of lactate at the end of experiment, measured by ^1H NMR to be 0.4 mM. Therefore the Faradaic efficiency is significantly lower than observed with the previous NAD^+ -red/LDH/BP/CP electrode with a value of 67%. This could be because the potential drifted into a region where there is unspecific NAD^+ reduction at the carbon.

The concentration of NADH is measured throughout the experiment (blue trace) and there appears to be a small amount of NADH in the bulk solution at the start (roughly 0.1 mM). This could be a result of some contaminant NADH at the start of the experiment, or that there is some generated with the cyclic voltammetry experiments performed before the chronopotentiometry. However, this is used by LDH and after 4 h, the concentration of NADH in the bulk solution is close to zero, as observed before. This would have an adverse positive impact on the reported Faradaic efficiency. This is because the concentration of lactate as a result of the electrosynthesis, measured by ^1H NMR indicated in Figure 5.10b, has not been corrected for the initial presence of NADH in the solution, and should therefore be at a lower concentration than indicated in the Figure, resulting in an overestimate in the Faradaic efficiency.

It seems that preparing NAD^+ -red/LDH/BP particles with a mass ratio of 9.5 has a negative effect on the stability of the NAD^+ -red/LDH/BP/CP electrodes. This electrocatalyst was only able to perform for several hours after the applied current was lowered to less than 10% of the i_{max} value, with it being very unstable

at larger reduction currents. The next section compares these three electrodes to each other and to that of a particle electrode at an analytical PGE electrode.

5.3.4 Comparative discussion on the effect of the BP nanoparticle mass ratio

This section compares the electrochemical characteristics of multiple BP nanoparticle electrodes. The data in Table 5.4 on page 161 compares electrochemical data from the NAD^+ -red/BP/PGE electrode described in Section 4.4.2 on page 109, electrode ‘Z’, with electrochemical data from the NAD^+ -red/LDH/BP/CP electrodes A, B and C with different BP mass ratios as described in this section.

The three different NAD^+ -red/LDH/BP/CP electrodes are compared to the simpler NAD^+ -red/BP/PGE RDE. The PGE-based electrode has a smaller geometrical surface area (0.03 cm^2) than the CP-based electrodes in the electrochemical flow cells (0.5 cm^2). However, the total surface area of the electrode is dominated by the mass of BP nanoparticles used for enzyme immobilisation. The total surface area of carbon on each electrode increases from the PGE to the CP electrode, but also when the $m_{\text{BP}}/m_{\text{NAD}^+-\text{red}}$ ratio increases. It is expected that the total amount of enzyme that can immobilise onto the particles increases as more BP is used for the enzyme immobilisation step.

The amount of NAD^+ -red enzyme exposed in preparation of the BP particles is the same for each electrode compared in the table ($42.0 \times 10^{-3} \text{ mg}$). For the NAD^+ -red/BP/PGE electrode, only approximately $1/3$ of the total particles were drop cast onto the electrode, therefore having a third of the total surface area of the electrode, 88.8 cm^2 , as compared to electrode B with a total surface area of 267 cm^2 .

The change of the total surface area does show some correlation with the trend of discharge capacitance for each electrode. There is a linear trend between the total surface area of the particle electrodes and the discharge capacitance, which is presented in Figure 5.11. The area-averaged value for the capacitance, C_A of these BP nanoparticle derived electrodes is calculated by taking the gradient of the plot,

$$C_A = \frac{C_d}{A_{\text{total}}} \quad (5.5)$$

Table 5.4: Comparing the current responses of different particle electrodes.

Electrode $m_{BP}/m_{NAD^{+}-red}$	NAD ⁺ -red/BP/PGE ^a			NAD ⁺ -red/LDH/BP/CP ^b		
	Z	A	B	C		
	4.8	2.4	4.8	9.5		
$A_{geometrical} / \text{cm}^2$ ^c	0.03	0.5	0.5	0.5		
A_{total} / cm^2 ^d	88.8	134	267	533		
$C_d / \mu\text{F}$ ^e	200	700	1000	3500		
$m_{NAD^{+}red} / 10^{-3} \text{ mg}$ ^f	4.7	42.0	42.0	42.0		
$i_{max} / \mu\text{A}$	-15.0	-15.0	-25.0	-35.0		
$i_{max}/m_{NAD^{+}red} / \mu\text{A mg}^{-1}$	-3191.5	-357.1	-595.2	-833.3		
$i_{max}/A_{geometrical} / \mu\text{A cm}^{-2}$	-500.0	-30.0	-50.0	-70.0		
$i_{max}/A_{total} / \mu\text{A cm}^{-2}$	-0.169	-0.113	-0.094	-0.066		

^aSee Section 4.4.2 on page 109.

^bSee Section 5.3, this section.

^cThe geometrical surface area of the working electrode.

^dThe total surface area of the working electrode using a BP surface area of $(1332 \pm 22) \text{ m}^2 \text{ g}^{-1}$, as calculated by BET gas adsorption analysis.^[76]

^eThe discharge capacitance of the electrode calculated from the blank cyclic voltammogram.

^fMass of NAD⁺-red on the electrode. For NAD⁺-red/BP/PGE electrodes roughly $\frac{1}{3}$ of the particles from the preparation are used each time.

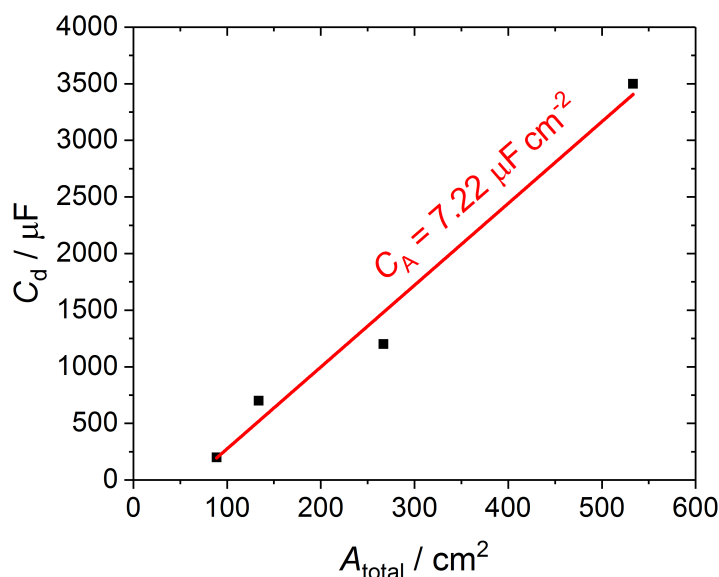


Figure 5.11: Plot of the discharge capacitance against total surface area for BP-derived electrodes. The area-averaged capacitance, C_A , for the carbon particle electrodes is calculated by taking the gradient of the plot. Data taken from Table 5.4 on page 161. $R^2 = 0.981$.

which is calculated to be $7.22 \mu\text{F cm}^{-2}$. When compared to the literature values of C_A at porous carbon electrodes,^[212] it is in line with previous results performed at high surface area carbon electrodes similar to BP.

Whilst the particles were prepared the same way, with the same amount of NAD^+ -red exposed to the varying amount of BP particles, the maximum reduction currents were measured and compared. Firstly, the NAD^+ -red/BP/PGE electrode does not have an NADH-dependent enzyme as part of the experiment. Therefore it does not have the NAD^+ concentration enhancement effect observed with using an NADH-dependent enzyme which reoxidises NADH to NAD^+ at the surface of the electrode. The i_{max} achieved for electrode Z is the same for electrode A, indicating that the electrocatalyst has the same total activity in each case, despite three times the mass of NAD^+ -red having been exposed to half the proportion of carbon in preparation of the NAD^+ -red/LDH/BP/CP particles. The activity per milligram of NAD^+ -red ($i_{\text{max}}/m_{\text{NAD}^+\text{-red}}$) is thus a third of the NAD^+ -red/BP/PGE (electrode Z). This indicates either that not enough NAD^+ -red has immobilised

onto the BP particles, or that the enzyme is immobilised in non-active orientations, or subject to poor mass transport.

Comparing the $i_{\max}$ for the three different NAD⁺-red/LDH/BP/CP electrodes, as the mass of BP used for the NAD⁺-red and LDH immobilisation increases, the maximum reduction current of NAD⁺ increases from 15.0 μA to $-25.0 \mu\text{A}$ and to $-35.0 \mu\text{A}$ at -0.56 V . This trend could be due to a few reasons. The increase in the surface area of the carbon could enable more of the exposed NAD⁺-red to immobilise, increasing the reduction current directly. More surface area could also mean that the immobilised NAD⁺-red would be less crowded, or could possibly be able to be arranged for a larger proportion of NAD⁺-red to allow for direct electron transfer to the carbon surface, increasing the reductive current. Another possibility could be that more LDH is able to absorb onto the surface of the carbon surface, therefore increasing the enhanced concentration of NAD⁺ at the surface of the electrode, as a result of the reoxidation of NADH cofactor. A third explanation could be that with the NAD⁺-red more spaced out on the carbon, there is microscopic hemispherical diffusion to each enzyme, thereby resulting in better mass transport. This also explains the trends in the $i_{\max}/m_{\text{NAD}^+\text{-red}}$ ratio for the increase in BP for NAD⁺-red/LDH/BP/CP electrodes A, B and C. There is a general increase in the magnitude of the reduction currents at the relative mass of BP is used for enzyme immobilisation.

The value of $i_{\max}/A_{\text{geometrical}}$ increases from $-30.0 \mu\text{A cm}^{-2}$, to $-50.0 \mu\text{A cm}^{-2}$ and to $-70.0 \mu\text{A cm}^{-2}$, with increasing the mass of BP used for the preparation of NAD⁺-red/LDH/BP/CP electrodes. This is because each of these electrodes has the same geometrical surface area and the total activity of the NAD⁺-red/BP particles is increasing with each increase of the relative mass of BP. However, the value of $i_{\max}/A_{\text{geometrical}}$ for electrode Z is much larger ($-500.0 \mu\text{A cm}^{-2}$) compared to any of the values for the NAD⁺-red/LDH/BP/CP electrodes. This is partly due to the better mass transport observed with a rotating PGE electrode, but also the catalytic particle layer on the surface of the PGE being more densely packed.

Finally, the $i_{\max}/A_{\text{total}}$ ratio can be used as a comparison. The maximum NAD⁺ reduction current generally decreases from $-0.113 \mu\text{A cm}^{-2}$, to $-0.094 \mu\text{A cm}^{-2}$ and to $-0.066 \mu\text{A cm}^{-2}$, as the relative mass of BP used for enzyme immobilisation increases for the NAD⁺-red/LDH/BP/CP electrodes. This indicates that there is less utilisation of the electrode surface as the carbon BP particle content is increased. Electrode Z on the other hand has a larger value of $-0.169 \mu\text{A cm}^{-2}$ when compared to the NAD⁺-red/LDH/BP/CP electrodes. This indicates that the NAD⁺-red enzymes are more densely packed on the surface of the BP nanoparticles than what is observed with the particles prepared for the NAD⁺-red/LDH/BP/CP electrodes. Of particular interest, this value should be the same as the $i_{\max}/A_{\text{total}}$ ratio for electrode B, but is nearly twice the magnitude. This could be due to the coimmobilised LDH displacing NAD⁺-red from the surface of the BP nanoparticles. Or it could be due to the individual BP nanoparticles being more spread out on the larger CP electrode, meaning that LDH enzymes on neighbouring particles cannot contribute to an enhanced NAD⁺ concentration on the surface of other NAD⁺/LDH/BP nanoparticles, which would increase the electrode activity. Another possibility is that the mass transport in the ‘mushroom’ electrochemical flow cell is poorer as compared to the RDE.

This section has offered a comprehensive discussion on the effect the mass ratio, $m_{\text{BP}}/m_{\text{NAD}^{+}\text{-red}}$, has on the electrochemical activities and behaviour of the different particle derived electrodes. NAD⁺-red/BP or NAD⁺/LDH/BP particles with a $m_{\text{BP}}/m_{\text{NAD}^{+}\text{-red}}$ ratio of 4.8, made by incubating 30 μL of NAD⁺-red (1.4 mg mL^{-1}) with 10 μL BP particle slurry (20 mg mL^{-1}), seem to give a good compromise between catalytic activity and electrode stability. Although the PGE particle derived electrodes might give the best catalytic currents relative to the amount of NAD⁺-red enzyme used, a larger total amount of enzyme can be immobilised on the larger CP electrodes and in particular with the case of the NAD⁺/LDH/BP/CP electrode with a $m_{\text{BP}}/m_{\text{NAD}^{+}\text{-red}}$ ratio of 4.8, is more stable. This electrode offers a compromise of a biological electrocatalyst having a high value of $i_{\max}/A_{\text{geometrical}}$

whilst maintaining a good stability. The next section discusses the use of a NAD^+ -red/LDH/BP/CP electrode within a different electrochemical flow cell, named the ‘star’ electrochemical flow cell.

5.4 Pyruvate reduction using the ‘star’ electrochemical flow cell

This section discusses using an NAD^+ -red/LDH/BP/CP electrode for pyruvate reduction to lactate, but this time using the ‘star’ electrochemical flow cell. It is expected that the different internal dimensions of the flow cell might have an effect on the mass transport or the stability of the catalytic film.

The NAD^+ -red/LDH/BP particles were prepared in the same fashion as described at the beginning of Section 5.3 on page 150. The mass ratio, $m_{\text{BP}}/m_{\text{NAD}^+\text{-red}}$, of BP (15 μL , 20 mg mL^{-1}) injected into to the NAD^+ -red enzyme (42.0×10^{-3} mg) this time is 7.1. After NAD^+ -red/BP particles were made, the supernatant was removed and the LDH (30 μL , 10 mg mL^{-1}) was added to the particles and incubated for 60 min at 4 °C to make the NAD^+ -red/LDH/BP particles. Once manufactured, the particle mixture was injected on to the CP electrode, which for the ‘star’ electrochemical flow cell is rectangular of area 0.6 cm^2 . Before the cell head was put in place, the particles were allowed to adsorb for 30 min at 4 °C on the surface of the CP.

Cyclic voltammetry was performed first on the NAD^+ -red/LDH/BP/CP electrode with 1 mM NAD^+ in solution, as shown in Figure 5.12a. The discharge capacitance is fairly large as compared to the similar electrodes described in Table 5.4, with a value of 4000 μF . The observed onset potential here is approximately -0.38 V, however this could be obscured by the slope in the non-redox region, which could arise from the increase in the working electrode resistance between the particles and the CP. The maximum NAD^+ reduction current achieved is approximately 36 μA at -0.56 V, a similar value to that achieved with the NAD^+ -red/LDH/BP/CP electrode with $m_{\text{BP}}/m_{\text{NAD}^+\text{-red}}$ ratio of 9.5.

A chronopotentiometry experiment was performed immediately after, with the results shown in Figure 5.12b. As it was observed previously that applying a current

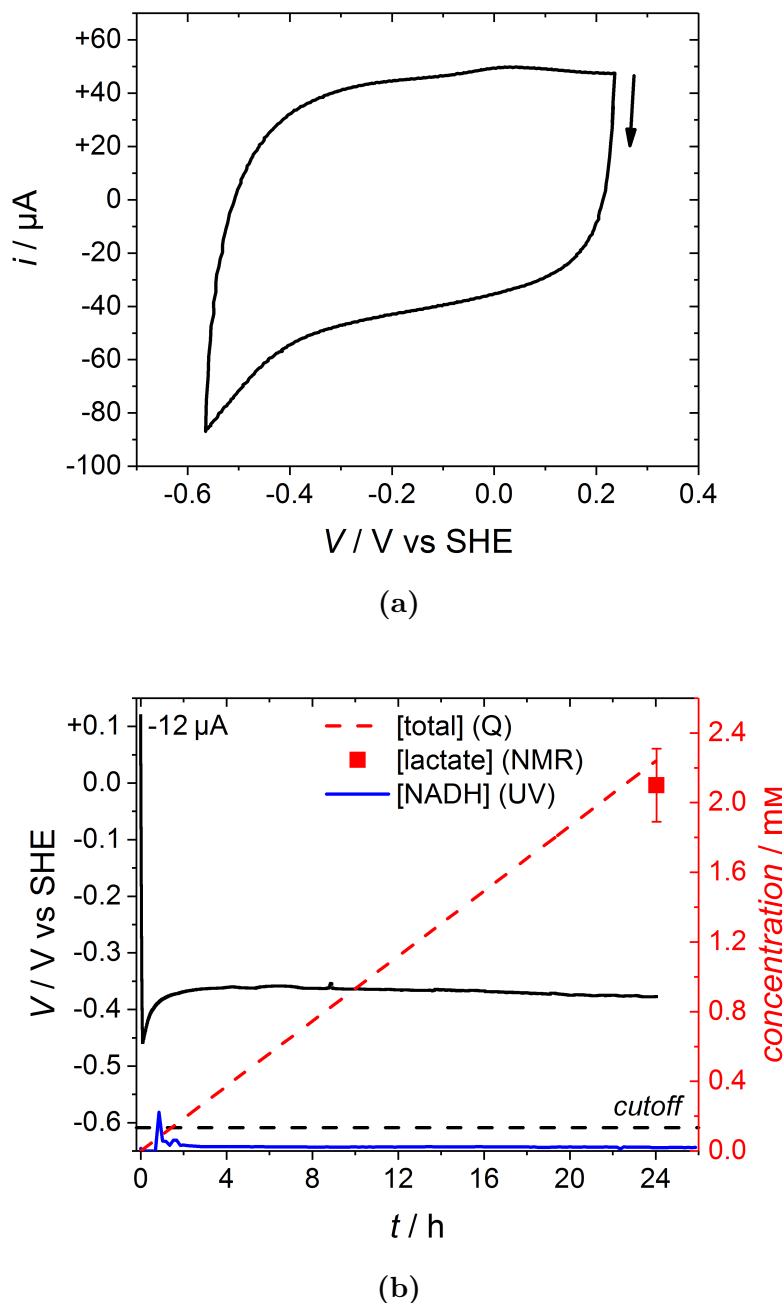


Figure 5.12: Electrochemistry experiments performed at a NAD^{+} -red/LDH/BP/CP electrode ($m_{\text{BP}}/m_{\text{NAD}^{+}\text{-red}} = 7.1$). **(a)** Cyclic voltammetry at this electrode with 1 mM NAD^{+} in solution. **(b)** Chronopotentiometry experiment performed with 1 mM NAD^{+} and 10 mM pyruvate in solution; applied currents are indicated on the plots. Black points: potential of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; red point: [lactate] calculated by ^1H NMR; blue trace: NADH calculated by in-line UV-Vis; black dashed line: potential cutoff at -0.61 V vs SHE . Conditions for **(a)** and **(b)**; flow rate: 2.08 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

of below approximately $1/4i_{\text{max}}$ enhances the stability of the potential response of the electrocatalyst, the applied current for the experiment was $-12\text{ }\mu\text{A}$, this time $1/3i_{\text{max}}$. Over the course of the 24 h experiment, the electrocatalyst exhibited excellent stability, with the potential of the cell not drifting from -0.36 V . This implies that the electrocatalyst should be able to be stable and survive beyond the 24 h demonstrated in this experiment.

The predicted total concentration of NADH and lactate generated by the NAD^+ -red/LDH/BP/CP electrocatalyst is shown by the dashed red trace, calculated from the charge passed through the working electrode. After 24 h it is predicted that 2.3 mM of product is generated. This is confirmed after taking an ^1H NMR measurement from a sample at 24 h, indicating that 2.1 mM of lactate has been produced by the electrocatalyst, which results in a high Faradaic efficiency of 91%. The concentration of NADH in the bulk solution was measured and it was observed that there was no accumulation of NADH in the bulk solution. This indicates that the reduced cofactor is being used immediately by the LDH.

By using the ‘star’ electrochemical flow cell, it appears the chronopotentiometry experiments are much more stable than the experiments performed in the ‘mushroom’ flow cell. This is more useful for electrocatalytic experiments, as the longer the electrocatalyst is stable, the more $\text{C}=\text{X}$ substrate it is able to reduce. Another method to improve $\text{C}=\text{X}$ conversions is to increase the catalytic current of the electrocatalyst. The next section investigates the effect of direct immobilisation of the NAD^+ -red enzyme onto the CP on the catalytic rate and stability of the electrocatalyst.

5.5 Investigating the effect of direct immobilisation of NAD^+ -red onto CP electrodes

This section investigates the effect of direct immobilisation of the NAD^+ -red enzyme on the surface of the CP electrode on the electrochemical response for NAD^+ and $\text{C}=\text{X}$ substrate reductions. It seems with the previous experiments that there is a large surface area of the BP nanoparticles that are not loaded with NAD^+ -red, which

leads to an unwanted large discharge capacitance. There is also a possibility that direct immobilisation of NAD⁺-red onto carbon paper will have a pronounced effect on the stability of activity of the enzyme. Relying on the CP only for NAD⁺-red immobilisation might also simplify the process of manufacturing the electrocatalyst. This section discusses different possible preparation methods for the manufacture of NAD⁺-red/CP electrodes and the effect of their electrochemical activity and stability within the ‘star’ electrochemical flow cell.

5.5.1 Using activated carbon paper as a working electrode and for NAD⁺-red immobilisation

As opposed to the previous type of carbon paper used in this section, *activated carbon paper*, ‘aCP’, is used for the direct immobilisation of NAD⁺-red. This is because activated carbon paper has vast numbers of small pores, which have been imaged frequently by SEM,^[146,213–217] increasing the surface area for NAD⁺-red immobilisation, in effect having similar properties to the BP nanoparticles, in increasing the surface area of the electrode.

The first experiment uses a NAD⁺-red/aCP and LDH in solution for pyruvate reduction. The electrode was prepared by pre-wetting the paper with Tris-HCl pH 8 buffer whilst thawing 10 μ L NAD⁺-red slowly to 4 °C. Then all of the NAD⁺-red was spotted onto the aCP when in position in the bottom of the flow cell and left to incubate for 30 min at 4 °C immobilise onto the aCP to generate the NAD⁺-red/aCP electrode. It was noted that the paper was a little hydrophobic when injecting the enzyme onto it. After incubating the NAD⁺-red on the aCP, the top of the cell was put in place and buffer with 1 mM NAD⁺ flowed through the cell. Electrochemical experiments were performed immediately.

Cyclic voltammetry was performed at the NAD⁺-red/aCP electrode with 1 mM NAD⁺ in solution, as shown in Figure 5.13a. The discharge capacitance is still relatively high as compared to the BP particle electrodes described in the previous sections of this chapter, having a value of 2000 μ F. Despite a slightly sloping voltammogram in the non redox region, the onset potential observed for NAD⁺

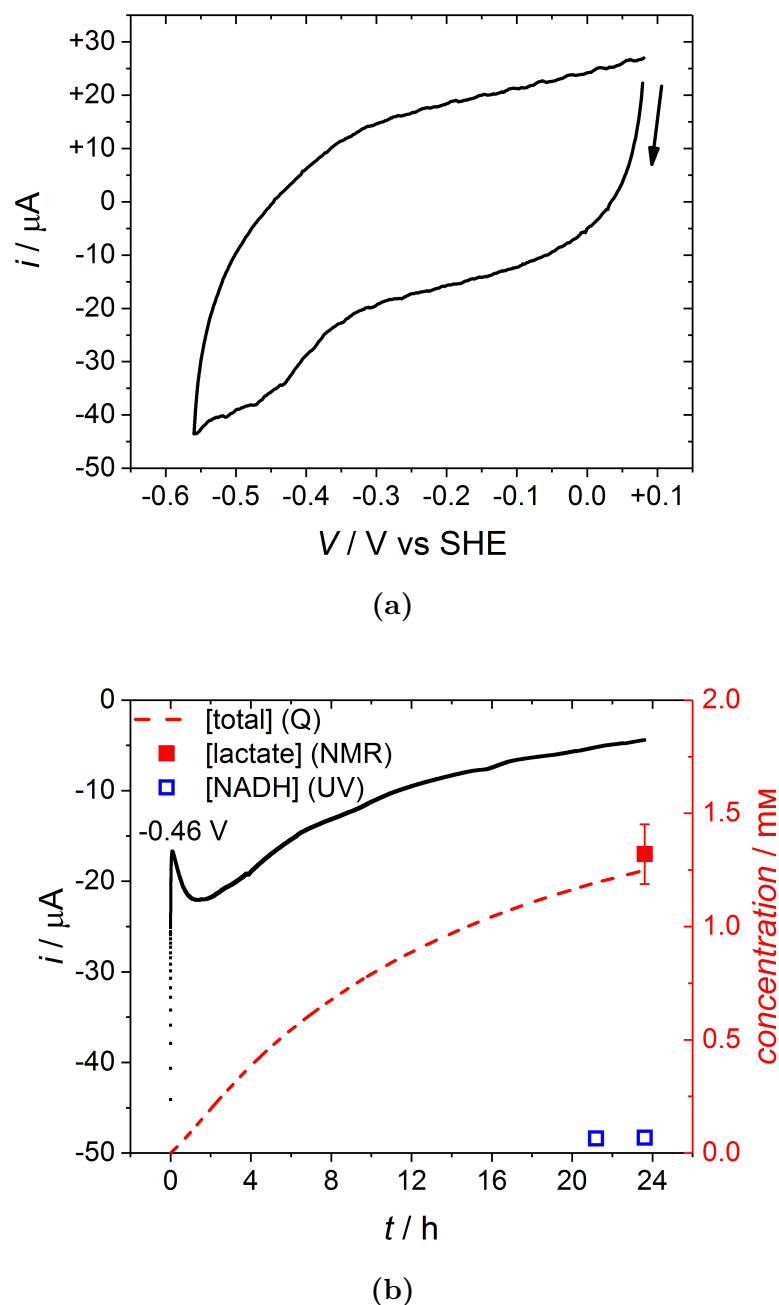


Figure 5.13: Electrochemistry experiments performed at an NAD^+ -red/aCP electrode. (a) Cyclic voltammetry at this electrode with 1 mM NAD^+ in solution. (b) Chronoamperometry experiment performed with 1 mM NAD^+ , 10 mM pyruvate and 0.4 mg mL^{-1} LDH in solution; potential applied: -0.46 V vs SHE . Black points: current of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; red point: [lactate] calculated by ^1H NMR; blue points: NADH determined from two samples by UV-Vis. Conditions for (a) and (b); flow rate: 2.68 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

reduction is -0.32 V . The maximum reduction current achieved is $-20\text{ }\mu\text{A}$ at -0.51 V , however this appears to be under mass transport limitation, as indicated by the change in shape of the catalytic wave at negative potentials. The flow rate was not increased from 2.68 mL min^{-1} to avoid greater destabilisation of the catalyst.

The chronoamperometry experiment (Figure 5.13b) performed immediately afterwards, but with 10 mM pyruvate and 0.4 mg mL^{-1} LDH in solution, shows the catalytic reduction current of the electrode at the applied potential of -0.46 V , and the electrode is stable enough to run an electrocatalytic reduction experiment for 24 h. The increase of the reduction current that is observed reaching a maximum of $-23\text{ }\mu\text{A}$ at 1 h is attributed to a lengthy mixing of the LDH and substrate in the flow system and also to lengthy wetting of the NAD^+ -red/aCP catalytic layer. After this maximum, the chronoamperometry behaves normally, decreasing slowly due to deactivation or loss of NAD^+ -red enzyme from the NAD^+ -red/aCP electrode. Perhaps also, the LDH is coimmobilising onto the aCP over time, reaching an equilibrium.^[87]

The predicted total conversion is shown by the red dashed trace, as calculated from the amount of charge passed through the working electrode. After 24 h, it is in good agreement with the total lactate concentration as measured by ^1H NMR (red point). For this experiment, it was not possible to have in-line UV-Vis measurements for constant NADH monitoring, however, the two blue points at 21 h and 23.5 h indicates that towards the latter part of the experiment, there is very little NADH in the bulk solution.

This experiment indicates that NAD^+ -red immobilisation directly on the aCP electrode is possible, however there is still a significant insulating effect, as indicated by the discharge capacitance of the electrode.

5.5.2 Investigating the effect on NAD^+ -red immobilisation after pretreating the activated CP electrode

In this section, an NAD^+ -red/aCP electrode is developed with a pretreatment step for the aCP electrode. Before NAD^+ -red immobilisation, the aCP is put in

concentrated HCl for 10 s before washing in ultrapure water. If the paper wasn't more hydrophilic, the HCl pretreatment step was repeated. This is to ensure the oxygen groups on the surface of the paper are protonated, as symbolised as 'aCP(H⁺)'. The paper was washed thoroughly and this time a blank cyclic voltammogram was recorded at this unmodified electrode in the 'star' electrochemical flow cell, as shown by the grey dashed line in Figure 5.14a. The blank does not show any redox active species and is in line with the blank experiments performed in Section 4.2 on page 97. It has been casually observed that performing a blank cyclic voltammetry experiment before the NAD⁺-red immobilisation step aids in immobilisation itself, possibly due to a low potential activation of the aCP(H⁺) electrode, or improved wetting.

The wet paper electrode was then cooled to 4 °C before 10 µL of NAD⁺-red (1.4 mg mL⁻¹), which had been thawed slowly to 4 °C as usual, was spotted onto the paper and left to incubate at 4 °C for 120 min. The top of the cell was put back in place and buffer with 1 mM NAD⁺ was flowed into the cell before performing further electrochemical experiments.

Figure 5.14a shows the cyclic voltammetry recorded at the NAD⁺-red/aCP(H⁺) electrode with 1 mM NAD⁺ in solution. The discharge capacitance is similar to that observed previously, 2000 µF, and the onset potential is similar to previous experiments. However the maximum reduction current of the electrode at -0.56 V is significantly larger ($i_{\text{max}} = -90 \mu\text{A}$) compared to the -20 µA observed at the previous electrode that was not pretreated with concentrated acid. A reason for this could be that the protonated oxygen groups form stronger hydrogen bonding with the NAD⁺-red so that more is immobilised onto the carbon paper, or the protonated oxygen groups enable better orientation of the NAD⁺-red enzymes for electron transfer. Additionally, if the paper wets better, the NAD⁺-red can penetrate better into the pores of the activated carbon better. At this flow rate, the NAD⁺ reduction again appears slightly mass transport limited, as indicated by the slope of the catalytic wave. Again, the flow rate was selected to balance loss of enzyme from the catalytic film with increasing the substrate concentration at the electrode.

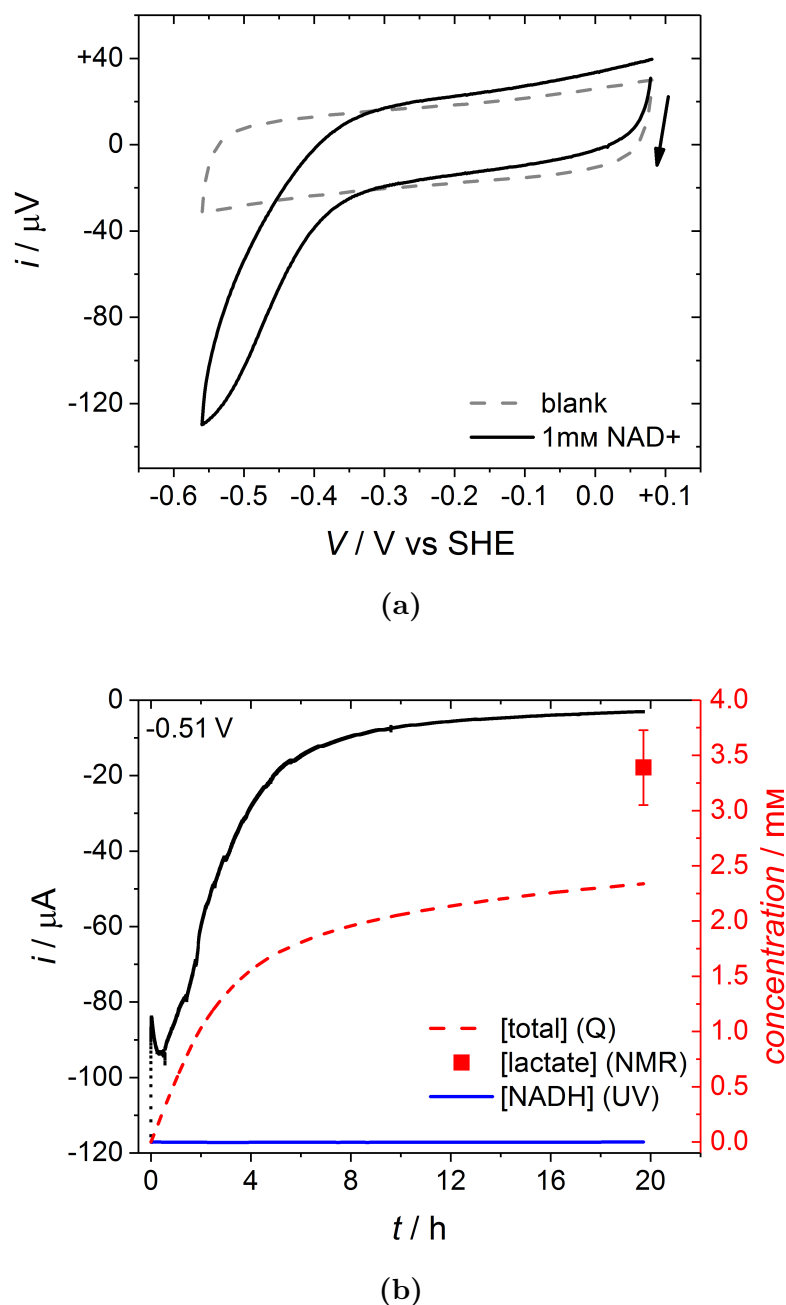


Figure 5.14: Electrochemistry experiments performed at an NAD^+ -red/aCP(H^+) electrode. **(a)** Cyclic voltammetry experiments. Grey dashed trace: unmodified CP electrode with blank electrolyte solution. Black trace: this NAD^+ -red/aCP electrode with 1 mM NAD^+ in solution. **(b)** Chronoamperometry experiment performed with 1 mM NAD^+ , 10 mM pyruvate and 0.57 mg mL^{-1} LDH in solution; potential applied: -0.51 V vs SHE. Black points: current of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; red point: [lactate] calculated by ^1H NMR; blue trace: NADH calculated by in-line UV-Vis. Conditions for **(a)** and **(b)**; flow rate: 4.17 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8

A chronoamperometry experiment was performed at the NAD^+ -red/aCP(H^+) electrode at a potential of -0.51 V vs SHE following addition of 10 mM pyruvate and 0.57 mg mL^{-1} LDH, with the results shown in Figure 5.14b. After the initial relaxation response, the reduction current increases to a value of about $-95\text{ }\mu\text{A}$ after 1 h , presumably as a result of slow mixing of the pyruvate and LDH through the flow system and due to increased wetting of the catalytic layer, or due to immobilisation of LDH onto the aCP(H^+) reaching an equilibrium. However, the current then decreases relatively rapidly, reaching a quarter of the initial current after only 4 h .

The predicted total conversion is shown by the red dashed trace and is calculated from the charge passed through the electrode over time. After 20 h , it is predicted that 2.3 mM lactate has been produced. However when compared to the ^1H NMR analysis of a sample taken from the bulk solution at 20 h , it seems that the ^1H NMR data gives a lactate concentration much larger than that predicted from the charge passed through the electrode. This could be due to improper mixing of the eluent and sampling the first extract from the electrochemical flow cell after the chronoamperometry, which would contain a higher concentration of lactate than the final sample to pass through the flow cell. Another possibility is that the sample had concentrated between the experiment and the ^1H NMR measurement, which would increase the concentration of lactate in the sample. The intermediate NADH is measured in-line with UV-Vis as indicated by the blue trace and as usual there is no build up of intermediate NADH in the bulk solution.

It is observed that there is a large benefit to pre-treating the aCP with concentrated acid, in that the maximum NAD^+ reduction current at the NAD^+ -red/aCP(H^+) is roughly five times larger as compared to when the aCP is not pre-treated with concentrated acid. Using the aCP for NAD^+ -red immobilisation shows that the enzyme is more mass transport limited when compared NAD^+ /BP electrodes, however this is partially due to the observed higher magnitude in catalytic reduction current. The next section investigates the effect of flow rate on the catalytic activity of the NAD^+ -red/aCP(H^+) electrode.

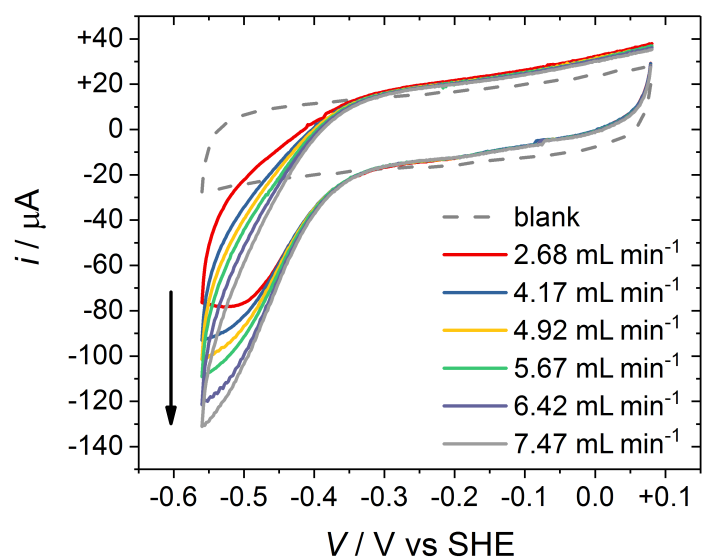
5.5.3 Investigating the effect of changing the flow rate on NAD^+ -red/aCP(H^+) electrodes

This section investigates the effect of flow rate on the electrochemical behaviour of a NAD^+ -red/aCP(H^+) electrode and also a chronopotentiometry experiment is performed on the electrode. The NAD^+ -red/aCP(H^+) is prepared the same way as before, with a blank cyclic voltammogram being recorded before NAD^+ -red immobilisation (grey dashed trace, Figure 5.15a). Once the top of the cell is put in place, buffer is flowed through the ‘star’ electrochemical flow cell with 1 mM NAD^+ in solution.

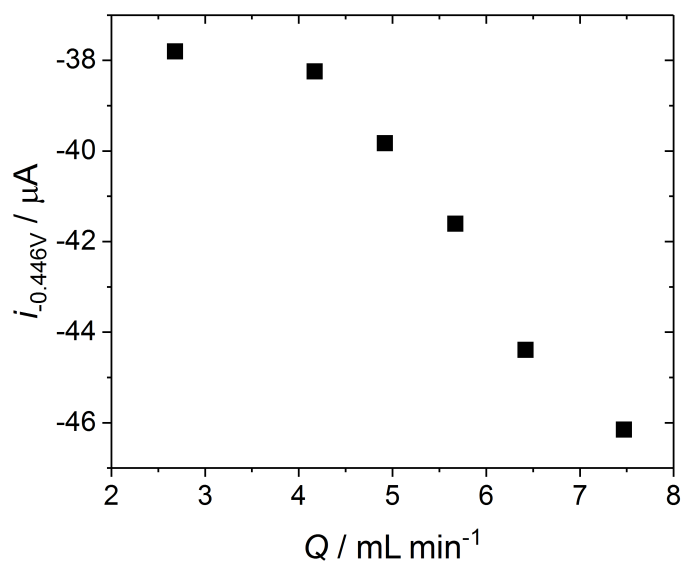
The cyclic voltammetry recorded at the NAD^+ -red/aCP(H^+) electrode is shown in Figure 5.15a. Except for the blank trace, all other traces are recorded with 1 mM NAD^+ in solution, but at different flow rates. The discharge capacitance for each trace is approximately 2000 μF , which is in line with what was observed before for similar same NAD^+ -red/aCP(H^+) electrodes. The red trace shows the cyclic voltammogram recorded at a flow rate of 2.68 mL min^{-1} , which indicates a maximum current of $-55 \mu\text{A}$. The increase in reduction current with increasing the flow rate is due to enhanced mass transport of substrate to the catalytic layer, increasing the activity of the NAD^+ -red/aCP(H^+) electrode. At a flow rate of 7.47 mL min^{-1} , the maximum flow rate for this pump and peristaltic flow tubing diameter, the maximum reduction current achieved at the electrode is $-90 \mu\text{V}$, as before.

The NAD^+ -red/aCP(H^+) electrode NAD^+ reduction current dependency on the flow rate through the ‘star’ electrochemical flow cell is plotted in Figure 5.15b. The reduction current was measured at a potential of -0.446 V vs SHE, a potential at which there is a maximum reduction gradient with no reduction peak showing. It appears that there is a linear correlation between NAD^+ reduction current of the NAD^+ -red/aCP(H^+) electrode and the flow rate, one that increases in magnitude with the flow rate. This is different to the relation with an RDE, where the current is related to the square root of the angular rotation rate of the electrode, as described by the *Levich equation*.

$$i_L = 0.620nFAD^{2/3}\nu^{-1/6}C\omega^{1/2} \quad (2.29 \text{ revisited})$$



(a)



(b)

Figure 5.15: Dependency on flow rate and chronopotentiometry of an NAD^+ -red/aCP(H^+) electrode. **(a)** Cyclic voltammetry at this electrode with 1 mM NAD^+ in solution at various flow rates. Grey dashed trace: recorded at an unmodified CP electrode with no substrate in solution. **(b)** Reduction current of the electrode at -0.446 V as a function of flow rate, Q . (cont.)

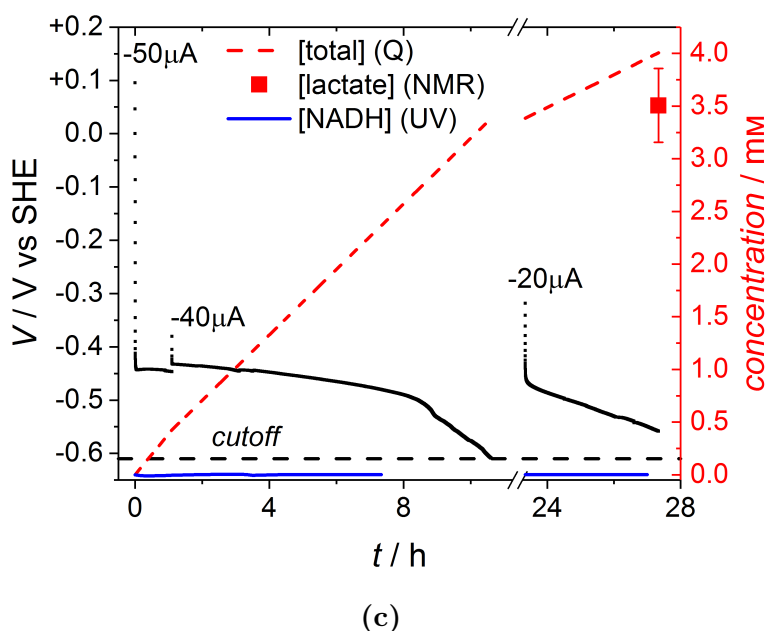


Figure 5.15: (cont.) Dependency on flow rate and chronopotentiometry of an NAD^+ -red/aCP(H^+) electrode. **(c)** Chronopotentiometry experiment performed with 1 mM NAD^+ , 10 mM pyruvate and 0.71 mg mL $^{-1}$ LDH in solution; applied currents are indicated on the plots. Black points: potential of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; red point: [lactate] calculated by ^1H NMR; blue trace: NADH calculated by in-line UV-Vis; black dashed line: potential cutoff at -0.61 V vs SHE . Flow rate: 7.47 mL min $^{-1}$. Conditions for **(a)** and **(c)**; electrolyte: 100 mM Tris-HCl buffer, pH 8.

A chronopotentiometry experiment was performed immediately after the cyclic voltammetry experiment, but with 10 mM pyruvate and 0.71 mg mL $^{-1}$ LDH injected into the bulk solution. The results are shown in Figure 5.15c, with the applied currents indicated on the plot. For the first hour, $-50 \mu\text{A}$ was applied, but the electrode was deemed to be becoming unstable after 1 h and so the applied current was changed to $-40 \mu\text{A}$ from 1 h onwards. The experiment was carried out over 12 h, during which the potential of the cell drifted from -0.43 V to the cutoff potential at -0.61 V . This presumably occurs due to deactivation of NAD^+ -red in the catalytic layer of the NAD^+ -red/aCP(H^+) electrode, or through losing NAD^+ -red into the bulk solution over time, causing the potential to drift more negative to be able to achieve the set current of $-40 \mu\text{A}$.

The experiment was restarted at about 24 h, after leaving the electrocatalyst inactive and under open circuit potential for 12 h. The $\text{NAD}^+\text{-red/aCP(H}^+)$ electrode was still electrochemically active, albeit at a lower applied current of $-20\text{ }\mu\text{A}$, which only lasted for a further 4 h.

The total predicted conversion, calculated from the charge passed through the electrocatalyst, is shown as a red dashed trace. After 28 h, it was predicted that a total of 4 mM of lactate was produced. This is in good agreement when compared to the ^1H NMR measurement taken from a sample at 28 h (red point), which indicates 3.5 mM conversion and a Faradaic efficiency of 87.5%. The concentration of NADH in the bulk solution was measured and it was found that there was no accumulation of NADH throughout the experiment.

This experiment indicates that, much like the RDE electrodes discussed in Chapter 4, the $\text{NAD}^+\text{-red/aCP(H}^+)$ electrodes in the ‘star’ electrochemical flow cell are highly dependent on the flow rate and require very high flow rates to alleviate mass transport limitations. However the direct immobilisation of the $\text{NAD}^+\text{-red}$ onto activated carbon paper that has been pretreated with concentrated HCl provides very high reduction currents and therefore more active electrocatalysts. It could also enable higher loading of the $\text{NAD}^+\text{-red}$ onto the electrode. The next section investigates the effect of increasing the amount of enzyme exposed to the $\text{aCP(H}^+)$ electrode for immobilisation.

5.5.4 Increasing the mass of $\text{NAD}^+\text{-red}$ exposed to the $\text{aCP(H}^+)$ for immobilisation

This section investigates the effect of exposing an $\text{aCP(H}^+)$ to an increased amount of $\text{NAD}^+\text{-red}$ for the enzyme immobilisation step. The $\text{NAD}^+\text{-red/aCP(H}^+)$ was prepared in the same way as described in Section 5.5.2 on page 170, except 20 μL of $\text{NAD}^+\text{-red}$ enzyme ($84.0 \times 10^{-3}\text{ mg}$) was exposed to $\text{aCP(H}^+)$ electrode instead of 10 μL .

After the $\text{NAD}^+\text{-red/aCP(H}^+)$ electrode was prepared, the top of the ‘star’ electrochemical cell was put in place and buffer was flowed through the electrochemical

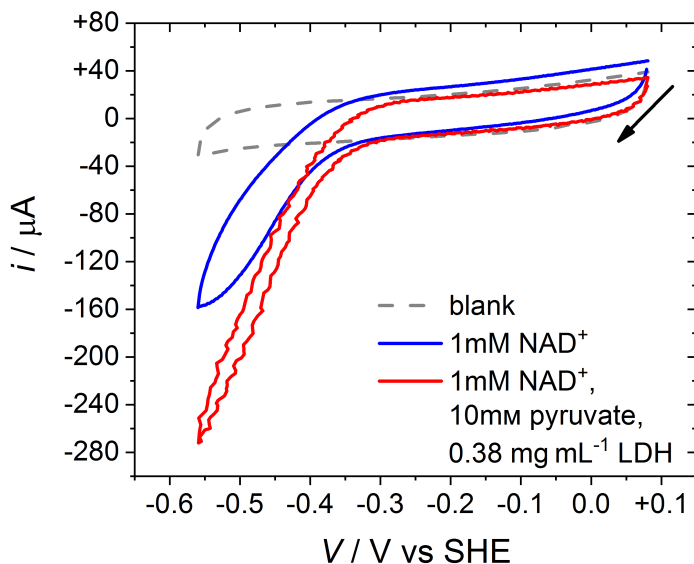


Figure 5.16: Cyclic voltammograms recorded at an unmodified aCP(H^+) (grey dashed trace), an aCP(H^+) electrode after being exposed to 20 μL NAD^+ -red with 1 mM NAD^+ in solution (blue) and then with 10 mM pyruvate and 0.38 mg mL^{-1} LDH injected into the solution (red). Flow rate: 7.47 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

flow cell with 1 mM of NAD^+ in solution. The blue trace in Figure 5.16 shows the cyclic voltammogram at this electrode with 1 mM NAD^+ in solution. The discharge current of the electrode is approximately 2000 μF as before and the onset potential for the reduction of NAD^+ is approximately -0.32 V. However, there is an increase in the maximum reduction current, from -90 μA as observed before, to -140 μA . This is due to more enzyme being exposed and therefore a higher loading on the aCP(H^+) electrode, or the aCP(H^+) electrode providing a more electrochemically active electrode. This maximum potential reached is limited by mass transport, as shown by the slope in the reduction wave. As this was the highest possible flow rate for this pump setup, the actual maximum current may be larger in magnitude.

The red trace shows the cyclic voltammogram recorded at the same NAD^+ -red/aCP(H^+) electrode, after 10 mM of pyruvate and 0.38 mg mL^{-1} of LDH was introduced into the bulk solution. This is the first presentation of a cyclic voltammogram recorded at an electrode with LDH present. The effect on the maximum reduction current is significant. The value for i_{max} increases from -140 μA to

$-250\text{ }\mu\text{A}$ at -0.56 V . One reason for this is that the LDH is using the catalytically produced NADH pushing the equilibrium forwards, which increases the drive for the reduction of NAD^+ . In addition, there is the enhanced NAD^+ concentration effect, as the LDH reoxidises NADH to NAD^+ at the surface of the electrode, whilst additionally there is mass transport of NAD^+ to the surface of the electrode supplied by the rate of flow of the bulk solution. Both of these effects probably contribute to the increase in the reduction current.

The turnover frequency related to the $250\text{ }\mu\text{M}$ reduction current, operational k_{cat} , is 8.19 s^{-1} , which is significantly larger than 0.24 s^{-1} calculated for the NAD^+ -red enzyme in Section 4.5.2.3 on page 123. This is a turnover number closer to that reported for similar heterogenous catalytic systems,^[52] and implies the enzyme is much more active under these conditions.

Another effect due to the presence of pyruvate and LDH in solution is that the electrode is no longer limited by mass transport, signified by the steep slope in the catalytic wave. This is due to the enhanced NAD^+ concentration effect at the surface of the electrode, as a result of reoxidation of NADH by LDH.

The electrochemistry in this section has implied that the exposure of aCP(H^+) to twice the amount of NAD^+ -red in preparation of the electrocatalyst nearly doubles the catalytic current. It also shows that for this electrode, the inclusion of LDH and pyruvate in solution vastly increases the reduction current.

5.5.5 Investigating the effect of increasing the immobilisation time for developing NAD^+ -red/aCP(H^+) electrodes

This section investigates the effect on increasing the duration of NAD^+ -red incubation onto aCP(H^+) electrodes. An NAD^+ -red/aCP(H^+) electrode was prepared in a similar fashion as described in Section 5.5.4 on page 177, however this time increasing the incubation time from 2 h to overnight. The top of the ‘star’ electrochemical flow cell was secured and buffer flowed through the cell with 1 mM NAD^+ in solution.

Cyclic voltammetry experiments were performed at the NAD^+ -red/aCP(H^+) electrode with the results shown in Figure 5.17. The grey dashed line shows a blank

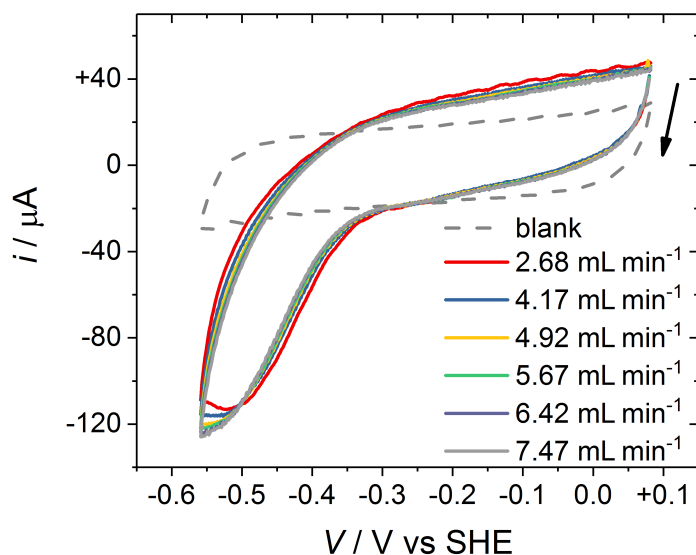


Figure 5.17: Cyclic voltammetry at an NAD^+ -red/aCP(H^+) electrode after incubating overnight, at various flow rates and with 1 mM NAD^+ in solution. Grey dashed trace: unmodified aCP(H^+) electrode with no substrate in solution. Electrolyte: 100 mM Tris-HCl buffer, pH 8.

recorded at an unmodified aCP(H^+) electrode with no substrate in solution. The other coloured traces are recorded at an NAD^+ -red/aCP(H^+) electrode with 1 mM NAD^+ in the bulk solution, with increasing flow rate. The discharge capacitance for the NAD^+ -red/aCP(H^+) electrode is approximately $2000 \mu\text{F}$, as observed with similar electrodes described earlier. The onset potential is again approximately -0.32 V vs SHE. At the lowest flow rate, 2.68 mL min^{-1} , the reductive wave peaks at -0.52 V with a current of $-90 \mu\text{A}$. This is not as high in magnitude as observed with the NAD^+ -red/aCP(H^+) electrode with a 2 h enzyme incubation time, as shown in Figure 5.16. This could be due to more enzyme deactivating after the longer incubation, leading to a less active NAD^+ -red/aCP(H^+) electrode.

As the flow rate is increased, the reduction current at potentials more positive than -0.46 V does not increase in magnitude. It is observed that the reduction current only increases at the most negative potentials. This suggests that mass transport only affects the slower rate processes at potentials more negative than -0.51 V .

Therefore in the preparation of any NAD^+ -red/aCP(H^+) electrode, an overnight

incubation time does not give a benefit in the catalytic activity of the NAD^+ -red/aCP(H^+) electrode and so the incubation time for NAD^+ onto aCP(H^+) electrodes was kept at 2 h. The next section investigates the effect of coimmobilising an LDH with the NAD^+ -red onto the aCP(H^+) electrodes on the electrochemical behaviour of the resultant LDH/BP/aCP(H^+)- NAD^+ -red/aCP(H^+) electrode.

5.5.6 Coimmobilising NAD^+ -red with LDH/BP particles onto aCP(H^+) electrodes

An aCP(H^+) electrode was prepared with NAD^+ -red enzyme and LDH/BP particles immobilised onto the surface. The electrode was prepared in the same fashion as a NAD^+ -red/aCP(H^+) electrode described in Section 5.5.4 on page 177. Meanwhile, LDH/BP particles were prepared by incubating 0.6 mg of LDH with BP nanoparticle slurry (10 μL , 20 mg mL^{-1}) for 2 h at 4 °C. After the first hour of NAD^+ -red incubation onto aCP(H^+) electrode, the LDH/BP nanoparticles were spotted onto the aCP(H^+) electrode to join the NAD^+ -red for the second hour of incubation. The ‘star’ electrochemical flow cell head was then put into place and buffer was flowed through the cell, with 1 mM NAD^+ in solution.

Electrochemistry experiments were performed immediately at this working electrode, as shown in Figure 5.18a. The grey dashed trace shows the cyclic voltammogram recorded at an unmodified aCP(H^+) electrode, with no cofactor or substrate in solution. The blue trace shows the response at the LDH/BP/aCP(H^+)- NAD^+ -red/aCP(H^+) electrode with 1 mM NAD^+ in solution. The discharge capacitance of this electrode is approximately 3000 μF , an increase compared to similar electrodes described previously in this section, consistent with the increase in the electrode surface area, with the presence of BP nanoparticles on the surface of the aCP(H^+). The onset potential for NAD^+ reduction is observed again at -0.32 V . The maximum magnitude of the reduction of NAD^+ (i_{max}) at this electrode is $-110\text{ }\mu\text{A}$ at -0.56 V , a similar magnitude to that observed at the NAD^+ -red/aCP(H^+) electrode in Section 5.5.4. It is also observed to be limited by mass transport, as the current drops off at more negative potentials.

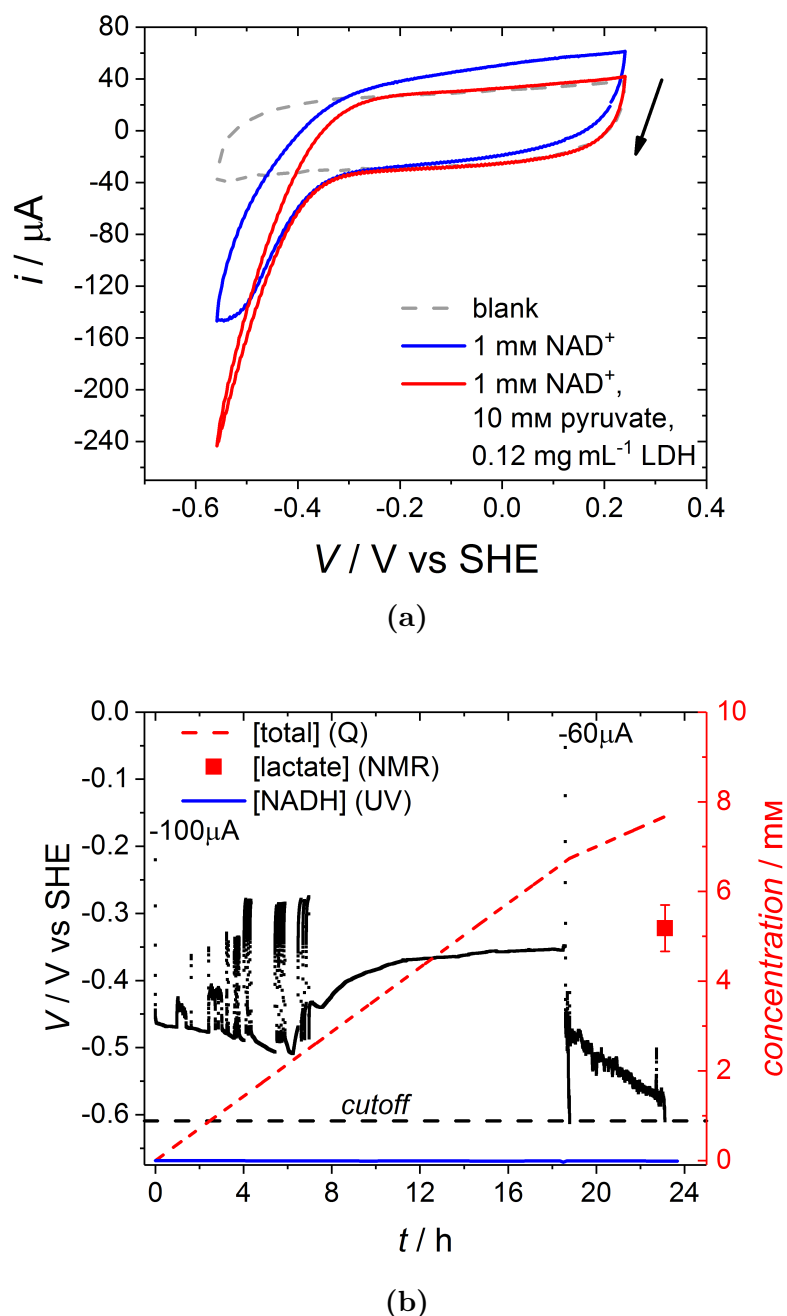


Figure 5.18: Electrochemistry of an LDH/BP/aCP(H⁺)-NAD⁺-red/aCP(H⁺) electrode in flow. **(a)** Cyclic voltammetry recorded at this electrode with 1 mM NAD⁺ in solution (blue trace) and then additionally with 10 mM pyruvate and -0.12 mg mL^{-1} in solution (red trace). Grey dashed trace indicates a blank recorded at the unmodified aCP(H⁺) electrode with no substrate in solution. **(b)** Chronopotentiometry recorded at $-100 \mu\text{A}$ at the same electrode, with 1 mM NAD⁺, 10 mM pyruvate and 0.12 mg mL^{-1} LDH in solution. Black points: potential of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; red point: [lactate] calculated by ^1H NMR; blue trace: [NADH] calculated by in-line UV-Vis; black dashed line: potential cutoff at -0.61 V vs SHE. Conditions for **(a)** and **(b)**; flow rate: 7.68 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

After addition of 10 mM pyruvate and 0.12 mg mL⁻¹ LDH is added into solution, the reduction peak of NAD⁺ increases in magnitude from -110 μ A to -210 μ A (Figure 5.18a, red trace), consistent with NAD⁺ concentration enhancement at the surface of the electrode.

It is also possible to observe the reversibility of the NAD⁺-red-derived electrodes for NAD⁺ reduction. The blue trace shows slight oxidation of NADH, with an onset potential of -0.32 V and an oxidation current of +30 μ A at +0.2 V. This oxidation is not observed with the red trace, which is identical to the blank cyclic voltammogram at more positive potentials than -0.32 V. This is because the presence of the substrate for LDH, pyruvate, also requires the NADH cofactor for the reduction of pyruvate to lactate, draining the accumulation of any NADH at the surface of the electrode and in the bulk solution.

A chronopotentiometry experiment was performed after the cyclic voltammetry experiments, with the results shown in Figure 5.18b, at first applying a current of -100 μ A which was achieved at a potential of -0.46 V which decreased over time. However, what can be seen is that this potential response is not stable at all, with many 'spikes' observed between 0 h and 8 h. This was also observed with other high current chronopotentiometry experiments. This is explained by the Pt counter electrode not having a large enough surface area to be able to balance the charge of electrons needed for reduction of NAD⁺ at the LDH/BP/aCP(H⁺)-NAD⁺-red/aCP(H⁺) electrode. Therefore the potentiostat protects itself from damage by tripping to open circuit potential, which is at more positive potentials compared to the NAD⁺ reduction potential. Between 8 h and 18 h, the potentiostat is not applying any current at all, and the potential of the cell relaxes to the open circuit potential. After 18 h, the experiment is reset and a smaller reductive current of -60 μ A is applied. From then on, the counter electrode is able to balance the charge from the cathode reaction and the experiment is able to run at -60 μ A for 5 h more.

This is also observable when comparing the total concentration of NADH and lactate (red dashed line) produced after 24 h to the analytically measured concentrations for lactate (red point, by ¹H NMR) and NADH (blue trace, by in-line

UV-Vis). The ^1H NMR indicates 5.2 mM of lactate has been produced, as compared to 7.8 mM as predicted by the charge transferred at the cathode, a Faradaic efficiency of 66.7%, assuming a constant current. This discrepancy is mainly due to the the period of open circuit potential between 8 h and 18 h, where the reductive current is effectively zero at the LDH/BP/aCP(H^+)- NAD^+ -red/aCP(H^+) electrode. Taking this period of inactivity into account, the corrected prediction is 4.2 mM lactate and NADH generated at the cathode. This is more in line with the ^1H NMR result now slightly overcalculating the amount of lactate produced. The blue trace indicates that there is not any accumulation of NADH in the bulk solution.

The observed effect of the potentiostat tripping if the counter electrode cannot balance the cathodic charge is problematic, if electrocatalytic reductions are to be performed with large currents well into the 100s of μA . The counter electrode which has been used, a Pt wire within a thin small volume counter electrode compartment, is not suitable for these high current experiments. Therefore a redesign of the counter electrode and the counter electrode compartment was necessary.

5.6 Development of the two chamber ‘star’ electrochemical flow cell

In the previous section, it was observed that the counter electrode that had been used, a Pt wire in a very small volume compartment, is not suitable to use as a counter electrode during very high current electrocatalytic reduction experiments. The wire is not able to balance the charge passing at the cathode, resulting in the potentiostat tripping to open circuit potential for protection. Therefore a different approach to the counter electrode is necessary to proceed with high current electrocatalytic reduction reactions.

5.6.1 Developing a carbon felt high surface area counter electrode

A new counter electrode was manufactured for use in the ‘star’ electrochemical flow cell. Carbon fibre material offers a very high surface area and is very malleable,

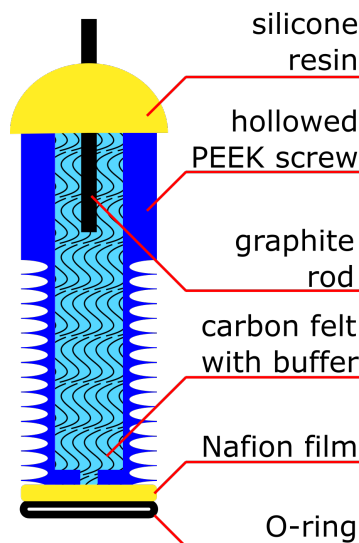


Figure 5.19: Diagram of the carbon felt counter electrode for the ‘star’ electrochemical flow cell.

so a lot can be put in small spaces. Therefore the plan was to use carbon felt as the counter electrode and to use a hollowed out PEEK screw as the second cell chamber for the carbon felt, as shown in Figure 5.19.

A PEEK screw fitting was hollowed out to increase the inside volume of the screw. The inside was then filled with carbon felt and filled the reaction buffer only (100 mM Tris-HCl, pH 8). A carbon rod was inserted through the top into the carbon felt, to form a connection between the carbon felt and the outside of the counter electrode compartment. The carbon rod was sealed in by use of a silicone resin. Before screwing the counter electrode compartment into the head of the ‘star’ electrochemical flow cell, a piece of Nafion™ 117 film, of thickness 183 μm , was placed between the bottom of the screw and an O-ring. Nafion™ is a type of proton exchange membrane, often used to separate the anode and cathode compartments of fuel cells. In this instance, it is replacing the use of the molecular sieve in the previous setup, in order to avoid reoxidation of the reduction products at the working electrode (cathode).

The next section discusses the use of carbon felt as a counter electrode, within a couple of electrocatalytic reduction experiments.

5.6.2 Using a carbon felt counter electrode with a two chamber ‘star’ electrochemical flow cell

Firstly, an NAD^+ -red/aCP(H^+) electrode was prepared in a similar fashion as described in Section 5.5.4. Cyclic voltammetry was performed immediately after incubation at this electrode, once the top of the ‘star’ electrochemical flow cell was secured in place, with the results shown in Figure 5.20a. The grey dashed trace shows a blank cyclic voltammetry experiment performed at an unmodified aCP(H^+) electrode, with no substrate in the solution. The blue trace shows the cyclic voltammetry performed at the NAD^+ -red/aCP(H^+) electrode with 1 mM NAD^+ in solution. The discharge capacitance of this electrode is approximately 2000 μF and the onset potential for NAD^+ reduction is -0.32 V , both values in line with previous experiments. The maximum reduction current achieved is $-70\text{ }\mu\text{A}$, although the sloping nature of the downwards arm of the reduction wave indicates that this current is limited by mass transport. It was observed that increasing the flow rate further did not affect the shape of the NAD^+ reduction peak. NADH oxidation is also observed, achieving an oxidation potential of $+10\text{ }\mu\text{A}$ at $+0.2\text{ V}$, with an onset potential of -0.32 V , indicating the reversibility of of this electrocatalytic reduction. The noise of the voltammogram is a result of fine tuning of the counter electrode position being necessary, to avoid impacting the flow inside the electrochemical flow cell chamber.

The red trace shows the cyclic voltammogram recorded at the same electrode, this time with an additional 10 mM pyruvate and 0.18 mg mL^{-1} of LDH in the bulk solution. There is a slight increase in the maximum reduction current, i_{max} , from $-70\text{ }\mu\text{A}$ to $-85\text{ }\mu\text{A}$ at -0.56 V , as a result of the enhanced NAD^+ concentration effect at the surface of the electrode as a result of LDH reoxidising the NADH to NAD^+ . This also continuously drives the equilibrium forward, increasing the activity of the electrocatalyst further. The NADH oxidation current is now not observed, as the electrocatalytically generated NADH is consumed immediately by the LDH.

A chronopotentiometry experiment was performed at this electrode, with an applied current of -40 A , with the results shown in Figure 5.20b. The potential of the

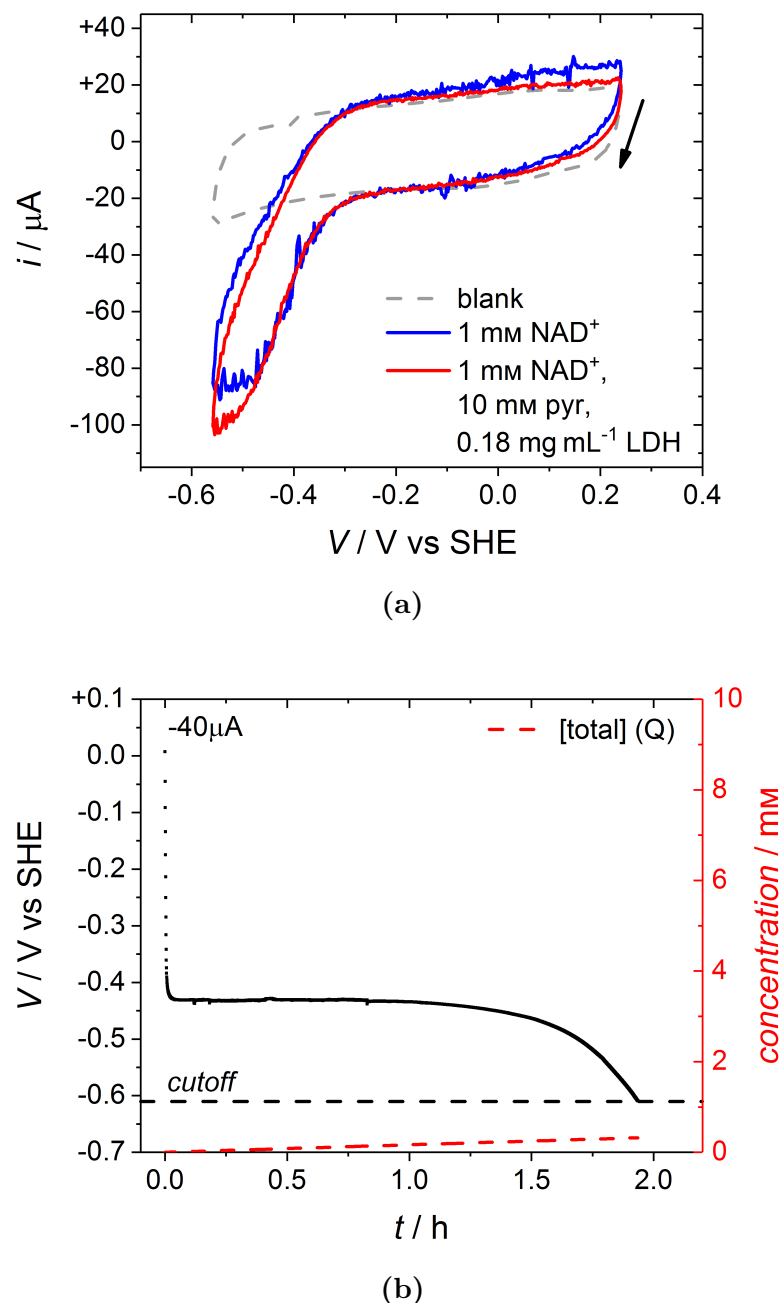


Figure 5.20: Electrochemistry of an NAD^+ -red/aCP(H^+) electrode in a two chamber electrochemical flow cell. **(a)** Cyclic voltammetry recorded at this electrode with 1 mM NAD^+ in solution (blue trace) and then additionally with 10 mM pyruvate and 0.18 mg mL^{-1} LDH in solution (red trace). Grey dashed trace indicates a blank recorded at the unmodified aCP(H^+) electrode with no substrate in solution. **(b)** Chronopotentiometry recorded at $-40 \mu\text{A}$ at the same electrode, with 1 mM NAD^+ , 10 mM pyruvate and 0.18 mg mL^{-1} LDH in solution. Black points: potential of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; black dashed line: potential cutoff at -0.61 V vs SHE . Conditions for **(a)** and **(b)**; flow rate: 7.47 mL min^{-1} ; electrolyte: 100 mM Tris-HCl buffer, pH 8.

cell is stable for the first 1.5 h, before drifting to the cutoff potential of -0.61 V at 2 h. This is attributed to a degradation of the catalytic layer inside the cell. The predicted total concentration of lactate and NADH calculated from the charge passed through the working electrode is indicated by the red dashed trace, which reveals only $0.4\text{ }\mu\text{M}$ of lactate and NADH is predicted after 2 h from the charge passed.

The important aspect of this experiment is to test the suitability of the carbon felt as a counter electrode and the preparation of the counter electrode compartment for high current experiments. Although this chronopotentiometry only lasted 2 h and the current was lower than before, the recorded potential was very stable using this counter electrode and there were no potential jumps as a result of potentiostat tripping. This indicates that this carbon felt counter electrode arrangement could balance the charge passing at the cathode and would be suitable for long term electrocatalytic experiments.

The next experiment, as shown in Figure 5.21, is a repeat of the previous experiment, however a different grade of activated carbon paper, aCP(H^+) of grade P75, is used.

A cyclic voltammetry experiment was performed at this electrode and the results are shown in Figure 5.21a. The grey dashed line shows a blank cyclic voltammogram. The blue trace shows the voltammogram recorded at the NAD^+ -red/aCP(H^+) electrode with 1 mM NAD^+ in solution. The discharge capacitance of this electrode made using P75 aCP(H^+) is slightly higher than before with a value of $3000\text{ }\mu\text{F}$ as compared to $2000\text{ }\mu\text{F}$. This is due to the different grade of activated carbon paper being used, generating a larger double layer capacitance effect. There is NAD^+ reduction observed, with the onset potential of reduction again at -0.32 V matching that of the equilibrium potential of the NAD^+/NADH couple. The maximum NAD^+ reduction current achieved is $-70\text{ }\mu\text{A}$, and this appears to be limited by mass transport, as the reduction peak is clearly visible at -0.48 V. Upon addition of 10 mM pyruvate and 0.19 mg mL^{-1} LDH (red trace), the reduction current only increases in magnitude a little to $-80\text{ }\mu\text{A}$, as a result of the enhanced NAD^+ concentration at the surface of the electrode, as discussed before. The magnitude

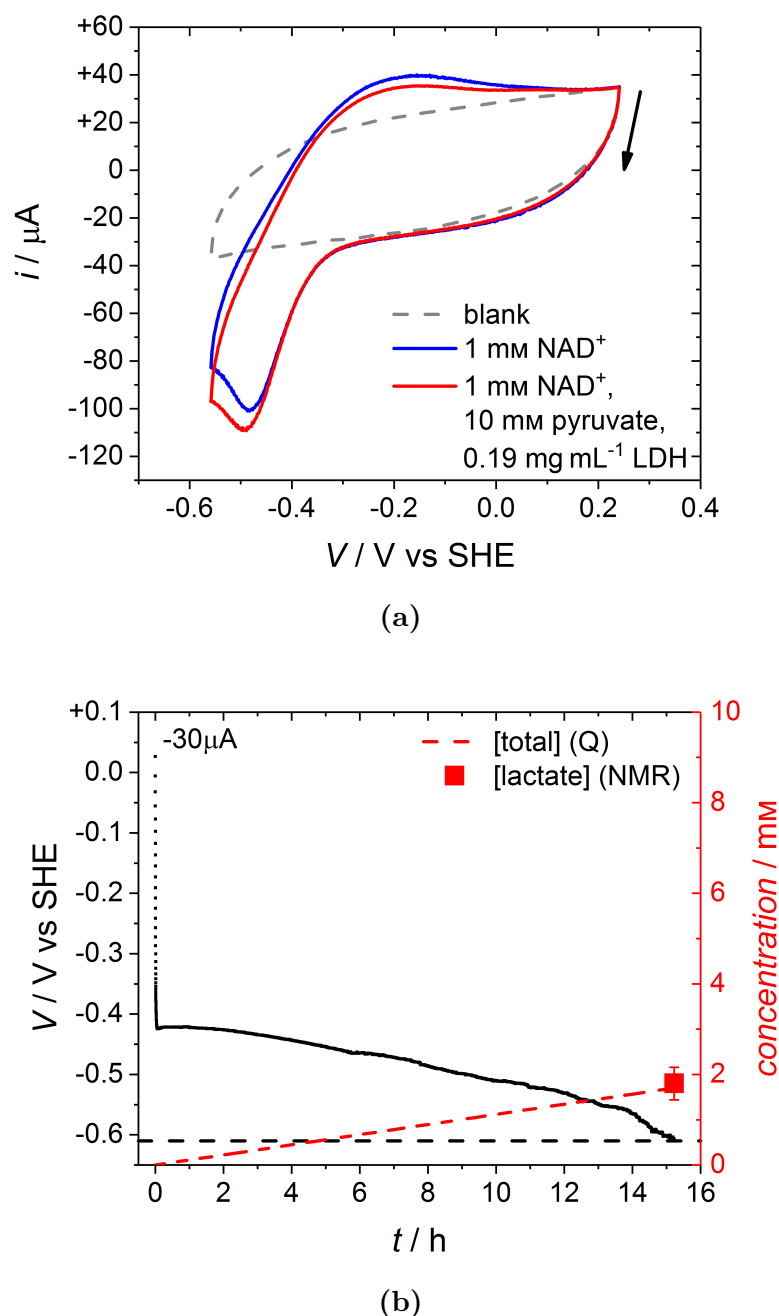


Figure 5.21: Electrochemistry of an NAD^+ -red/aCP(H^+) electrode in a two chamber electrochemical flow cell with P75 aCP. **(a)** Cyclic voltammetry recorded at this electrode with 1 mM NAD^+ in solution (blue trace) and then additionally with 10 mM pyruvate and 0.19 mg mL^{-1} LDH in solution (red trace). Grey dashed trace indicates a blank recorded at the unmodified aCP(H^+) electrode with no substrate in solution. **(b)** Chronopotentiometry recorded at $-30 \mu\text{A}$ at the same electrode, with 1 mM NAD^+ , 10 mM pyruvate and 0.19 mg mL^{-1} LDH in solution. Black points: potential of cell; red dashed trace: total lactate and NADH generated as predicted by the charge passed; red point: [lactate] calculated by ^1H NMR; black dashed line: potential cutoff at -0.61 V vs SHE . Conditions for **(a)** and **(b)**; flow rate: $11.17 \text{ mL min}^{-1}$; electrolyte: 100 mM Tris-HCl buffer, pH 8.

of the reduction peak did not increase with a further increase in the flow rate of the bulk solution. These observations could possibly be due to the different topology of the P75 grade of the aCP(H⁺), severely limiting the mass transport of the enzyme substrates to the enzymes.

There is an oxidation peak observed at a potential of -0.18 V , which could be attributed to NADH reoxidation to NAD⁺. This could be due to cofactor becoming stuck at the surface of the P75 aCP(H⁺) electrode, or perhaps with reduced mobility to move between the NAD⁺-red immobilised on the surface of the electrode and the LDH in solution.

A chronopotentiometry was performed at this electrode, with an applied current of -30 h and the results shown in Figure 5.21b. At this current, the potential of the cell drifted over 15.5 h from an initial potential of -0.43 V to the cutoff potential of -0.61 V . There were no potential spikes as observed frequently with the previous Pt counter electrode, though the applied current was not as high before. The total predicted concentration of NADH and lactate after 15.5 h calculated from the charge passed at the working electrode, is in agreement with the measured concentration of lactate measured by ^1H NMR, giving a high Faradaic efficiency of 100%. The concentration of NADH in the bulk solution was not measured in line, but it can be assumed there was no accumulation of intermediate NADH because of the high Faradaic efficiency.

These experiments indicate that the carbon felt counter electrode, with the higher surface area, is able to balance the high magnitude of charge being passed at the cathode. It is anticipated now that this is a good setup for further chronopotentiometry experiments that run for many hours at high reduction currents.

5.7 Summary

This chapter has discussed the application of the NAD⁺-red-LDH bioelectrocatalyst to electrochemical flow cells, for the selective reduction of pyruvate, a prochiral C=X containing substrate, to lactate. The use of the electrochemical flow cell brings good mass transport of the substrates to the electrode and is similar to

the induced convection at rotating RDEs. The use of the ‘star’ electrochemical flow cell brings greater stability of the potential of the cell to be able to run high reduction currents for many hours.

For the development of particle based NAD⁺-red/BP/CP electrodes, it was found that immobilising the NAD⁺-red enzyme with the BP slurry (20 mg mL⁻¹) with a mass ratio of 4.8 produced particle derived electrodes with high reduction currents and good catalytic stability. With a lower mass of BP, the reduction current of the electrocatalyst was lower, indicating less NAD⁺-red immobilisation or more crowding of the enzyme on the particles. With a greater mass of BP, the discharge capacitance was much larger and overwhelmed the reduction current. The electrocatalyst was also less stable.

It was calculated that using carbon paper, which has a larger geometrical surface area than PGE electrodes, meaning that more enzyme can be loaded onto the carbon electrodes. This generates a greater reduction current overall. However, the reduction current per enzyme or per geometrical surface area was not as efficient. This highlights the ideality of the PGE RDE for electrochemical analysis and indicates the larger and cheaper carbon paper electrodes for use in the electrochemical flow cells are not as current dense as the PGE RDE. However, this compromise is necessary in developing an electrosynthetic system. It was also found that the mass of the BP particles was linearly correlated to the average discharge capacitance of porous carbon electrodes, therefore the BP nanoparticles is the main contributor to the total surface area of the electrodes, rather than the PGE electrode or the carbon paper electrodes.

Direct immobilisation of NAD⁺-red onto activated carbon paper that had been pretreated with concentrated HCl generated NAD⁺-red/aCP(H⁺) electrodes that provided very large reduction currents, up to $-140\text{ }\mu\text{A}$. This reduction current was increased further up to $-250\text{ }\mu\text{A}$ when LDH and pyruvate were injected into the bulk solution, showing the increased activity of the NAD⁺-red/aCP(H⁺) electrode due to the enhanced NAD⁺ concentration effect at the surface of the electrode. With these electrodes it was also noticed that the reduction current increased in

magnitude linearly with the increase in flow rate. It was possible to coimmobilise LDH/BP particles onto the NAD^+ -red/aCP(H^+) electrodes.

Finally, the development of the carbon felt electrode, with a counter electrode compartment in a PEEK screw fastening alleviated potential cutouts at the working electrode. This is due to the increase in the surface area of the counter electrode, enabling the counter electrode to balance the charge from the large reduction currents at the cathode, therefore completing the circuit safely.

The next chapter takes further analysis of the ‘star’ electrochemical flow cell and looks at the reduction current dependency of the NAD^+ -red/aCP(H^+) electrodes on the potential applied to the cell. In addition, cofactor product analysis was performed to investigate the selectivity of the cell as a response to potential.

6

Flow electrochemistry for cofactor, product and potential optimisation

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The previous two chapters have discussed the development of an enzymatic electrocatalyst and its application to flow electrosynthesis. In this chapter, the flow cell setups described in Chapter 5 are utilised for the optimisation of the cell potential by analysing the cofactor and products of the electrocatalytic reductions.

6.1 Introduction to the experimental setups for comparison

Figure 6.1 shows the two electrocatalytic systems on which electrochemical experiments and their results are directly compared. Both the electrodes in Fig-

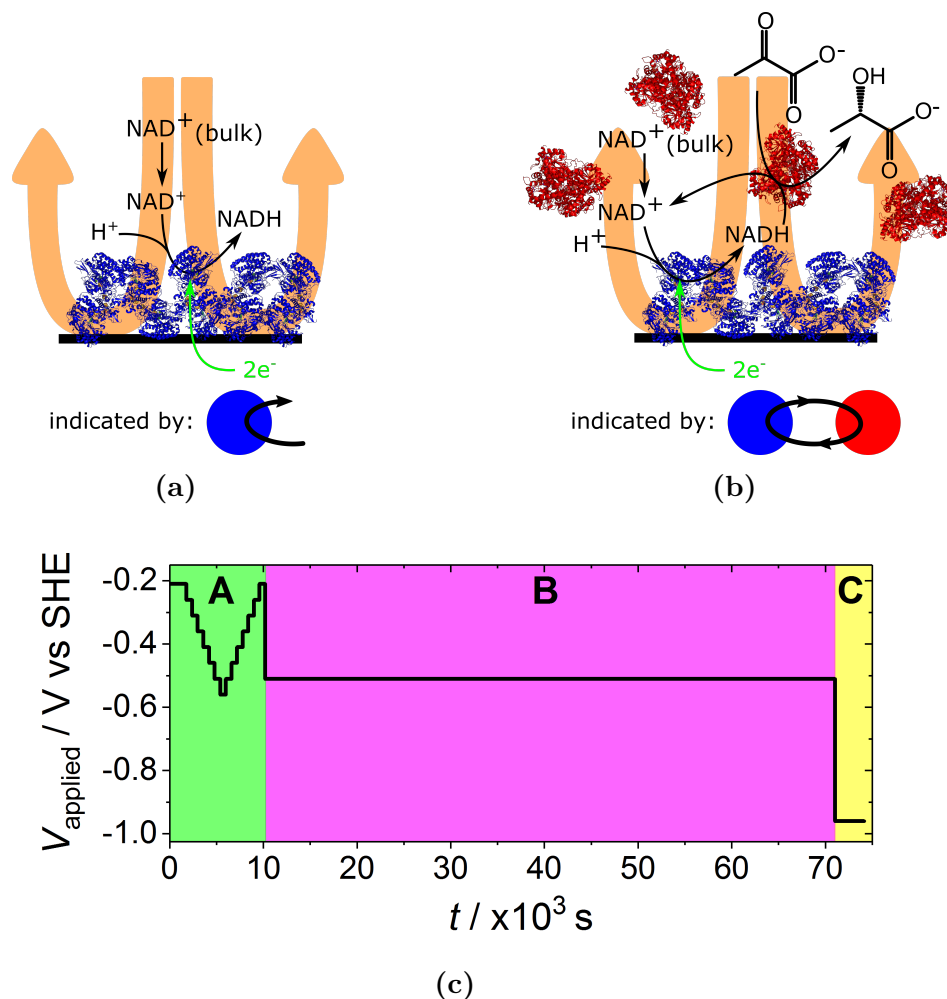


Figure 6.1: Electrochemical flow cell experiment setups in this chapter. (a) NAD^+ reduction using NAD^+ -red immobilised on P75 aCP(H^+). (b) Pyruvate reduction using NAD^+ -red immobilised on P75 aCP(H^+) and LDH in solution. (c) The potential profile for the experiments performed in this Chapter. **A**: potential step experiment, **B**: chronoamperometry at -0.51 V vs SHE, **C**: chronoamperometry at -0.96 V vs SHE.

Figure 6.1a and Figure 6.1b are identical and made in the same way. They are both NAD^+ -red/aCP(H^+) electrodes made with P75 activated carbon paper, prepared by incubating $20 \mu\text{L}$ of NAD^+ -red (1.4 mg mL^{-1}) for 2 h at 4°C on aCP(H^+), activated carbon paper of grade P75 which has been pretreated with concentrated HCl. After incubation, the top of the ‘star’ electrochemical flow cell was put into place and 100 mM Tris-HCl pH 8 buffer with 1 mM NAD^+ was flowed through the top of the cell. For electrode (b), LDH and pyruvate is additionally provided in the bulk solution.

For the experiment shown in Figure 6.1a, this is the only preparation performed for the observation of NAD^+ reduction. However in the second experiment as shown in Figure 6.1b, there is additionally a second NADH-dependent enzyme, 1 mg LDH, present in solution. This allows for the consumption and reoxidation of NADH to NAD^+ during the reduction of pyruvate to lactate in solution.

Figure 6.1c shows the potential profile of the experiments performed in this Chapter. Period **A** shows the multi-potential step experiment performed at both electrodes **(a)** and **(b)**, as described in Section 6.2. Period **B** shows the potential profile for the chronoamperometry performed at -0.51 V vs SHE and period **C** the chronoamperometry performed at -0.96 V vs SHE, both described in Section 6.3.

This chapter investigates the effect of the electrode potential on the current response, with and without a second enzyme reoxidising the NADH cofactor. The electrocatalyst reduction products are also analysed with UV-Vis spectroscopy, HPLC and ^1H NMR spectroscopy. The next section investigates the catalytic activity at a range of potentials, from a region of no reductive activity, to -0.51 V vs SHE.

6.2 Potential step chronoamperometry: potential profile A

The effect of the change in applied potential to each electrode on the electrocatalytic activity on NAD^+ reduction was investigated. The potential applied to the cell was stepped successively more negative from a region of no catalytic activity down to -0.51 V, before again increasing the potential of the cell stepwise to the ‘non-catalytic’ region. Solution samples were taken after a short pause of the electrocatalyst at each potential and the cofactor concentrations were determined by UV-Vis spectroscopy. First, the electrochemistry results are discussed.

6.2.1 Electrochemistry

First, cyclic voltammetry was performed at each electrode, as shown in Figure 6.2. For each electrode, a blank cyclic voltammogram (grey trace) was recorded at the

unmodified aCP(H⁺) electrode, with no cofactor or substrates in solution. The discharge capacitance was similar for both electrodes 500 μ F for **(a)** and 700 μ F for **(b)**, but the difference is likely to be due mainly by electrode-to-electrode variation.

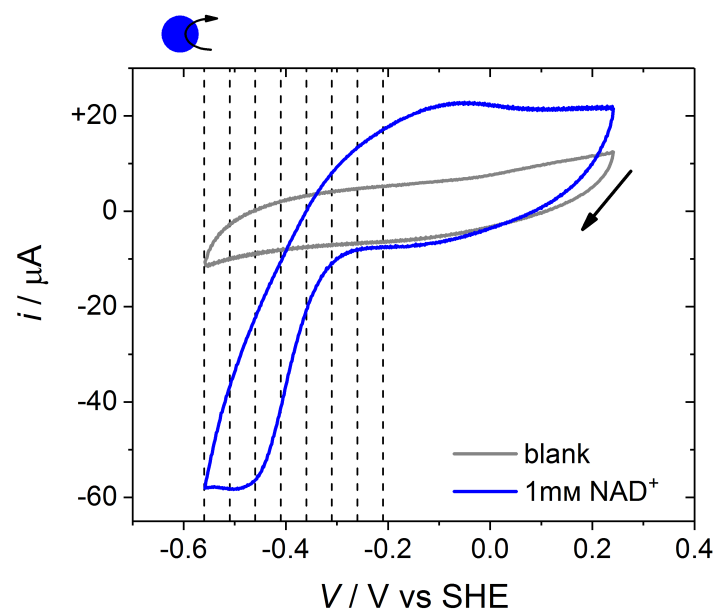
A cyclic voltammogram was recorded at the NAD⁺-red/aCP(H⁺) electrodes with 1 mM NAD⁺ in solution, as shown by the blue traces in Figures 6.2a and 6.2b. Both electrodes show a clear catalytic wave for 1 mM NAD⁺ reduction, which have an approximate onset potential of -0.32 V. The maximum reduction current was found to be mass transport limited in both cases, with the catalytic current dropping off at negative potential (high driving force). It was observed that on increasing the flow rate the current response did not change, therefore the flow rate of $11.17 \text{ mL min}^{-1}$ was chosen. This effect was noticed at the previous electrodes constructed from aCP(H⁺) with grading P75, and suggests the mass transport limitation occurs within the electrode itself. Nevertheless the high currents and stabilities make this carbon electrode material worth pursuing.

The i_{max} for **(a)** was approximately $-48 \mu\text{A}$ and the i_{max} for **(b)** is approximately $-30 \mu\text{A}$. The difference in reduction currents could be aligned with the higher discharge capacitance for the electrode in **(b)**, which suggests that there is less active enzyme on the surface of the electrode. Alternatively, there could be a small variance in the way the electrode was prepared.

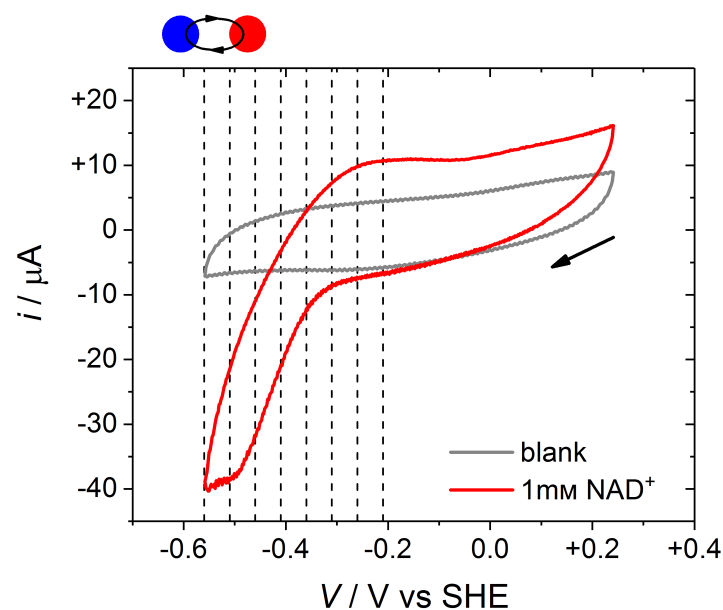
Also shown on the plots in Figure 6.2 are the potentials that will be applied at each electrode in the multi-step chronoamperometry experiment (Figure 6.4), as shown by the grey dashed vertical lines. This is discussed in further detail later on.

For electrode **(b)**, the current was measured when the substrate for LDH, pyruvate, was injected into the bulk solution. Figure 6.3 shows the current response at this electrode to different applied potentials shown on the plot, and upon the injection of 10 mM pyruvate as indicated by the single-headed arrow on the plot. The red shaded section indicates the region where pyruvate is present in solution.

The first potential applied is -0.21 V, a potential more positive than that for the NAD⁺/NADH couple, meaning that there is no reduction current as a result of NAD⁺ reduction and negligible NADH oxidation current. This provides a baseline



(a)



(b)

Figure 6.2: Cyclic voltammetry plots of two NAD^+ -red/aCP(H^+) electrodes in a two chamber electrochemical flow cell with an aCP of grade P75, (a) without, and (b) with LDH in the bulk solution. Blue trace: with 1 mM in solution; grey trace: black voltammetry recorded at an unmodified aCP(H^+) electrode with no NAD^+ or pyruvate in solution. Vertical dashed lines indicate the potentials that were applied to the electrode for the multi-step chronoamperometry experiment (Figure 6.4). Flow rate: $11.17 \text{ mL min}^{-1}$; electrolyte: 100 mM Tris-HCl buffer, pH 8.

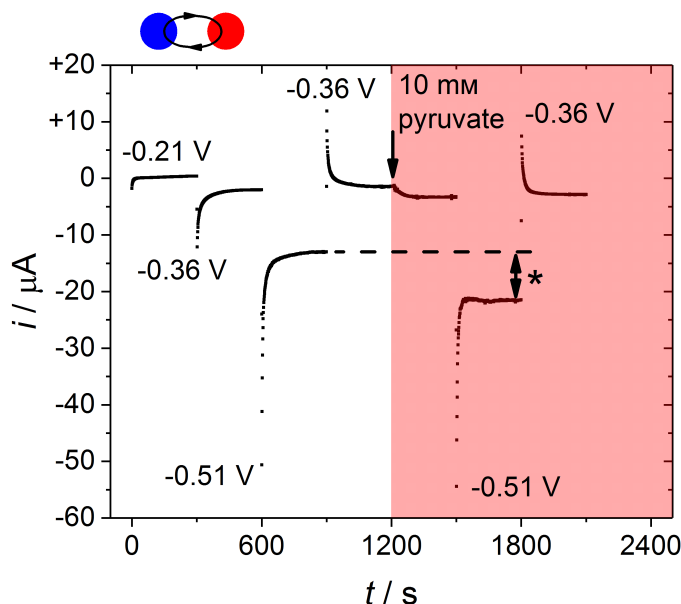


Figure 6.3: Chronoamperometric response to an injection of pyruvate at an NAD^+ -red/aCP(H^+) electrode with LDH in solution.

current very close to $0 \mu\text{A}$. A potential of -0.36 V is then applied at 300 s, a potential chosen that has a minimal response in reduction current. The current response steadies out at about $-3 \mu\text{A}$, before a potential of -0.51 V is applied at 600 s. This significantly increases the current response of the NAD^+ -red/aCP(H^+) electrode to $-13 \mu\text{A}$, consistent with the greater driving force for catalysis.

The potential is then set back to -0.36 V at 900 s, where the current restores to a slightly lower value of $-2 \mu\text{A}$ as a result of the catalyst deactivating or NAD^+ -red enzyme falling into the bulk solution, decreasing the activity of the catalyst. At 1200 s, 10 mM pyruvate is introduced into the bulk solution, increasing the reduction current from $-2 \mu\text{A}$ to $-5 \mu\text{A}$. This indicates that the LDH is reoxidising the cofactor, NADH, to NAD^+ whilst also reducing pyruvate to lactate, thus creating an enhanced concentration of NAD^+ at the surface of the electrode.

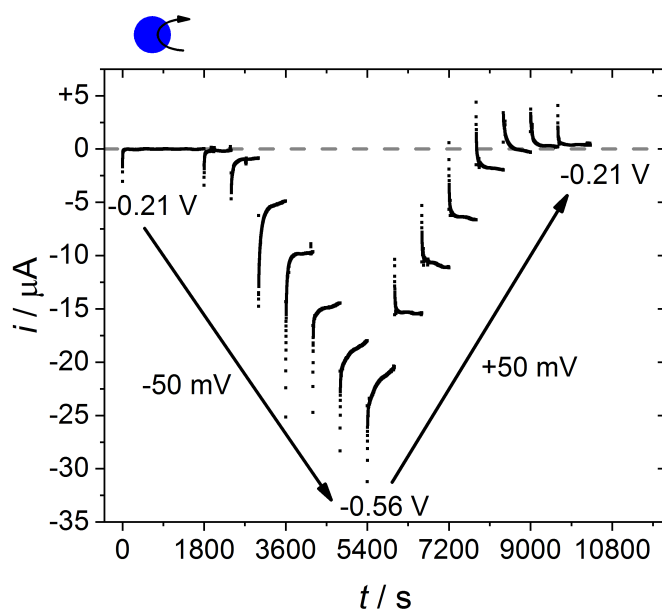
When a potential of -0.51 V is reapplied, the value where the first electrosynthesis experiments are performed in this Section, the reduction current increases from $-13 \mu\text{A}$ (without pyruvate) to $-22 \mu\text{A}$ (with pyruvate, red region), marked by an asterisk on the plot. This further highlights the enhanced NAD^+ concentration effect at the surface of the electrode, enhancing the reduction current. Also, it indicates

that the electrocatalyst is more substrate limited without LDH as compared to the more positive applied potential of -0.36 V. It is noticeable that the current response stabilises a lot more quickly than before, due to the constant removal of NADH at the electrode and in solution, meaning that the concentration of NAD^+ in the bulk solution and at the surface of the electrode is higher in magnitude than before and not depleting. When the potential is returned to -0.36 V at 1800 s the current returns to a smaller value of -4 μA .

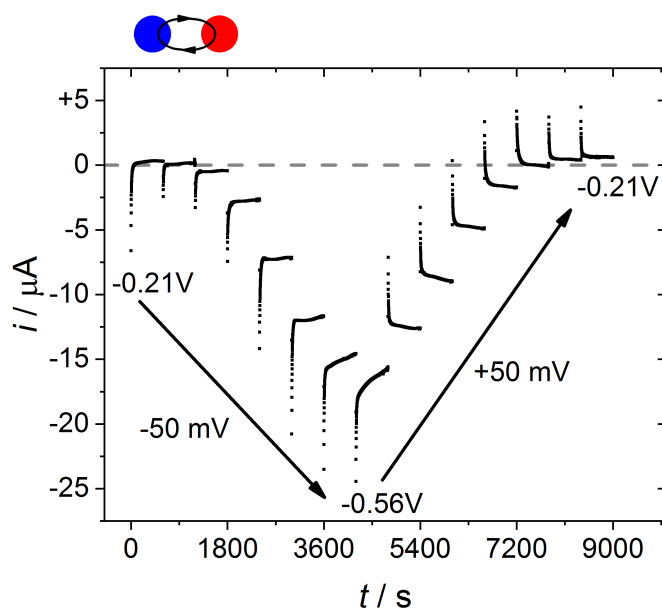
These chronoamperometric responses have shown the current enhancement when using an NADH-dependent enzyme such as LDH with the NAD^+ -red/aCP(H^+) electrode, constantly pushing the NAD^+/NADH equilibrium forwards by constantly removing NADH and reoxidising to NAD^+ , which also enhances the surface concentration of NAD^+ .

Next, the current response was investigated with multi-point chronoamperometry experiments at both electrodes **(a)** and **(b)** as shown in Figure 6.4. This experiment follows the potential profile in period **A**, as shown in Figure 6.1c. For each electrode, the potential was first held at -0.21 V vs SHE, a potential at which there is no redox activity at the electrode. This gives rise to a 0 μA current response at each electrode. The reduction potential is then increased stepwise from -0.21 V by a value of -50 mV each time until a potential of -0.56 V is reached, holding the potentials for 600 s each time. The current response of the electrocatalyst is recorded each time. At -0.56 V, the potential of the electrode is then changed stepwise in a positive direction in $+50$ mV intervals until the original potential of -0.21 V is reached.

The general trend in this chronoamperometric plot is that as the applied potential is taken more negative, the reduction current increases in magnitude as the driving force for electrocatalysis increases. Here, i_{max} for **(a)** is -22 μA at a potential of -0.56 V. For electrode **(b)**, the i_{max} with pyruvate present is -17 μA . For each electrode, the reduction current does not reach a plateau, but continues to increase in magnitude from -0.21 V to -0.56 V. Therefore, it could be assumed that the catalyst can be driven at even more negative potentials to generate



(a)



(b)

Figure 6.4: Multi-step chronoamperometry experiments (potential profile **A** as shown in Figure 6.1c) performed at an NAD^+ -red/aCP(H^+) electrode **(a)** without, and **(b)** with LDH and 10 mM pyruvate in the bulk solution. Cofactor: 1 mM NAD^+ ; flow rate: $11.17 \text{ mL min}^{-1}$; electrolyte: 100 mM Tris-HCl buffer, pH 8.

a higher electrocatalytic rate. However, at much lower potentials, the enzyme becomes deactivated, in effect destroying the electrode.^[54,195] This is investigated later in Section 6.3.

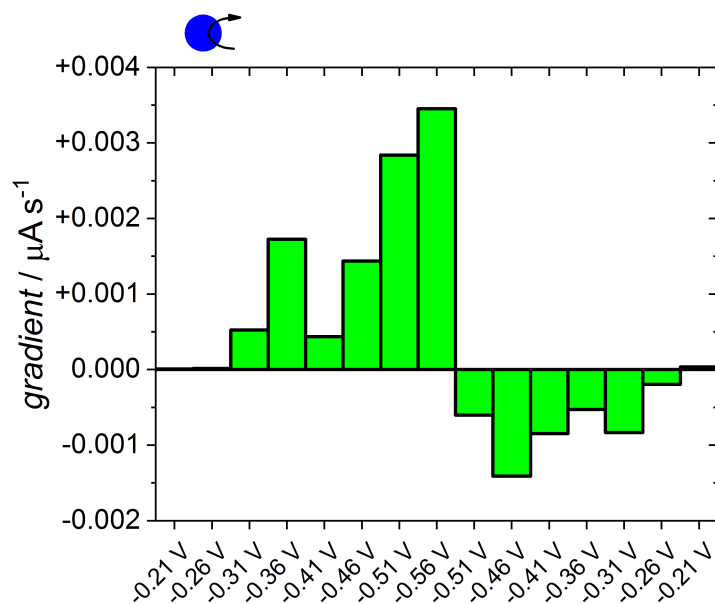
6.2.2 Discussion on stability and cofactor analysis

The overall stability of the NAD^+ -red/aCP(H^+) electrode can be investigated by looking at the gradients of the chronoamperometric plots at each potential. Figure 6.5 shows the gradients of these plots at each potential. For electrode (a) it can be seen that there is a general increase in the gradient from -0.21 V of $0\text{ }\mu\text{A s}^{-1}$ to a maximum gradient at -0.56 V of $+0.0035\text{ }\mu\text{A s}^{-1}$. There are slight deviations from this trend due to slight chemical instability of this electrode. The reason for the decrease in stability as the potential reaches more negative values is likely due to a faster depletion of NAD^+ so the electrode becomes more substrate limited and less active more quickly.

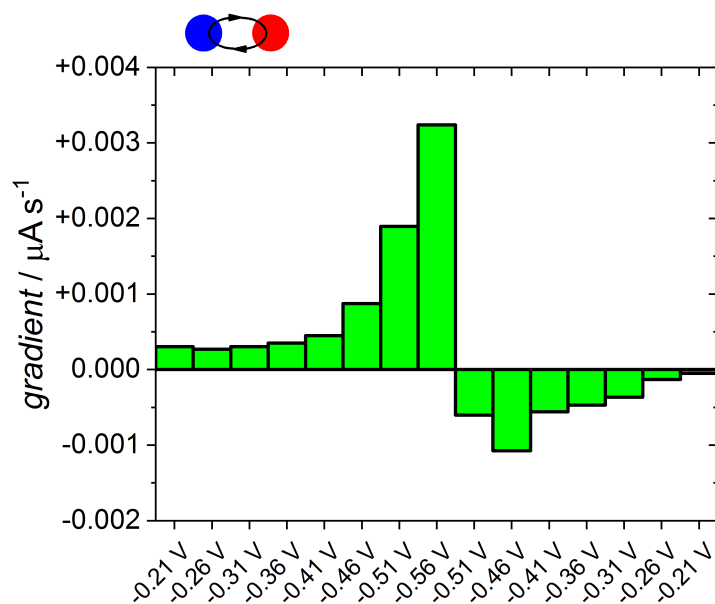
The trend in the gradient is similar for electrode (b), although the trend is more coherent. This could simply be due to the electrode-on-electrode differences as a result of the preparation of the electrodes. Or it could indicate that the presence of the NADH-dependent enzyme LDH, which reoxidises the NADH cofactor to NAD^+ , driving the reaction forwards, somehow has a chemical stability effect on the NAD^+ -red/aCP(H^+) electrode.

The observation noted in Chapter 5 that running a chronopotentiometry experiment at $1/4$ to $1/3$ of i_{max} allows for a more stable electrocatalyst is supported by the observations here. For electrode (b), $1/3$ of i_{max} is approximately $-7\text{ }\mu\text{A}$, which relates to a potential of -0.38 V , which is a similar potential to that recorded with the ‘star’ electrochemical flow cell experiment in Section 5.4 on page 165. At this potential, approximately from 3600 s to 4200 s in Figure 6.4a, the current response is more stable than that observed at -0.56 V .

This can be seen more clearly by looking at electrode (b). Roughly $1/3$ of i_{max} is $-5\text{ }\mu\text{A}$, relating also to a potential of -0.38 V . The gradient here is a much lower value at approximately $+0.0005\text{ V}$ as compared to $+0.0035\text{ V}$ at -0.56 V .



(a)



(b)

Figure 6.5: Gradients of the current responses recorded in the multi-step chronoamperometry shown in Figure 6.4, for NAD^+ -red/aCP(H^+) electrodes (a) without, and (b) with LDH and 10 mM pyruvate in the bulk solution.

This means that at the lower potential of -0.38 V , the electrocatalyst $\text{NAD}^+\text{-red/aCP(H}^+)$ is much more stable. The positive current-time slopes are indicative of the electrocatalyst becoming less electrochemically active over time.

Next, the conversion of NAD^+ to NADH as predicted by the charge, Q , passed through the electrode is compared to the actual concentration of NADH as measured by UV-Vis spectroscopy. Samples were taken after the electrodes were held at each potential for 600 s, with the results shown in Figure 6.6. It can be seen for electrode **(a)** there is a good correlation between the conversion of NAD^+ to NADH , indicating very high Faradaic efficiency.

The general trend is sigmoidal, as the reductive potential is increase stepwise from -0.21 V to -0.56 V , increasing the catalytic current, and then the applied reductive potential is decreased stepwise back to -0.21 V , decreasing the catalytic current. The slight decrease in the concentration of the predicted NADH at the last few steps, rather than a horizontal plot, is a result of oxidation of the generated NADH , albeit at small currents.

When looking at the cofactor conversions at electrode **(b)**, it can be seen that there is no correlation between the predicted cofactor conversions and the measured concentrations by UV-Vis spectroscopy. This is because there is an NADH -dependent enzyme, LDH, in solution with 10 mM pyruvate substrate. Therefore this is using the NADH for the reduction of pyruvate to lactate, reoxidising the cofactor back to NAD^+ . It has been observed before that this occurs immediately at the surface of the electrode, as the extended time electrosynthesis experiments with in-line UV-Vis spectroscopy measurements (see Chapter 5) indicate even at higher reduction currents there is no accumulation of the NADH cofactor in solution. Therefore, as shown in Figure 6.6b, whilst the predicted concentration of reduced species increases sigmoidally with the varying applied potential, the concentration of NADH as measured by UV-Vis spectroscopy remains approximately zero.

This section has showed the dependency of the NAD^+ reduction current of the $\text{NAD}^+\text{-red/aCP(H}^+)$ electrode on the applied potential of the working potential. Increasing the magnitude of the reductive potential increases the current response

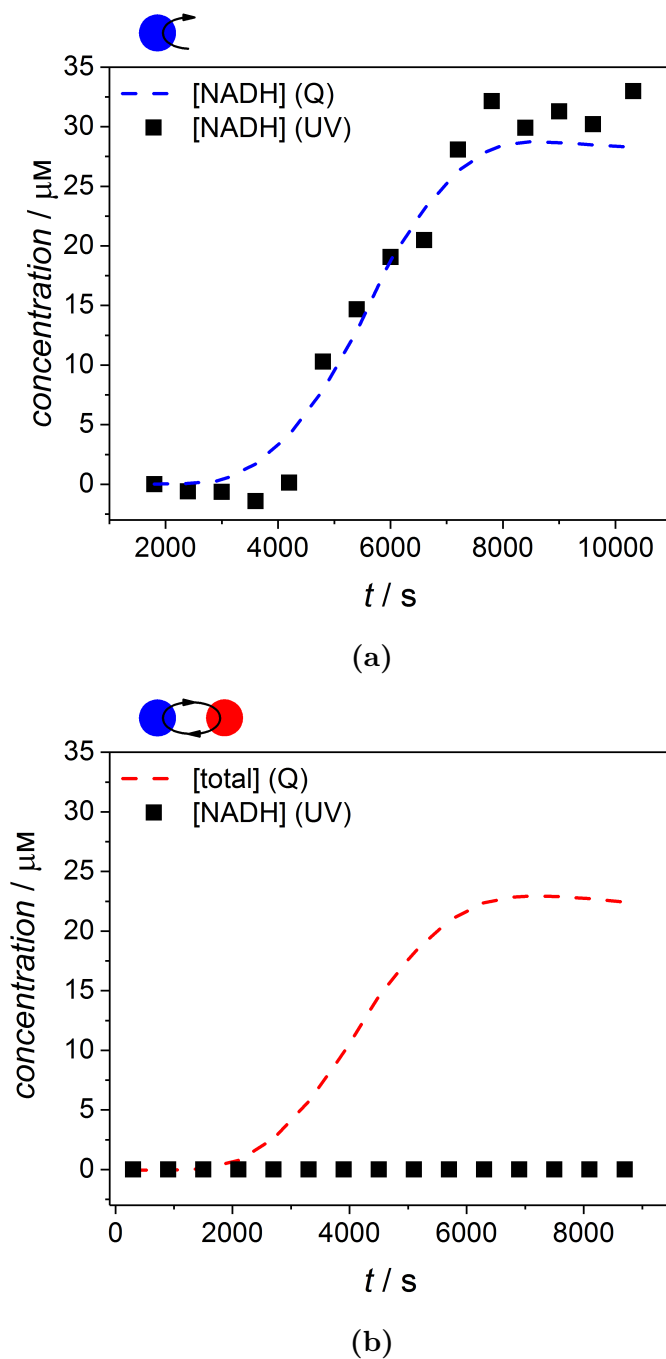


Figure 6.6: Concentration of cofactor reductions for the multi-step chronoamperometry shown in Figure 6.4, for NAD^+ -red/aCP(H^+) electrodes (a) without, and (b) with LDH and 10 mM pyruvate in the bulk solution. Dashed lines: predicted by the charge passed through each electrode; black points: measured by UV-Vis spectroscopy.

of the electrode. However in doing so, the stability of the current response at the electrode decreases. The next section discusses analysis of the cofactor formed at electrodes **(a)** and **(b)** during extended electrocatalytic reductions at a modest applied magnitude potential and at a potential that is expected to deactivate the enzyme.

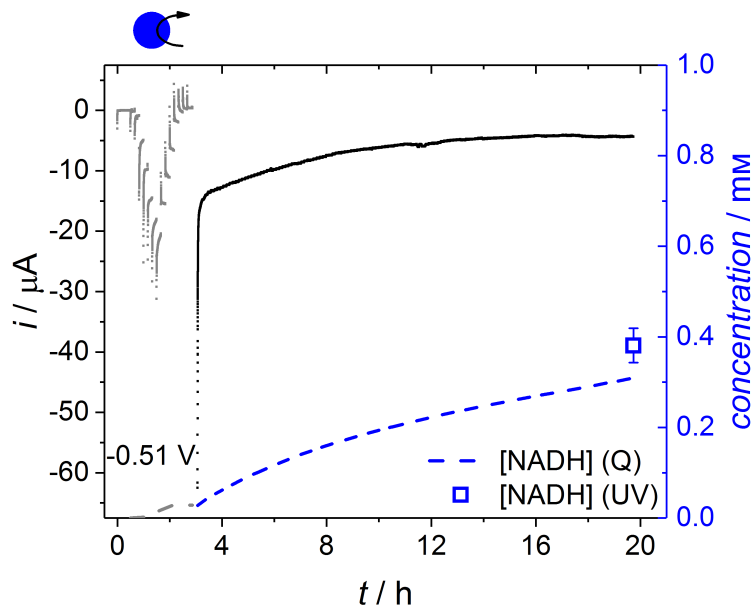
6.3 Extended time electrocatalysis: potential profiles **B** and **C**

Next, the effect of the potential applied was investigated during extended electrocatalytic reduction experiments. First, cofactor product analysis was performed after a chronoamperometry experiment with -0.51 V (profile **B**) applied. Second, a chronoamperometry experiment was performed at a much more negative potential (-0.96 V, profile **C**), a value where the enzyme becomes irreversibly deactivated^[54] and NAD^+ is reduced directly at carbon (see Section 4.2.1 on page 98). The cofactor was analysed by UV-Vis spectroscopy, HPLC and ^1H NMR spectroscopy after both of these experiments.

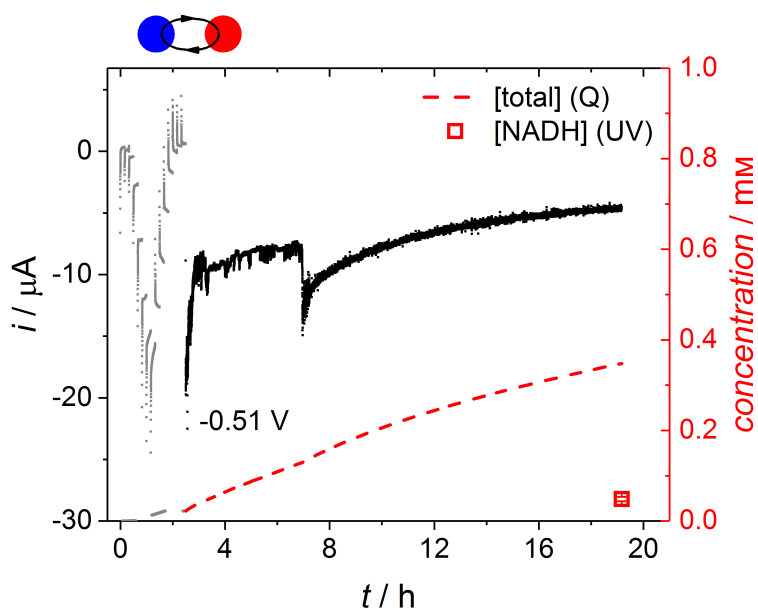
6.3.1 Electrochemistry

The chronoamperometric plots for the -0.51 V experiments are shown in Figure 6.7. As these experiments were a continuation from the multi-step chronoamperometry as shown in Figure 6.4, this part of the chronoamperometry is shown in grey on the plots. The dashed lines indicated the predicted cofactor NAD^+ to NADH conversions as calculated from the charge passed through the electrodes. The point at around 20 h at the end of the experiments indicates the concentrations of NADH at the end of each electrocatalysis experiment determined by UV-Vis spectroscopy.

Electrode **(a)** shows a current response that relaxes to an initial value of $-15\ \mu\text{A}$, which slowly decreases over 17 h to $-7\ \mu\text{A}$. The predicted concentration of NADH after this experiment is calculated from the charge passed to be $0.32\ \text{mM}$ (blue dashed line). Compared to the point at 20 h representing the concentration of NADH calculated by UV-Vis spectroscopy (absorption at 340 nm) to be $0.38\ \text{mM}$ NADH. Either the predicted value underestimates, or the UV-Vis spectroscopy



(a)



(b)

Figure 6.7: Extended time chronoamperometry experiments at -0.51 V (potential profile **B** as shown in Figure 6.1c) performed at NAD^+ -red/aCP(H^+) electrodes **(a)** without, and **(b)** with LDH and 10 mM pyruvate in the bulk solution. Dashed line: [NADH] (blue) or [total] (red) as predicted by the charge passed through the electrodes; point: [NADH] as determined by UV-Vis spectroscopy. Cofactor: 1 mM NAD^+ ; flow rate: $11.17 \text{ mL min}^{-1}$; electrolyte: 100 mM Tris-HCl buffer, pH 8.

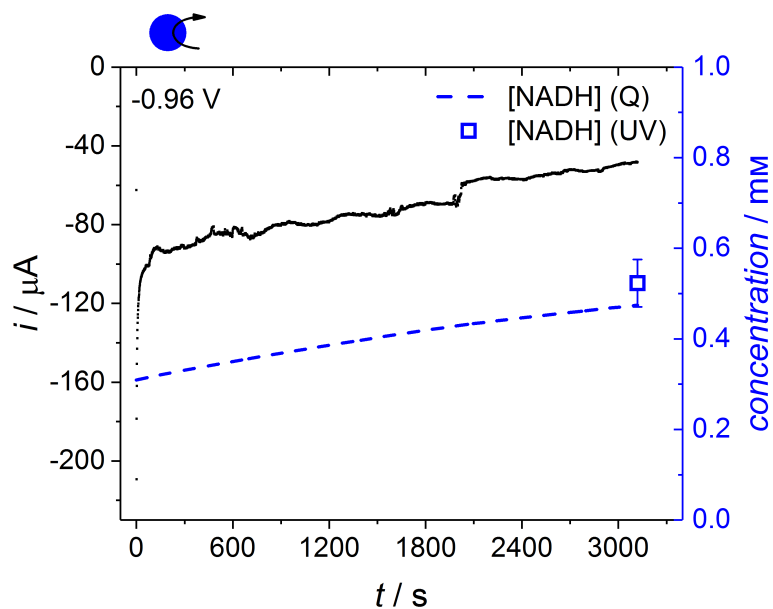
sample overdetermines the concentration of the reduced cofactor. However, both values are roughly in agreement showing high Faradaic efficiency.

For electrode **(b)**, the chronoamperometric response indicates an initial current of $-10\ \mu\text{A}$, which gradually decreases to $-7\ \mu\text{A}$ at the end of the experiment at 19 h. There is a jump in the current at about 7 h, which could be attributed to a movement of a trapped air bubble out of the electrochemical flow cell, perhaps up to that point not allowing for a perfect connection between the electrodes and the bulk solution. This also appears to make the electrocatalytic response more stable. The dashed line indicates the predicted concentration of the reduced species calculated from the charge passed. After the 19 h experiment it is predicted that 0.36 mM of reduced species has been produced by the electrocatalyst. This is much higher than the value measured by UV-Vis spectroscopy (point) of 0.04 mM. This is due to the presence of the NADH-dependent enzyme, LDH, reoxidising the NADH cofactor back to NAD^+ , whilst reducing the pyruvate substrate to lactate. Therefore there is little accumulation of NADH in solution.

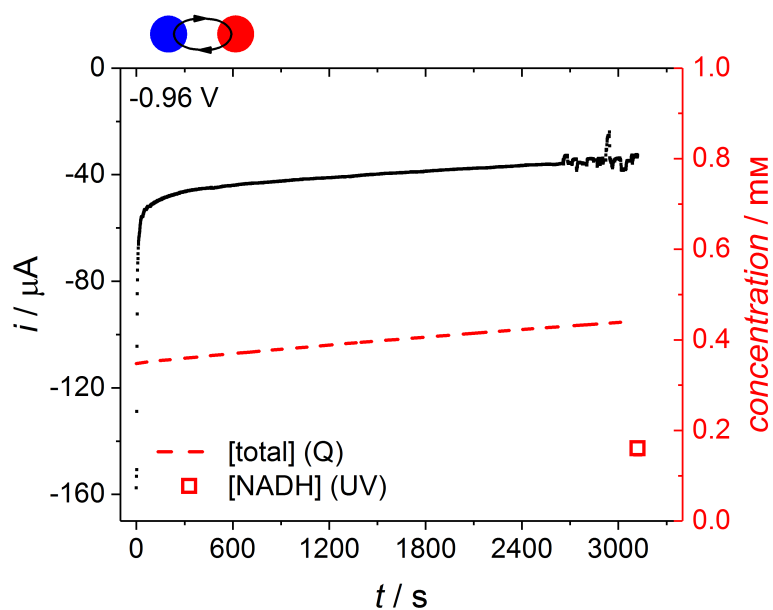
These observations are as expected, where electrode **(a)** generates NADH which is accumulated, and with electrode **(b)**, the cofactor NADH is used immediately by the NADH-dependent LDH for the reduction of pyruvate to lactate. Next, the effect on the cofactor during a chronoamperometry experiment holding the potential at a much more negative value is investigated.

Chronoamperometry experiments were continued at electrodes **(a)** and **(b)**, this time at $-0.96\ \text{V}$ (see Figure 6.8). The experiment performed with electrode **(a)** has an initial reduction current of $-90\ \mu\text{A}$, with the current decreasing to $-50\ \mu\text{A}$ at the end of the 3000 s experiment. The current response is not smooth, indicating that there could be H_2 evolution at the working electrode, or that the aCP working electrode is losing integrity. The reduction current is significantly higher as compared to the experiment at $-0.56\ \text{V}$.

On the other hand, electrode **(b)** shows a current response slightly smaller than the reduction current observed in **(a)**, with an initial current of $-50\ \mu\text{A}$, reaching $-40\ \mu\text{A}$ at the end of the 3000 experiment. These lower reduction currents could be



(a)



(b)

Figure 6.8: Extended time chronoamperometry experiments at -0.96 V (potential profile **C** as shown in Figure 6.1c) performed at NAD^+ -red/aCP(H^+) electrodes **(a)** without, and **(b)** with LDH and 10 mM pyruvate in the bulk solution. Dashed line: $[\text{NADH}]$ (blue) or $[\text{total}]$ (red) as predicted by the charge passed through the electrodes; point: $[\text{NADH}]$ as determined by UV-Vis spectroscopy. Cofactor: 1 mM NAD^+ ; flow rate: $11.17 \text{ mL min}^{-1}$; electrolyte: 100 mM Tris-HCl buffer, pH 8.

a result of some LDH in solution becoming immobilised on the aCP(H⁺) electrode and blocking sites on the carbon.^[87]

These experiments are a continuation from the chronoamperometry experiments in Figure 6.7, therefore the predicted NADH generation is taken as the total produced from all chronoamperometry experiments in this chapter. For clarity, the whole chronoamperometry is not shown and only the -0.96 V section is shown in Figure 6.8.

The predicted conversion of NAD⁺ to NADH for both electrodes **(a)** and **(b)** as calculated from the charge passed through the electrodes are shown as dashed lines in Figures 6.8a and 6.8b. For electrode 6.8a, the predicted concentration of NADH generated by the electrocatalyst has now increased from 0.32 mM to 0.48 mM, with the measured concentration of NADH by UV-Vis spectroscopy (point) in agreement and increasing by roughly the same amount, from 0.38 mM to 0.54 mM.

For electrode **(b)**, the predicted NADH concentration increases from 0.36 mM to 0.44 mM, in line with the charge passed through with the electrode. However, what has been observed is the NADH concentration measured by UV-Vis spectroscopy also increases by approximately the same amount from 0.04 mM to 1.6 mM. This is unexpected for this electrode with an NADH-dependent enzyme, LDH, in the bulk solution, as previously it has been observed that this enzyme would immediately reoxidise the NADH back to NAD⁺, therefore not observing an accumulation of the NADH cofactor in the bulk solution. This could be a result of LDH becoming denatured by the low potential applied at the electrode, however the LDH in the bulk is unlikely to be exposed to the electrode surface for long. Therefore it is likely due to a non-biological form of the NADH cofactor being generated, which is incompatible with the NADH-dependent oxidoreductases such as the LDH used here. This is discussed in more detail in Section 6.3.2.

Figure 6.9 shows the cyclic voltammetry experiments performed for both electrodes **(a)** and **(b)** after the chronoamperometry at -0.96 V (green trace) and compared to the cyclic voltammograms recorded before the chronoamperometry

experiments (blue and red traces, taken from Figure 6.2). The green traces were recorded at each electrode after 3000 s chronoamperometry experiment at -0.96 V.

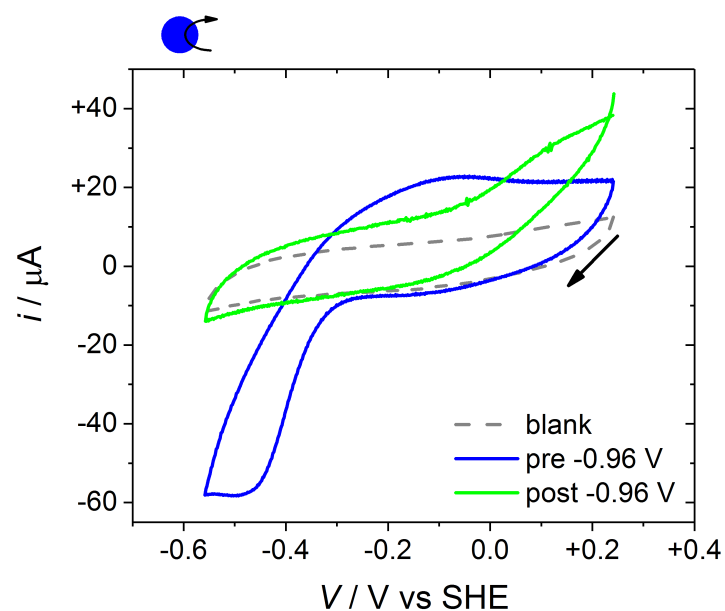
For electrodes **(a)** and **(b)**, there is a clear catalytic reduction wave, of onset potential -0.32 V. Electrode **(a)** reaches a value of $i_{\max}$ of -48 μA and electrode **(b)** reaches a value of $i_{\max}$ of -30 μA . However after the chronoamperometry experiments held at a high reductive potential of -0.96 V, the catalytic wave attributed to NAD^+ reduction by NAD^+ -red immobilised onto aCP(H^+) is not present in the subsequent cyclic voltammograms, shown in green. This indicates that the low potential chronoamperometry experiment has denatured or deactivated the NAD^+ -red enzymes, therefore there is no reductive catalytic response shown in the voltammogram within this potential window. Therefore any charge passed through the -0.96 V chronoamperometry experiments shown in Figure 6.8 is likely to be attributed to uncatalysed NAD^+ reduction directly at the surface of the aCP(H^+) working electrode, leading to unspecific cofactor reduction products.

There is some cofactor oxidation observed at both electrodes **(a)** and **(b)** with onset potential of -0.10 V at each electrode. This could be attributed to reoxidation of the cofactor that was reduced during the -0.96 V chronoamperometry experiments.

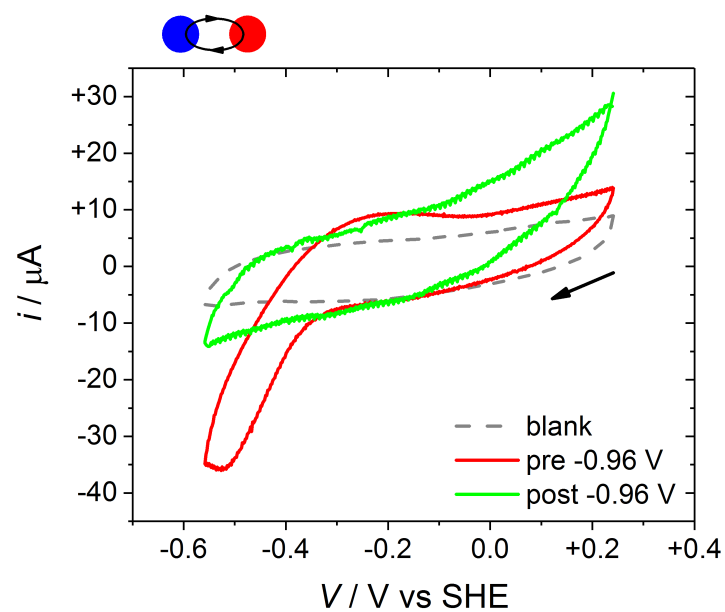
This section has shown that enzymatic NAD^+ reduction at NAD^+ -red/aCP(H^+) electrodes is possible at -0.51 V, but for chronoamperometry experiments held at -0.96 V, it appears that the reduced product as a result of the electrosynthesis is not compatible with the NADH-dependent LDH. The next section analyses the cofactor produced after each of these chronoamperometry experiments using UV-Vis spectroscopy, HPLC and ^1H NMR spectroscopy.

6.3.2 Cofactor analysis

This section uses UV-Vis spectroscopy, HPLC and ^1H NMR spectroscopy analytical techniques to analyse the cofactor products after the -0.51 V and -0.96 V chronoamperometry experiments performed at both the electrodes.



(a)



(b)

Figure 6.9: Cyclic voltammograms recorded before (blue/red) and after (green) performing chronoamperometry experiments at -0.96 V at NAD^+ -red/aCP(H^+) electrodes (a) without, and (b) with LDH and 10 mM pyruvate in the bulk solution. Cofactor: 1 mM NAD^+ ; flow rate: $11.17 \text{ mL min}^{-1}$; electrolyte: 100 mM Tris-HCl buffer, pH 8.

As there is the possibility of biologically incompatible NADH being generated during the -0.96 V chronoamperometry, it is necessary to look at the raw UV-Vis data, as shown in Figure 6.10, taken of the bulk solutions after the -0.51 V (blue/red trace) and -0.96 V (green traces) chronoamperometry experiments. The absorbance at 260 nm is contributed to by both NAD^+ and NADH, with the peak at 340 nm only contributed by NADH (see Section 2.6 on page 59 and Section 5.1.3 on page 144). It is noticeable that after the -0.51 V chronoamperometry experiment, there is an accumulation of NADH with electrode **(a)** indicated by a peak at 340 nm (blue trace). On the other hand, using electrode **(b)** with LDH and the substrate pyruvate in solution, there is no absorbance at 340 nm (red trace), indicating the generated NADH is being used immediately by the LDH.

After the -0.96 V chronoamperometry, the peak at 340 nm increases in intensity for the experiment performed at electrode **(a)**. This is also observed for the experiment at electrode **(b)**, indicating an accumulation of a reduced species generated during the -0.96 V chronoamperometry.

Also of note is the increase in the absorbance at 300 nm after the -0.96 V chronoamperometry experiment, for the experiments performed at both **(a)** and **(b)** electrodes. This could also be attributed to a generation of a species that is not NAD^+ or 1,4-NADH. However UV-Vis spectroscopy does not give enough information to make any conclusions on the nature of the reduced species created by the electrodes during the -0.96 V chronoamperometry. People often only use UV-Vis as a spectroscopic tool to identify whether the correct form of NADH has been produced, which often doesn't provide enough information on the nature of the reduced NAD^+ products. Therefore for these experiments, HPLC was used to give more information on the nature of the reduction products.

Samples from the end of each chronoamperometry experiment were also analysed using HPLC, with the chromatograms shown in Figure 6.11. The blue and red traces relate to the samples analysed after the -0.51 V chronoamperometry at the electrodes **(a)** and **(b)** respectively. It can be seen that after the -0.51 V

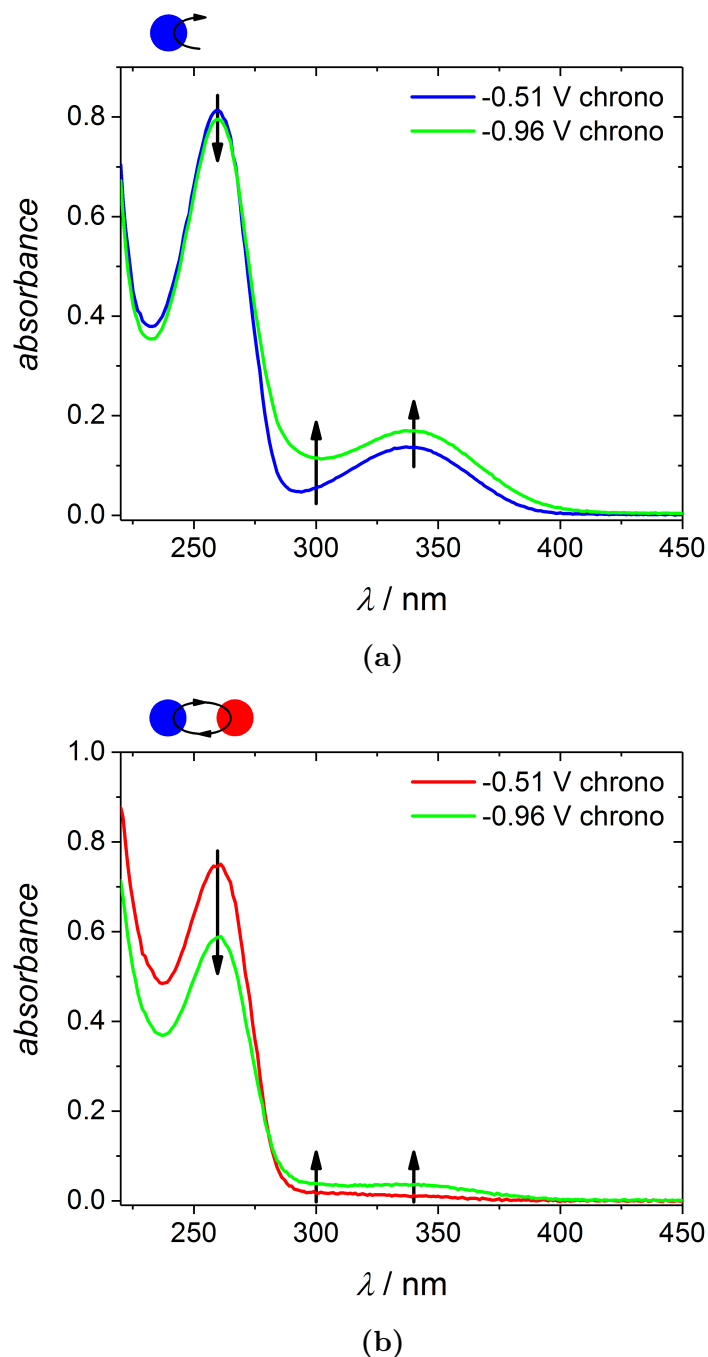


Figure 6.10: UV-Vis spectra of the bulk solution after -0.51 V (blue/red trace) and -0.96 V (green traces) chronoamperometry experiments performed at NAD^+ -red/aCP(H^+) electrodes (a) without, and (b) with LDH and 10 mM pyruvate in the bulk solution.

chronoamperometry with electrode **(a)** there is accumulation of the biologically active 1,4-NADH cofactor, as shown by the peaks (blue trace) in the chromatograms recorded at 260 nm and 340 nm in Figure 6.11a at a residence time of 19 min. For the equivalent experiment performed at electrode **(b)** (red trace), it can be seen that there is no 1,4-NADH present at the end of the experiment, as a result of reoxidation by the LDH in solution.

After the -0.96 V chronoamperometry experiment at electrode **(a)**, the sample measured by HPLC (green trace) shows three peaks in the 260 nm absorbance chromatogram. The first peak at a residence time of 19 min implies the presence of 1,4-NADH, though at a lower concentration than that before the -0.96 V chromatogram. The 1,4-NADH generated by the -0.51 V chronoamperometry could have been destroyed electrochemically by the low reducing potential experiment. This is also indicated by the decrease in the absorbance at 340 nm. After the -0.96 V pulse, the concentration of NAD^+ has also decreased, as indicated by a decrease in the absorbance of the NAD^+ peak at a residence time of 20.5 min in the chromatogram recorded at 260 nm. The presence of another peak at a residence time of 24 min in both the 260 nm and 340 nm chromatograms is attributed to the accumulation of biologically inactive forms of the reduced NADH cofactor, possibly 1,2-NADH, 1,6-NADH, or the dimer NAD_2 .^[56,96,218–221] Therefore an inactive form of the reduced NADH cofactor has an absorbance at 340 nm, which is why it appears in the UV-Vis spectrum in Figure 6.10a as though 1,4-NADH had been generated during the -0.96 V chronoamperometry.

In fact, the inactive NADH peak appearing at a residence time 5 min longer than that for 1,4-NADH (19 min) implies the inactive NADH is significantly less soluble than 1,4-NADH and therefore has a different structure. Therefore, it would be possible that the peak at a residence time of 24 min could be the dimer, NAD_2 , which is biologically inactive and generated directly at the carbon electrode.

After the -0.96 V chronoamperometry experiment at electrode **(b)**, it can be seen in the HPLC chromatogram (green trace) that there is still no 1,4-NADH present after the chronoamperometry, as indicated by the absence of a peak at a

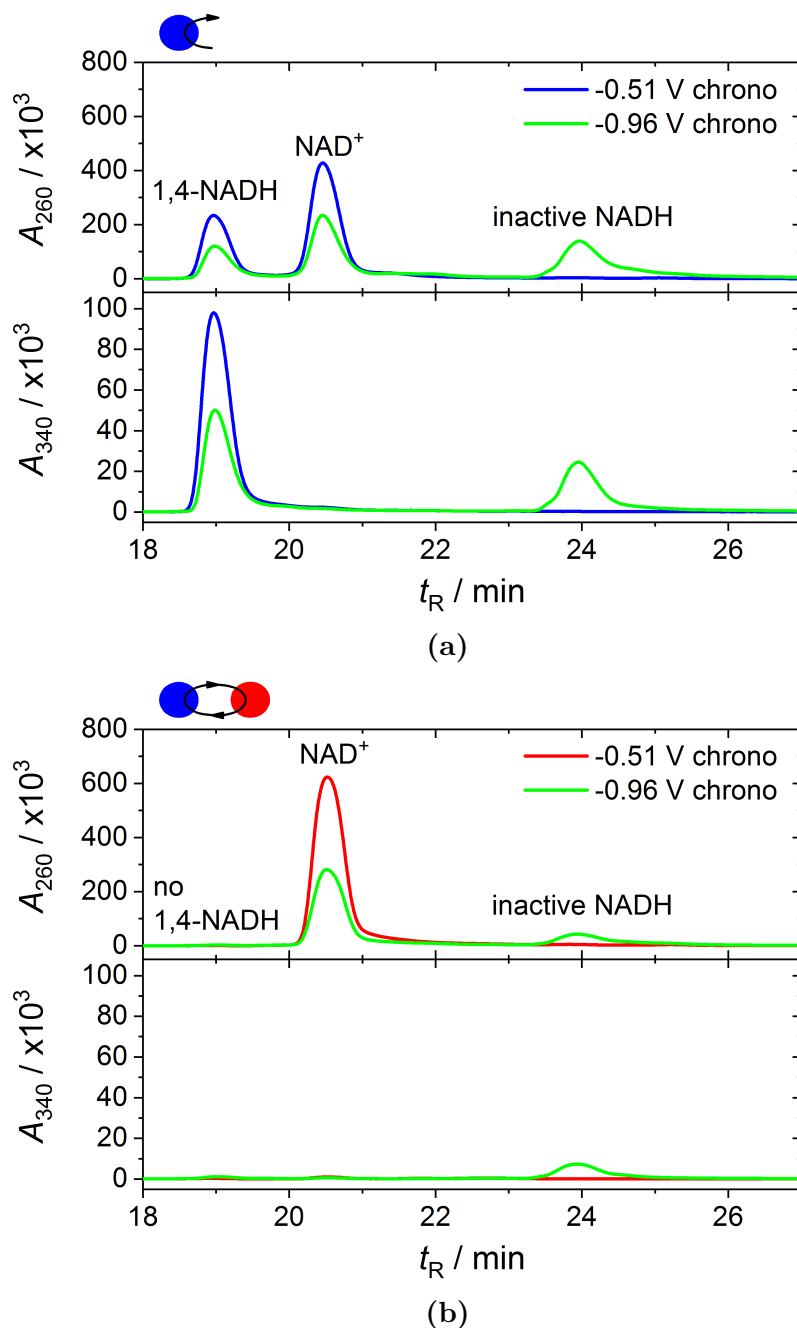


Figure 6.11: HPLC chromatograms of the bulk solution after -0.51 V (blue/red trace) and -0.96 V (green traces) chronoamperometry experiments performed at NAD⁺-red/aCP(H⁺) electrodes (a) without, and (b) with LDH and 10 mM pyruvate in the bulk solution. Top chromatograms: absorbance at 260 nm; bottom chromatograms: absorbance at 340 nm.

Table 6.1: Relative absorptions of HPLC peaks for electrodes **(a)** and **(b)**.

Molecule	t_R / min	Integration at 260 nm / 10^{-3} min^{-1}			
		(a)		(b)	
		−0.51 V	−0.96 V	−0.51 V	−0.96 V
1,4–NADH	19	109	58	0	0
NAD ⁺	20.5	232	141	349	159
Other	24	0	136	0	51
Total		341	335	349	210

residence time of 19 min. However, there is a decrease in the absorbance of the NAD⁺ peak at a residence time of 20.5 min as a result the reduction of NAD⁺ to inactive forms of NADH, possibly the dimer NAD₂ as discussed above, or as a result of further electrochemical destruction of the molecule. The presence of a peak at a residence time of 24 min in the 260 nm and 340 nm chromatograms is attributed to the accumulation of biologically inactive form of the cofactor.

Table 6.1 shows the integrations of the three peaks for electrodes **(a)** and **(b)**. These values can be assumed to be relative concentration values for the three species, as the molar absorption at 260 nm for NAD⁺ ($17.4 \text{ mM}^{-1} \text{ cm}^{-1}$) and 1,4–NADH ($14.1 \text{ mM}^{-1} \text{ cm}^{-1}$) is approximately the same, and it is assumed the biologically inactive nicotinamide species at 24 min is also a similar structure so would have a similar molar absorption coefficient.

For electrode **(a)**, the total integration of the NAD⁺ and 1,4–NADH peaks after the −0.51 V chronoamperometry experiment is $341 \times 10^{-3} \text{ min}^{-1}$. After the chronoamperometry experiment performed at −0.96 V, the peak integrations for NAD⁺ and 1,4–NADH decrease to $58 \times 10^{-3} \text{ min}^{-1}$ and $141 \times 10^{-3} \text{ min}^{-1}$ respectively. The peak assumed to be inactive NADH at a residence time of 24 min appears with an integration value of $136 \times 10^{-3} \text{ min}^{-1}$. The total of all the peak integrals after the −0.96 V chronoamperometry makes $335 \times 10^{-3} \text{ min}^{-1}$, which is roughly matches the total of the peak integrals after the −0.51 V chronoamperometry. This implies there is a mass balance and that the creation of the inactive NADH peak only comes from the loss of NAD⁺ and 1,4–NADH.

Table 6.2: ^1H NMR proton assignments for NAD^+ and 1,4-NADH standards.

Label	δ , ^1H NMR spectroscopy (400 MHz, D_2O)	
	NAD^+	1,4-NADH
N2/N2'	9.23 (s, 1H)	6.85 (s, 1H)
N5/N5'	9.04 (d, $J = 6.1$ Hz, 1H)	6.02 (d, $J = 5.5$ Hz, 1H)
N6/N6'	8.73 (dt, $J = 8.2, 1.6$ Hz, 1H)	5.92–5.85 (m, 1H)
N4/N4'	8.06 (s, 1H)	2.76–2.53 (m, 2H)

For electrode **(b)**, the mass balance between the peak integrals after the -0.51 V ($349 \times 10^{-3} \text{ min}^{-1}$) and -0.96 V ($210 \times 10^{-3} \text{ min}^{-1}$) chronoamperometry steps is not so clear. This could be a result of a dilution in the sample from the -0.96 V chronoamperometry experiment, or that more of the cofactor is adsorbed to the aCP(H^+) electrode as compared to the sample after the -0.51 V step.

The presence of the biologically inactive NADH cofactor peaks in the chromatograms for electrodes **(a)** and **(b)** shows that the reduction current during the -0.96 V chronoamperometry experiments is a result of reducing the NAD^+ cofactor to biologically inactive reduced forms of NADH, possibly the dimer NAD_2 . Next, ^1H NMR spectroscopy was performed for each of the samples to analyse the cofactor structure.

Figure 6.12 shows the ^1H NMR spectra for 4 mM NAD^+ and 1,4-NADH standards in water. Peaks between a chemical shift of 5 and 3 are dominated by water and Tris-HCl buffer peaks. The region between a chemical shift of 9.5 and 5.8 can be entirely assigned to the cofactor. ^1H NMR spectroscopy peaks are assigned for the protons on the nicotinamide ring as shown in Table 6.2.^[222,223] For ease of comparison, the peaks labelled as N2 and N2' are used as diagnostic peaks for determination of NAD^+ and 1,4-NADH respectively.

^1H NMR spectra were then recorded for samples taken from solution after both the -0.51 V (blue) and the -0.96 V (red) chronoamperometry experiments at electrode **(a)**. For the -0.51 V spectrum, as shown in Figure 6.13 it can be seen that the spectrum has peaks contributing from both NAD^+ and 1,4-NADH, with

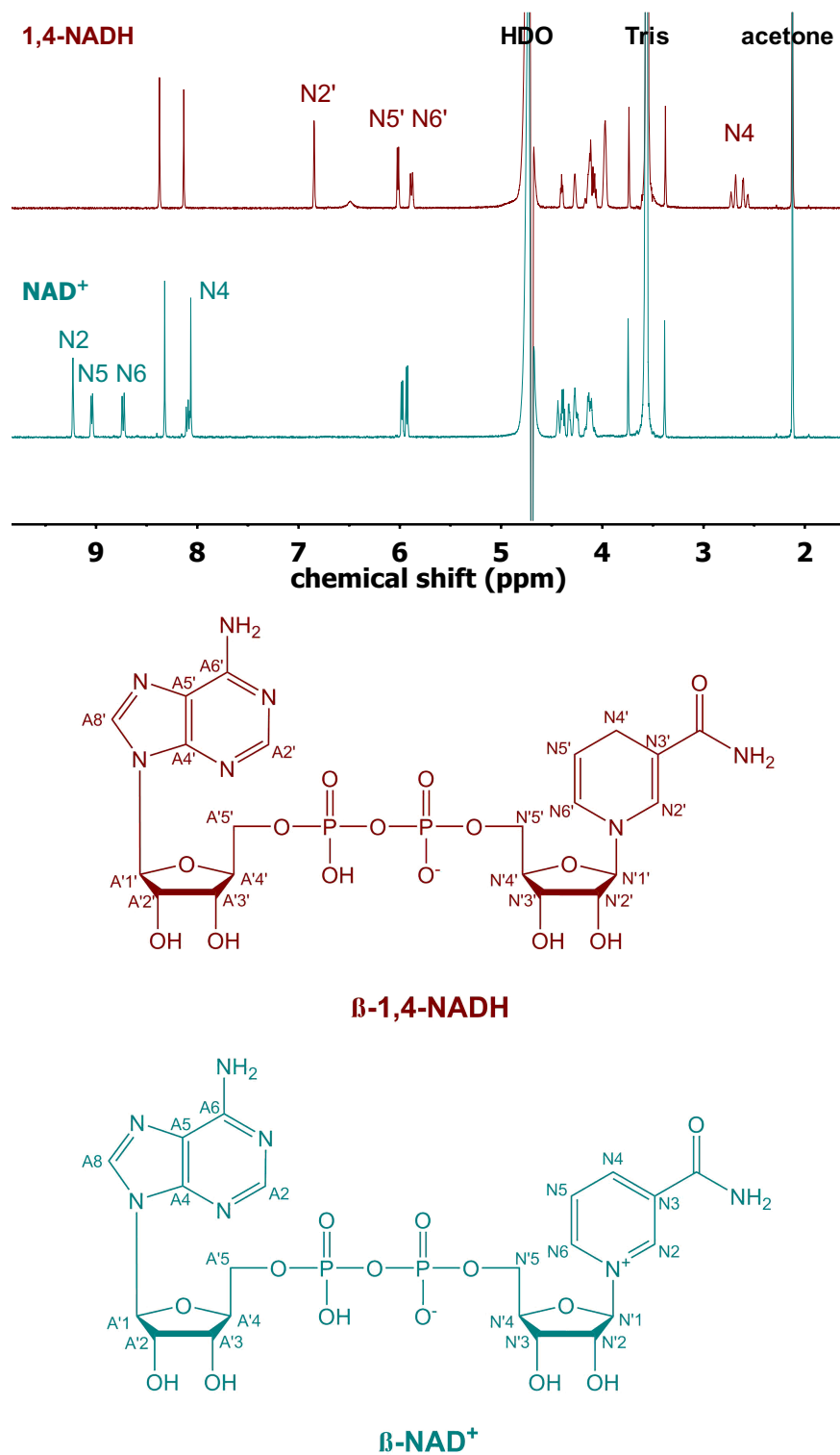


Figure 6.12: ^1H NMR spectroscopy standard spectra for NAD^+ (turquoise) and 1,4-NADH (maroon), each at a concentration of 4 mM.

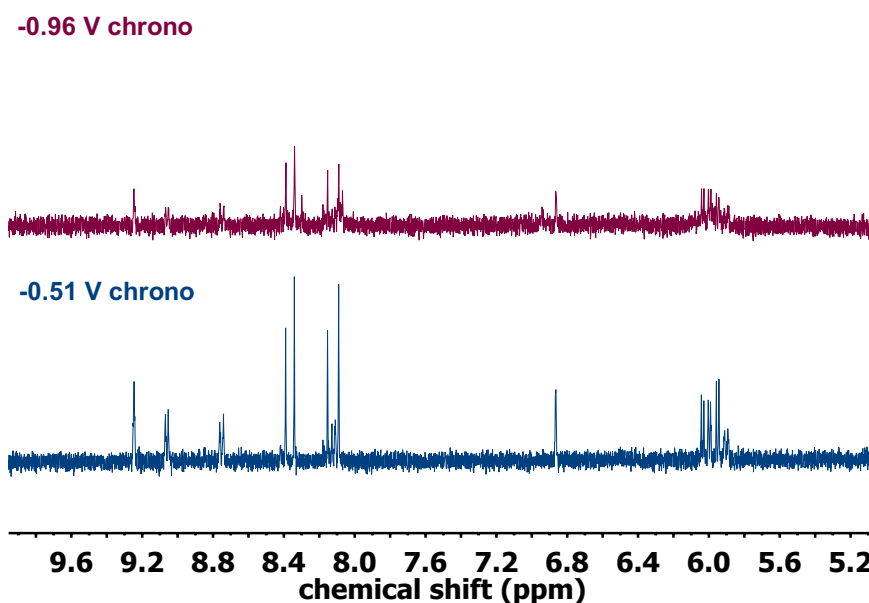
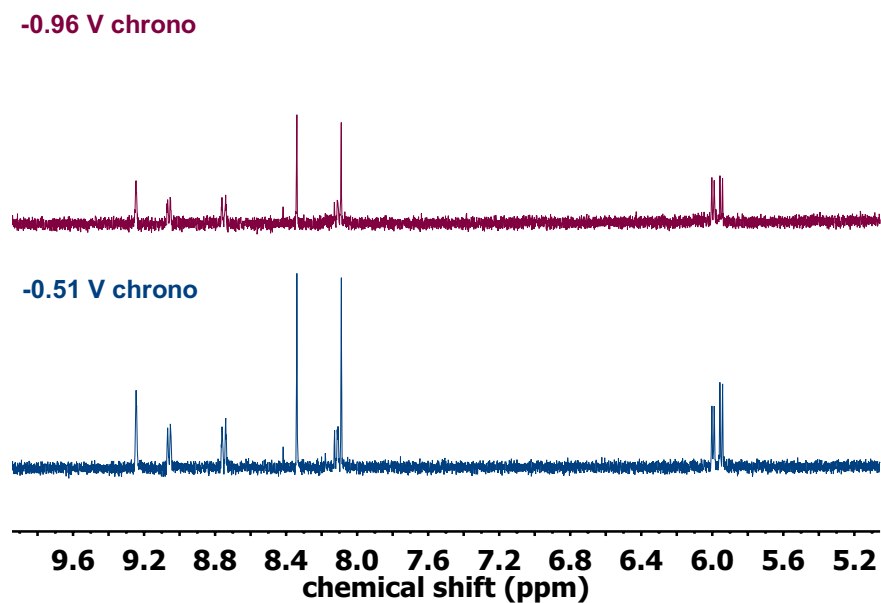


Figure 6.13: ^1H NMR spectra in the cofactor region for the (a) electrode after the -0.51 V (blue) and -0.96 V (red) chronoamperometry experiments.

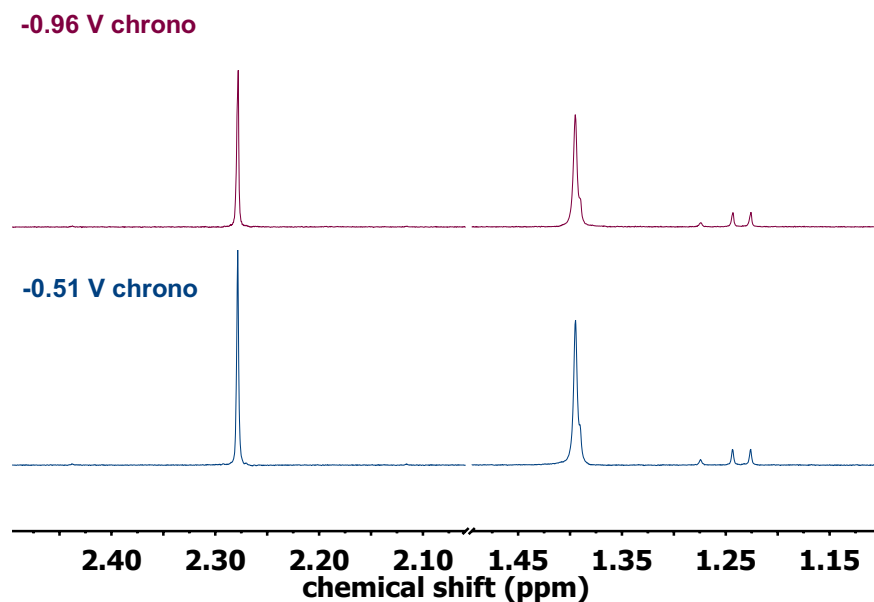
the peaks at a chemical shift of 9.25 (s), N2, relating clearly to the NAD^+ and the peak at 6.86 (s), N2', being characteristic for 1,4-NADH.

After performing the -0.96 V chronoamperometry experiment, the spectrum changes slightly. Although none of the peaks change in chemical shift or multiplicity, the magnitude of all the peaks decrease slightly. This indicates that the more negative potential of -0.96 V has destroyed some of the cofactor in solution, decreasing the overall concentration of NAD^+ and NADH. The reason peaks of the inactive forms of the cofactors are not visible in the spectrum is likely due to their presence at very low concentrations after the relatively short 3000 s chronoamperometry step. There are no other peaks in other areas of the spectra.

Figure 6.14a shows the ^1H NMR spectra within the cofactor region after the -0.51 V (blue) and -0.96 V (red) chronoamperometry steps performed at electrode (b). For the -0.51 V spectrum, it can be seen clearly that there is only NAD^+ present in the sample solution, with the characteristic N2' peak for 1,4-NADH not being present. This indicates that there is not an accumulation of the 1,4-NADH cofactor in solution, supporting the results found by HPLC.



(a)



(b)

Figure 6.14: ^1H NMR spectra in (a) the cofactor region, and (b) the substrate region, for the '(b) electrode' after the -0.51 V (blue) and -0.96 V (red) chronoamperometry experiments.

After performing the -0.96 V chronoamperometry experiment, again the spectrum changes slightly. Like before with electrode **(a)**, none of the peaks change in chemical shift or multiplicity, but the magnitude of all the peaks decrease slightly. This indicates the electrode has destroyed the overall concentration of NAD^+ and NADH , as also observed with electrode **(a)**. There are no other cofactor peaks in other areas of the spectra.

Figure 6.14b shows the same ^1H NMR spectra for electrode **(b)** in the pyruvate ($\delta 2.36, 1.48$) and lactate ($\delta 1.23$) region. Lactate is produced after the -0.51 V chronoamperometry experiment (blue), implying successful 1,4- NADH production. After the -0.96 V experiment (red) the relative magnitude of the lactate peak remains small implying that the 1,4- NADH cofactor was not being produced.

6.4 Summary

This chapter has discussed the results of two comparative chronoamperometry experiments, performed at NAD^+ -red/aCP(H^+) electrodes, without and with an NADH -dependent enzyme, LDH, included in solution with pyruvate as a substrate. For the electrode with LDH in solution, it was observed that the reductive current increases as pyruvate is injected into the electrolyte.

The potential dependency of the reduction current was investigated. It was found that as the applied potential of the electrode moves to more negative potentials, the reduction current increases in magnitude as expected. The Faradaic efficiency was determined by using UV-Vis spectroscopy and was determined to be close to 100%. It was also noticed that the stability of the electrode decreased when a more negative potential was applied, as determined by an increase in the gradients, or current decay.

Two chronoamperometry experiments were performed, at a reduction potential of -0.51 V vs SHE and at the much more negative -0.96 V vs SHE, without and with the LDH and pyruvate in the bulk solution. It was found that at -0.51 V the NAD^+ was reduced with good Faradaic efficiency by the NAD^+ -red/aCP(H^+) electrode to generate the biologically active 1,4- NADH , which was confirmed by UV-Vis spectroscopy, HPLC and ^1H NMR spectroscopy. This was also confirmed

with the experiment with LDH and pyruvate in solution, where the generated 1,4-NADH was reoxidised immediately, in turn reducing pyruvate to lactate.

With the chronoamperometry experiments performed at -0.96 V , it was found that the NAD^+ -red/aCP(H^+) electrode was deactivated or destroyed, as shown by the lack of a NAD^+ reduction wave during cyclic voltammetry experiments. It was observed that for the experiment with LDH and pyruvate, there was a reduced species accumulating, which was unable to be used by LDH for the reduction of pyruvate to lactate. This time, inactive forms of NADH (possibly 1,2-NADH, 1,6-NADH or the dimer NAD_2) were generated, which was confirmed by UV-Vis spectroscopy and HPLC. The use of ^1H NMR spectroscopy confirmed these cofactor structures of NAD^+ and 1,4-NADH and indicated that the -0.96 V chronoamperometry electrochemically destroyed NAD^+ and 1,4-NADH in the bulk solution, decreasing the overall cofactor concentration.

The experimental methods demonstrated in this chapter, changing one reaction parameter over time, with continuous monitoring of the electrochemical behaviour of the electrocatalyst and possibly the continuous monitoring of the cofactor and products with in-line analytical techniques such as UV-Vis spectroscopy, offer vast amounts of cohesive data. Instead of changing the potential of the electrode, the reaction can be optimised for other conditions, like cofactor concentration, flow rate, substrate concentration and LDH concentration in solution. The wealth of information provided by the electrocatalyst in tandem with in-line analytical methods can provide a vast array of time labelled data for electrocatalyst refinement and instant product analysis.

In summary, a value of $1/4$ to $1/3$ of the measured i_{max} is a good compromise between the catalytic rate and having a potential that provides reasonable stability. The use of an immobilised NAD^+ -red enzyme is necessary for selective generation of the biologically active 1,4-NADH cofactor, which is efficiently taken up by LDH, either coimmobilised or in solution. There is no evidence of a biologically inactive form of the NADH cofactor being generated by the NAD^+ -red electrocatalyst at potentials more positive than -0.56 V .

7

Comparing the developed bioelectrocatalyst to existing cofactor regeneration techniques in biosynthesis

In this Thesis, a bioelectrochemical catalyst has been developed for the enantioselective reductions of ketones. An NADH-dependent oxidoreductase is coupled with an electrochemically driven NAD^+ -red enzyme, which recycles the cofactor. This chapter compares the turnover numbers and space-time yields of the electrocatalytic system developed in this Thesis with common industry cofactor regeneration techniques, hydrogen-powered regenerations and a similar bioelectrocatalytic NADPH regeneration technique.

Table 7.1 contains turnover data and space-time yields from various different cofactor recycling techniques. Entries 1 and 2 are examples of using a dehydrogenase, fed with a sacrificial carbon-based substrate, to regenerate NAD^+ to NADH for use with an oxidoreductase in a homogeneous biocatalytic reduction. This is a very common technique used in industrial biosynthesis. The example of using an FDH in flow was discussed earlier in Section 1.2.4 on page 14 for the biosynthesis of a Trandolapril precursor.

Entries 3 and 4 are cases of using HydRegen particles, developed within the Vincent Group, where an immobilised hydrogenase is used to power a coimmobilised

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Table 7.1: Comparing the catalytic characteristics of selected biosyntheses with NAD(P)H regeneration systems.

Entry	Cofactor regeneration	$C_{\text{NAD(P)}^+}$ / mM	Duration / h	TOF / s^{-1} ^a	TON ^b	TTN ^c	STY / $\text{g L}^{-1} \text{d}^{-1}$ ^d
1	GDH in batch ^[224]	1.5	25	3.17	367	285 714	260
2	FDH in flow ^[49]	0.2	4.6	0.01	900	218	165
3	HydRegen particles (batch) ^[52]	1	21.5	1.41	10	135 000	1.34
3a		0.01	-	-	655	-	-
4	HydRegen particles (flow) ^[58]	1	26	0.58	5	54 000	0.41
5	Armstrong Group ^[86]	0.02	19.3	0.22	2085	15 595	0.32
6	This work (Chapter 5)	1	16	1.69	4	97 350	0.21
(6a)	(−250 μA for 24 h)	1	24	8.19	51	707 803	4.58

^aTOF, Turnover frequency: Moles of product per second per mole NAD(P)⁺-reductase.

^bTON, Turnover number: Moles of product per mole cofactor.

^cTTN, Total turnover number: Moles of product per mole NAD(P)⁺-reductase.

^dSTY, Space-time yield: Grams of product per litre catalyst volume per day.

NAD⁺-reductase enzyme to regenerate the NADH cofactor. This has been discussed previously in Section 1.2.5 on page 18. These particles are included within this comparative discussion as they use a similar NAD⁺-reductase enzyme to what is utilised in the bioelectrocatalyst developed in this Thesis. Two cases of the HydRegen particles are discussed: one when used in batch and another in flow.

Entry 5 is the case of using an NADP⁺ reductase enzyme immobilised on ITO surfaces, for the electrochemical recycling of the NADPH cofactor. Although not the same cofactor as the other entries, this bioelectrochemical system is similar to that demonstrated in this Thesis for NADH recycling. The system developed by the Armstrong Group has been discussed in Section 1.3 on page 25. Finally, entry 6 shows the turnover parameters associated with the work demonstrated in Chapter 5.

It can be seen that for the GDH cofactor recycling system, the turnover parameters are very high. A TOF of 3.17 s⁻¹ is the highest value in the table and is generally high for biocatalytic NADH generation. This indicates that the GDH is very fast at being able to regenerate the NADH cofactor. The TON, the number of times a cofactor is used per mole of product generated, is also reasonably high, at 367. The total turnover number, TTN, is 285 714 and the largest TTN value in the table. This indicates that it has the highest efficiency in moles of product generated compared to the moles of the NAD⁺-reductase used in the reaction.

The space-time yield, STY, for the GDH cofactor recycling system is high (260 g L⁻¹ d⁻¹) This indicates that the GDH is the best system for NADH regeneration in terms of product produced per reaction volume per unit time. With these parameters, and the fact that GDH is relatively stable in solution and requires glucose as a cheap substrate, it is clear to see that the GDH has faster cofactor and product turnovers, saving time and money for use in industrial synthesis.

An alternative dehydrogenase cofactor recycling system is using FDH in a flow reactor, as shown in entry 2. Of particular note, there is a lower initial concentration of NAD⁺ (0.2 mM) and a shorter reaction time of 4.6 h, compared to most entries in the table. This has generated a higher TON value of 900 as compared to the GDH system. However the TOF (0.01 s⁻¹) and TTN (218) values are much lower,

due to a much higher concentration of the NAD^+ -reductase being used, which may increase costs and decrease the carbon efficiency. However, the STY is still high at $165 \text{ g L}^{-1} \text{ d}^{-1}$, which is an indication of a high yielding cofactor regeneration method for the reaction volume used.

The heterogeneous HydRegen particles can be used in a batch or flow reactor, and both cases are included in the table. Both have the same initial NAD^+ concentration and both have similar reaction times, however the TOF for the batch reaction (1.41 s^{-1}) is higher than that for the flow reaction (0.58 s^{-1}). This indicates that the HydRegen particles are working slower when used in a packed-bed reactor. The TON in batch is double that for the flow reactor (10 compared to 5) which indicates that the cofactor is not being regenerated fast enough in the flow reactor. The TTN for the batch reactor is reasonably high at 135 000, which is within the same magnitude of the TTN for the GDH cofactor recycling (285 714). The TTN for the HydRegen particles in the flow reactor is also reasonably high at 54 000.

A problem with the HydRegen particles, as with all the cofactor regeneration systems under early development (entries 3 to 6), is that they have very poor STY values. For the batch method the STY is $1.34 \text{ g L}^{-1} \text{ d}^{-1}$, whereas the STY for the slow reactor is worse at $0.41 \text{ g L}^{-1} \text{ d}^{-1}$. It is anticipated that with scale-up development, the STY values could improve and an advantage of using a flow reactor is that single-pass reactions are achievable with further development. Therefore it is anticipated that the STY values for flow reactions increase once developed into single-pass reactions.

It is worth noting that there have been reactions performed with HydRegen particles in batch under different cofactor concentrations. When the initial NAD^+ concentration was lowered from 1 mM to 0.01 mM, the cofactor TON increases to 655 (entry 3a), which is in line with the GDH and FDH cofactor regeneration techniques. However, the other turnover parameters suffer as a result of a decrease in cofactor concentration.

The Armstrong Group's electrochemical NADPH regeneration technique is presented in entry 5. It is noticeable that they often only use a very low initial

7. Comparing the developed bioelectrocatalyst to existing cofactor regeneration techniques in biosynthesis

concentration of NADP^+ for their reactions, which leads to an extremely high cofactor TON of 2085, which is even higher than the cofactor TONs for the GDH and FDH cofactor regeneration techniques. However the TOF (0.22) and TTN (15 595) values are fairly low compared to the other techniques in the table. This could be attributed to an overestimation of the amount of electrochemically active NADP^+ -reductase immobilised on their ITO electrodes. Indeed when there is a smaller amount of NADP^+ -reductase immobilised onto the ITO electrodes, the TTN increases. The STY is also fairly low, having a value of $0.32 \text{ g L}^{-1} \text{ d}^{-1}$.

Finally, the bioelectrochemical NADH recycling technique developed in this Thesis, is compared to the existing cofactor regeneration techniques (see entry 6). This data is taken from the electrosynthesis experiment performed in Section 5.5.3 on page 174. It can be seen that there is a fairly good TOF achieved of 1.69 s^{-1} . This is not quite as good as the GDH cofactor recycling method used in industrial biosynthesis, however it is in-line with the HydRegen particles in batch synthesis, which uses a similar NAD^+ -reductase enzyme, and better than the TOF shown by the Armstrong Group, which is a similar bioelectrochemical cofactor regeneration technique. The TTN of 97 350 is also relatively high compared to the other cofactor regeneration systems presented in the table.

The flow bioelectrocatalyst developed in this Thesis seems to have a significantly low cofactor TON (4) and STY ($0.21 \text{ g L}^{-1} \text{ d}^{-1}$) when compared to most other cofactor regeneration methods presented in the table. These values are similar to the cofactor TON and STY using HydRegen particles in a flow reactor (5 and $0.41 \text{ g L}^{-1} \text{ d}^{-1}$). This could be a reflection of the amount of cofactor being too high for a flow reactor, decreasing the cofactor TON, or the reactions not being single-pass flow reactions, which would significantly decrease the STY. Lowering the concentration of the cofactor and developing the bioelectrocatalyst into a single-pass flow cell would help to avert these limitations.

It can be seen that electrochemically powering the immobilised NAD^+ -red enzyme directly is a very efficient electrochemical cofactor regeneration method. However, the electrosynthesis providing the turnover data in entry 6 in the table

does not necessarily demonstrate the power of the bioelectrochemical catalyst developed in this Thesis. The largest catalytic reduction current measured was $-250\text{ }\mu\text{A}$, with twice the amount of NAD^+ -red immobilised onto the carbon electrode (see Section 5.5.4 on page 177). The predicted turnover numbers for a sustained electrosynthesis at $-250\text{ }\mu\text{A}$ for 24 h at this electrode, assuming a constant reduction potential, is presented in entry 6a. Without changing the cofactor concentration it can be seen that there is an increase in magnitude in the cofactor TON from 4 to 51. Decreasing the cofactor concentration will improve this further. The STY is also improved, from $0.21\text{ g L}^{-1}\text{ d}^{-1}$ to $4.58\text{ g L}^{-1}\text{ d}^{-1}$, however compared to the GDH and FDH cofactor recycling systems, it appears that this system is not necessarily economically viable for industrial synthesis at this time. Developing this bioelectrocatalyst into a single-pass system rather than a semi-continuous flow synthesis will improve the STY value.

The TOF and the TTN are also predicted to provide very high values, from 1.69 s^{-1} to 8.19 for the TOF and the cofactor TTN increasing from 97 350 to 707 803. These are extremely high values and indicates that the NAD^+ -red is working very fast to regenerate the NADH cofactor. This also indicates that this bioelectrocatalyst could be a viable system for industrial electrosyntheses in flow.

The turnover numbers and space-time yield of the bioelectrocatalyst developed in this Thesis has been compared to existing industrial cofactor regeneration techniques and to other cofactor regeneration techniques in development. Although it does not have the high STYs demonstrated by the GDH and FDH regeneration techniques, or the very high cofactor TON demonstrated by the Armstrong bioelectrochemical regeneration technique, the TOF and TTN are promisingly high. Further development in transforming the bioelectrocatalyst into a single-flow catalyst and reducing the relative concentration of the cofactor will aid in improving the STY and cofactor TON values accordingly. For now, at least, this flow bioelectrocatalyst for enantioselective reductions is a promising synthetic technique and further development could transform it into an applicable industrial process.

8

Outlook for biocatalytic electrosynthesis

The development of the NAD^+ -red electrocatalyst, in tandem with LDH or alaDH enzymes, and in flow, has opened up many possible research routes.

Chapter 4 to 6 of this Thesis have demonstrated the development of an enantioselective bioelectrocatalyst implemented in flow systems, using an NAD^+ -reductase enzyme immobilised on carbon electrodes to selectively regenerate the biologically active 1,4- NADH cofactor. It has been shown that alaDH or LDH enzymes efficiently take up the cofactor for enantioselective reductions of $\text{C}=\text{X}$ substrates. Therefore, it should be straightforward to extend to other commercially available NADH -dependent oxidoreductases for other enantioselective reductions. This would open up the possibility of a modular electrocatalyst design, where any NADH -dependent oxidoreductase can plug-and-play with the NAD^+ -red electrocatalytic cofactor regeneration system, opening a library of possible enantioselective reductions using this electrocatalyst design.

For the biocatalytic electrochemical reductions demonstrated in this Thesis, protons are used from the solvent to generate the hydride and proton for the reduction of the $\text{C}=\text{X}$ substrate. Replacing this with a deuterated solvent would provide an electrocatalytic labelling system for $\text{C}=\text{X}$ reduction products with high enantiomeric purity, as has been demonstrated with operation of the Vincent Group's H_2 -driven NADH recycling system in D_2O .^[225]

The flow electrosynthesis experiments presented in this Thesis were performed under ‘semi-continuous’ flow, where the solution was repeatedly cycled through the electrochemical cell. It should be possible to develop this into a single pass electrochemical flow system, where the solution is only required to pass through the electrocatalyst once. This has been demonstrated for a similar heterogeneous catalytic particle system^[58] and could be applied to the electrocatalyst. This would enable easier development of sequential bioelectrocatalytic modules, coupling other electroenzymatic reactions to form a fully realised electrosynthesis process.

With the development of high reduction currents at the cathode, greater attention should be paid to the reaction that occurs at the anode. This electrode could be designed so that a H_2 oxidation reaction could occur, which would generate 2H^+ , maintaining pH neutrality. As it has been shown that the NAD^+ -red enzyme is reversible, the anode could also perform an electroenzymatically-driven oxidation of organic molecules with a similar cofactor regeneration system with no overpotential necessary. Similar to this suggestion, a deracemization device could be developed with sequential oxidation, then re-reduction of an organic molecule to generate an enantiopure product.

It has been shown that a genetic variant of the NAD^+ -red used in this system can be used for the regeneration of the related NADPH cofactor.^[226] This would unlock another library of NADPH-dependent oxidoreductases for a wider reaction scope, again using a carbon support. Using a separate cofactor pool, this NADPH-recycling system could operate as part of the same biological electrocatalytic flow module as another NADH-dependent enzyme. An alternative NAD(P)H recycling system has been developed by Armstrong and coworkers, but an ITO surface is required for electron exchange with the NADP^+ -reductase, FNR. The extent to which the ITO is silent towards redox transformations of relevant enzyme substrates and products, over the potential range of interest has not been fully investigated.

Overall, the work presented in Chapters 4 to 6 of this Thesis advance the field of bioelectrosynthesis with a promising device demonstrated.

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