Nanoporous gold electrodes for application in trace metal sensors, biosensors and biofuel cells

Thesis presented for the award of
Doctor of Philosophy (PhD)

by

Till Siepenkötter

Under the supervision of Professor Edmond Magner
Submitted to the University of Limerick
Declaration of academic integrity

With this statement I declare, that I have independently completed the following work entitled with “Nanoporous gold electrodes for application in trace metal sensors, biosensors and biofuel cells”. The information and thoughts taken directly or indirectly from external sources are properly marked as such. This work has not been previously submitted to this or any other university.

_________________________________________________________________________

Place and Date                          Till Siepenkötter
Abstract

Nanoporous Gold (NPG) is a material of emerging interest for immobilization of biomolecules and especially enzymes. NPG materials provide a high surface area onto which biomolecules can either be directly physisorbed, covalently linked after first modifying the NPG with a self-assembled monolayer (SAM) or entrapped in a polymer matrix. The immobilization of enzymes while using NPG substrate material is being pursued for applications in sensors, assays, supported synthesis, catalysis and biofuel cells. NPG materials can be prepared by using many different approaches. However, the most common method used is the dealloying of a low carat gold alloy containing between 20-50 atomic % gold in a strong acid (70% HNO₃), which oxidizes the least noble metal, removing it from the alloy. The rapid rearrangement of the gold atoms at the solid/liquid interface leaves behind the characteristic surface morphology. The resultant structure consists of interconnected ligaments and pores with typical widths between 5-200 nm. The surface area of these materials can be up to 500 times higher than their geometric area.

Surface addressability of NPG is crucial for functionalization and surface modification for the use in sensors, biosensors and biofuel cells. Full addressability of the surface area of NPG was observed with small molecules such as sulphuric acid. The surfaces could also be modified using bulky anthraquinone functional groups attached on activated diazonium salts throughout the whole structure. Surface modification of NPG has been achieved using a variety of strategies, such as through SAM formation of thiol compounds, electro-reduction of in situ synthesized diazonium compounds and the drop-casting or electro-polymerization of osmium redox polymers and hydrogels.

Surface functionalized NPG could be used for a variety of applications. Bulky negatively charged sulfonate groups could therefore attract positively charged free trace metal ions (such as Cu²⁺) in solutions for direct detection at the electrode surface. The sensor displayed a detection range from 0.2 to at least 25 µM which is within the legal concentration limit of 20.5 µM (1300 ppb) in drinking water (United States, EPA). The sensitivity and limit of detection (LOD) were found to be 8.18 µA cm⁻² µM⁻¹ and 18.9 nM (~1.2 ppb) respectively. The BDS surface functionalization was also capable of blocking biofouling material from the electrode surface, making it possible to measure in complex media such as artificial human serum. Fructose dehydrogenase (FDH) could be covalently attached to carboxylic acid terminated diazonium compounds for the precise detection of D-fructose concentrations in a range of natural sweeteners and
beverages. The sensor was able to give accurate readings within 5 seconds with a linear range of 0.05 - 0.3 mM D-fructose concentration, a sensitivity of $3.7 \pm 0.2 \, \mu A \, cm^{-2} \, mM^{-1}$ and a LOD of 1.2 μM. When combining anodic enzymes, such as glucose dehydrogenase (GDH) and FDH, with cathodic enzymes such as bilirubin oxidase (BOD), enzymatic biofuel cells with considerable power outputs can be obtained. GDH/MvBOD EFCs generated power densities of up to 17.5 and 7.0 μW cm$^{-2}$ in PBS and artificial serum, respectively, at an OCV of ~0.45 V (vs Ag/AgCl) with a concentration of 5 mM D-glucose. These EFCs retained over 60% of their initial power density after 8 hours of continuous operation. FDH/BpBOD EFCs generated power densities of up to 13 μW cm$^{-2}$ at an operating potential of 0.18 V vs Ag/AgCl at a concentration of 10 mM D-fructose. The half-life was found to be $ca. \, 19 \, h.$
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“And now here is my secret, a very simple secret: It is only with the heart that one can see rightly; what is essential is invisible to the eye.”

- Antoine de Saint-Exupéry, The Little Prince
Publications and Presentations

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Submitted manuscripts:


Manuscripts in preparation:

- Siepenkoetter T, Mastin H, Salaj-Kosla U, Magner E, “Benzene Diazonium Sulfonate modified NPG electrodes for precise Copper Detection in Water Samples and Artificial Human Serum”


- Xiao X, Siepenkoetter T, Ó Conghaile P, Leech D, Magner E, “Enzymatic biofuel cells utilising nanoporous gold electrodes on contact lenses”

- Lopez F, Siepenkoetter T, Xiao X, Magner E, Schuhmann W, Salaj-Kosla U, “Potential pulse-assisted immobilization of Myrothecium verrucaria Bilirubin oxidase at unmodified planar and nanoporous gold electrodes.”
Oral presentations:

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<th>Description</th>
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<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>AFM</td>
<td>Atomic force microscopy</td>
</tr>
<tr>
<td>Ageo</td>
<td>Geometric surface area</td>
</tr>
<tr>
<td>AnGOx</td>
<td>Aspergillus niger Glucose Oxidase</td>
</tr>
<tr>
<td>AQ</td>
<td>Anthraquinone functional groups</td>
</tr>
<tr>
<td>Areal</td>
<td>Electrochemically addressable surface area</td>
</tr>
<tr>
<td>ASV</td>
<td>Anodic stripping voltammetry</td>
</tr>
<tr>
<td>BDS</td>
<td>Benzene diazonium sulfonate</td>
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<tr>
<td>BFC</td>
<td>Enzymatic biofuel cells</td>
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<tr>
<td>BOC</td>
<td>Tert-butyloxycarbonyl protecting group</td>
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<tr>
<td>BOD</td>
<td>Bilirubin oxidase</td>
</tr>
<tr>
<td>BOKU</td>
<td>Universität für Bodenkultur, Vienna</td>
</tr>
<tr>
<td>BpBOD</td>
<td>Bacillus pumilus bilirubin oxidase</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>C</td>
<td>Carbon</td>
</tr>
<tr>
<td>CAG</td>
<td>Contact angle goniometry</td>
</tr>
<tr>
<td>CBN</td>
<td>Carbon based nanostructures</td>
</tr>
<tr>
<td>CC</td>
<td>Carbon cloth</td>
</tr>
<tr>
<td>CDH</td>
<td>Cellobiose dehydrogenase</td>
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<tr>
<td>Cdl</td>
<td>Double layer capacitance</td>
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<tr>
<td>CL</td>
<td>Crosslinker</td>
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<tr>
<td>CMC</td>
<td>N-cyclohexyl-N’-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate</td>
</tr>
<tr>
<td>CNRS</td>
<td>Centre National de la Recherche Scientifique, Bordeaux</td>
</tr>
<tr>
<td>CtCDH</td>
<td>Corynascus thermophilus cellobiose dehydrogenase</td>
</tr>
<tr>
<td>CV</td>
<td>Cyclic voltammogram/Cyclic Voltammetry</td>
</tr>
<tr>
<td>Cyt. c</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>DEE</td>
<td>Diethyl ether</td>
</tr>
<tr>
<td>DET</td>
<td>Direct electron transfer</td>
</tr>
<tr>
<td>DIN</td>
<td>Deutsches Institut für Normung e.V.</td>
</tr>
<tr>
<td>DIPEA</td>
<td>N,N-Diisopropylethylamine</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DMS</td>
<td>Dimethyl suberimidate, disuccinimidyl tartrate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DM™</td>
<td>Digital Micrograph™</td>
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</table>
DNA: Deoxyribonucleic acid
DPV: Differential pulse voltammetry
EcGDH: *Escherichia coli* glucose dehydrogenase
EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDX: Energy-dispersive x-ray spectroscopy
EFC: Enzymatic fuel cells
EPA: The Environmental Protection Agency
ET: Electron transfer
FAD: Flavin adenine dinucleotide
FAD-GDH: *Glomorella cingulata* FAD dependent glucose dehydrogenase
FDH: D-Fructose dehydrogenase from *Gluconobacter* sp.
FFT: Fast fourier transform
GC: Glassy carbon
GcGDH: *Glomorella cingulata* FAD dependent glucose dehydrogenase
GDH: Glucose dehydrogenase
GOx: Glucose oxidase
HBF₄⁻: Tetrafluoroboric acid
HCF: Hexacyanoferrate
HFCS: High-fructose corn syrup
ICP-AAS: Inductively coupled plasma-atomic emission spectrometry
ICP-MS: Inductively coupled plasma-mass spectrometry
IR: Infrared spectroscopy
IUPAC: International Union of Pure and Applied Chemistry
Kₘ: Michaelis-Menten constant
Lₑ: Characteristic length scale
LED: Light-emitting diode
LI method: Linear intercept method
LOD: Limit of detection
LSV: Linear sweep voltammetry/voltammogram
M: Metal
MCO: Multicopper oxidase
MES: 4-Morpholineethanesulfonic acid
MET: Mediated electron transfer
MFC: Microbial fuel cell
MOPS: 3-(N-morpholino) propanesulfonic acid
MPA: 3-Mercaptopropionic acid
MPS: 3-Mercapto-1-propanesulfonate
MWCNT: Multi-walled carbon nanotubes
*Mv*BOD: *Myrothecium verrucaria* bilirubin oxidase
NA: 6-Amino-2-naphthoic acid
NA-DS: 2-Carboxy-6-naphtoyl diazonium salt
NBD: 4-Nitrobenzene diazonium salt
NHE: Normal hydrogen electrode
NQ: Naphthoquinone
NPG: Nanoporous gold
NUIG: National University of Ireland, Galway
OCV: Open circuit voltage
PAMAM: Poly(amidoamine) dendrimer
PB: Phosphate buffer
PBS: Phosphate buffer saline
PEG: Poly ethylene glycol
PEGDGE: Poly(ethylene glycol) diglycidyl ether
PQQ-GDH: Pyrroloquinolinequinone-dependent glucose dehydrogenase
Pyr(NQ)$_2$: 1-[Bis(2-naphthoquinonyl)aminomethyl]pyrene
R$_f$: Roughness factor
SAM: Self-assembled monolayer
SANS: Small-angle neutron scattering
SAXS: Small-angle X-ray scattering
SC: Semiconductor
SEM: Scanning electron microscope
SEM-EDX: Scanning electron microscopy- energy dispersive x-ray spectroscopy
SPGE: Screen printed graphene electrodes
SPR: Surface plasmon resonance
STM: Scanning tunneling microscopy
SWCNT: Single-walled carbon nanotubes
TB: Toluidine blue polymer
TBATFB: Tetrabutylammonium tetrafluoroborate
TEM: Transmission electron microscope
THF: Tetrahydrofuran
TP: Toray paper
TTF: Tetrathiafulvalene
TvLac: Laccase from *Trametes versicolor*
UPD: Underpotential deposited
UV: Ultraviolet
UV-Vis: Ultraviolet–visible spectroscopy
WHO: World Health Organization
XPS: X-ray photoelectron spectroscopy
XRD: X-ray diffraction
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Chapter 1: 

Literature Review
1.1. Proteins in electrochemistry

1.1.1 Introduction

Proteins are biological molecules that can be found in all cells of living creatures. These molecules are the most abundant biological molecules and occur in great variety and with very specific functions. Proteins can act as structural elements, signal receptors, transporters that can carry specific substances in and out of a cell, or most interestingly, as catalytic active species, are perhaps the most versatile of all biomolecules [1]. These biomolecules consist of polymers of amino acids. Amino acids are molecules which possess a central carbon atom, an amino group, a carboxylate and an R group (R groups differ from each amino acid due to hydrophobic and hydrophilic effects). Twenty amino acids occur naturally and form proteins via the formation of peptide bonds between the amino (N) and carboxyl (C) groups (Figure 1.1 A-B) forming primary and secondary structures [2]. Primary structures constitute a linear sequence of amino acid in the polypeptide chain. Secondary structures (alpha helix and beta sheets) are formed by defined hydrogen bonds between the peptide groups throughout the main chain of the structure (Figure 1.1 B).

More complex forms occur via the formation of tertiary (Figure 1.1 C) and quaternary (Figure 1.1 D) structures. Tertiary structures are formed by the folding of alpha-helixes and pleating of beta sheets into consolidated three-dimensional structures.

Figure 1.1: schematic representation of possible organizations of protein structures with (A) primary structure, (B) secondary structure, (C) tertiary structure and (D) quaternary structure, copied from reference [2].
Quaternary structures are delineated by multiple subunits of tertiary structures. The protein structure is stabilized by disulfide bonds, hydrogen bonds, ionic bonds as well as Van der Waals and hydrophobic interactions [2]. The final protein structures depend on the amino acid sequences and the interactions among the amino acids [2].

Proteins that possess the ability to either accept or donate electrons, resulting in an increase or decrease in the oxidation state [3] are also referred to as redox proteins. They consist of a redox center which usually is surrounded by a polypeptide structure. The specific properties of the protein define its function and ultimately its potential application for electrochemical sensors, fuel cells or biomedical devices [4]. Redox proteins and enzymes can basically be ascribed as more complex and fragile three-dimensional proteins [5], which facilitate [2] the ability to catalyze biological reactions. In general a reaction occurs by a substrate S reacting to a product P that possess a lower free energy ground state G (as seen in Figure 1.2 A). The difference in the free energy $\Delta G^0$ is available when the reaction occurs. In order to trigger the reaction a transition energy state has to be overcome which requires introducing activation energy into the system (as seen in Figure 1.2 A). The general reaction mechanism can be seen in equation 1.1 [1].

$$E + S \xrightleftharpoons{\text{ES}} \xrightleftharpoons{\text{EP}} E + P \quad (1.1)$$

In some cases the transition state presents a high energy barrier. This problem in cells is overcome by the ability of enzymes to lower the activation energy as seen in Figure 1.2 B. The mechanism (as demonstrated in equation 1.1 [1]) involves two transition states (ES) and (EP). This demonstrates the advantage of enzymes in biological systems. The exploitation of this effect has resulted in a range of applications
of enzymes in industries such as biofuels and biological detergents, brewing and dairy industry, food processing and in the pharmaceutical industry as selective biocatalyst [6].

Stabilization of enzymes is crucial for the preparation of durable biosensors and biofuel cells. Protein engineering is one of the most frequently used approaches to improve stability and activity of proteins/enzymes. Protein engineering can be performed by [5]:

1. **Directed evolution** [7]: where usually a library of random mutants with particular properties are constituted, attributes of interest are defined, the mutants of particular interest are selected and afterwards this process is repeated until the enzyme engineer is satisfied with the specific properties of the mutated enzyme.

2. **Site-directed mutagenesis** [8]: in contrast to the random mutation of directed evolution, site-directed mutagenesis applies a more organized approach to alter the specific properties of the mutated enzymes, such as activity, stability or thermal compatibility. Here the protein structure is examined extensively and individual sites of interest are targeted for mutagenesis. It is important to note that this method requires a deep knowledge on the structure of the protein. The site mutagenesis is then evaluated for effectiveness to the desired new attributes of the proteins.

3. **Peptide chain extension** [9]: As the name indicates this method uses the lengthening of either nitrogen or carbon terminated polypeptide chains of the enzyme which is usually used to improve the stability of the enzyme.

Understanding biological electron transfer in these macromolecular structures is somewhat complex. A wide range of theoretical and experimental studies have been performed to develop a better understanding of biological electron transfer. In general the electron transfer rate constant is measured between electronically localized donor and acceptor states of the protein [10].

Numerous materials are reported to be suitable supports for the immobilization of enzymes. Carbon [11] (glassy carbon [12], carbon nanotubes [13], carbon cloth [14]), Au [15], Ag [16], Pt [17], Bi [18], Hg [19], Al [20] and alumina oxide [21] are among the most common used supports for enzyme immobilization. Lin et al. also showed that cyanuric chloride is an adaptable surface activating reagent for the immobilization of enzymes on glassy carbon, pyrographite and metal oxides such as TiO$_2$, In$_2$O$_3$ and SnO$_2$[22].
1.1.2 Enzymes stabilization and immobilization

Enzymes are macromolecular structures that are quite sensitive to temperature, pH and concentration effects that can initiate denaturation processes. Denaturation of enzymes can be understood as a process of unfolding of the enzyme’s tertiary or quaternary structure. This usually leads to a disordering of the polypeptide chains, where the key components of the structure are not able to continue their participation in active functions of the protein [23]. Numerous methods and strategies have been developed and applied to enhance the stability of enzymes and avoid denaturation. Enzyme immobilization and enzyme stabilization are the two most important components of increasing the lifetime of these catalytic proteins, which also make them interesting in bioelectrochemical studies. Even though the mechanisms involved in immobilization and stabilization may differ substantial from one another, they are still related, as both involve either changing the specific configuration of the proteins or the application of binding/trapping of the protein. Enzyme stabilization can be ascribed as a method of enhancing the active catalytic lifetime of the protein, while enzyme immobilization involves techniques to chemically or physically bind the protein to a defined surface. Enzyme immobilization can therefore be defined as a method of enzyme stabilization. Stabilization methods involve immobilization, chemical modification, addition of additives and protein engineering (previously described in section 1.1.1 above) [5]. Chemical modification is one of most common technique to achieve better enzyme stabilization. The most common used representative in terms of chemical modification is the adding of cross-linking reagents to the protein solution. These crosslinking additives induce intramolecular crosslinking of the peptide chains between the individual proteins, forming connected agglomerate structures (as seen in Figure 1.3). Glutaraldehyde has a long history in the use as cross-linking reagent for protein [24]. Originally used by crystallographers for the determination of crystal structures of proteins, they are also used in protein stabilization [25]. The carbonyl groups of the glutaraldehyde can react with free amine and hydroxyl groups of the protein, resulting in a crosslinked network. Dimethyl suberimidate, disuccinimidyl tartrate (DMS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) are also reported to be effective crosslinking reagents [5]. Water soluble polymers, such as polyethylene glycol (PEG), are also often used as crosslinking reagents for enzymes as discussed by Veronese et al. [27]. Veronese describes these PEGylating reagents as molecules with N- or C-terminated end groups that react with the individually desired amino acid of the protein [27].
Another commonly used method for enzyme stabilization is the use of additives. Usually additives fall into 4 general categories. These can be delineated as small molecules (trehalose [28]), surfactants, polymers (PEG [27]), proteins (BSA [29], human albumin [30]) and micelles [31], respectively [5]. Upon dehydration, trehalose sugar produces a gel that is capable of protecting the protein structure from external influences [5]. Due to the fact that proteins tend to denature at interfaces, surfactants can be introduced. Surfactants can establish stabilization of the protein as they introduce competition at the electrode interface [5]. If enzymes are confronted with harsh environments such as extremes of temperature, large shifts in pH or to organic solvents, micelles can be used as stabilization agents. In aqueous solutions micelles typically form aggregates with hydrophilic head groups at the interface while the hydrophobic tails points toward the center of the aggregate. Micelles can protect proteins by binding to the protein with the hydrophobic and hydrophilic reacting with their respective counterpart. This form of layer formation on the protein can provide protection to foreign influences [5].

Enzyme immobilization is another approach that can be used to stabilize and utilize the native catalytic activity of the protein structures. Cao very vividly considered enzyme immobilization to be a form of art, as much as it can be considered to be science [32]. Enzyme immobilization can be broken down into three main sections as displayed in Figure 1.3. These immobilization techniques are: binding onto support material, entrapment and cross linking (described in section 1.1.2) of the proteins.
We generally distinguish between three different mechanisms involved when proteins are bound to a support material. This can be due to physical forces, such as adsorption, hydrophobic and van der Waals interactions, ionic interactions, or chemical bindings, such as covalent attachments or cross linking [33]. Adsorption is basically caused through intermolecular forces resulting in the accumulation of proteins on the solid interface [5]. However, binding by physisorption is generally very weak and may result in random orientations of the enzymes on the surface, which might not be desirable. Therefore adsorption may not be very suitable in real life application. Ionic binding presents more stable systems. Chemical bonding however results in the most stable forms of immobilized enzymes, when supported on a stable substrate material. The major drawback of this approach is that the protein structure can be altered, which may affect its catalytic properties. Covalent attachment of enzyme is often performed via the terminal functional group of self-assembled monolayers (SAMs) on a support material. However, through the covalent attachment of an enzyme to a SAM the activity can be dramatically decreased. The methods used are comprehensively discussed in section 1.2. Solid substrates can be a range of materials. Besides obvious conductive metal substrates, organic polymers (such as Eupergit® C [34]), biopolymers (polysaccharides such as cellulose, starch, agarose and chitosan [35]), hydrogels (polyvinyl alcohol [36]) and inorganic supports (mesoporous silicas [37]) are among the most common used supports [33].

Once proteins are immobilized on supports the question of electron transfer arises. We differentiate between two major electron transfer mechanisms, direct electron transfer (DET) or mediated electron transfer (MET).

### 1.1.3 Direct electron transfer

The mechanism involved to achieve electron transfer from an enzyme to an electrode mainly depends on the distance the electrons have to overcome as seen in equation 1.2, where $k$ is the electron transfer constant, $\alpha$ is a constant directly dependent on the medium the electrons have to travel through, $r$ is the distance between electron acceptor and donor, $\Delta G$ is the Gibbs energy of activation, $R$ is the gas constant and $T$ represents the temperature. In a system with same medium, temperature
and reaction the only variable affecting the electron transfer rate is therefore the distance between the electron donor and acceptor [38].

The most desired electron transfer mechanism between a protein and an electrode is DET, due to its simplicity. The simplified schematic scheme can be seen in Figure 1.5 A. When DET occurs, the tightly bound cofactor of the active site inside the redox protein can directly deliver electrons to and from the electrode [39]. The simplest approach of DET is if the electroactive area of the enzyme simply comes in contact with the electrode and is therefore capable of delivering the electrons directly [41] (Figure 1.4 C). This mechanism however occurs rarely due to the fact that the electroactive sites of the enzyme are usually buried within the enzyme structure. This “direct” electron transfer is subject to significant constraints and is dependent on the distance between the redox active site and the electrode together with the orientation of the active site to the support material. This can schematically be seen in Figure 1.4. If the active site of the enzyme faces away from the electroactive area (Figure 1.4 A) no electron transfer can be observed, while if the redox center is close to the electroactive area or even touches it (Figure 1.4 B) DET can be achieved [40].

For DET, without direct contact of the redox center to the electrode, two different mechanisms can occur. The mechanism involved is highly dependent on the properties of the proteins used. A tunneling mechanism is often involved. Usually complex bridging molecules, exhibiting different functional groups enables electrons to tunnel through the energy barrier to establish ET. Tunneling is the consequence of higher potential energy acting on the electron in the space between the reacting pair than the energy of the electron in its localized site as demonstrated by R.A. Marcus et al.

![Figure 1.4](image-url)  
**Figure 1.4:** Schematic illustration of the effect of orientation of an immobilized enzyme at an electroactive substrate with (A) hindered ET and (B) tunneling of electrons from the active center to the electrode and (C) DET of electrons to the electrode, modified from reference [40].
Figure 1.5: Schematic illustrating of the mechanism involved in (A) direct electron transfer (DET) and (B) mediated electron transfer (MET) between an enzyme active site and an electrode in the oxidation of a substrate, modified from reference [44].

The electron transfer of the redox protein to the electrode is not only dependent on the difference in potential between them, and the distance between the electroactive surface and the redox center, but also on the rigidity of the structure [39,43]. DET via tunneling can become difficult when the distance between the prosthetic group of the enzyme and the electrode surface is long, due to the shielding that is provided by the protein shell.

Another process that induces DET is the superexchanging of electrons. Superexchange of electrons is a mechanism that is often observed for structures that possess a long distance to the support surface, where DET via direct tunneling of the active site to the electrode surface becomes less likely [39]. Superexchanging involves relay molecules in the protein structure that are within tunneling distance to one another and to the electrode surface that are able to internally shuttle electrons.

Another method to force orientation on the enzymes is to immobilize the enzymes to the surface and therefore reducing the distance of the redox active area of the enzyme from the electrode. ET responses from redox centers that are further away than 2 nm from the electroactive area can be expressed as negligible [39]. If it not possible to constrain the redox center close to the electroactive surface, DET will not be feasible. However it is important to note that the immobilization of enzyme can also result in blocking of the access to the active site of either the co-substrate or the substrate which will also results in no measurable catalytic activity, due to mass
transport issues. Also, denaturation of the enzyme can be observed, leading to diminished catalytic activities and therefore lower currents [39].

This demonstrates the need to design optimal electrode configurations to ensure that the ET distance between an immobilized redox protein and a suitable electrode surface is made as short as possible but with favorable orientation [39]. If it is not possible to generate these conditions, alternative strategies have to be explored such as the introduction of mediators that are capable of shuttling generated electrons from the active site of the enzyme to the electrode surface, i.e. MET.

### 1.1.4 Mediated electron transfer

Alternative electrode shuttling mechanisms are necessary for redox proteins that do not exhibit the above mentioned features. Mediated electron transfer (MET) is a common alternative to DET in bioelectrochemistry. Typically a co-substrate or an electrochemically active species, such as redox mediators, are used to shuttle electrons between the active site of a redox protein and the electrode surface. The schematic representation of the MET mechanism is displayed in Figure 1.5 B. During the shuttling process of the electrons between the active site and the electrode, the mediator is cycled between its oxidized and reduced forms [45]. Mediators can have high diversity in structure, redox potentials and specific properties. Therefore, MET can generally be performed using two different classifications, homogenous- and heterogeneous mediated transfer. In homogenous mediated systems the mediator and the enzyme are free in solution. They diffuse freely through the medium, while after electron transfer the free mediator interacts with the electrode [39]. In contrast, in heterogeneous mediated systems either the mediator or the enzyme is immobilized on the electrode surface and the electron transfer is established between them. This usually is achieved by adding the mediator to a bulk solution, with the goal to reach an immobilized enzyme, or by immobilizing the mediator onto the electrode in a solution that contains free enzyme [47].

The requirement for the use of MET becomes obvious when the physical properties of enzymes are observed (Figure 1.5). The dimensions of enzymes are in the range of 7-20 nm [46] and the redox centers can be buried deep within the protein matrices. These structural properties result in the spatial separation of the biocatalytic redox sites from the electrode, which results in hindered electrical contact of the enzyme with the conductive substrate. This results in diminished or disabled energy harvesting.
Figure 1.6: Schematic representation of heterogeneous MET strategies with (A) application of a diffusional electron transfer mediator, (B) introduction of redox-relay units to the protein associated with the electrode, (C) immobilization of the enzyme in a redox polymer associated with the electrode, (D) reconstitution of an apo-enzyme on a relay-cofactor unit linked to the electrode, modified from reference [46].

from the biocatalytic reaction, thus demonstrating the necessity for MET. MET can generally be performed using a wide range of approaches. A summary of the most common strategies is displayed in Figure 1.6. The easiest way to achieve MET is by introducing diffusional electron mediators that transport electrons between the redox center of the enzyme and the electrode, as displayed in Figure 1.6 A [46]. Another strategy is to affix redox mediating relays to the enzyme structure (on the periphery as well as inner protein sites) which can effectively shorten the electron transfer distance leading to MET between the biocatalytic redox centers and the electrode (Figure 1.6 B) [46]. This approach however can alter the structure of the enzyme as the relays are usually covalently bound to the enzyme, which may result in significantly decreased catalytic activity. A very commonly used technique is the immobilization of the redox enzymes in electroactive polymer matrices [48] or redox active hydrogels [15] that transports electrons between the enzyme active sites and the electrodes. This transport is made possible by flexible charge carrying redox-active segments within the polymer matrices (Figure 1.6 C). Heller and coworkers have extensively studied the electrical
contacting of redox enzymes integrated in redox active active Os$^{2+/3+}$ polypyridine or poly-bi-imidazole complexes [15,48]. These studies led to a variety of amperometric biosensors with “electrochemically wired” enzymes [46]. The redox potential of Os$^{2+/3+}$ can be tuned within a wide range by exchanging the ligands bound to the complexes.

For the use of Os-complexes (i.e. polymers or hydrogels) in bio fuel cells (for instance a glucose/O$_2$-bio fuel cell) the anode should operate at the lowest possible potential (close to that of the FAD redox site of glucose) while the biocatalytic anode should operate at the most positive potential. This necessitates the use of tunable redox potentials of the Os-complexes. Figure 1.7 displays two examples of a redox polymer and a redox hydrogel. PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$ (Figure 1.7 A) is a redox polymer that is capable of wiring bilirubin oxidase (BOD) to an electrode [49]. Figure 1.7 B displays a poly[N-vinylpyridine Os(N,N'-dialkylated-2,2''-bimidazole)$_3$]$^{2+/3+}$ polymer hydrogel. The long alkyl chains retaining the Os$^{2+/3+}$ complex to the polymer backbone provides flexibility to the charge carrier units, leading to an enhanced electron transfer throughout the hydrogel structure [46].

Another strategy for achieving MET is the reconstitution of an apo-enzyme (an enzyme from which the cofactor has been removed) on relay/cofactors that are covalently bound to the electrode surface as seen in Figure 1.6 D. The native cofactor is extracted from the enzyme and reconstitution of the apo-enzyme with the relay-cofactor linked to the electrode results in electron transfer to or from the electrode [46].

Figure 1.7: Schematic representation of cathodic redox polymer (A) PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$ which electrochemically wires BOD to an electrode [49] and anodic redox hydrogel (B) ([Os(N,N'-dialkylated-2,2''-bi-imidazole)$_3$]$^{2+/3+}$) that electrically wires GOx to the anode of a BFC [46],[50].
1.2 Self-assembled Monolayers (SAMs)

1.2.1 Introduction

Significant attention has focused on self-assembled monolayers (SAMs) since Zinsman et al. first described the adsorption of a monomolecular layer on a metal surface in 1946 [51,52]. SAMs are distinguished by the ability of a chemical compound to spontaneously adsorb onto a substrate material in a highly ordered manner (without the presence of interfering substances as metal atoms, metal oxides or organic compounds, which lead to defects in the ordered structure [53]). These adsorbates decrease the free energy at the interface between the substrate material and the surrounding environment, which explains the spontaneous formation of SAMs [54]. The substrate material is usually composed of metal atoms or metal oxides as indicated in Figure 1.8. As seen in Figure 1.8, SAMs covalently attached to a substrate material typically consist of 4 components. These components typically consist of 1) planar surfaces (such as glass or silicon slabs supporting thin films of metal, metal oxides, metal foils or single crystals [53]) or highly structured (such as colloids, nanocrystals or nanorods [53]) which acts as substrate material on which 2) the ligand or head group, often composed of sulfur (as seen in Figure 1.8) or carbon. 3) Usually SAMs are composed of a 1-3nm alkane chains with the purpose to act as spacer or physical barrier. These spacers can alter the physical properties of the substrate material (e.g. the conductivity, wettability and corrosion resistance) [53]. The final component is 4) the

![Figure 1.8: Schematic diagram of an ideal, single-crystalline SAM of alkane thiolates supported on a gold surface. The specific characteristics of the SAM are highlighted, modified from reference [53].](image)

Figure 1.8: Schematic diagram of an ideal, single-crystalline SAM of alkane thiolates supported on a gold surface. The specific characteristics of the SAM are highlighted, modified from reference [53].
terminal/functional group which determines the surface properties and possesses functional groups that can be used for enzyme immobilization.

SAM modified surfaces find applications in many fields of chemistry and physics. A frequent use for SAM modified conductive surfaces is reported to be the use as sensor [55]. The voltammetric sensing of pH using SAM modified electrodes was first reported by Wrighton et al. [56]. They were able to set up a cell system, where quinone (pH sensitive) and ferrocene (pH insensitive) were co-immobilized on SAMs as working and reference electrode respectively. With changing pH in solution the oxidation and reduction peaks of quinone shift linearly. The authors found these pH measurements to give precise result in a range from pH 1-11 [56]. SAMs are also used as support for the sensing of inorganic compounds [55]. The electrodes are modified using surface active SAM for the detection of redox active or redox inactive inorganic compounds. Turyan et al. were among the first that presented SAMs with carboxylic acid terminated end-groups immobilized on electrodes to detect cadmium ions [57] and pyridine terminated SAM to determine Cr(VI) in the presence of Cr(III) [58] in solution. However the most interesting application of SAM modified electrodes is the detection of organic and biochemical compounds [55].

1.2.2 Sulphur based SAMs

Due to their strong affinity to bind on clean metal surfaces, sulfur [59] and selenium [60] are among the most common used ligands for the formation of SAMs. Sellers et al. attributes this fact to the ability of these two ligands to form multiple covalent bonds with metal surfaces [61]. Some organosulfur compounds which are known to spontaneously form SAMs on gold are listed in Figure 1.9 [62]. The particular sulfur compound used depend on the proposed application. Octadecanethiol for instance shows excellent protection of the metal surface towards oxidation. Laibinis

![Figure 1.9: Organosulfur compounds that form SAMs on gold based substrate material, modified from reference [62].](image-url)
et al. showed that an octadecanethiol SAM covalently attached to silver prevented oxidation of the surface and that copper coated with the same SAM protects the surface from corrosion when submersed in nitric acid solution [65].

Bain et al. comprehensively studied the formation and properties of monolayers prepared by spontaneous binding of organic thiols onto gold [66]. Using x-ray photoelectron spectroscopy (XPS) analysis they were able to show, that the thiols bind to the metal surface via the sulfur group and expose the terminal group to the monolayer-air/monolayer-liquid interface as seen in Figure 1.8. This fact makes it possible, by altering the terminal group, to create hydrophobic (hydrocarbon terminated thiols) or hydrophilic (alcohol/ carboxylic acid terminated thiols) surface structures.

Figure 1.10 shows some known specific trends of SAM with differing end-groups which were determined by ellipsometry, contact angle goniometry (CAG), IR spectroscopy, XPS, electrochemical redox probing and capacitance measurements [63,64,67]. They were also able to show that longer chain thiols bind faster and more orientated to the metal surface in contrast to shorter chain thiols. Longer chains therefore lead to a more closely packed film. This effect is proposed to be based on the faster kinetics for the adsorption of longer chain layers, which are due to the increased Van-der-Waals interactions [62,66].
**1.2.3 Carbon based SAMs**

While the spontaneous formation of thiol based SAMs can be a convenient way to achieve binding chemistry, this method also suffers from the disadvantage that the gold-sulphur bond does not form a very stable covalent bond. This bond is more likely to break under external influences such as UV radiation [68], thermal influences [69] and mechanical stresses. Thiol based SAMs can also change structure over time [69]. A more stable covalent bond is formed between gold and carbon. The binding energy of sulphur and gold lies between 167-184 KJ/mol [70]. Gooding and coworkers have compared the electrochemical performance and compared the stability of 4-carboxyphenyl surface modified gold and carbon electrodes as well as 3-mercaptopropionic acid surface modified gold electrodes. They were able to show that significantly higher stability could be seen from the aryl diazonium modified electrodes in comparison to the equivalent alkanethiol electrodes [71].

One method of achieving covalent attachment of carbon based monolayers on gold surfaces is the electrochemical reduction of aryl based diazonium salts onto gold surfaces. This method has been widely used in the surface modification of carbon [72,73], metals such as Au [71,74] and semiconductor such as on H-terminated Si(111) [75] electrodes over recent years. **Figure 1.11** displays the schematic reaction of the electrochemical reduction of aryl based diazonium salts on the support material.

Diazonium salts are capable of bearing a wide range of functional groups that possess high stability over a wide potential window [74], which make them interesting for biochemical applications.

![Schematic reaction of the electrochemical reduction of aryl based diazonium salts](image)

*C: Carbon, SC: Semiconductor, M: Metal*

**Figure 1.11:** Schematic representation of aryl based diazonium salts covalently attached on conductive or semi-conductive support material [76].
1.3 High surface area materials for electrochemical applications

1.3.1 Introduction

Porous materials are of continuous interest in modern technological applications such as catalysis, sensing, and filtration. Ding et al. categorize these highly porous structures into three different categories [77]. The first category is comprised of intrinsic porous structures, as seen in zeolites for instance. These structures exhibit open molecular scaffolds. An example of a naturally occurring zeolite is zeolite A (Figure 1.12). As seen in Figure 1.12 the molecular scaffold holds void spaces, which in this regard can be regarded as a natural occurring, pore bearing molecule. Zeolites are of significant interest as they possess three properties especially of interest in heterogeneous catalysis. (1) The ability to exchange cations, which allows for their use as catalysts. (2) In some cases these cation sites are exchangeable by H-terminated functional groups, which make it possible to generate a high number of very strong acidic sites. (3) Their pore diameter is very generally speaking around 10 Å and they possess pores that have one or more discrete size (as can also be observed in Figure 1.12 A [78]. The pores from zeolites show reactant (Figure 1.12 B) and/or product (Figure 1.12 C) selectivity depending on the reaction under study. This is probably the most interesting feature of these natural occurring macromolecules. The second category is defined as synthetic porous structure, where a self-assembly process is employed during fabrication. Ding and Erlebacher categorize synthetic structures of the second category to be those materials made by casting in the intervening spaces microphase-separated block copolymers, colloidal crystals, self-assembled surfactants and even biologically formed skeletal structures [77].

![Figure 1.12](image.png)

**Figure 1.12:** (A) Schematic structure representation of naturally occurring zeolite A (reproduced from reference [79]), (B) reactant selectivity of natural pore structure [78] and C: product selectivity of natural pore structure (both modified from reference [78]).
The last category can also be considered as a synthetic created porous structure. Here, porosity is formed by a three-dimensional spinodal decomposition mechanism of one or more [80] selected phases from a multi-phase bulk material, which then results in a heterogeneous [81] or bicontinuous [82] porous structure [77]. The mechanism includes a solution of two or more components which can separate into distinct phases with distinctly different chemical compositions and physical properties [83]. Heterogeneous porous structures exhibit non-uniform porosity throughout the structure while bicontinuous porous structures have the same pore size, distribution and alloy composition of the material. Therefore bicontinuous structures are more desirable as better control of the pore formation is achieved. In contrast heterogeneous structures are less controllable and extensive characterization has to be performed to ensure reliable reproducibility.

1.3.2 Porous structures

A porous material or structure has to show void (i.e. empty) spaces throughout the structure. Porosity defines the fraction of the volume of the void spaces as a function of the total volume [84]. The value of the porosity usually lies between 0 and 1 while the value for solids being closer to 0 and for porous structures closer to 1. This porosity parameter $\phi$ can be calculated using L. F. Athy’s definition [84]:

$$
\phi(z) = \phi_0 e^{-kz}
$$

where $\phi_0$ is the surface porosity, $k$ is the compaction coefficient (m$^{-1}$), that basically denotes the density of the pores and $z$ displays the depth of the pores (m). Porosity can alternatively be calculated from the bulk density $\rho_{\text{bulk}}$ and particle density $\rho_{\text{particle}}$:

$$
\phi = 1 - \frac{\rho_{\text{bulk}}}{\rho_{\text{particle}}}
$$

The term porosity originates from the fields of earth sciences and soil mechanics, however nowadays it is used in pharmaceutics, ceramics, metallurgy, materials, manufacturing, and engineering. There are numerous definitions of porous materials. One accepted definition involves [85]:

- The material must contain relatively small spaces (pores or voids) which are free of solids and are embedded in the solid or semi-solid matrix [85].
- It must be permeable to a variety of fluids, which means that fluids are able to penetrate through the pore matrix from one side to the other [85].
The term nanoporosity covers a wide field of pore sizes. For that reason the International Union of Pure and Applied Chemistry (IUPAC) described different categories that are summarized in Figure 1.13. IUPAC differentiates between micropores (0.2-2 nm), mesopores (2-50 nm) and macropores (50-1000 nm) [87]. Figure 1.13 also displays common measurement techniques to observe and characterize the porous structure and their approximate range of sensitivity [88].

In this research project the main focus is on the preparation and characterization of nanoporous gold for applications in electrochemistry and bio-electrochemistry, i.e. for the use as trace metal sensors, biosensors and bio fuel cells. The mechanism, preparation and characterization methods used are described in the following sections.

### 1.3.3 Preparation of nanoporous gold

NPG can be achieved by dealloying precursors that are prepared using a wide range of methods. These include electrodeposition of gold on a substrate material [89], using of commercially available gold leaf with predefined thickness and alloy composition [77] or evaporation techniques, such as thermal evaporation [90] and
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sputter coating of gold on a suitable substrate [91]. Sputter deposition of binary [91] or ternary [92] alloys can be performed to manufacture different alloys.

Magnetron sputtering has the great advantage of giving the freedom to adjust the alloy composition and thickness compared to commercially available gold leaf. Sputtered alloy samples can be prepared on glass slides as substrate material. The glass slides are cleaned in an ultrahigh vacuum chamber by argon plasma. Afterwards a thin titanium adhesion layer is sputtered on the pre-cleaned glass slide. A pure layer of gold is then deposited for better adhesion and suppression of an electrochemical response from the titanium layer. The desired alloy composition and thickness can then be sputtered deposited as the final layer [91,93].

These alloys, usually composed of gold and a less noble metal such as silver, are dealloyed either chemically [91] or electrochemically [94,95] in strong acids or even in neutral pH [96]. Chemical dealloying is performed using a strong oxidizing substance such as concentrated nitric acid or perchloric acid, while electrochemical dealloying involves using a diluted oxidizing substance and applying a potential for a defined period of time. The morphology of the NPG layer strongly depends on the conditions used with the alloy composition, thickness, dealloying time, temperature, concentration of oxidizing substance and the addition of electrolytes all playing a role in determining the composition and structure of the final material. Figure 1.14 shows the mechanism of the dissolution of less noble metal from an alloy compound. Once the alloy comes in contact with the dealloying reagent or localized low pH (A) lateral removal of less noble metal begins which then leads to cluster formation of the noble atoms (shown grey in Figure 1.14); (B) the formed clusters are supplied from remaining noble metals from dissolution, which leads to coarsening of the clusters until the next alloy layer is attacked by the oxidation reagent. This also leads to the forming of the characteristic length $\lambda$ between the pores; (C) hills formed of noble metal atoms emerge from the dissolution surface as the oxidation reagent penetrates through the second layer of the alloy. $\lambda$ remains the same throughout the process. While the continuous dissolution of less noble metals proceeds throughout the alloy-electrolyte interface, less noble metal atoms form at the base of the hills due to the widening of perimeters and the shrinking of alloy area between them; (D) the formed hills are undercut (the dotted lines indicate the undercutting of the hills in Figure 1.14 D). This leads to an increase in the average distance of the individual hills; while dissolution continuous, nucleation of new noble metal atom clusters occurs at the same characteristic length $\lambda$. As seen in Figure 1.14 D the distance between the two primal hills is approximately 2 times the characteristic
length $[\lambda]$ along the alloy-electrolyte interface [98]. This pore generation mechanism has been comprehensively studied by Erlebacher et al. [94,99,100]. While the etching continues to rearrange the surface, the porosity starts to extend into the bulk and therefore forms an inherent porous network that possesses a core shell microstructure composed of the less noble metal (silver) and the noble metal (gold) as seen in Figure 1.14 E. Secondary coarsening processes (as shown in Figure 1.14 F), will further coarsen the microstructure behind the dissolution front, leading to enrichment of the noble component (gold) in the porous metal and therefore larger pore sizes [97]. These...
secondary coarsening processes can be thermal annealing, which drives a capillary surface relaxation (coarsening), resulting in a decrease of surface area, or by simply adding components to the starting alloy that segregate to the surface during dealloying [97].

Depending on the conditions used, the pore sizes and therefore the resultant surface morphology may differ substantially from one another. The composition of the alloy has the highest impact on the surface morphology. The material forms nanopores after dealloying, when the gold content of the alloy lies between 20 and 50 at. %. Below a gold content of 20 at. % the porous structure collapses as the support is too unstable, while above 50 at. % only surface pitting occurs [102]. During the dealloying procedure, the temperature and time of exposure to the oxidizing reagent play a major role in controlling the sizes of the pores and ligaments, while also controlling the residual silver content of the substrate material. For a commercial gold-silver alloy of 50 at. % of each compound, the pore sizes were around 5 nm for low temperature dealloying and short etching times, while high contents of residual silver were detected [95,103]. By altering the conditions, the pore sizes could be increased to 50 nm [104]. The pore sizes and therefore the surface morphology can be tuned to a certain extent.
[105], which makes this substrate material a very interesting material for a wide range of electrochemical applications.

The preparation of nanoporous gold is not always necessarily the result of etching in harsh conditions with high temperatures or strong oxidizing substances [96]. Erlebacher et al. also described a method of preparing nanopores that involves neutral etching conditions. They report an etching mechanism based on a Pourbaix (potential-pH) diagram of silver (Figure 1.15), which shows schematic representation of passivation and corrosion regions under special conditions [106]. Further examination of the diagram unfolds that at neutral pH (~pH 7) lower dealloying potentials lead to corrosion of silver (Figure 1.15 A). A potential of 1.55 V vs NHE leads to the formation of pores even though it is well in the passivation area of the Pourbaix diagram, which raises the question of why this is happening. Erlebacher and coworkers expect this to be due to a localized pH drop at the etching front (Figure 1.15 B). The proposed pH drop at the etching front is responsible for the dissolution of the less noble content (silver) into the electrolyte.

The created pores can be sectioned into three different regions as seen in Figure 1.16. These are (1) the dissolution front, (2) the oxide formation region and (3) the passivated nanoporosity region. To understand the processes occurring on the etching front it is necessary to determine which reactions are occurring. These are the oxidation of Ag to AgO or Ag2O as seen in equation 1.5 and 1.6 [101].

\[
\begin{align*}
2\text{Ag} + \text{H}_2\text{O} & \rightleftharpoons \text{Ag}_2\text{O} + 2\text{H}^+ + 2e^- \\
\text{Ag}_2\text{O} + \text{H}_2\text{O} & \rightleftharpoons 2\text{AgO} + 2\text{H}^+ + 2e^- 
\end{align*}
\] (1.5) (1.6)

In both reactions two protons are formed and released into the solution, due to the oxidation of silver at the etching front. These protons lead to a decrease of pH which in return shifts the region (Pourbaix diagram Figure 1.15) from the passivation state to the corrosion state leading to continued dealloying and formation of pores [101]. Etching is complete when the exposed area is either covered with Ag2O components (as the exposure of more Ag to the dissolution interface is hindered), or at higher potentials, when full layers of AgO are formed and no further oxidation is possible [101]. This mechanism however proposes that a high silver content, in form of silver oxide, still remains on the surface structure, which may require sub-sequential removal depending on the intended use.

Another commonly method used to alter the pore morphology of nanoporous
gold structures is to thermally anneal existing pores, which leads to coarsening of the cracks, ligaments and pores. This thermal coarsening method involves a mechanism, which increases the surface diffusion of gold atoms at the surface-electrolyte interface, which in turn leads to ligament growth and thereby increases the average pore sizes throughout the structure [107-109]. Thermal treatment can be an effective method to tune pore morphology to a desired zone. Using this annealing method pore sizes ranging from 5 nm to a few microns can be prepared [107].

1.3.4 Characterization of nanoporous structures

The chemical and physical properties of the resulting alloy structure are of high significance, when these substrates are evaluated for their possible applications, and therefore need to be studied extensively. Many methods are used to characterize and determine pore formation and morphology. These methods include electron microscopy, elemental composition determination, electrochemical methods and image processing.

The most common used techniques to clarify the physical properties are scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The imaging used from these two techniques immediately show the surface morphology.
obtained after etching. Other common techniques involve atomic force microscopy (AFM) and scanning tunneling microscopy (STM), which are able to display three-dimensional depth profiles of the surface morphology. However, these are only able to display the outer most layers without providing information throughout the whole porous structure. These methods can be complemented with x-ray diffraction (XRD), small angle x-ray diffraction (SAXS) and small angle neutron scattering (SANS). The pore structure is directly proportional to the remaining less noble metal content of the alloy after dealloying. Therefore, it is inevitable to determine the chemical compound composition of the material. The most common techniques involve scanning electron microscopy-energy dispersive x-ray spectroscopy (SEM-EDX) and x-ray photoelectron spectroscopy (XPS). Depending on the composition of the alloy compound and the etching conditions used, the silver content of the resulting nanoporous material were reported to be as low as 1 at. % [104].

The length scale of the nanopores, ligaments and cracks can be determined by a number of methods. A very conventional and straightforward method to determine the length scales involves a linear intercept (LI) method [81]. Here the intensity profiles from an SEM or TEM image along random defined lines are plotted. Then the distances are measured between the high and low intensity phases. This gives a very rough estimation of the average pore size, when performed in a statistically reproducible manner. A more precise technique has been described by Fujita and Chen [82]. Here, a method derived from a fast fourier transform (FFT) spectra is proposed, that can measure the characteristic length scale of a bicontinuous nanoporous structure. By using a mathematical model to rotationally average the FFT power spectra [110] of an SEM or TEM image, the resultant significant intensity peak can be used to calculate the average pore size of the nanoporous structure. It is worth noting however, that this method is only applicable for bicontinuous structures, which show the same pore distribution and morphology throughout the whole observed sample.

1.3.5 Application of nanoporous gold material

Due to their specific properties in terms of high specific surface area and nanometer scale cracks, ligaments and pores, NPG is a very interesting material which finds a wide range of use in many fields. Besides their optical applications due to the differing surface plasmon resonance (SPR) response from nanostructured metals compared to their bulk counterparts, they find applications in heterogeneous catalysis,
electrocatalysis, supported and unsupported sensing and as support for enzymatic Biofuel cells (BFCs).

As a dynamic, thermal and mechanical stable, self-supported and chemically inert material it is only comprehensible that gold has found a lot of attention in recent years. Haruta et al. have shown in their pioneering work in 1987 that gold, despite its general inertness [111], is capable of acting as a catalyst in heterogeneous reactions, such as the oxidation of CO to CO$_2$ at low temperatures [112]. Later Haruta showed that the catalytic activity is highly dependent on the particle sizes used for catalysis [113]. Activation energies of a number of reactions could be decreased significantly, leading to new green energy processes. Gold catalyst show advantages over conventional platinum group metals in pollution and emission control, chemical processing, clean energy technologies such as potential use in fuel cells [114]. Haruta later referred to the new use of gold as the beginning of a modern catalytic gold rush [115].

Zielasek et al. were the first to show that nanoporous gold shows remarkable catalytic activity for the epoxidation of propene and the oxidation of CO, which before was only detectable from fine dispersed gold nanoparticles in solution or on a substrate material [111]. This response differs immensely from the bulk properties of gold, where no catalytic activity can be observed [111]. The ability of porous gold to act as a substrate material with high surface area for the immobilization of various enzymes and the fact that it is highly conductive makes it the ideal material to perform electrocatalysis. This is one of the advantages of nanoporous gold in comparison to immobilized gold nanoparticles [114]. This fact has been extensively utilized by numerous research groups by coupling a wide variety of proteins and enzymes onto these porous structures. Among others these comprise laccase [116-118], glucose oxidase [116,119], alcohol dehydrogenase [119], xylanase [120], acetylcholine esterase [121], cytochrome c [91], lignin peroxidase [122], lipase [123], catalase [123], horseradish peroxidase [123], bovine serum albumin [124], and immunoglobulin G [124]. Strategies of binding these bulky molecules onto nanoporous gold include physical, electrostatic and covalent attachments [103]. Due to the high surface to volume ration of nanoporous gold a larger amount of proteins/enzymes can be loaded on the support, which in the use as enzyme based biosensor lead to more precise and detectable signals. When used as an enzymatic BFC, these higher loadings on the substrate material result in higher power output and stability.
1.3.6 Influence of pore size and structure on immobilized proteins

Porous structures in general are perfect substrate materials for a wide variety of immobilizations. These can vary from long polymeric chains up to bulky proteins, such as enzymes or even DNA strands. Wang et al. showed how the nanoporous structured gold can act as a good support for the immobilization of lipase, catalase and horseradish peroxidase [123]. These macromolecules with about 5 nm, 10 nm and 4 nm molecule diameters respectively were immobilized on nanoporous gold with an average pore size of 35 nm. They report the catalytic performance and stability to be remarkably high in each case. They propose that the confined environment the pores exhibit can help stabilize the enzymes in denaturing environments such as high temperatures and organic solvents [103,118,123]. A schematic representation of lipase immobilized on and in nanoporous gold can be seen in Figure 1.17.

Qiu et al. adsorbed laccase on nanoporous gold films with differing pore sizes of 10-20 nm, 40-50 nm and 90-100 nm attached to glassy carbon electrodes (NPG-GC). The results of the experiments showed, that the pore sizes of 40-50 nm showed the best results in terms of immobilization and mass transport of the enzyme and the substrate to the inner pores, which are the limiting parameters for the activity of the immobilized enzyme. The 10-20 nm pores are too small for the approximately 7 nm in diameter laccase to penetrate deep into the pores and to guarantee efficient mass transport of the substrate, while the 90-100 nm pores lead to smaller addressable surface areas, which in return are responsible for a lower response [118].

Figure 1.17: Schematic representation of the immobilization of lipase onto nanoporous gold, reprinted from reference [123].
1.4 Applications

Modified or unmodified nanostructured electrodes are used for a wide range of electrochemical applications such as in batteries [125], capacitors [126], high-performance pseudocapacitors [127] and solar cell applications [128]. Nanostructured electrodes are also capable of detecting trace metal concentrations in solution [129], which is discussed in detail in section 1.4.1. The applications for immobilized enzyme on conducting or semiconducting substrate material are numerous. In the scope of this literature review the focus will lie on enzymatic biosensors and on enzymatic biofuel cells (BFC), which are introduced in section 1.4.2 and section 1.4.3 respectively.

1.4.1 Trace heavy metal sensors

The accumulation of heavy metals, due to their non-biodegradability, in the environment is a major concern, arising through increased industrial activities in a globalizing world. Heavy metals such as Hg, Pb, Cu, Cd, As, etc. can be highly toxic and pose a serious threat for the environment and human health [130,131]. A variety of analytical methods have therefore been developed for the sensitive and selective determination of trace metal concentrations in drinking water, nutrition and biological samples, such as blood and serum [131]. Established methods include AAS (atomic absorption spectroscopy), UV-Vis (ultraviolet–visible spectroscopy), ICP-MS (inductively coupled plasma–mass spectrometry) and ICP-AAS (inductively coupled plasma–atomic emission spectrometry) [131]. These instrumentations however are very expensive and require trained personnel and the techniques demand thorough sample preparation. These drawbacks make the development of rapid detection devices obvious. There has been a wide range of development in the field diagnostic devices. These can roughly be segmented into microfluidic, opto-fluidic and point-of-care devices [131]. In the scope of this literature review the focus will lie on point-of-care devices with special attention toward electrochemical sensors and the role of nanostructured electrode materials.

Electrochemical sensors have the decisive advantage that the sensing signals are collected through wires and therefore do not require complex detectors like optical or fluorescent based sensing systems. Electrochemical sensors can therefore be prepared in compact systems [131]. The principle function of an electrochemical sensor is based on the defined redox potentials of trace metal ions in solution. Electrochemical signals of trace metals in solution can be achieved utilizing a variety of techniques, such as
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voltammetric, amperometric, potentiometric, impedometric, and conductometric. However the most frequently used technique is anodic stripping voltammetry (ASV). ASV involves two steps. In a first step the trace metal is electrochemically reduced to the electroactive surface of the bulk or nanostructured electrode. Usually this step is done by utilizing underpotential deposition (UPD), which results in a monolayer coverage of the trace metal on the surface [129]. The second step involves the subsequent stripping of the prior deposited trace metal. The stripping results in a detectable peak, which can be used to quantify the trace metal concentration in solution.

Surface modification with functional groups utilizing thiols [132] or diazonium compounds [71] have been reported to increase the detection of trace metals. In addition the use of nanostructured electrodes was also reported to increase sensitivity of trace metal sensors as well as decrease the limit of detection (LOD) in comparison to bulk electrodes [133]. Nanoparticle modified electrodes, nanostructured electrodes, microelectrodes and nano-electrode arrays have frequently been used for this purpose.

1.4.2 Biosensors

Enzymatic biosensors are devices with high selectivity, due to the high substrate specificity and interference free indication of reaction products. First proposed by Clark and co-workers in 1962 [134], enzyme modified electrodes can be utilized as electrochemical detectors of specific analytes. These biosensors embody the possibility for real time analysis of rapid measurements in clinical analysis of body analytes, for instance [45]. A working biosensor can mainly be divided into 3 main components. It usually comprises an immobilized biologically active compound with a signal transducer coupled with an electronic amplifier.

Figure 1.18 shows the schematic setup of biosensors. The output signal displayed by the transducer is typically converted from the physico-chemical change in the biologically active layer on the substrate, which results from the detection of the analyte [45]. These changes usually arise from the change caused by chemical substances, however other physical properties such as heat, light or sound can also induce a measurable change in the biologically active material. Therefore the type of transducer used can be electrochemical, piezoelectric, calorimetric or optical. Biosensors can be constructed using any of the above mentioned transducers. Electrochemical biosensors have emerged as the most common used biosensor [45]. In comparison with the above mentioned biosensors they overcome most of the known
disadvantages. Optical biosensors for instance cannot be used in turbid media, while thermal biosensors cannot be utilized for small heat changes and light and sound sensitive biosensors are generally not easy to handle [45]. Examples for the advantageous of electrochemical biosensors are that they deliver rapid response, they are easy to handle, they can be used in turbulent solutions and they are of low cost [45].

Electrochemical biosensors can be divided into conductimetric, potentiometric and amperometric biosensors. Conductimetric biosensors make use of the change in conductance between two metal electrodes due to the change of the biological component as already demonstrated by Sukeerthi and co-workers in 1994 [135]. In contrast, potentiometric biosensors detect this change by measuring the difference in potential with respect to a reference electrode [136]. Amperometric biosensors measure the change in current on an electrode that results from the direct oxidation of the products of the respective biochemical reaction. These type of biosensors have the great advantage that the response is linearly dependent on the analyte concentration, leading to an accurate sensing in a dynamic measuring range [45].

A common use of immobilized enzymes is as a biocatalyst for the production of specific compounds. Non-selective noble metal catalysts such as platinum and palladium, which are currently the main catalytic components of low temperature fuel cells, pose certain disadvantages, which are mainly the very high costs of these noble metals. Therefore the interest in replacing these catalyzing reagents by enzymatic systems, for specific needs, is increasing. These enzymatic biofuel cells (BFCs) are described in section 1.4.3.

Figure 1.18: General configuration of a biosensor, based on the type of transducer used, modified from reference [45].
1.4.3 Biofuel Cells

1.4.3.1 Fundamentals

A fuel cell in its most simple setup consists of a fuel and an oxidant which are, ideally in a continuously flow, passed over two electrodes: A fuel oxidizing anode and an oxidant reducing cathode, which are connected to one another by an external load and separated by an electrolyte [44]. Traditionally fuel cells rely on non-selective noble metal catalyst, such as platinum, to perform fuel oxidation and oxidant reduction. However these materials are expensive and vulnerable to catalyst poisoning. These are just a few examples of why significant research has been focused on new catalytic systems, which find application in the use of fuel cells.

Enzymatic biofuel cells (EFCs) are devices that are composed of an enzyme modified (as shown in section 1.1.2) anode and cathode for fuel oxidation and oxidant reduction. The advantageous of the biologically derived catalyst used in EFCs are numerous. These advantages range from the capacity to produce a wide range of catalysts based on sustainable (biological) processes, the versatility of the catalysts produced to oxidize/reduce a wide range of substrates under moderate conditions of pH and temperature and the specificity of the catalytic reactions [44]. Another important advantage of BFCs that the need to separate the individual compartments of the fuel cell is not necessary. This is due to the fact that electrocatalytically inert electrodes, such as carbon or gold, can be immobilized with enzyme catalysts that are specific for one reaction in contrast to noble metal catalysts [44,50]. This approach decreases the specific energy density (Joule per gram in terms of device weight) of these devices even more, leading to a wider range of possible application, since it makes it possible to miniaturize the devices while still receiving satisfying power outputs.

The general setup of a membraneless, direct electron transfer based EFC is shown in Figure 1.19. Enzyme 1 which is immobilized or free enzyme in solution oxidizes the fuel (which can be conventional or biodegradable fuels) at the anode, releasing electrons and in specific cases protons (such as FAD dependent GOx). At the cathode, where enzyme 2 is immobilized or free in solution, the generated electrons are consumed to reduce the oxidant. In some cases the generated protons can also play a part in reducing the oxidant (i.e. laccase immobilized on the cathode), or they are consumed by regenerating the cofactor (i.e. FAD in GOx: \[ \text{GOx} \text{FAD} + 2e^- + 2H^+ \rightarrow \text{GOx} \text{FADH}_2 \]) [137]. The reaction and therefore the power output of such devices are
highly dependent on the enzymes that are chosen. Dependent on the utilized enzyme on the anode and cathode the catalysis can be performed at relatively mild conditions with regard to pH and temperature [44].

1.4.3.2 Types of biofuel cells

BFCs can be subdivided into two categories of fuel cells. They are segmented into BFCs, which utilize:

(1) living cells, such as bacteria or algae, so called microbial fuel cells (MFCs).
(2) catalysts extracted from cells, such as enzymes, enzyme cascade and more recently mitochondria, so called enzymatic biofuel fuel cells (EFCs).

MFCs use bulky organic material such as bacteria to perform catalysis and to generate electrons, from the degradation of organic matter. One of the significant advantages of MFCs is that they are able to degrade a wide range of biodegradable organic matter to generate power. These can be simple molecules such as carbohydrates or proteins, but they can also be complex mixtures of organic matter, as they arise from
human, animal and food processing waste waters [138]. This feature makes MFCs a promising approach for renewable bioelectricity generation from biomass [138]. However, the conversion of biomass by a bacteria or algae is not solely an efficient catalytic step. As catalysis is being performed by a living microorganism, which derives energy for self-preservation from the catalyzed reaction, an energy loss has to always be expected [138]. In addition to this major problem, the overall low performance of MFCs in comparison to other more established fuel cell technologies (such as EFCs) and the high cost of its components (typically based on Pt on carbon composite materials as substrate) compared to the low amount of the wastewater that is treated, are the main obstacles to the commercial development of MFCs. The performances of MFCs could be improved by the development of cheaper, nano-composite substrate materials (such as nano-structured carbon). However these materials can also introduced additional problems as the materials can be detrimental to the viability of the microbes and may reduce the output [139]. In comparison to MFCs, EFCs use extracted enzymes from cells, which are immobilized on anode and cathode to generate a power output.

1.4.3.3 Potential applications

Early EFCs were constructed to harness electric power output from biofuels such as glucose and lactose. The idea was to power artificial hearts or pacemakers. However these prototype devices were not sustainable in terms of stability of the oxidizing and reducing enzymes as well as in terms of power output. Batteries, which received significant attention in the 1970s, pose a more promising approach [44]. Since then research in the field of EFCs has focused on achieving higher power outputs and on increasing enzyme stability. With new technological developments in recent years, EFCs have received increased attention, as potential power sources for low-energy consuming devices, such as devices to monitor glucose levels in biological fluids [50]. Other researchers have focused on using EFCs as power sources for external electronic devices. Wang and coworkers demonstrated the use of flexible bioanodes and biocathodes on human skin, which are able to power an external connected load, by metabolization of human perspiration [140].

Glucose based bioanodes are among the most often used systems for the harvesting of power from body liquids, due to availability of glucose in tissue and blood at reasonable concentrations (ca. 4-6 mM). Glucose oxidase (GOx) from *Aspergillus niger* is the predominately used enzyme for enzyme based glucose oxidation. GOx is an
enzyme with two identical tightly bound FAD cofactor containing subunits, which catalyzes the oxidation of β-d-glucose to gluconolactone whilst reducing oxygen to hydrogen peroxide [143]. The FAD center of native GOx however is buried deeply in the enzyme, which makes DET impossible, as recently pointed out by G.S. Wilson [144] and P.N. Bartlett [145]. Through the addition of mediators, electrons can shuttle from the active site to the electrode surface. In the case of GOx this is often done by ferrocene derivates, which results in the loss of cell voltage, due to the difference in redox potential, but facilitates electron transfer [44,146]. To establish a driving force for the electrons in MET based EFCs the redox potentials of the mediators has to be more positive at the anode and more negative on the cathode. Depending on the potential differences between the oxidative and reductive, the resulting EFC cell voltage loss may increase. Therefore a compromise has to be made between driving force (potential) and the current for optimal power output [44].

GOx based bioanodes and laccase based bioanodes have quite frequently been used as the two major components of EFCs. Cosnier and Cinquin were the first to successfully implant such a device into small mammals abdomen such as rats [141]. The setup for such a device can be seen in Figure 1.20. A power output of 24.4 µW mL⁻¹ was generated by this implemented EFC [147]. This shows the potential that these devices are capable to constitute.
Figure 1.21: proposed miniaturized implantable submarine EFC with a substrate permeable membrane propelled by a rotating flagella, for sensing or drug delivering applications [148].

In a very futuristic mind-set, one could imagine constructing a miniaturized submarine like device, which is powered by an EFC and capable of travelling through the human blood stream and converting body own energy, in from of glucose, to power this movement or a tool/ sensor, as displayed in Figure 1.21. This device would then be conveniently able to measure glucose concentration in the body, which is a very interesting application for diabetes patients, or be able to deliver drugs to the specific site of interest.
1.5 Scope of this Project

The goal in this project is to prepare and characterize nanoporous gold as a basis for high surface area electrodes for the use in bioelectrochemical applications such as trace metal sensing, bio-sensing and energy harvesting via bio fuel cells under the aspect of developing biologically powered implantable nanodevices. The results obtained and the setups realized will be shown in the following chapters and include following highlights:

- The surface structure and morphology of nanoporous gold sputtered on glass sheets was optimized by altering the alloy composition and etching conditions of the dealloying procedure. A method was developed to prepare electrodes with defined properties can be reproduced in a highly manner. These electrodes are characterized using SEM and EDX. Pore sizes were determined by manual readout, LI intercept and by rotationally averaging an FFT power spectra using image J software. The immobilization of cytochrome c (cyt. c) and BOD on NPG with differing pore sizes is discussed.

- Various immobilization techniques such as covalent attachment to thiol and diazonium film modified NPG surfaces and the surface modification with polymer films are displayed and discussed in detail.

- Benzene diazonium sulfonate (BDS) modified NPG electrodes are utilized for the detection of trace metals, shown on the case of copper, in various solutions, such as water samples (tap water, rain water and river water), as well as the use in complex organic media (artificial human serum) is demonstrated. In addition fructose dehydrogenase (FDH) was covalently attached to diazonium film modified NPG electrodes and utilized as a biosensor for the precise detection of D-fructose in a variety of natural sweeteners and beverages.

- Enzyme modified bioanodes and biocathodes on NPG support material have been developed for use as EFCs. FAD-GDH (*Glomorea cingulata* FAD dependent glucose dehydrogenase) modified bioanodes and *Mv*BOD (*Myrothecium verrucaria* bilirubin oxidase) modified biocathodes were utilized as a biofuel cell to operate in artificial human serum. In addition FDH modified bioanodes and *Bp*BOD (*Bacillus pumilus* bilirubin oxidase) biocathodes were connected to prepare EFCs that could utilize fructose in solution for power generation.
1.6 References


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Chapter 1 Literature Review


Chapter 1 Literature Review


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Chapter 2:

Nanoporous gold electrodes with tunable pore sizes for bioelectrochemical applications
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2.1 Introduction

Gold, a noble metal already in focus in ancient human history, has been receiving immense attention over the past 15 years which can be referred to as modern “Gold rush” for catalytic applications [1]. Hundreds of papers are published annually on innovative uses of gold in modern chemistry [2]. Traditionally gold has always been regarded as an inert and inactive metal, when present in its bulk form. It was not until 1976 that Thomas et al. first demonstrated the ability of gold to catalyze oxidation reactions of alkynes, arenes, and cyclopropanes [3]. Later, it was found that gold could act as a chemical catalyst for heterogeneous reactions in both the gas and liquid phase, when present in nanoparticulate form [2]. This discovery has triggered a wide range of uses of gold as a catalyst.

NPG has been the focus of many electrochemical investigations, such as the effect of residual Ag in the alloy on the electrocatalytic properties [4], the electrochemical properties of NPG in biofouling solutions [5] or more recently the potentiometric responses of microdroplet redox compounds on NPG [6]. Nanostructured gold can be prepared using a wide variety of methods [7]. Common preparation methods involve templating and dealloying processes [8]. NPG, manufactured by applying dealloying processes, has already been described in section 1.3.3. The three-dimensional nets of channels fabricated in nanostructures make it an ideal material for high quality catalysts [9,10], sensors (enzymatic [11] or non-enzymatic [12]), actuators [13,14], etc. The processes involved in dealloying have been extensively studied by Erlebacher et al. [15-18]. However due to a number of issues, mainly being the expensive nature of gold and the complexity of the manufacturing process, its use has been limited to research laboratories [19]. These drawbacks have favored the use of carbon based materials, such as graphite, graphene, carbon nanoparticles, carbon nanotubes (single- or multiwalled SWCNT/MWCNT), carbon fibers (such as carbon cloth (CC) and Toray paper (TP)) or carbon composite materials. The advantages of carbon based electrodes comprise low cost, wide potential window, relatively inert electrochemistry and electrocatalytic activity for a variety of redox reactions [20]. Nevertheless, despite all these advantages they possess a decisive disadvantage. Magrez et al. already tested the toxicity of these carbon based nanostructures (CBNs) in vitro on lung tumor cells. They concluded that these CBNs are not only toxic, while the hazardous effect is size-dependent, moreover the cytotoxicity is enhanced when the surface of the particles is functionalized after acid treatment [21]. In addition to Magrez
work, Davoren et al. demonstrated how SWCNT show toxic response on human A549 lung cells. Due to their size, SWCNT can easily be dispersed in air and can therefore be easily inhaled [22].

The main scope of this project is the manufacture and evaluation of the use of NPG in devices such as sensors, biosensors and biofuel cells that could ultimately be used as implanted devices. Due to the toxic nature of CNBs, such carbon based materials are not suitable. Here lies the main advantage of gold based electrodes. Though gold nanoparticles have also been reported to possess toxic characteristic, these have been limited to mechanical effects in the human body due to the small nature of nanoparticles. nanoparticles of 12 nm diameter for instance may be able to cross the blood brain barrier [23,24], while objects of 30 nm or less can undergo endocytosis by living cells [25]. However, there is to date no reported toxicity effects of bulk gold toward the human body, which makes NPG an ideal substrate material for implanted biodevices. For the application of NPG in biodevices many obstacles need to be overcome.

The pore sizes of the prepared NPG electrodes need to be suitable for the intended application. If the pore sizes of the NPG material are less than the diameter of an intended biomolecule, the pores will be inaccessible for immobilization and therefore only pose unused space. If the pore sizes exceed the diameter of the biomolecule, the resultant lower active surface areas can lead to lower electrochemical responses. Wettability and surface addressability of these nanostructures are also crucial. Surface modification can only occur if the modifying particles in solution come in direct contact with substrate material. The possibility of pore size tuning is therefore an inevitable necessity and thoroughly discussed in the following subchapters. In addition to the morphology tuning of NPG electrodes, the electrodes were used as substrate for protein immobilization. In this study cytochrome c from equine heart was used as a model protein to evaluate the pore size effect on the electrochemical response. In addition the ‘blue copper’ protein bilirubin oxidase from *Myrothecium verrucaria* (MvBOD), that reduces oxygen to water, was physically adsorbed to the NPG electrodes and the differing NPG morphologies were monitored in regard to the electrochemical responses.
2.2 Experimental

2.2.1 Reagents and materials

Nitric acid 70% (HNO$_3$), sulfuric acid 95-98% (H$_2$SO$_4$), potassium chloride, potassium phosphate monobasic and dibasic, poly(ethylene glycol) diglycidyl ether (PEGDGE), ethanol 96 % (v/v), 6-mercapto-1-hexanol, 11-mercaptopoundecanoic acid, N-cyclohexyl-N’-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMC), sodium fluoride (NaF) and cytochrome c from equine heart (type VI) were obtained from Sigma-Aldrich Ireland, Ltd.. All chemicals were used as received unless stated otherwise. _Myrothecium verrucaria_ bilirubin oxidase (MvBOD) was kindly provided by Novozymes. Deionized water with a resistivity of 18.2 MΩ cm was obtained from an Elgastat maxima-HPLC (Elga, UK).

2.2.2 Electrode manufacture

To manufacture high surface NPG electrodes, commercially available glass sheets (microscope slides, J. Melvin Freed Brand, USA) were sputtered using magnetron sputtering in an ultra-high vacuum chamber (ORION-5-UHV custom sputtering system) at room temperature. The chamber was equipped with 3 metal targets for substrate deposition *i.e.* Au, Ag from AJA International Inc., USA and Ti from Kurt J. Lesker Company Ltd., UK with 99.99% purity. Before depositing the metal layers, the glass sheets were exposed to argon plasma under vacuum to ensure a clean surface for adhesion and to prevent impurity inclusions. During the magnetron sputtering process, the metal targets are battered with energetic ions created by the collision of free electrons in the argon plasma. This causes atoms to discharge from the targets and then to condense into a uniform layer on the surface of the glass sheets [26]. The setup of the custom magnetron sputtering system allows to deposit up to three individual metals simultaneously, allowing the design a wide range of alloys and composites.

As seen in Figure 2.1 A, magnetron sputtering was carried out using a 10 nm Ti adhesion layer, followed by a pure Au layer that is a third as thick as the alloy layer, to suppress any electrical activity of the Ti layer and to improve adhesion of the alloy layer. A 50 nm to 1000 nm thick Ag$_x$/Au$_{1-x}$ alloy layer is sputtered on top of this layer (Figure 2.1 B). Concentrations of silver above 35 at. % in the alloy was found to be the limit of what is possible to use for sputtering. Lower concentrations of silver led to uncontrollable sputtering rates which made it impossible to reproduce sample sheets and
were therefore not used. In this study silver concentrations of 70, 50, 35 and 30 at. % were used, while 30 at. % were not used due to the uncontrollable sputtering rates. The sputtered glass sheets were then cut into uniform pieces of approximately 0.5 - 0.7 cm$^2$ in size using a circular saw. The sheets were dealloyed using 70% HNO$_3$ at a defined temperature and for a defined period of time, respectively. This chemical etching procedure leads to the formation of a nanoporous structure through the continuous dissolution of the Ag content from the alloy and the rearrangement of the gold atoms at the solid-liquid interface as seen in Figure 2.1 C.

After the formation of nanopores a silver wire was soldered to the surface using a 99.99% pure indium wire (Sigma-Aldrich Ltd.). The soldering point was supported by a two component epoxy glue, composed of a resin and a hardener fraction, (EVO-STIK, Bostik Industries Ltd) to ensure adhesion to the surface. After drying over night, dielectric paste (Gwent Group, UK) with a solid content of ca. 53 % was used to insulate the electrode components and to define an electroactive surface area, as indicated in Figure 2.1 D. The paint was dried under vacuum to enhance penetration through the pores and to accelerate the drying process.
2.2.3 Characterization of NPG electrodes

Various methods and characterization techniques were used to determine the specific properties of the manufactured nanoporous structures. The methods used are summarized below.

2.2.3.1 Electrochemical characterization

Electrochemical measurements were performed using a CHI1030C multichannel potentiostat or a CHI620A potentiostat. The manufactured electrodes were used as a working electrode in a three electrode setup. For counter and reference electrodes a Pt wire (ALS Co. Ltd., Tokyo, Japan) and Ag/AgCl in 3 M KCl (IJ Cambria Scientific Ltd., UK) were used respectively. The NPG electrodes were cleaned by cycling in H$_2$SO$_4$ (0.1 to 0.5 M) in the potential range of -0.2 V to 1.6 V at a scan rate of 0.1 V s$^{-1}$ for two full cycles. Afterwards the NPG electrodes were rinsed with deionized water for approximately 30 s. For double layer capacitance ($C_{DL}$) measurements electrodes were immersed in 0.1 M phosphate buffer saline (PBS) with 0.1 M KCl supporting electrolyte at pH 7.0 and cycled between 0 V and 0.8 V for 3 full cycles at scan rates between 0.01 V s$^{-1}$ and 0.1 V s$^{-1}$.

2.2.3.2 Determination of electrochemical addressable surface area $A_{real}$ and roughness factor $R_f$

$A_{real}$ and $R_f$ values were determined using an gold oxide stripping technique. The NPG electrodes are cycled in 0.1 M - 0.5 M H$_2$SO$_4$ in the potential range of -0.2 V to 1.6 V at 0.1 V s$^{-1}$ for two full cycles. These parameters were chosen due to the fact that increasing concentration of H$_2$SO$_4$ and/or number of cycles will lead to a continuance of the etching procedure, which results in altered surface structure. This continued etching is not desired when pore sizes are being tailored. During this cycling procedure gold oxide is formed and afterwards directly reduced to crystalline gold. This process produces a characteristic reduction peak in the cyclic voltammogram (CV), from which the electric charge can be calculated by integrating the scan rate normalized peak. $A_{real}$ was calculated using a values of 390 µC cm$^{-2}$ for the capacitance of gold [28].

$R_f$ values can be calculated by the ratio of $A_{real}$ to the geometric surface area ($A_{geo}$). $A_{geo}$ differs for every electrode due to the unreproducible nature of the electrode manufacturing process. $A_{geo}$ is therefore precisely determined for each electrode by taking a high resolution picture (Nikon D7100 SLR-equipped with Nikon AF-S DX 18-
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140 mm VR-Objective) on a millimeter grid. After specifying the ratio of pixels per millimeter using ImageJ software [29], $A_{geo}$ of the electrodes can be precisely determined. Such accuracy is crucial for the preparation of reproducible electrodes.

2.2.3.3 Morphology characterization

Determination of the morphologic structure of the nanoporous is essential to validate its physical properties. Electron microscopy is a useful tool in determining surface structures. For the characterization a scanning electron microscope (SEM) is used (Hitachi SU-70). SEM is capable of generating high resolution images by scanning the surface using a focused beam of electrons. The emitted electrons interact with the atoms of the sample which result in detectable signals that can give information on the topography and composition of the bombarded sample. One common issue occurring from emitting high loads of electrons on a surface is surface charging, which results in a low contrast profile and therefore poor imaging. To overcome this problem samples are connected to the SEM stage by a fast drying silver paint (SCP, Electrolube, UK) that guarantees efficient charge transport. SEM images were taken at three different magnifications: 25K, 80K and 200K magnification at a working distance of 2 - 15 mm and at 10 kV.

2.2.3.4 Silver content determination

The remaining silver in the alloy after dealloying is an intrinsic piece of information that indicates how far the etching has proceeded. For the determination energy-dispersive x-ray spectroscopy (EDX) integrated in the Hitachi SU-70 SEM was used (Oxford Instruments). EDX was performed under following specifications: 15 mm working distance, 20 kV and ca 25-30% deadtime were used. To ensure comparable results the same specifications were used on all sample sheets.

2.2.3.5 Pore size determination

Besides the information obtained from the determination of $R_f$ values the actual pore sizes and pore distribution of the NPG are of utmost significance. The most straight forward method for pore sizes and pore size distribution determination is to manually readout of the pore size by measuring many pores of different SEM images. For this method the image is converted into a black and white image using the ISODATA function of ImageJ. The dimensions of the pore sizes were obtained by
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Figure 2.2: Manual readout of pore sizes using ISODATA corrected ImageJ SEM example picture at multiple regions.

manually measuring the distances between the pores. For this to be accurate the distance has to be measured at least 30 different positions as seen in Figure 2.2.

Another commonly used method is the linear intercept method (LI method) for the determination of pore sizes [30]. An SEM or transmission electron microscopy (TEM) image is used for the measurement (Figure 2.3 A). At different sites of the image intensity profiles are plotted in a random manner. These intensity profiles (Figure 2.3 B) are then used to manually determine the pore size. By measuring the distance at a number of sites of interest, a very accurate average pore size profile of the

Figure 2.3: Linear intercept method (A) visualized on an example SEM picture using ImageJ and (B) the resulting intensity plot with the measured pore sizes, indicated by the red lines.
sample can be obtained. However the pore sizes and the pore distribution are very
dependent on where the lines are plotted, as this can significantly change the results. It
is therefore important to impartially approach the plotting of the intensity profiles.

Another method is to rotationally average a fast Fourier transform (FFT) power
spectra generated from a SEM or TEM picture of the sample. The contrast differences
in the image enables to measure the characteristic length scale of a bicontinuous
nanoporous structure. The method, first described by Fujita et al. [31], uses a
mathematical model implemented by Mitchel et al. [32] utilizing the commercially
available software Digital Micrograph™ (DM™). As seen in Figure 2.4 A, a random
square of the, in this case, SEM image is chosen to create a FFT power spectra (Figure
2.4 B). After defining the picture center, the length scale and rotationally averaging
(Figure 2.4 C) of the FFT power spectra, it shows a much clearer intensity profile
throughout the spectra. By plotting an intensity profile over the whole longitude through
the middle of the averaged spectra, a reciprocal signal is generated as seen in Figure 2.4
D. The intensity profiles for the spectra before and after rotationally averaging are
displayed here. $L_C$, the characteristic length scale of the nanopores, can be calculated

![Figure 2.4](image_url)

**Figure 2.4:** Average pore size determination using the method of rotationally
averaging FFT power spectra with (A) example SEM image with chosen region of
interest, (B) FFT power spectra before, (C) after rotationally averaging, (D) reciprocal
spectra before and after rotationally averaging.
from the distance between the origin of the FFT power spectra and the first scattering peak \( g_0 \) (from Figure 2.4 D) in the reciprocal space, by applying Equation 2.1 [31].

\[
L_C = \frac{1}{g_0} \\
\tag{2.1}
\]

The characteristic length \( L_C \) scale is dependent on the length of the pores and the length of the ligaments as seen in Equation 2.2 [31].

\[
L_C = L_{pore} + L_{Lig}. \tag{2.2}
\]

In a bicontinuous structure, for which this method has originally been proposed by Fujita et al, the ligament and pore length scale are approximately the same as shown in Equation 2.3. Bicontinuous structures are defined to be quasi-periodic, resulting in same pore size distribution and pore sizes throughout the whole nanoporous structure [31].

\[
L_{Lig.} = L_{pore} \approx \frac{L_C}{2} \tag{2.3}
\]

It is important to note that this approximation is only valid for bicontinuous structures and is not applicable for heterogeneous structures in which length scales of ligaments and pores differ from each other.

2.2.4 Electrode optimization

The alloy compositions and etching conditions for the manufacture of NPG electrodes were altered to achieve optimized electrodes in terms of \( A_{real} \) and \( R_f \) values. The alloy compositions used were 70% Ag/30% Au, 50% Ag/50% Au and 35% Ag/65% Au respectively. Dealloying conditions were varied in terms of dealloying time and dealloying temperature. The dealloying time was varied from 1 minute to 60 minutes, while the dealloying temperatures were varied from 0 °C to 60 °C.

2.2.5 Cytochrome c immobilization on NPG

NPG electrodes were exposed to a solution of 6-mercapto-1-hexanol (1 mM) and 11-mercapto-undecanoic acid (1 mM) in a 60 % ethanol and 40 % water (v/v) solution. The ethanol concentration was chosen to ensure the thiols were soluble while not dissolving the dielectric paste on the NPG electrodes. After 24 hours the gold electrodes were rinsed with ethanol and a 4.4 mM PBS pH 7.0 solution and then dried in a stream of \( \text{N}_2 \) to remove loosely attached thiol molecules. The dried electrodes were placed in a 5 mM solution of CMC in 100 mM PBS at pH 7.0 for 30 minutes at 4 °C.
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The electrodes were afterwards immediately placed in buffer solution (4.4 mM PB, pH 7.0) containing 50 µM cytochrome c, as determined by photo spectrometric validation, for 1 hour at 4 °C. The modified electrodes were then rinsed carefully with buffer (4.4 mM PB, pH 7.0). Any electrodes which were not used immediately were stored dry at 4 °C.

2.2.6  MvBOD immobilization on NPG

MvBOD was adsorbed on NPG electrodes. Herein a 10 µL aliquot of the enzyme solution containing 0.35 mg ml\(^{-1}\) was placed on a clean, dry NPG electrode and allowed to adsorb in the vacuum chamber under pressure (~10\(^{-2}\) mbar) for at least 3 min. The electrode was removed from the vacuum chamber, covered with 10 µL of 15 mg ml\(^{-1}\) PEGDGE solution and allowed to air dry at room temperature for approximately 15 min.

2.3  Results and discussion

All results obtained are summarized and extensively discussed in the following subchapters.

2.3.1  Electrode manufacturing and surface area optimization

The alloy composition and thicknesses were varied to clarify etching influences on the resulting porous structure. By altering the etching conditions in terms of dealloying temperature and time of dealloying, the resulting porous structure was observed using a variety of characterization methods.

2.3.1.1  Influence of alloy composition

By altering the alloy compositions during magnetron sputtering the porous structures after dealloying may differ substantially from one another. The alloy compositions used were Ag\(_{70}\)/Au\(_{30}\), Ag\(_{50}\)/Au\(_{50}\), Ag\(_{30}\)/Au\(_{70}\) and Ag\(_{35}\)/Au\(_{65}\) all in at.%. Adjustment of the alloy composition during sputtering requires delicate skills during the operation of the sputtering and was performed by Dr. S. Belochapkine. During the operation of the sputtering procedure many parameters, such as sputtering rates of gold and silver and the pressure have to be controlled simultaneously. The silver target in the vacuum chamber has approximately twice the size of the gold target. Therefore
controlling the silver amount at low sputtering rates becomes very difficult. The alloy composition of Ag$_{35}$/Au$_{65}$ appeared to be the limit of what was possible to construct in a reliable and reproducible manner. Therefore the Ag$_{30}$/Au$_{70}$ alloy was not investigated further. After dealloying of the differing alloy compositions at 40 °C for 15 min., electrodes were constructed from the resulting glass sheets and prepared for characterization. As seen from the SEM images (Figure 2.5) the resultant structures differ substantially from one another. Figure 2.5 A shows an Ag$_{70}$/Au$_{30}$ alloy after dealloying with pores continuously distributed alongside the whole image. In comparison with Figure 2.5 B (Ag$_{50}$/Au$_{50}$ alloy) the pores of the Ag$_{70}$/Au$_{30}$ alloy are much larger. The Ag$_{50}$/Au$_{50}$ alloy however does not show such a continuous distribution of the pores, instead occurring as islands of material surrounded by pores. The size of these pores is much smaller than those in Figure 2.5 A. These results can also be seen in Table 2.2. The $R_f$ value for the Ag$_{50}$/Au$_{50}$ alloy at these dealloying conditions is roughly 2 times higher than those of Ag$_{70}$/Au$_{30}$, which make them appear to be the most suited substrate for high area surfaces. However in additional trials they were found to be very inconsistent and the results were not reproducible. As shown in Table 2.1 the $R_f$ values differ substantially from one another with roughness values between 12.5 and 44.4, despite the same dealloying conditions being used.

In contrast to this the Ag$_{70}$/Au$_{30}$ alloy shows a much wider span for $R_f$ values from 4.1 and 9.3 for 2 different electrodes. The samples taken for these results were at completely different positions of the sputtered glass sheet. Electrode 1 was taken from the edge of the sheet while electrode 2 was from the center. Herein we can clearly see that the origin of the cut piece from the full sheet is of crucial importance. This is due to the fact that the gold target in the sputtering chamber is smaller than the silver target and they are both again smaller than the 2 glass sheets. Since the sputtering rate is measured and controlled over the values received in the middle of the “to sputter” area, the
Table 2.1: Summary of $R_f$ value results for a 300 nm thick Ag\textsubscript{50}/Au\textsubscript{50} alloy dealloyed using the same dealloying conditions.

100 nm Ag\textsubscript{50}/Au\textsubscript{50} electrodes:

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Dealloying time [min]</th>
<th>Dealloying temp [°C]</th>
<th>$A_{geo}$ [cm\textsuperscript{2}]</th>
<th>$A_{real}$ [cm\textsuperscript{2}]</th>
<th>$R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.01</td>
<td>5</td>
<td>0.5</td>
<td>0.24</td>
<td>7.77</td>
<td>33.1</td>
</tr>
<tr>
<td>1.02</td>
<td>5</td>
<td>0.5</td>
<td>0.31</td>
<td>10.85</td>
<td>35.3</td>
</tr>
<tr>
<td>1.03</td>
<td>5</td>
<td>0.5</td>
<td>0.33</td>
<td>5.40</td>
<td>16.2</td>
</tr>
<tr>
<td>1.04</td>
<td>5</td>
<td>0.5</td>
<td>0.21</td>
<td>3.10</td>
<td>14.5</td>
</tr>
<tr>
<td>1.05</td>
<td>5</td>
<td>0.5</td>
<td>0.31</td>
<td>5.02</td>
<td>16.4</td>
</tr>
<tr>
<td>1.06</td>
<td>5</td>
<td>0.7</td>
<td>0.26</td>
<td>11.50</td>
<td>44.4</td>
</tr>
<tr>
<td>1.07</td>
<td>5</td>
<td>0.7</td>
<td>0.33</td>
<td>4.07</td>
<td>12.5</td>
</tr>
<tr>
<td>1.08</td>
<td>5</td>
<td>0.7</td>
<td>0.29</td>
<td>3.89</td>
<td>13.3</td>
</tr>
<tr>
<td>1.09</td>
<td>5</td>
<td>0.7</td>
<td>0.26</td>
<td>4.23</td>
<td>16.0</td>
</tr>
<tr>
<td>1.10</td>
<td>5</td>
<td>0.7</td>
<td>0.31</td>
<td>5.81</td>
<td>18.9</td>
</tr>
</tbody>
</table>

Sputtering rates can vary significantly leading to different “internal” alloy compositions across the sheet. This effect is extensively discussed in section 2.3.1.2. Figure 2.5 C shows the surface structure of an Ag\textsubscript{35}/Au\textsubscript{65} alloy after dealloying. One can clearly see that no nanoporous are evident. With this low concentration of silver in the alloy only surface pitting occurs. This can be clearly seen on the SEM image (Figure 2.5 C) as black dots on the surface. Such results are in agreement with literature reports [33-35].

In addition to the investigation of the influence of alloy compositions the same approach was used to examine the real surface area of commercially available flat gold electrodes. The results for 4 different electrodes are shown in Table 2.2. With $R_f$ values varying between 3.6 and 4.6 these electrodes are clearly far away from being flat gold electrodes [36]. This can mainly be attributed to the preparation procedure of the electrodes. They are polished using alumina powder with an average diameter of 50 nm. The polishing leaves channels in the gold surface about 50 nm wide and deep, which results in increased surface roughness. Therefore it is inevitable that the real surface areas of any material used are properly characterized before use.
Table 2.2: Summarization of $R_f$ value results for differing alloy compositions, dealloyed at 40 °C for 15 min and for commercially available macro gold electrodes.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Charge Q [μC]</th>
<th>Real area [cm$^2$]</th>
<th>Geometric area [cm$^2$]</th>
<th>$R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag$<em>{70}$/Au$</em>{30}$</td>
<td>1</td>
<td>382.6</td>
<td>0.981</td>
<td>0.240</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>910.2</td>
<td>2.334</td>
<td>0.250</td>
</tr>
<tr>
<td>Ag$<em>{50}$/Au$</em>{50}$</td>
<td>3</td>
<td>914.6</td>
<td>2.345</td>
<td>0.227</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1116.0</td>
<td>2.862</td>
<td>0.238</td>
</tr>
<tr>
<td>Ag$<em>{30}$/Au$</em>{70}$</td>
<td>5</td>
<td>145.9</td>
<td>0.374</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>199.1</td>
<td>0.511</td>
<td>--</td>
</tr>
<tr>
<td>Ag$<em>{35}$/Au$</em>{65}$</td>
<td>7</td>
<td>220.0</td>
<td>0.564</td>
<td>0.255</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>168.9</td>
<td>0.433</td>
<td>0.223</td>
</tr>
<tr>
<td>Electr. T1</td>
<td>T1</td>
<td>44.3</td>
<td>0.114</td>
<td>0.0314</td>
</tr>
<tr>
<td>Electr. T3</td>
<td>T3</td>
<td>49.6</td>
<td>0.127</td>
<td>0.0314</td>
</tr>
<tr>
<td>Electr. T5</td>
<td>T5</td>
<td>56.0</td>
<td>0.144</td>
<td>0.0314</td>
</tr>
<tr>
<td>Electr. T6</td>
<td>T6</td>
<td>55.8</td>
<td>0.143</td>
<td>0.0314</td>
</tr>
</tbody>
</table>

2.3.1.2 Internal sputtering differences on glass sheets

As already indicated in section 2.3.1.1, the alloy composition from one part of the glass sheet differs at different points on the sheet, especially when the edges are compared to the center of the sheet. Therefore for every experiment the sputtered glass sheets were cut as shown in Figure 2.6 into pieces with an area of approximately 0.65 cm$^2$. The red areas in Figure 2.6 represent uncovered areas due to the mounting of the glass sheet during sputtering and can therefore not be used for the preparation of electrodes.

After dealloying this differing “internal” alloy composition leads to substantial different roughnesses measured for different cut pieces. This behavior was observed throughout this research project, as can be seen in an example shown in Table 2.3. The

Figure 2.6: Representative scheme for the cutting of glass sheets into 26 parts after dealloying, while the red areas represent uncovered areas due to the mounting of the holders.
R_f obtained in dealloyed cut sheets at the same conditions (i.e. 5 minutes at ≈ 0 °C) decreased gradually from electrode 1.17 (R_f ≈ 49.3) to 1.26 (R_f ≈ 33.4). Because of this, for the comparison of electrodes, only adjacent pieces of the cut glass sheet were used to minimize the probability of comparing different alloy compositions. In order to obtain comparable results the edge pieces of the sputtered sheets (i.e. cut sheets 1, 2 and 23-26) were used as test pieces and only adjacent pieces (i.e. cut sheets 3-22) were used to compare the morphological properties in the following experiments, as they showed the most reproducible results.

**Table 2.3:** Summary of results obtained from a 300 nm Ag$_{70}$/Au$_{30}$ alloy from electrodes manufactured from sheets of different areas of the glass sheet.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Dealloying time [min]</th>
<th>Dealloying temp [°C]</th>
<th>A$_{geo}$ [cm$^2$]</th>
<th>A$_{real}$ [cm$^2$]</th>
<th>R_f</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.17</td>
<td>5</td>
<td>0.4</td>
<td>0.20</td>
<td>9.86</td>
<td>49.3</td>
</tr>
<tr>
<td>1.18</td>
<td>5</td>
<td>0.4</td>
<td>0.30</td>
<td>13.13</td>
<td>43.2</td>
</tr>
<tr>
<td>1.19</td>
<td>5</td>
<td>0.4</td>
<td>0.32</td>
<td>14.51</td>
<td>44.8</td>
</tr>
<tr>
<td>1.20</td>
<td>5</td>
<td>0.4</td>
<td>0.25</td>
<td>11.49</td>
<td>45.2</td>
</tr>
<tr>
<td>1.21</td>
<td>5</td>
<td>0.4</td>
<td>0.22</td>
<td>9.37</td>
<td>42.8</td>
</tr>
<tr>
<td>1.22</td>
<td>5</td>
<td>0.4</td>
<td>0.25</td>
<td>10.05</td>
<td>40.2</td>
</tr>
<tr>
<td>1.23</td>
<td>5</td>
<td>0.4</td>
<td>0.37</td>
<td>13.71</td>
<td>37.6</td>
</tr>
<tr>
<td>1.24</td>
<td>5</td>
<td>0.4</td>
<td>0.31</td>
<td>10.76</td>
<td>35.2</td>
</tr>
<tr>
<td>1.25</td>
<td>5</td>
<td>0.4</td>
<td>0.28</td>
<td>9.43</td>
<td>34.1</td>
</tr>
<tr>
<td>1.26</td>
<td>5</td>
<td>0.4</td>
<td>0.28</td>
<td>9.50</td>
<td>33.4</td>
</tr>
</tbody>
</table>

**2.3.1.3 Influence of dealloying temperature and time on NPG morphology**

The pore sizes, distribution and general morphology of the generated nanoporous structure depend significantly on the dealloying conditions used. As a basis for dealloying, sputtered Ag$_{70}$/Au$_{30}$ alloys with differing alloy thicknesses of 50, 100 and 300 nm were prepared. The electrodes prepared from these sheets were optimized towards addressable surface area, by altering the etching conditions in terms of dealloying temperature and dealloying time. A characteristic example of the results can be seen in Figure 2.7. The SEM images were obtained by dealloying a 100 nm Ag$_{70}$/Au$_{30}$ alloy at different conditions (at A: 1 min/ 0.5 °C, B: 1 min/ 20.8 °C, C: 5
min/ 0.5°C, D: 5 min/ 20.8 °C, E: 1 min/ 40.2 °C, F: 1 min/ 59.9 °C, G: 5 min/ 40.2 °C and H: 15 min/ 60.5 °C respectively, as indicated in Table 2.4). It is visible to the unaided eye that the pore sizes increase from Figure 2.7 A to Figure 2.7 H. By manually reading out the pore sizes from the SEM images in Figure 2.7 the pore sizes span from 4.4±0.9 nm (Figure 2.7 A) to 77.9 ± 4.6 nm (Figure 2.1 H) as displayed in Table 2.4. This increase in pore size entails a decrease in roughness (Rf) from A: 44.2 ± 0.9 to H: 3.7 ± 0.2. This decrease in Rf is due to the fact that the larger pores provide less surface area than the smaller pores, as they possess more void space.

As expected, the results are highly dependent on the NPG film thickness. The highest Rf values for 50 nm film thickness were found to be 6.1 ± 0.9 while for 100 nm thickness it was 44.2 ± 0.9 and for 300nm 130.5 ± 12.1 respectively (as seen in Table 2.4). These results were obtained at the same dealloying conditions of ≈0°C and 1 minute dealloying time.

Figure 2.7 also gives insight into the morphological transformations during continuous dissolution of silver and rearrangement of the gold structure on the surface interface. Due to the rigid structure of the sputtered alloy on the glass sheets, the structure loses its ability to flexibly move during the dissolution process, leading to fracturing of the surface, as seen in Figure 2.7 A-D. This is in contrast to gold-silver alloy leaves which can be stabilized on glassy carbon (GC) electrodes after dealloying. The leaves, which are floated on HNO3 for dealloying [37], possess the flexibility needed to contract during dealloying, showing no cracks or ligaments post dealloying [38]. It can also be observed that these cracks, which are generated from the instant the sheets come in contact with the oxidizing agent, grow and extend (Figure 2.7 A-D) by ongoing etching, to the point when they are no longer distinguishable from pores (Figure 2.7 E-H). These results are summarized in Table 2.4 with the average crack lengths ranging from 120 nm for sheets dealloyed at 0.5 °C and 1 min to 754 nm for sheets dealloyed at 20.8 °C and 60 min (100 nm film thickness). Under the latter conditions, the cracks form pores by continuous rearrangement of the gold atoms on the surface interface [17].
Figure 2.7: NPG surfaces with differing morphologies obtained by altered etching condition with (A) 1 min/0.5 °C (~4 nm), (B) 1 min/20.8 °C (~9 nm), (C) 5 min/0.5 °C (~8 nm), (D) 5 min/20.8 °C (~15 nm), (E) 1 min/40.2 °C (~24 nm), (F) 1 min/59.9 °C (~41 nm), (G) 5 min/40.2 °C (~51 nm) and (H) 15 min/60.5 °C (~78 nm) for 100 nm thick films. (Note: numbers in brackets show the average pore size)
Table 2.4: Summary of results obtained from 50, 100 and 300 nm alloy films in regard to roughness factor, silver content remaining after dealloying and the average pore size.

<table>
<thead>
<tr>
<th>Dealloying time [min]</th>
<th>Dealloying temp. [°C]</th>
<th>Rf</th>
<th>Ag content [at. %]</th>
<th>Average Pore size [39]</th>
<th>Average crack length [39]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>50 nm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.3</td>
<td>6.1 ± 0.9</td>
<td>12.6 ± 0.5</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>0.3</td>
<td>4.8 ± 0.1</td>
<td>4.6 ± 0.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>15</td>
<td>0.5</td>
<td>4.9 ± 0.3</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>15</td>
<td>20.8</td>
<td>2.7 ± 0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>15</td>
<td>40.2</td>
<td>2.6 ± 0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>15</td>
<td>59.9</td>
<td>2.0 ± 0.4</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>100 nm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1</td>
<td>0.5</td>
<td>44.2 ± 0.9</td>
<td>40.5 ± 0.5</td>
<td>4.4 ± 0.9</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>20.8</td>
<td>11.7 ± 0.7</td>
<td>7.5 ± 0.3</td>
<td>8.7 ± 1.2</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>40.2</td>
<td>4.4 ± 1.1</td>
<td>2.8 ± 0.4</td>
<td>24.2 ± 3.4</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
<td>59.9</td>
<td>2.8 ± 0.4</td>
<td>2.5 ± 0.4</td>
<td>51.0 ± 3.8</td>
</tr>
<tr>
<td>C</td>
<td>5</td>
<td>0.5</td>
<td>9.6 ± 0.7</td>
<td>7.7 ± 0.4</td>
<td>8.1 ± 1.6</td>
</tr>
<tr>
<td>D</td>
<td>5</td>
<td>20.8</td>
<td>8.2 ± 0.5</td>
<td>3.4 ± 0.4</td>
<td>15.2 ± 2.2</td>
</tr>
<tr>
<td>G</td>
<td>5</td>
<td>40.2</td>
<td>3.6 ± 1.0</td>
<td>1.6 ± 0.5</td>
<td>41.7 ± 3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.9</td>
<td>2.4 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>61.8 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.6</td>
<td>8.0 ± 0.5</td>
<td>12.5 ± 0.3</td>
<td>14.4 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>14.8</td>
<td>6.6 ± 1.0</td>
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<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>19.7</td>
<td>6.4 ± 0.2</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>25.3</td>
<td>5.3 ± 1.2</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>30.0</td>
<td>4.7 ± 1.9</td>
<td>--</td>
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<tr>
<td></td>
<td></td>
<td>15</td>
<td>34.8</td>
<td>4.7 ± 0.4</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>40.2</td>
<td>4.1 ± 0.4</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>44.8</td>
<td>4.5 ± 0.6</td>
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</tr>
<tr>
<td></td>
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<td>15</td>
<td>49.7</td>
<td>3.6 ± 0.3</td>
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<tr>
<td></td>
<td></td>
<td>15</td>
<td>55.1</td>
<td>3.6 ± 0.4</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td><strong>H</strong></td>
<td>15</td>
<td><strong>60.5</strong></td>
<td><strong>3.7 ± 0.2</strong></td>
<td><strong>6.4 ± 0.4</strong></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.5</td>
<td>4.9 ± 0.3</td>
<td>3.5 ± 0.5</td>
<td>28.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>20.8</td>
<td>3.3 ± 0.4</td>
<td>1.9 ± 0.3</td>
<td>43.2 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>40.2</td>
<td>3.3 ± 0.4</td>
<td>0.6 ± 0.5</td>
<td>63.7 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>59.9</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td><strong>300 nm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.5</td>
<td>130.5 ± 12.1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>0.3</td>
<td>44.9 ± 2.2</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>15</td>
<td>0.5</td>
<td>33.8 ± 2.0</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>15</td>
<td>20.8</td>
<td>24.8 ± 1.1</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>15</td>
<td>40.2</td>
<td>12.5 ± 3.5</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>15</td>
<td>59.9</td>
<td>5.9 ± 0.7</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

[a] The cracks have been filled through continuous rearrangement of gold, leading to no measurable average crack size for these samples.
The mechanism for dealloying, as already extensively been discussed in section 1.3.3, involves the acid etching of a defined alloy, which comprises the removal of the less noble metal from the alloy. Therefore the amount of silver left in the alloy after dealloying could be able to predict resulting pore sizes and roughness created. When observing Table 2.4 it becomes clear that this assumption is applicable. The lower the amount of silver left in the alloy the bigger the pores and the lower the roughness. When examining the samples shown in Figure 2.7 the silver content decreased from A: 40.5±0.5 at. % to G: 1.6±0.5 at. % as determined by SEM-EDX.

All the results obtained herein showed very high reproducibility when adjacent cut sheets were tested for at least 3 different electrodes. The results from Table 2.4 were visualized in 2 graphs for 100 nm alloy thickness with dependence on dealloying

Figure 2.8: Summary of results achieved by altering etching conditions and the influence on the roughness $R_f$ with (A) $R_f$ dependence on dealloying temp, (B) $R_f$ dependence on dealloying time and (C) combined results in a 3D matrix, respectively and (D) the average pore size dependency on the dealloying conditions as determined by manual readout.
temperature (Figure 2.8 A) and dealloying time (Figure 2.8 B). The excellent reproducibility generated from the electrodes is indicated by the error bars in the two graphs. The trend observed is also displayed herein. The highest $R_f$ values can be achieved by keeping the etching time and temperature as low as possible. For 100 nm thick films the highest roughness achieved was $44.2 \pm 0.9$ (Table 2.4). By altering the conditions by just increasing the temperature or the time ever so slightly the $R_f$ is decreased (1 min to 5 min) to $9.6 \pm 0.7$ or (0.5 °C to 20.8 °C) to $11.7 \pm 0.7$ respectively.

The influence of dealloying time and temperature for 100 nm thick alloy layers of an Ag$_{70}$/Au$_{30}$ is clearly evident from the 3-dimensional plots in Figure 2.8. An exponential decrease in $R_f$ value was observed with small increases in dealloying time and temperature (Figure 2.8 C). The opposite effect was observed for the average pore size (Figure 2.8 D). The same specific trends were observed (see Table 2.4) for 50 and 300 nm thick films respectively. The plots in Figure 2.8 C-D can be used as a “blue print” model to determine the dealloying conditions needed to generate electrodes with a specific roughness or pore size. Due to the high level of reproducibility of the preparation procedure, these electrodes provide the opportunity to prepare enzymatic bio fuel cells and biosensors where the optimum loading of enzyme and therefore efficient usage of electroactive area can be achieved by tuning the surface roughness and the pore size of the support.

2.3.2 Pore size determination

Three different methods for pore size determination were performed using SEM images. These methods consist of the manual readout of pore sizes of an in ImageJ adjusted SEM image (ISODATA adjustment) at ~35 different sites; linear intercept of a, in ImageJ, ISODATA modified SEM image by plotting random intensity profile lines for manual readout and the rotationally averaging of FFT power spectra generated from a SEM image by DM™ software for pore size calculation. All the methods used showed certain advantages as well as disadvantages.

The method of manually reading out the pore size is very time consuming. However it gives insight into the areas of interest and allows specific pore properties to be analyzed as one can observe only cracks, ligaments, pores or combined properties. This main advantage of the manual readout however also poses its main drawback. The analyst runs the risk of determining pore sizes with a bias in mind. When this method is the chosen method it has to be ensured that these “preferred” results are minimized.
danger of receiving “favored” results is diminished by using the LI method. However it is still present as the intensity profile lines cutting through the image are still placed by the analyzer. So again it is important to ensure random placement of these lines for reliable results. By using the rotationally averaged FFT power spectra for pore size determination this problem can be completely ruled out, as large areas are used for analysis that represent a very good average of intensity profiles throughout the image. However when comparing the results obtained for one SEM picture (Figure 2.3) with all three methods, the results can differ substantially from one another as seen in Table 2.5. For manual readout the values were 58.0±6.2 nm, for the LI method 105.6±20.5 nm and for the rotationally averaged FFT power spectra 118.3±9.9 nm.

While the values for the FFT analysis and the LI method are very close to one another, the value for the manual determined pore size differs immensely. The method of analyzing FFT spectra is in general the same method as the LI method, with the difference that there are much more “intensity profile lines” used. One can imagine the method as intensity line after intensity line plotted over each other. This method is therefore based on the same principle as the LI method, but in a much more precise manner. This can also be seen by the standard deviation generated from both methods as seen in Table 2.5. However these two methods pose a decisive drawback. These methods are only applicable if the pores are well distributed throughout the whole structure. This is not the case in any of the analyzed electrodes, due to the inflexibility of the porous structure, which creates cracks and ligaments on the surface. This lack of free movement at the surface is due to the static attachment of the alloy layer on the glass sheets. This is in contrast to a gold-silver leaf which is placed on a conductive electrode (such as glass carbon electrodes). It can freely contract and expand during the etching and therefore no cracks are generated which results in bicontinuous structures [40,41]. Because of this reason all the results regarding pore sizes obtained in Table 2.4

Table 2.5: Summary of results obtained from three different pore size determination techniques from the same SEM sample picture.

<table>
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<tr>
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<tbody>
<tr>
<td>Manual readout</td>
<td>58.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Linear intercept</td>
<td>105.6</td>
<td>20.5</td>
</tr>
<tr>
<td>Rotationally averaging of FFT power spectra</td>
<td>118.3</td>
<td>9.9</td>
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</tbody>
</table>
were determined by the manual readout method. The problem of biased readout could be diminished by measuring the pore size at random places of the SEM image while uniformly distributing the measurement points throughout the SEM image and by using the hiding function of ImageJ until a sufficient amount of points have been measured. This hiding of the results can reduce operator bias on the results.

It is pivotal to emphasize that to this date, no such detailed study of tuning roughness’s and pore sizes of NPG generated from magnetron sputtering has been reported in the literature, thus demonstrating the novelty of this work. Tremendous opportunities could be unfolded by morphology tuning of nanoporous structures, hence making them the ideal substrate for the manufacture of enzymatic or non-enzymatic sensors, enzymatic biofuel cells or super capacitors, which are only a few of the possible applications. Finding optimal pore sizes for specific enzyme immobilization or encapsulation could also increase the stability of the enzymes based on the inability of protein unfolding due to volume constraints when immobilized inside the pores. To investigate these possibilities the addressability of these pores needed to be investigated, as well as the modification of these surfaces to prepare for enzyme immobilization and encapsulation.

### 2.3.3 Surface addressability

Surface addressability is a common problem with regard to nanostructured electrodes. These issues are commonly caused by molecules that are too bulky to be able to penetrate through the pores, thus making the generated area inaccessible for the intended applications. These surface addressability issues can also be caused by the inability of the surface area to become fully wet due to diffusion, mass transport issues, capillary forces or pressure differences. For this reason it was necessary to test the NPG electrodes for surface addressability by eliminating the main causes of incomplete wetting. To minimize these issues the NPG electrodes were rinsed thoroughly with deionized water and afterwards dried under vacuum in a desiccator to ensure that the pores are as free from water as possible. The electrodes were then used directly, so that capillary forces that would otherwise draw water from the air, by condensation of water (dependent on air humidity) from the air and subsequent capillary pull, are used in a constructive manner to wet the electrodes with the sulphuric acid solution. This capillary pull has been referred to the adjustment of a capillary equilibrium that porous solids occupy, as demonstrated with sand in the early 1940’s by Leverett et al. [42].
range of pore structures at different film thicknesses were prepared to examine the influence of thickness on surface addressability. Under the assumption that surface addressability should be most difficult for the smallest pores, NPG electrodes were manufactured with average pore sizes of ~5 nm. A sample plot of NPG electrodes at different times (T=0°C, with 1, 5 and 15 min dealloying time) and differing film thicknesses (50-1000 nm) can be seen in Figure 2.9. The experiments showed that $R_f$ values increases linearly up to a film thickness of at least 500 nm for the 3 different setups. An example can be seen with an approximate 3 fold $R_f$ increase from 44 ± 1 to 131 ± 12 for electrodes dealloyed at 0 °C for 1 min at 100 and 300 nm alloy sheets. It is noteworthy that upon increasing the film thickness to 1000 nm, the surfaces were no longer fully addressable, leading to reduced measureable roughnesses. Therefore the issues of wettability can be assumed to play no major role for NPG electrodes which have been dried in a vacuum chamber before testing for up to 500 nm film thickness. Therefore the method of pre-drying the electrodes under external force, i.e. under vacuum, was used before using the electrodes for any application. An alloy thickness of 100 nm was chosen for all the following experiments, as this thickness is fully addressable and the setup is thriftier in regards to resources.

Figure 2.9: Surface addressability of nanopores manufactured at differing dealloying conditions for Ag$_{70}$/Au$_{30}$ alloys with 50, 100, 300, 500 and 1000 nm alloy thickness at 0 °C, dealloyed for 1 min (green ■), 5 min (blue ●) and 15 min (red ▲), respectively.
2.3.4 Pore size effect on immobilized cytochrome c

Cytochrome c was chosen as a model redox probe to monitor the influence of pore size on the electrochemical response as it has very well defined electrochemical responses and due to the simplicity of covalently attaching it to a surface interface. Another advantage of cytochrome c is the nearly spherical nature of the redox probe with unit cell parameters of A: 5.4 nm B: 5.4 nm and C: 4.2 nm [43]. After covalently attaching cytochrome c on a 50/50 mol % mixed thiol of 6-mercapto-1-hexanol and 11-

![Figure 2.10: (A) Baseline corrected DPV responses of cytochrome c immobilized on a 50/50 molar % mixed thiol of 6-mercapto-1-hexanol and 11-mercaptoundecanoic acid for NPG electrodes prepared using differing dealloying conditions and (B) peak current density (geometric) of immobilized cytochrome c plotted against the average pore size of NPG electrodes.](image)

Figure 2.10: (A) Baseline corrected DPV responses of cytochrome c immobilized on a 50/50 molar % mixed thiol of 6-mercapto-1-hexanol and 11-mercaptoundecanoic acid for NPG electrodes prepared using differing dealloying conditions and (B) peak current density (geometric) of immobilized cytochrome c plotted against the average pore size of NPG electrodes.
mercaptoundecanoic acid the electrochemical response was monitored in a 4.4 mM PBS solution containing 100 mM NaF as supporting electrolyte using DPV. It can clearly be observed that the response achieved by DPV is highly dependent on the dealloying conditions used in the electrode manufacturing process Figure 2.10 A. The responses range from approximately 30 to less than 3 µA cm$^{-2}$. The highest current densities were obtained for electrodes with an average pore size of ~9 nm, where the pores are sufficiently large to be accessible to the redox probe while also possessing a large electroactive surface area. When the pore size was decreased to ca. 5 nm, the current density decreased substantially. While this electrode provides the largest electroactive surface area, the average pore size is close to the actual size of the redox protein and the surface area in the pores is not accessible. When the average pore size of the NPG electrodes was increased, a steady decrease in current density was observed. As cytochrome c should be able to penetrate through this porous structure without difficulty, the response will be limited by the electroactive surface area. This area is gradually decreased by coarsening of the generated pores, leading to the loss of electrochemical response due to lower loadings of the protein. However, as a redox protein, cytochrome c is not in the main focus of this work, as the aim is the use of NPG for energy extraction of biological reactions. Therefore its use was limited to the use as a model system. To further clarify the pore size effect on enzymatic systems, the direct faradaic response of MvBOD was examined and is thoroughly discussed in section 2.3.5.

2.3.5 Pore size effect on immobilized MvBOD

MvBOD is a ‘blue copper’ protein that reduces oxygen to water. The enzyme possesses high catalytic activity at neutral pH while utilizing relatively low overpotentials for the reduction of O$_2$. Direct electron transfer (DET) of MvBOD adsorbed on planar and also unmodified, bare gold electrode is difficult with an unstable faradaic response reported previously [44]. However, the high roughness factor of the support enables significant improvement in the faradaic response and stability of MvBOD when compared to a flat gold electrode. As previously described, NPG of roughness factor 26 was used as a support for MvBOD. The high response arises from the porous morphology of NPG and the high loading of enzyme obtained under the conditions used [45]. Current densities of ca. 65 µA cm$^{-2}$ were observed at MvBOD NPG electrodes prepared by dealloying at 0.5 °C for 5 min (Figure 2.11 B). The size of
the pores of 8 ± 2 nm for this surface is similar to the size of $Mv_{BOD}$ (A: 4.0 nm B: 5.0 nm and C: 6.0 nm) [46].

On plotting the current densities (using the real electroactive surface area rather than the geometric area) against the applied potential, it can be observed that higher enzyme loadings could be achieved with smaller pore sizes, *i.e.* electrodes prepared at 0 °C/ 5 min and 40 °C/ 1 min (**Figure 2.11 C**). This change is indicative of a more favorable orientation of $Mv_{BOD}$ on the surface of these electrodes. The other preparation conditions lead to similar faradaic responses for each system, indicating a more favorable orientation of $Mv_{BOD}$ occurred on such surfaces. Similar results have previously been reported by Shleev *et al.* on the influence of Au nanoparticle size on the $Mv_{BOD}$ response after immobilization [47].

On increasing the temperature of dealloying to 20 °C (**Figure 2.11 B**) (and therefore increasing the pore sizes), the current density decreased. Further increases in the temperature of the process using the same dealloying time of 5 min resulted in a slight decrease in the response. The average pore sizes of NPG fabricated on dealloying at 40 °C and 60 °C are ~42 nm and ~62 nm respectively, much smaller than the size of the enzyme.

When the dealloying temperature was maintained at 40°C and the time varied from 1 to 5 and to 15 min (pore diameters of 24, 42 and 78 nm, respectively), a similar trend was observed (**Figure 2.11 A**). Current densities of ca. 23 µA cm$^{-2}$ were obtained for NPG dealloyed for 1 min. These conditions of dealloying enable the fabrication of NPG electrodes with an average pore size of 24 nm which is larger than the size of the enzyme. On increasing the time of dealloying to 5 and 15 min, the average pore size doubled to 42 nm and 46 nm, respectively, again resulting in a decrease in the current density (**Figure 2.11 A**).

From the results obtained with $Mv_{BOD}$, it appears that the faradaic response occurs from enzyme molecules adsorbed at the surface of the electrode. With cyt c in contrast, the optimum response was obtained with electrodes of pore sizes that were larger than the protein, where the protein was incorporated in the pores [48]. To stabilize the response, the electrodes were coated with PEGDGE-epoxy polymer. This cross-linking agent stabilizes the adsorbed $Mv_{BOD}$ by creating covalent bonds with nucleophilic groups such as amine, thiol and hydroxyl groups on the surface of the enzyme [49]. However a decrease in the response was observed, with a 20% response obtained after 4 hours (**Figure 2.11 D**).
Figure 2.11: Direct electrochemical response of MvBOD on PEGDGE polymer modified NPG electrodes prepared at (A) 40 °C and different dealloying times, (B) 5 min dealloying time and different temperatures, (C) current densities (calculated from $A_{real}$) and (D) stability test of MvBOD with a NPG electrode dealloyed at 0 °C for 5 min, inset: decline of response as a function of time. (Note: numbers in brackets show the average pore size)
2.4 Conclusions

NPG electrodes were manufactured using a magnetron sputtering technique. The sputtered glass sheets were dealloyed using concentrated HNO$_3$. The etching conditions chosen had a significant impact on the generated porous structure making it possible to tune the pore morphology to specific needs. When tuning the pore size the following conditions were changed: i) atomic alloy composition, ii) dealloying time, iii) dealloying temperature and iv) the film thickness. The dealloying temperature and time were found to have the highest impact on the roughness and therefore the pore sizes generated. By choosing short dealloying times at low temperatures, the pores were found to be as small as <5 nm, which resulted in surface roughnesses that were 45 times higher than planar gold for 100 nm thick NPG films. A model was presented that can be used to pinpoint etching conditions to receive desired morphologies for specific purposes such as enzyme immobilization, sensing or capacitor applications.

Pore size determination throughout the differing pore structures was achieved using three different characterization methods; i) manual readout, ii) linear intercept method and iii) the rotationally averaging of FFT power spectra generated from DM™ software. The manual readout method was found to be the most feasible approach as the other two methods become very unprecise when the porous structure does not exhibit bicontinuous pore structures. However it is important to note that the manual readout method is prone to biased influences from the analyst and therefore actions must be undertaken to reduce these to a minimum.

The nanoporous structures showed outstanding addressability for sulphuric acid. The pores were fully addressable for thicknesses up to at least 500 nm. This was evident through the linear relationship displayed by the results received for the calculation of the $R_f$ values. Wettability and surface addressability were enhanced by drying the electrodes before use in a vacuum chamber to use capillary forces for constructively “pulling” substrate or liquid into the pores.

The influence of the pore size on the bioelectrochemical response of cyt c and *Myrothecium verrucaria* bilirubin oxidase was evaluated. Maximal current densities of ca. 30 µA cm$^{-2}$ were observed at cyt c modified NPG electrodes with an average pore size of $\approx$10 nm. With bilirubin oxidase, high current densities of ca. 65 µA cm$^{-2}$ were detected at *MvBOD* modified NPG electrodes with the response likely arising from surface adsorption of the enzyme.
References


Chapter 3:

Surface modification techniques for NPG substrates
3.1 Introduction

Surface modification for bioelectrochemical applications, such as biosensors and biofuel cells on gold substrates can be achieved using many different approaches. The use of self-assembled monolayers (SAMs), composed of functionalized thiols, is a common used technique for surface modification and functionalization of gold [1]. The spontaneous formation of the gold-sulphur bond, the simple and quick manufacturing, the relatively well structured assembly of the molecular chains, the structural rigidity and the controllable interface properties are among the main reasons why they have received so much attention in the past [2]. The chain length used determines the functionality of the SAM [3]. While long chains form well organized monolayers, the distance to the electroactive surface becomes larger, which results in a hindered electron transport. Short chains in contrast form a surface structure with much more defects but enable for better electron transport [4]. Despite all the convenient properties of thiol based SAMs, they also hold a decisive drawback. With a binding energy of 184 kJ mol\(^{-1}\), the chemisorbed gold-sulphur bond is not stable enough for long term stability [5]. The potential window in which the gold-sulphur bond is stable lies at best between +1.0 and -1.0 V [vs SCE], while the potential window highly depends on the chain length, terminal group and quality of the subjacent gold surface [6].

A more promising approach is reported in the use of aryl diazonium salt modified electrode surfaces. Gooding et al. report this surface modification method to appear to be suitable for all electrode types [6]. The formation of the surface modified layer is due to radical formation and assembly. This mechanism results in a modifying layer that may be a few molecules thick [7]. Despite these multilayers the stability of the layer is much more stable than the SAM formed by thiols (stable potential window of about 2.9 V) [8]. The electro-grafting of aryl diazonium salts occurs through the forming of radicals (by stripping of the diazo functional group), by applying negative potentials [9]. Utilizing electro-grafting of diazonium films on conductive substrate can enable surface functionalization’s with alkyl, halogeno-alkyl, perfluoro-alkyl, carboxylic, ester, cyanide, halides, nitro, amino, alcohols and thiol functional groups [10].

Another stable method of surface functionalization and modification is the modification with Osmium redox polymers. Herein osmium complexes especially bipyridines, terpyridines, and phenanthrolines, have particularly high self-exchange rates [11] and are among the most common used for stable enzymatic biosensors and
biofuel cells [12]. In general these complexes are bound to a backbone structure which can be designed to be hydrophilic or hydrophobic, pose functional groups capable of crosslinking (e.g. epoxy functional groups) and have long or short spacer groups to implement flexibility or rigidity to the system. The resulting redox potential of the osmium polymers can be notably shifted to more positive potentials by exchanging one or two labile chloro ligands at a bis(chelate) Os complex [13]. By altering the groups linked to the chelate ligands a wide range of redox potentials can be achieved, linked with the electron donating or withdrawing capabilities [13]. Enzymes can be immobilized in the herein formed polymer matrices. Here, multicopper oxidases (MCO), usually laccases and bilirubin oxidases, are often used for the electrochemical four-electron reduction of O₂ to H₂O without the forming of disruptive H₂O₂ [14]. Bilirubin oxidase from Bacillus pumilus (BpBOD) is a bacterial enzyme with a molecular weight of 59 kDa containing four copper ions that are arranged in a T1 and T2/T3 site embedded in the protein structure [15]. The capability of BpBOD to undergo DET/MET have been extensively described by Lojou et al., where the interactions on functionalized (amine/carboxylic acid) and un-functionalized carbon nanotubes have been studied [16].

Here the use of a variety of diazonium surface modification techniques on NPG substrates are evaluated. The synthesis and characterization of functionalized aryl diazonium compounds electro-grafted on NPG substrate (amine, nitro, hydroxylamine, carboxylic acid and sulfonate functional groups) is discussed. In addition the surface modification with redox polymers and hydrogels are discussed. In particular Os(bpy)₂(PVI)₁₀Cl⁺²⁺ (NUI-Galway) and PAA-PVI-[Os(dcl-bpy)₂Cl]⁺²⁺ (CNRS Bordeaux) redox polymers as well as Aru032-TB (RU Bochum) redox hydrogel were used for surface functionalization and are extensively discussed. In addition the immobilization of laccase and BpBOD is discussed.
3.2 Experimental

3.2.1 Reagents and Materials

Nitric acid (70%), sulfuric acid (95-98%), potassium chloride, potassium phosphate monobasic and dibasic, sodium nitrite, tetrafluoroboric acid solution (HBF₄), 4-[N-BOC] aminomethylanilin (97%), 4-nitrobenzenediazonium tetrafluoroborate (97%) tetrabutylammonium tetrafluoroborate (TBATFB), hydrochloric acid (37%), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diaminonickel salt (ABTS), dimethyl sulfoxide (DMSO), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC), N-hydroxysuccinimide (NHS), N,N-Dimethylformamide (DMF), N,N-diisopropylethylamine (DIPEA), glucose oxidase from *Aspergillus Niger* (GOx) (100,000 – 250,000 U g⁻¹) laccase from *Trametes versicolor* (*TvLac*) 1.0 U mg⁻¹ and 2,2′-(ethylenedioxo)bis(ethylamine) (98 %) were obtained from Sigma-Aldrich Ireland, Ltd. Acetonitrile (ACN) was purchased from Fisher Scientific Ireland Ltd. Anthraquinone-2-carboxylic acid was purchased from Tokyo Chemical Industry UK Ltd. All chemicals were used as received unless stated otherwise. Deionized water with a resistivity of 18.2 MΩ cm was obtained from an Elgastat maxima-HPLC (Elga, UK).

3.2.2 Preparation of planar and NPG electrodes

NPG electrodes were prepared as already described in section 2.2.2. Glass sheets were sputtered with a 10 nm thick Ti adhesion film, followed by a 35 nm thick pure Au layer and by a 100 nm Ag₇₀/Au₃₀ alloy layer [17,18]. After sputtering the sheets were cut into uniform pieces of approximately 0.5-0.7 cm⁻² using a circular saw. The sheets were dealloyed in nitric acid following the dealloying times and temperatures for the desired average pore size distribution as identified in chapter 2. A silver wire was soldered to the surface of the sputtered sheet using an indium wire and the soldering point then supported by a two component epoxy glue. The electroactive surface area was defined by a dielectric paste (Gwent Group, UK).

Planar Au electrodes were also prepared utilizing magnetron sputtering. After sputtering a 10 nm Ti adhesion layer a pure gold layer of 100 nm was sputtered. Electrodes were prepared identically to the preparation of NPG. Alternatively, polycrystalline planar Au-disk electrodes (CHI) (φ=2 mm) were thoroughly polished utilizing 0.05 µm alumina powder dispersed in deionized water on a polishing pad. After rinsing the electrodes were placed in a solution of 0.5 M H₂SO₄ cell and the potential cycled between -0.2 and 1.65 V for 10 cycles at 100 mV s⁻¹. In a final step
they were extensively cleaned in an ultrasonic bath for 5-10 minutes. After cleaning and determining $A_{\text{real}}$ and $A_{\text{geo}}$ of NPG electrodes, (section 2.2.3), the electrodes were ready for surface modification which was performed utilizing a variety of techniques.

### 3.2.3 Diazonium film modifications

#### 3.2.3.1 Synthesis and surface modification of gold with anthraquinone functional groups

The functionalization of NPG with anthraquinone (AQ) functional groups was achieved by following published procedures [19,20]. 4-[N-BOC] aminomethyl benzene diazonium salt was synthesized by adding 300 mg of 4-[N-BOC] aminomethylaniline into a two neck round flask. After extensive deoxygenation with Ar or N$_2$, 200 $\mu$L HBF$_4$ in 3 mL H$_2$O was added to the flask under stirring to dissolve the amine and activate the reaction. Once the amine was dissolved, an inert gas reservoir was attached to ensure an O$_2$ free environment throughout the reaction. Afterwards the flask was immersed in an ice bath. To start the reaction, 95 mg NaNO$_2$ dissolved in 350 $\mu$L H$_2$O was added dropwise to the system using a syringe. Dropwise adding of the reducing agent is necessary as explosive components can be formed. It is therefore also necessary to keep the reaction volume low. After addition, the ice bath was removed and the reaction was left for 2 hours under continuous stirring. The solution formed a yellowish precipitate as the reaction proceeded. After the reaction was complete, the flask with the precipitate was left at -18 °C over night. The next day the precipitate was filtered from the solution and washed several times with diethyl ether (DEE). The diazonium salt was fully dried in a vacuum chamber and stored at -18 °C. The compound was found to be stable for approximately one month. To verify the correct compound was synthesized and purified NMR experiments were performed using a JEOL Delta-GSX 270 NMR.

![Figure 3.1: Coupling of diazonium salts on gold surfaces and electrochemical AQ surface modification.](image)
NPG electrodes with differing pore sizes were used for surface modification. NPG electrodes were used for the attachment of the diazonium compound and modification using AQ functional groups. The diazonium compound was electrochemically reduced at the NPG electrodes. A solution of diazonium salt (5 mM) and TBATFB (0.1 M) dissolved in 10 ml ACN were prepared and the potential was cycled in the range of 0 to -0.7 V vs Ag/AgCl in 3 M KCl at 50 mV s\(^{-1}\) for 8-12 full cycles (Figure 3.1 1-2). After rinsing in ACN the BOC protective group was removed by immersion in 1 M HCL for 2 h (Figure 3.1 3). AQ-functional groups were attached to the activated amine groups (activated with 0.1 M EDC and 0.06 M NHS) by adding 0.025 M AQ-2-carboxylic acid in ACN (Figure 3.1 4).

### 3.2.3.2 Surface mod. with carboxylic acid functional groups

Covalent attachment of carboxylic acid functional groups was achieved by electro-grafting of diazonium species. 2-Carboxy-6-naphtoyl diazonium salt (NA-DS) was synthesized \textit{in-situ} by mixing a 2 mL solution (20 mM in acetonitrile) of 6-amino-2-naphthoic acid (NA) with 2 mL of a solution of NaNO\(_2\) (2 mM) in 1 M HCl in an ice bath (Figure 3.2 1) [21,22]. A single potential scan from 0 to -0.5 V at 200 mV s\(^{-1}\) was performed to electrochemically reduce NA-DS (Figure 3.2 2-3). After rinsing with deionized water, 3-mercaptopropionic acid (MPA) was attached to the void spaces on the electrode surface by immersion of the electrodes in a 1 mM MPA solution over night.

![Figure 3.2: Schematic reaction mechanism for the functionalization of gold surfaces with carboxylic acid functional groups and subsequent filling of void spaces with MPA.](image)

### 3.2.3.3 Surface mod. with nitro and amine functional groups

Surface functionalization utilizing diazonium species was also performed for nitro and amine functionalization. Functionalization was achieved following a published procedure [23]. NPG electrodes were immersed in the \textit{in-situ} reaction solution of 10 mL ACN containing 2 mM 4-nitrobenzenediazonium tetrafluoroborate and 100 mM
Figure 3.3: Schematic reaction mechanism for the functionalization of gold surfaces with nitro and amine functional groups.

TBATFB as reducing agent and supporting electrolyte (Figure 3.3 1-2). The *in-situ* synthesized nitro terminated 4-nitrobenzene diazonium salt (NBD) was electrochemically reduced at the gold surface by cycling the potential from 0.6 V to -0.6 V at a scan rate of 200 mV s\(^{-1}\) for two cycles (Figure 3.3 3). The exposed nitro groups could be partially reduced to amine groups by performing two CVs in a solution of 1:9 EtOH/H\(_2\)O and 0.1 M KCl supporting electrolyte from 0 to -1.4 V at 200 mV s\(^{-1}\) (Figure 3.2 4).

### 3.2.3.4 Surface mod. with sulfonate functional groups

Surface modification with sulfonate functional groups was performed by electrografting of freshly synthesized benzene diazonium sulfonate (BDS). BDS is an intermediate product in the synthesis of the pH indicator methyl orange. BDS was synthesized by dispersing 0.03 mol sulfanilic acid in 150 mL 1 M HCl. After cooling of the dispersion to about 4 °C, 16.5 mL 1 M NaNO\(_2\) was added dropwise to the reaction solution. After addition the white precipitate dissolved completely and the ice bath was removed. The reaction was continued for another 1 hour. BDS formed a spongy white precipitate after approximately 25 minutes. The reaction mechanism is displayed in Figure 3.4. The addition of NaNO\(_2\) to HCl leads to the formation of nitrosonium ions (NO\(^+\)) (Figure 3.4 A). Sulfanilic acid exhibits an internal proton transfer when in solution (Figure 3.4 B). The previously formed nitrosonium ion then reacts with the amine group of the sulfanilic acid, leading to the diazonium compound [24]. The synthesized BDS was dried and washed with deionized water and afterwards stored in a dark container at -18 °C. Due to the reactive nature of the diazo group (N≡N\(^+\)) the synthesized BDS was not kept longer than 7 days. BDS modified electrodes were prepared by electrochemically reducing BDS to the gold surface (BDS-NPG). CV was performed from 0.3 to -0.5 V vs Ag/AgCl (3 M KCl) at 200 mV s\(^{-1}\) in a solution of 5 mM BDS and 0.1 M tetrabutylammonium tetrafluoroborate (TBATFB) as supporting.
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**Figure 3.4:** Proposed reaction mechanism for the electrochemical reduction of benzene diazonium sulfonate (BDS) onto gold surfaces with (A) formation of the nitrosonium ion, (B) formation of the BDS and (C) the electrochemically induced reduction onto a gold electrode.

Electrolyte in ACN (Figure 3.4 C). To avoid excessive multilayer formation of the BDS film this step was limited to one scan [6].

### 3.2.4 Polymer modification of gold surfaces

#### 3.2.4.1 Drop-cast modification

Os(bpy)$_2$(PVI)$_{10}$Cl$^{2+/3+}$ was kindly provided by Dr. PÓ Conghaile and Prof. D. Leech and was synthesized following a published procedure (the proposed structure is displayed in Figure 3.5) [25]. Os(bpy)$_2$(PVI)$_{10}$Cl$^{2+/3+}$ modified NPG electrodes were prepared by drop-casting a mixture of PBS buffer containing 8 µL of the redox polymer Os(bpy)$_2$(PVI)$_{10}$Cl$^{2+/3+}$ at a concentration of 6 mg ml$^{-1}$, 4.8 µL enzyme solution (glucose
dehydrogenase) at a concentration of 10 mg mL\(^{-1}\) and 1.9 \(\mu\)L of the crosslinker PEGDGE at a concentration of 15 mg mL\(^{-1}\). 20 \(\mu\)L of this freshly prepared enzyme/polymer/crosslinker solution were then drop-casted on the electrodes. To promote wetting of the electrode surface, the electrodes were exposed to a vacuum of maximum 0.098 MPa for approximately 3 min and allowed to dry at 4 °C over night.

### 3.2.4.2 Electrochemical deposition of redox hydrogel and polymer

NPG electrodes were also modified using electrodeposition techniques. Here the modification with Aru032-TB redox hydrogel (Figure 3.6 A) and PAA-PVI-[Os(dcl-bpy)\(_2\)Cl\(^{2+/3+}\) redox polymer (Figure 3.6 B) is described. Aru032-TB redox hydrogel was
kindly provided by Dr. A. Ruff and Prof W. Schuhmann and synthesized following a published procedure [26]. Aru032-TB redox hydrogel was electrodeposited together with glucose oxidase and crosslinker 2,2-(ethylenedioxy)bis(ethylamine). The solution for electrodeposition contained 20 mg mL\(^{-1}\) Aru032-TB redox hydrogel, 10 mg mL\(^{-1}\) glucose oxidase and crosslinker 20 mg mL\(^{-1}\). Electrodeposition was carried out using chronoamperometry: -1.8 V/0.02 s pulse width and 0 V for 30-60 s as rest period to allow for sufficient diffusion. 50 to 100 cycles were used with a compound mixture of (1: 3: 0.5) (enzyme solution: TB polymer: CL), non-diluted, diluted 1:10 and 1:50 in H\(_2\)O, respectively.

PAA-PVI-[Os(dcl-bpy)\(_2\)Cl]\(^{+/2+}\) redox polymer and Bacillus pumilus bilirubin oxidase (BpBOD) was kindly provided by Dr. N. Mano. Electrodeposition was achieved following a published procedure [27]. In summary a 30 µL solution with 1 mg mL\(^{-1}\) PAA-PVI-[Os(dcl-bpy)\(_2\)Cl]\(^{+/2+}\) redox polymer and 1 mg mL\(^{-1}\) BpBOD enzyme solution in McIlvain buffer 0.1 M at pH 5.0 was drop-casted on each NPG electrode. Pulsed deposition was performed by pulsing the potential from 0.9 V (2 s) to -0.4 V (3 s) vs Ag/AgCl in 3 M KCl for 1-6 min.

### 3.2.5 Electrochemical measurements

NPG electrodes were cleaned in 0.5 M H\(_2\)SO\(_4\) by cycling the potential between -0.2 and 1.6 V at 0.1 V s\(^{-1}\) vs Ag/AgCl (3 M KCl). To minimize changes to the structure of the nanopores this step was limited to 2 cycles [17]. The electrodes were rinsed with deionized water and dried in a vacuum chamber. All electrochemical measurements were performed using a CHI620A, CHI802 and CHI1030C multichannel potentiostat operating in a standard three electrode configuration with NPG, Ag/AgCl (in 3 M KCl) and Pt wire as working, reference and counter electrodes, respectively.
3.3 Results and Discussion

3.3.1 Results and Discussion of diazonium modified NPG

The electro-grafting of diazonium species on gold substrate can be achieved by \textit{in-situ} synthesis of the diazonium salts and subsequent electrochemical reduction to the surface. The \textit{in-situ} approach has the advantage of avoiding the stability issues of these compounds as the diazo group is highly reactive. In this study a wide variety of functionalized diazonium salts have been utilized and are depicted in Figure 3.7. 4-[N-BOC] aminomethyl benzene diazonium salt was the first diazonium species used as the bulky BOC group prevents the formation of multilayers on the gold surface which assists in the evaluation of the formation on the NPG surface [19].

![Figure 3.7: A wide variety of diazonium modifications has been developed throughout this work, being BOC- protected and unprotected amine, nitro, carboxylic acid and sulfonate functional groups (left to right).](image)

3.3.1.1 Surface addressability and functionalization with AQ

4-[N-BOC] aminomethyl benzene diazonium salt shows a characteristic reduction peak at approximately -450 mV (vs Ag/AgCl) when electrochemically reduced to the surface of the gold substrate (Figure 3.8 A). Upon continuous scanning it can be observed that the capacitance decreases gradually. This can also be observed in Figure 3.8 A. The decrease in capacitance is a further indication of blocking of the surface with the bulky diazonium species. Near monolayer coverage can be assumed as the bulky BOC group prevents the radicals, which are formed by applying negative potentials, from attacking the meta position of the molecule, therefore successfully preventing the formation of dimers and trimers [19]. If the electrochemical reduction of 4-[N-BOC] aminomethyl benzene diazonium salt is performed on NPG electrodes this trend can also be observed (Figure 3.8 B), however is not as distinctive as on planar...
Figure 3.8: Cyclic voltammograms of NPG electrodes (300 nm alloy thickness) with (A) 10 full CV scans between 0 to -0.8 V at 50 mV s\(^{-1}\) at a planar gold electrode, (B) 10 CV scans between 0 to -0.8 V at 50 mVs\(^{-1}\) at a NPG electrode (C) response of AQ functional group covalently attached to the diazonium at varying scan rates and (D) comparison of modified planar gold and NPG electrodes at 100 mVs\(^{-1}\) in 100 mM PBS pH 7.0.

Au. Typically the responses obtained were very noisy which might be due to the diffusion of the diazonium species throughout the nanoporous structure. For the attachment of AQ functional groups to the diazonium film the solvent dimethylformamide (DMF) had to be exchanged as DMF dissolves the dielectric paint used for the preparation of the NPG electrodes. A variety of solvents were screened to obtain a solvent that was compatible with the paint. A summary of the obtained results can be seen in Table 3.1. It was found that all components are soluble in polar aprotic solvents. The dissolution in polar protic solvents such as water and ethanol was found to be difficult and therefore not further investigated. Dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), and acetonitrile were found to dissolve all reactants while not substantially affecting the paint of the electrodes. Acetonitrile was used for all further experiments. Acetonitrile was found to produce similar surface coverages of AQ as in DMF (calculated by equation 3.1).

\[
\Gamma = \frac{Q}{nF^*A^*Q} \tag{3.1}
\]
AQ functionalization was achieved through the activation of the amine group with EDC and NHS of the de-protected 4-[N-BOC] aminomethyl benzene diazonium salt. The typical redox reaction of AQ at differing scan rates can be seen in Figure 3.8 C. Upon increasing the scan rate the current increased following Randles-Sevcik equation. The main advantage of using NPG in comparison to planar Au electrodes can be seen in Figure 3.8 D. The responses obtained at planar Au electrodes are in a magnitude smaller than those obtained from NPG due to the large surface area NPG electrodes possess.

The surface coverages calculated by changing the NPG morphology, in terms of average pore size and film thickness, are listed in Table 3.2. Under the assumption that near full surface coverage is the most difficult to achieve with small pores, they have been utilized to evaluate the process. This assumption is made as surface addressability becomes more difficult if the channels are narrow and if bulky molecules hinder access. For comparison the surface coverages achieved on planar Au electrodes are also depicted. A coverage of 15.6 ± 1.1 pmol cm⁻² was achieved for planar Au electrodes. NPG electrodes with approximately 5 nm average pore size showed heavily reduced value of about 4-6 pmol cm⁻². Upon increasing the average pore size to ca. 9 nm, the surface coverage of AQ approached the values achieved by planar Au electrodes. Therefore surface modification was found to be easier if the pores are fully accessible for the solution. This is also indicated by altering the film thickness utilized. Upon increasing the film thickness from 50 to 300 nm by keeping the average pore size roughly the same the surface coverage decreases from 5.6 ± 1.9 to 4.6 ± 1.8 and
Table 3.2: Surface coverages of NPG electrodes with differing pore sizes and film thicknesses as determined by the redox responses obtained through CV of AQ modified electrodes.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Average pore size [nm]</th>
<th>Film thickness [nm]</th>
<th>AQ surface coverage [pmol cm(^{-2})][(^{[a]})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planar Au</td>
<td>-</td>
<td>-</td>
<td>15.6 ± 1.1</td>
</tr>
<tr>
<td>NPG</td>
<td>5 ± 2</td>
<td>50</td>
<td>5.6 ± 1.9</td>
</tr>
<tr>
<td>NPG</td>
<td>4 ± 1</td>
<td>100</td>
<td>4.6 ± 1.8</td>
</tr>
<tr>
<td>NPG</td>
<td>9 ± 2</td>
<td>100</td>
<td>14.8 ± 2.6</td>
</tr>
<tr>
<td>NPG</td>
<td>5 ± 3</td>
<td>300</td>
<td>4.4 ± 2.2</td>
</tr>
</tbody>
</table>

\(^{[a]}\) calculated utilizing electrochemically addressable surface area

4.4 ± 2.2 pmol cm\(^{-2}\) respectively. The still relatively large values of the 300 nm film thickness can be explained through the many cracks that are present in thick NPG electrodes with small pore sizes as already extensively evaluated in section 2.3.1.3.

3.3.1.2 Surface functionalization with carboxylic acid, nitro and amine functional groups

The in-situ synthesis and functionalization of NPG electrodes with carboxylic acid functional groups was performed utilizing NA-DS. The synthesized compound is not very stable and therefore cannot be stored. The electrochemical reduction of NA-DS therefore needs to be performed at low temperatures (ca. 0 °C) and directly after synthesis. A typical reductive scan of NA-DS can be seen in Figure 3.9 A. NA-DS shows a characteristic reduction peak at a potential of approximately 0.1 V vs Ag/AgCl. To prevent excessive multilayer formation potential cycling was limited to one full scan [21]. Since the electrochemical reduction is performed in a solution of the diazonium salt, hydrogen evolution can be observed at a potential of -0.4 V vs Ag/AgCl, due to the low pH of the solution. NA-DS modified NPG electrodes were utilized for fructose biosensors in chapter 4 and for enzymatic biofuel cells (EFCs) in chapter 5.

To modify NPG electrodes with positively charged functional groups (i.e. amine groups) a two-step surface synthesis was applied. In a first step the freshly synthesized NBD was electrochemically reduced to the NPG surface, while forming a characteristic broad reduction peak at around 0.3 V vs Ag/AgCl. The reduction of the capacitive current after the first reductive scan is obvious and can be seen in Figure 3.9 B. The nitro terminated diazonium film formed on the surface of the electrode can further be
Figure 3.9: Cyclic voltammograms of (A) reductive scan of Na-DS on NPG substrate ($\nu=200$ mV s$^{-1}$), (B) the electro-grafting of 4-Nitrobenzyl diazonium salt ($\nu=200$ mV s$^{-1}$), (C) electrochemical reduction of the nitro groups to amine and hydroxylamine functional groups ($\nu=100$ mV s$^{-1}$) and (D) enzymatic reduction of O$_2$ by laccase modified NPG electrodes in saturated N$_2$ and O$_2$ McIlvain buffer solution (150 mM, pH 5.0) and after adding of 10 µM ABTS ($\nu=5$ mV s$^{-1}$ all vs Ag/AgCl).

reduced to obtain amine and hydroxylamine groups. This was achieved by cycling the potential of the modified NPG electrodes in a solution of EtOH and H$_2$O (ratio 1:9) with 100 mM supporting electrolyte KCl. The reduction leads to the formation of amine and hydroxylamine functional groups that show characteristic reduction peaks as displayed in Figure 3.9 C [23]. To evaluate the surface modification technique, the prepared NPG electrodes were used to covalently attach laccase by crosslinking with CMC. As can be seen in Figure 3.9 D, no DET of laccase could be observed in O$_2$ saturated solutions of 150 mM McIlvain buffer solution at pH 5.0, when compared to N$_2$ saturated buffer solution. However upon addition of 10 µM of ABTS a typical catalytic wave of laccase can be observed. Therefore utilizing this system did not result in DET most likely due to the low concentration of enzyme used (1 U mg$^{-1}$) and/or an unfavorable orientation of laccase toward the electrode material. The catalytic current obtained from MET was too little to be further considered for bioelectrochemical applications and was not further investigated. It however showed that the modification technique was successful in binding laccase to NPG electrodes. It is expected that higher current densities can be obtained utilizing this surface modification strategy if the laccase solution can be further.
3.3.1.3 Synthesis of sulfonate terminated diazonium salts and surface modification

Sulfonate functional groups are very bulky negatively charged groups that can be utilized for a variety of applications. The bulky group could prevent the electrode surface from surface contamination as they block bulky molecules from getting in contact with the surface through steric hindrance. Also the strongly negative charged sulfonate group has the ability to orientate proteins in such a way that the positive sites are orientated towards the electroactive surface. BDS mainly however finds application as intermediate product in the synthesis of methyl orange [24]. A characteristic CV of the electrochemical reduction of BDS on NPG substrate can be seen in Figure 3.10 A. BDS shows a typical broad reduction peak at around 0 V vs Ag/AgCl. To avoid multilayer formation of the BDS on the electrode, CV was limited to one scan. However if multiple scans are performed the reduction in capacitance can be observed, which can be related to the subsequent filling of the void spaces available on the NPG electrode Figure 3.10 B. The modification of NPG electrodes with BDS was utilized to prepared heavy metal sensors, presented in the case of copper, and is shown in detail in section 4.3.1.

Figure 3.10: (A) Example cyclic voltammogram of the deposition of benzene diazonium sulfonate (concentration of 5 mM) on a NPG electrode at 200 mV s⁻¹ (vs Ag/AgCl in 3 M KCl). Supporting electrolyte was 0.1 M TBATFB. (B) Cyclic voltammograms of the reduction of BDS to NPG surfaces (i.e. 18 nm average pore size) with multiple scans (n=15) recorded vs Ag/AgCl in 3 M KCl reference electrode at 200 mV s⁻¹.
3.3.2 Results and Discussion of Polymer modified NPG

A variety of redox polymers and hydrogels have been utilized throughout this work. Upon encapsulating enzymes in redox polymers the stability and electron transfer from the catalyzed reaction can be enhanced. Here Aru032-TB, a toluidine based redox hydrogel with a hydrophobic backbone [26], and two osmium complex based redox polymers Os(bpy)$_2$(PVI)$_{10}$Cl$^{2+/3+}$ [25] and PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+2+}$ [27] were utilized.

3.3.2.1 Toluidine blue polymer

The toluidine hydrogel (Figure 3.11 B) could be electrodeposited on NPG electrodes by applying a series of potential pulsing in combination with crosslinking agents (Figure 3.11 A) and enzyme solution. This technique results in an encapsulated enzymatic film on the surface of the electrode. Here Aspergillus niger glucose oxidase (AnGOx) (Figure 3.11 C) and cellobiose dehydrogenase from Corynascus thermophilus (CtCDH) were utilized to encapsulate in the deposited film. Polymerization can occur at the toluidine group of the hydrogel as shown in Figure 3.11 D. In addition to the polymerization, crosslinking of the bi-functional crosslinker happens at the epoxy group in the backbone of the hydrogel structure (Figure 3.11 B). By applying potential pulses, it is therefore possible to form an insoluble and stable film on the electrode surface.

Figure 3.11: Compounds used in the crosslinking of GOx in electrodeposited toluidine blue polymer with (A) in-active bi-functional crosslinker, (B) Aru032-TB redox hydrogel and (C) Aspergillus niger GOx (AnGOx) at pH 5.6. (D) schematic mechanism of the polymerization of toluidine blue [28]
Figure 3.12: Current densities of TB modified NPG electrodes (~40 nm average pore size) at 100 mV s⁻¹ with differing dilution rates of the depositioning solution of 1:50 (black line), 1:10 (red line) and without dilution (blue line) in 100 mM PBS pH 7.0.

To optimize the deposition, the pulse widths, dilution rates of the hydrogel and recovery times of the pulsed deposition, to allow more time for diffusion, were varied. The response of deposited hydrogel at different dilution rates is shown in Figure 3.12. At high dilution rates (1:50) no obvious redox peaks of the hydrogel could be observed. Upon decreasing the dilution a rise in capacitance could be observed indicating a thicker redox hydrogel film. If the solution is used without dilution very well define redox peaks are observed followed again by an increase in capacitive current. However in general, electrodeposition of Aru032-TB proved to be difficult as it was not possible to evaluate if the polymer can fully address the available nanoporous network. GOx and CdCDH redox hydrogel modified NPG electrodes however did not show any catalytic responses in 5 mM glucose solutions. Aru032-TB modification has therefore not been further progressed.

3.3.2.2 Osmium polymer Os(bpy)$_2$(PVI)$_{10}$Cl$_{2+/3+}$

Os(bpy)$_2$(PVI)$_{10}$Cl$_{2+/3+}$ redox polymer has also been utilized for the encapsulation of enzymes, in this case with flavin dependent glucose dehydrogenase (FAD-GDH). Os(bpy)$_2$(PVI)$_{10}$Cl$_{2+/3+}$ redox polymer was drop-casted on a variety of NPG substrate with differing average pore size and nanoporous film thickness. A summary of the obtained results can be seen in Figure 3.13. When the same quantities of the mixture, containing polymer, crosslinker and enzyme, are drop-casted on NPG electrodes there is not a significant change in current densities when calculated by the
geometric surface area $A_{geo}$ with the exception of ~18 nm electrodes that showed increased current.

**Figure 3.13:** Cyclic voltammograms obtained at Os(bpy)$_2$(PVI)$_{10}$Cl$^{2+/3+}$ drop-casted on NPG electrodes with differing average pore sizes and differing NPG film thicknesses of (A-B) 100 nm and (C-D) 300 nm. Current densities were calculated utilizing geometric (A-C) and electrochemically addressable (B-D) surface areas at a scan rate of 5 mV s$^{-1}$ in N$_2$ saturated PBS pH 7.0.

densities (Figure 3.13 A). If the electrochemically addressable surface area $A_{real}$ is used for the calculation there again is no obvious distinction, besides that the electrodes with the largest pores show the highest response (Figure 3.13 B). This is likely due to the enhanced addressability of the viscous mixture of polymer, crosslinker and enzyme solution. While increasing the film thickness of NPG from 100 to 300 nm the peak current densities calculated by $A_{geo}$ could be roughly doubled (i.e. from ~40 µA cm$^{-2}$ to ~80 µA cm$^{-2}$ for NPG electrodes with average pore sizes of 18 ± 4 nm and 16 ± 2 nm, respectively) (Figure 3.13 C-D). The effect of pore accessibility becomes more apparent when the $A_{real}$ of 300 nm electrodes are considered. The highest responses were obtained with electrodes of ~44 nm average pore size and decreases gradually with decrease in pore size. Os(bpy)$_2$(PVI)$_{10}$Cl$^{+/2+}$ redox polymer modified NPG electrodes were utilized in biofuel cells and are extensively described in chapter 5.
3.3.2.3 Osmium polymer PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$

In addition to Os(bpy)$_2$(PVI)$_{10}$Cl$^{+/2+}$, PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$ redox polymer was used for immobilization of enzyme, in this case BpBOD. PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$ can be electrodeposited by applying potential pulses and can be tuned by increasing the depositioning time [27]. A characteristic CV of immobilized redox polymer PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$ can be seen in Figure 3.14 A, with a depositioning time of 5 minutes and a NPG electrode with ~62 nm average pore size in N$_2$ saturated 100 mM McIlvain buffer at pH 5.0. PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$ depicts an apparent high redox potential of ~350 mV vs Ag/AgCl, making it an interesting mediator for enzymatic fuel cell, due to its capability to increase the open circuit voltage (OCV) of an enzymatic biofuel cell. This might increase the potential use and application of the biofuel cell. A range of differing depositioning conditions in terms of pulsing time and average pore size is shown in Figure 3.14 B-D. The highest current densities ($A_{geo}$) were obtained for NPG electrodes with large average pore sizes of ~62 nm in O$_2$ saturated McIlvain buffer. At a deposition time of 5 minutes it was possible to

![Figure 3.14: (A) Characteristic cyclic voltammogram obtained with PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$ modified NPG electrode (electrodeposited for 5 min) with average pore size of 62 nm at a scan rate of 50 mV s$^{-1}$ in N$_2$ saturated 0.1 M McIlvain buffer. Cyclic voltammograms obtained at differing depositioning time of PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$ and BpBOD at NPG electrode with (B) 15 nm, (C) 42 nm and (D) 62 nm average pore size in O$_2$ saturated 0.1 M McIlvain buffer at a scan rate of 5 mV s$^{-1}$.](image-url)
obtain ~515 μA cm\(^{-2}\) current density (Figure 3.14 D). By decreasing the depositioning time the obtained current densities were gradually decreased as expected in each case. The accessibility of NPG electrodes with large pore size of ~62 nm is therefore obviously improved when compared to smaller pore sizes (Figure 3.14 B). The \(Bp\)BOD/ PAA-PVI-[Os(dcl-bpy)\(_2\)Cl]\(^{2+/3+}\) redox polymer modified bioelectrode was utilized as a biocathode in an EFC setup, where D-fructose is oxidized at an bioanode and is extensively described in chapter 5.
3.4 Conclusions

A wide variety of surface modifications have been utilized throughout this chapter. For the covalent attachment of enzymes on surface functionalized NPG electrodes the focus was directed on innovative diazonium compound in contrast to thiol compounds. While thiol-gold bonds form spontaneously in a highly orientated manner dependent on the functional groups and chain length of the spacer groups, diazonium compounds can be electrochemically reduced to an electroactive surface following subsequent synthesis. The carbon-gold bond is more stable than the thiol-gold bond making it an interesting alternative for a variety of applications. In particular, diazonium compounds with amine, nitro, carboxylic acid and sulfonate functional groups were in focus, as they are known to facilitate covalent bonds, post crosslinking, in near proximity to the electron shuttling vicinity of the protein structures. Surface coverage calculations of near monolayer AQ functionalized diazonium films showed that NPG electrodes with sufficient average pore sizes of ~10 nm were similar to planar Au electrodes modified utilizing the same strategy. Following this conclusion a wide variety of synthesis strategies were adapted and developed for NPG substrate. Diazonium modified NPG was utilized for enzyme immobilization, i.e. laccase and MvBOD. Laccase experiments did not show any promising results and was not further continued, while MvBOD results are extensively described in chapter 5.

In addition to diazonium modifications redox hydrogel (Aru032-TB) and redox polymers (Os(bpy)$_2$(PVI)$_{10}$Cl$^{2+/3+}$ and PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$) were utilized to establish MET of an enzyme to the NPG electrodes. This was done either by simply drop-casting of the components on the surface of NPG electrodes or by electrodeposition. It was found that NPG electrodes with larger pores were more accessible for the viscous solutions, leading to higher loadings of redox polymer/hydrogel and therefore enzymes. The optimal conditions for enzymatic loading is of crucial importance for the development of long term stable and high energy conversion bioelectrodes in the use of biofuel cells. PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+/2+}$ redox polymer modified NPG electrodes were electrodeposited to encapsulate BpBOD. The modified electrodes showed high current densities of ~515 $\mu$A cm$^{-2}$ towards the reduction of O$_2$ to H$_2$O. The capability of this promising biocathode to utilize generated electrons from a bioanode was used in a biofuel cell setup and is further evaluated in chapter 5.
3.5 References


Chapter 3  Surface modification techniques for NPG substrates


Chapter 4:  

Modified NPG electrodes and their application as trace metal sensor and biosensor
4.1 Introduction

Surface modified NPG electrodes with a wide range of average pore sizes can be used for a variety of applications. In this chapter the use of these electrodes as a trace metal sensor, for the detection of Cu$^{2+}$, and as biosensor, for the detection of D-fructose, is presented.

The detection of trace metals is of crucial importance, due to the toxicity they exhibit in environmental matrices and biological materials [1]. Metals such as lead [2], copper [3], zinc [4], cadmium [5], mercury [6] and arsenic [7] are among the most destructive pollutants that can be present in samples such as drinking water, foods, soil samples and in the human body [8-10]. Copper is indispensable in the human body for a number of functions, mainly being needed as a cofactor for a range of enzymes. However variations in the copper concentrations can indicate health issues such as Menkes’ syndrome [11], an extreme form of copper deficiency that can cause seizures and intellectual disability, or Wilson’s disease, a genetic disorder that causes the accumulation of copper, which can cause liver diseases and neuropsychiatric symptoms [11]. A variety of techniques are used for the detection of trace metals in biological samples with atomic adsorption spectroscopy (AAS) [12,13] and inductively coupled plasma-mass spectroscopy (ICP-MS) [14] being the main methods used. However these techniques require expensive instrumentation and highly trained technicians. Therefore the developments of inexpensive, disposable and straightforward quantification techniques are desirable. A promising approach is the electrochemical determination of underpotential deposited (UPD) trace metals in solution through stripping voltammetry [15].

Gold has often been used as an electrode material for the detection of heavy metals in solution utilizing the UPD process. Nanoporous gold in particular exhibits favorable characteristics such as large surface areas [16], tunability of the surface morphology [17] and the capability to protect from biofouling materials [18]. UPD deposition has frequently been used in conjunction with various thiol terminated self-assembled monolayers (SAMs) [19]. It was found that the chain lengths [20,21] and functional groups [22] of these SAMs have significant influence on the detection of the trace metal of interest. However the spontaneous formation of the sulphur-gold bond demonstrates lower stability than that of carbon gold bonds [23]. Therefore, stripping of the trace metal from the electrode surface can result in the partial stripping of the thiol monolayer. Due to this decisive drawback a carbon based system is preferential. The
electrochemical attachment of aryl diazonium salts on conductive electrodes was first demonstrated by Pinson and co-workers [24] and has been extensively described in section 3.3.1. A mono-or multilayer of the compound is electrochemically reduced at the electroactive surface by applying very low reductive potentials to trigger the one electron reductive adsorption process [25]. Oligopeptide modified diazonium films on gold electrodes have previously been used for the detection of trace metals (Cu$^{2+}$, Cd$^{2+}$ and Pb$^{2+}$) in solution [26,27]. Although these systems are very sensitive toward low concentrations of free metal ions, these relative complex systems utilize expensive oligopeptides and are limited by the amount of available complexation sites for the free metal ions [27]. The determination of Cu$^{2+}$ through complexation of Cu$^{2+}$ at a multilayer of methoxy groups linked to an electroactive surface through diazonium reduction has also been previously demonstrated by Geneste et al. [28]. In contrast, the direct detection on the electrode surface can pose a promising approach.

Here we demonstrate an alternative surface modification technique by utilizing inexpensive benzene diazonium sulfonate (BDS), an intermediate product of the methyl orange synthesis [29]. By utilizing NPG as the substrate for the sensor we are able to improve blocking of biofouling materials in solution to precisely measure free copper concentrations in water samples (river, rain and tap water samples) and in artificial human serum. The schematic representation of the Cu$^{2+}$ sensor is displayed in Figure 4.1 A.

In addition to the use of trace metal sensor, surface modified NPG electrodes can be utilized to immobilize enzymes through covalent attachment. Here we will demonstrate the use of immobilized D-fructose dehydrogenase (FDH) to detect levels of D-Fructose in a variety of natural sweeteners and beverages. Fructose is a monosaccharide that does not occur naturally in human diet, but is produced by digestive enzymes in intestinal mucosa [30]. As the initial step of its metabolism in the human body is insulin independent, fructose has been proposed as a “healthier” alternative in comparison to natural sugars and is widely used in artificial sweeteners [31]. Due to the inexpensive production of sweeteners such as high-fructose corn syrup (HFCS), via the enzymatic isomerization of fructose and glucose in starch isolated corn [32], sweeteners have gradually replaced natural sugars in the production of foods and beverages [31]. These sugar replacements have been widely linked with the epidemic of obesity [33]. Large intakes of fructose have been linked to hepatic steatosis, impaired insulin resistance and glucose tolerance, increased blood pressure and enhanced plasma triglyceride concentrations [31,34]. In addition to these metabolic effects, high levels of
fructose intake have also been reported to have substantial effects on the metabolism of minerals [31,35].

Through the ubiquitous presence of sweeteners (in form of fructose), there is a need for the development of rapid and accurate analytical methods to monitor daily sugar intake. Established methods for the quantitative and qualitative detection of D-fructose include chromatographic (such as TLC [36], GC coupled with MS [37] and HPLC [38]) electrophoretic [39], titration [40], gravimetric [41], calorimetric [42], fluorimetric [43] and spectrophotometric [44] methods. While these methods are sensitive they are time consuming and require trained laboratory staff. Biosensors pose an alternative method of analysis that can enable rapid and accurate analysis with high levels of sensitivity and substrate specificity [45]. For the enzymatic detection of fructose in solutions, biosensors have often utilized the redox enzyme D-fructose dehydrogenase from *Gluconobacter species* (FDH) [46]. FDH was first described by Adachi et al. [47]. The enzyme (EC Nr.: 1.1.99.11) has an approximate molecular weight of ~140 kDa and is comprised of 3 subunits; a flavin adenine dinucleotide (FAD) subunit (MW: 67,000 Da), a heme C (cytochrome c) subunit (MW: 50,800 Da) and a peptide domain of unknown function (MW: 19,700 Da) [47,48]. The FAD subunit of the enzyme is linked to the catalytic conversion of D-fructose to 5-dehydro-D-fructose. The reduced form of FAD is then oxidized in an intramolecular electron transfer step to the heme subunit [48,49]. Direct electron transfer (DET) of the heme subunit can then occur at an electrode [50]. Various fructose biosensors based on DET have been reported in the literature. A wide variety of substrate materials based on carbon (carbon paste [51-53], screen printed graphene electrodes (SPGE) [54], graphite [55,56], glassy carbon (GC) [57], porous carbon [58] multi and single walled nanotubes (MWCNT [59] & SWCNT [60], Pt [52] and colloidal Au [61,62] have been used for enzymatic fructose biosensors.

NPG has been used as an electrode in a number of biosensors and biofuel cells [63]. NPG can be prepared by dealloying an Au alloy through the continuous etching of a less noble metal (such as Ni or Ag) from the alloy [16,64]. This etching procedure results in the formation of a well-defined three-dimensional nanostructure that has shown to be a beneficial substrate for the immobilization of redox enzymes [63,65,66]. In addition to being conductive, mechanically stable, and free of surface contaminants (through the etching procedure), NPG was found to increase the storage, thermal and operational stability of redox enzymes such as xylanase [67], laccase [68], lipase [69] and bilirubin oxidase [66]. NPG electrodes can be easily modified using alkanethiol
based self-assembled monolayers (SAMs). However the chemical stability of thiol based SAMs is easily affected by physiological changes in the test solution (such as pH, temperature, ionic strength and applied potentials) [70]. A more suitable method has been introduced by Pinsen et al. in 2001 through the electrochemical reduction of aryl diazonium salts onto electrodes [71]. In this report, the use of NPG as an electrode support for FDH in the development of a biosensor for fructose is examined. A representative schematic representation of the presented biosensor is displayed in Figure 4.1 B. The sensor could be used to accurately determine the fructose concentration in a range of food samples.

Figure 4.1: Schematic representation of (A) a BDS modified NPG for use as a sensor for Cu$^{2+}$ and (B) a fructose biosensor, with FDH immobilized on NPG substrate.
4.2 Experimental

4.2.1 Reagents and Materials

Potassium chloride, sodium phosphate dibasic, citric acid (anhydrous), nitric acid (70%), sulfuric acid (95-98%), hydrochloric acid (35-37%), sodium 3-mercaptop-1-propanesulfonate (90%) (MPS), sulfanilic acid (99%), tetrabutylammonium tetrafluoroborate (99%), copper(II) sulfate pentahydrate (≥98%), sodium sulfate (≥99.0%), uric acid, calcium sulphate dehydrate, magnesium sulphate, ethanol (98%) 6-amino-2- naphthoic acid (NA), 3-mercaptopropionic acid (MPA), sodium nitrite, N-cyclohexyl- N’-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMC), D-(-)-fructose, D-(+)-glucose, D-galactose, D-mannitol, L-ascorbic acid, D-lactose, urea, L-cystine, ascorbic acid and albumin from bovine serum were obtained from Sigma-Aldrich Ireland, Ltd. Acetonitrile anhydrous >99.8%, sodium chloride and sodium bicarbonate were purchased from Fischer Scientific. Cu²⁺ standard with 1000 ppm (µg/mL) was purchased from Inorganic Ventures. All chemicals were used as received unless stated otherwise. Food samples i.e. honey, agave, maple syrup, cola and sports drink were purchased from a local supermarket and were used after appropriate dilution. D-Fructose dehydrogenase from Gluconobacter sp. (FDH) was purchased from Sorachim SA and used at a concentration of 4 mg ml⁻¹. Deionized water with a resistivity of 18.2 MΩ cm was generated by an Elgastat Purelab Pulse system (Elga, UK).

4.2.2 Preparation of planar and NPG electrodes

NPG electrodes were prepared as already extensively described in section 2.2.2. In brief, glass sheets were sputtered with a 10 nm thick Ti adhesion film, followed by a 35 nm thick pure Au layer and by a 100 nm Ag₇₀/Au₃₀ alloy layer (Figure 4.2 A) [65,66]. After sputtering the sheets were cut into uniform pieces of approximately 0.5-0.7 cm² using a circular saw. The sheets were dealloyed in nitric acid following the dealloying times and temperatures for the desired average pore size distribution as identified in chapter 2 (Figure 4.2 B). Afterwards a silver wire was soldered to the surface of the sputtered sheet using an indium wire. The soldering point was then supported by a two component epoxy glue and an electroactive surface area was defined by a dielectric paste (Gwent Group, UK).
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Polycrystalline planar Au-disk electrodes (CHI) (ø=2 mm) were thoroughly polished utilizing 0.05 µm alumina powder dispersed in deionized water on a polishing pad. After rinsing they were cycled in 0.5 M H₂SO₄ for 10 cycles at 100 mV s⁻¹. In a final step they were extensively cleaned in an ultrasonic bath.

4.2.3  NPG electrode characterization

The electrochemical stripping of gold oxide by cyclic voltammetry from -0.2 to 1.6 V at 0.1 V s⁻¹ in 0.5 M H₂SO₄ was used to calculate the electrochemically addressable surface area of each NPG electrode by applying a conversion factor of 390 µC cm⁻² [72]. This method also cleans the Au surface of contaminants. However, continuous scanning also promotes successive etching of the NPG surface resulting in increased average pore sizes, which is not desirable in this case. To avoid substantial changes to the nanopores this step was limited to 2 full cycles. The geometric surface areas (Ageo) of the NPG electrodes were determined by recording a high resolution photograph of each electrode on a millimeter grid and measuring the area using ImageJ software [73]. The average pore sizes and surface morphology of the NPG electrodes were determined, as previously described (section 2.2.3) [65,66], by taking high resolution SEM images (Hitachi SU-70) of the electrodes. SEM images were taken at a magnification of 120,000 for each sample and were afterwards converted into black and white images with the ISODATA function of ImageJ software. These images were used to determine the average pore sizes distribution of NPG. The pore sizes were determined by manually measuring the distances between the pores, with at least 35 different measurements performed for each electrode, on at least 3 SEM images.

4.2.4  Experimental section for Cu²⁺ trace metal sensor

NPG electrodes were cleaned by cycling the potential between -0.2 and 1.65 V at 0.1 V s⁻¹ vs Ag/AgCl (in 3 M KCl). To avoid the continuous etching of the Ag/Au alloy this step was limited to 2 full cycles. After rinsing in deionized water and subsequent drying in a vacuum chamber the electrodes were ready for surface modification. For MPS modified NPG (MPS-NPG) a solution of 5 mM MPS in deionized water was prepared and the electrodes were immersed over night. The MPS forms a dispersed monolayer on the surface of the NPG due to the bulky negative charge of the sulfonate group [74]. BDS modified electrodes were prepared by electrochemically reducing BDS to the gold surface (BDS-NPG) as already extensively described in section 3.2.3.4. CV was performed from 0.3 to -0.5 V vs Ag/AgCl (3 M
KCl) at 200 mV s\(^{-1}\) in a solution of 5 mM BDS and 0.1 M tetrabutylammonium tetrafluoroborate (TBATFB) as supporting electrolyte in acetonitrile. To avoid excessive multilayer formation of the BDS film this step was limited to one fast scan [25].

All electrochemical measurements were performed using a CHI802A potentiostat operating in a standard three electrode configuration with NPG or planar Au, Ag/AgCl (in 3 M KCl) and Pt wire as working, reference and counter electrodes, respectively. UPD was achieved using amperometry at 0.15 V vs Ag/AgCl (3 M KCl) for 10 minutes. Subsequent stripping of Cu was achieved using square wave voltammograms (SWV). SWV was performed at an increment of 1 mV and an amplitude of 50 mV vs Ag/AgCl (3 M KCl) at a frequency of 25 Hz. Current densities were calculated by the electrochemical addressable surface area (\(A_{\text{real}}\)) unless stated otherwise.

Tap water samples were taken from the tap after allowing it run for about 1 minute. Rain water samples were collected close to a roof drainage of a building with partial copper roof paneling. River water samples were taken from the Shannon River, Limerick, Ireland. The water samples were stored at 4 °C when not being tested. For the comparison of the obtained results from the copper sensor atomic adsorption control experiments were performed with a Varian SpectrAA 220 AAS. Water samples were used without prior pretreatment.

Artificial serum samples were prepared by mixing following quantities of the following compounds, which represent the average in a healthy human body: uric acid (0.36 mM), L-ascorbic acid (0.054 mM), D-(-)-glucose (5 mM), D-(-)-fructose (0.2 mM), D-lactose (15 µM), urea (4.45 mM), L-cystine (0.075 mM), calcium sulphate dehydrate (2.38 mM), magnesium sulphate (0.87 mM), sodium chloride (103 mM), sodium bicarbonate (25.5 mM) and 7% of bovine serum albumin (BSA) [11]. BSA was added to the solution after the serum was injected with the appropriate amount of Cu\(^{2+}\) to ensure proper dissolution in the serum.

### 4.2.5 Preparation and characterization of D-Fructose biosensor

Functionalization of the NPG electrodes with carboxylic acid group was achieved by electrochemical reduction of 2-carboxy-6-naphtoyl diazonium salt (NA-DS). NA-DS was prepared in situ by mixing a 2 mL solution (20 mM in acetonitrile) of 6-amino-2-naphthoic acid (NA) with 2 mL of a solution of NaNO\(_2\) (2 mM) in 1 M HCl
in an ice bath [75,76]. After deoxygenation of the reaction solution, the potential was scanned from 0.6 to -0.5 V at 200 mV s\(^{-1}\) vs Ag/AgCl (3 M KCl). To avoid multilayer formation of the NA-DS on the NPG surface this step was limited to one single potential scan [66,75]. Due to the single scan full coverage of the NA-DS on the NPG surface cannot be achieved. Therefore the void spaces were blocked using a short chained thiol, \(i.e.\) MPA (1 mM) to reduce substrate oxidation at the bare electrode by immersing in thiol solution over night in the fridge at 4 °C (Figure 4.2 C). Thiol modified NPG electrodes were prepared by immersing NPG electrodes in a 2 mM MPA solution over night at 4 °C (Figure 4.2 D). Prior to enzyme modification the surface modified NPG electrodes were thoroughly rinsed and dried in a vacuum chamber. Afterwards 20 μL of 4 mg mL\(^{-1}\) FDH solution was drop cast on to the electrode surface and left in a vacuum chamber (maximum 0.098 MPa) for approximately 3 minutes to enhance the loading of the enzyme [77]. It is important that the enzyme solution does not dry during this process in order to avoid loss of activity. Afterwards the electrodes were left at 4 °C for 1 h. For the following crosslinking of the FDH on the electrode surface the electrodes were immersed in a 5 mM CMC solution at 4 °C for 2 h. Afterwards the NPG electrodes were tested in 100 mM McIlvain buffer at different temperatures and pH’s.

Electrochemical measurements were conducted using CHI potentiostat’s (models CHI620A, CHI802C and a CHI1030C multichannel). All measurements were performed using a standard three electrode configuration with NPG, Ag/AgCl (in 3 M

![Figure 4.2:](image)

Figure 4.2: (A) Sputtered glass sheet with a layer of titanium, pure gold and gold/silver alloy (bottom to top). (B) NPG electrode surface post dealloying using concentrated nitric acid. (C) Electrochemical reduction of NA-DS on the NPG surface utilizing a single scan and subsequent filling of the void spaces with MPA. (D) Preparation of a MPA SAM on NPG substrate by immersion over night at 4 °C. After adsorption of FDH on the two modified electrodes the enzyme was crosslinked with CMC (sizes not to scale).
KCl) and Pt wire as working, reference and counter electrodes, respectively. All current densities were calculated utilizing the geometric surface area. Stability measurements were performed at defined periods of time after storage in 100 mM McIlvain buffer pH 5.5 at 4 °C. Calibrations were performed at a potential of 0.15 V vs Ag/AgCl in 3 M KCl, a potential just above the onset potential of ca. 0.0 V for the oxidation of FDH immobilized on the electrode, but not too high to oxidize interfering compounds at the bare electrode.

Samples of honey, agave and maple syrup were heated to 60 °C under continuous stirring before preparing stock solutions of 1 % (w/v %). Cola and sports drink were used without prior pre-treatment. For the determination of the D-fructose content using a commercial available enzymatic kit (R-biopharm, Cat. No. 10 139 106 035) the samples were diluted to yield a glucose + fructose concentration of between 0.15- 1.0 g L⁻¹. Samples were diluted in McIlvain buffer [78] in the range 0.1 – 0.3 mM to reduce the concentration of fructose to that of the linear range of the fructose biosensor and then tested directly. The limit of detection (LOD) was determined by multiplying the absolute standard deviation of three blank samples by 3 and dividing by the sensitivity of the biosensor [79].
4.3 Results and Discussion

4.3.1 Results and Discussion of the Cu\(^{2+}\) trace metal sensor

4.3.1.1 MPS modified planar Au electrodes

After extensive polishing and cleaning, commercially available planar electrodes were modified with MPS, a short chained thiol with a sulfonate functional group. MPS has frequently been reported to improve the sensing capabilities of trace metal sensors as the bulky negative charged sulfonate group is capable of attracting positively charged ions [22,80]. Surface modification with MPS leads to a disorganized monolayer of the thiol film on the electrode surface, which can be associated with the bulky sulfonate group as well as the short spacer group (i.e. 3 carbon atoms) that does not allow for much flexibility during the formation of the monolayer [22]. This disorganized monolayer is preferential as it allows for enough void spaces in the monolayer for the deposition of the trace metal on the surface of the electrode. Cyclic voltammograms of unmodified and MPS modified planar Au electrodes can be seen in Figure 4.3. At high concentrations of Cu\(^{2+}\) (1 mM), the unmodified planar Au electrodes shows redox peaks (Figure 4.3 A) characteristic for Cu\(^{2+}\) which are associated with underpotential deposition and stripping (a1,c1) as well as bulk depositioning and stripping (a2,c2). When compared with MPS modified planar electrodes, it becomes apparent that the peaks are more defined for unmodified electrodes which can be associated with the reduced accessible electrode area from the surface modified electrodes. When the concentration of Cu\(^{2+}\) is reduced to 1 µM, the unmodified planar electrode displays no apparent redox process, while the MPS modified planar electrodes displayed well

![Figure 4.3:](image)

**Figure 4.3:** Cyclic voltammograms at planar Au electrodes in the presence of (A) 10\(^{-3}\) M Cu\(^{2+}\) and (B) 10\(^{-6}\) M Cu\(^{2+}\) in 0.1 M H\(_2\)SO\(_4\) which were unmodified (black) and MPS surface modified (red) at a scan rate of 20 mV s\(^{-1}\) vs Ag/AgCl (3 M KCl).
defined underpotential deposition and stripping (a1,c1) peaks (Figure 4.3 B). Interestingly no bulk deposition and stripping could be observed which might be due to the depletion of Cu\(^{2+}\) through the UPD process. From the MPS modified electrodes a favorable underpotential deposition potential of 0.15 V (vs Ag/AgCl in 3 M KCl) was applied for all following experiments.

### 4.3.1.2 Dependency of pore size to electrochemical response

Through altering the etching conditions used the pore sizes of the resulting NPG can be finely tuned [17]. In this study NPG with average pore sizes of 15 ± 2, 42 ± 3 and 62 ± 4 nm were used respectively. The conditions used for dealloying are shown in Table 4.1. To improve sensitivity of the trace metal sensor the average pore size of the NPG electrodes which results in the highest responses needs to be determined. Electrodes which exhibit too small pores might be inaccessible for the bulk solution and therefore the trace metal ions. Surfaces with very large pores result in smaller active surface area, therefore limiting sensitivity and reducing the LOD. As seen in Figure 4.4 the stripping voltammograms from MPS modified NPG in 5 µM Cu\(^{2+}\) (after deposition for 10 minutes at 0.15 V) differ substantially from one another. The clearest response is given by NPG electrodes with 42 nm average pore size (NPG-42) when the electrochemical addressable surface areas are considered for current density calculations. Even though the responses received with NPG electrodes of 15 nm average pore size (NPG-15) were lower than those of the 62 nm NPG electrodes (NPG-62), the NPG-15 electrodes displayed great sensitivity towards the detection of Cu\(^{2+}\). The detection with NPG-62 electrodes was somewhat difficult as the stripping peak could easily be overlayed by the relatively large capacitance signal obtained with the NPG electrodes. This can also be seen in the noise in the response obtained with the NPG-62 electrode. Therefore for all following experiments, electrodes with average pore

<table>
<thead>
<tr>
<th>Dealloying temp. [^\circ C]</th>
<th>Dealloying time [^{\text{min}}]</th>
<th>Average pore size [^{\text{nm}[a]}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.8</td>
<td>5</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>40.2</td>
<td>5</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>59.9</td>
<td>5</td>
<td>62 ± 4</td>
</tr>
</tbody>
</table>
Figure 4.4: Square wave voltammograms of MPS modified NPG electrodes with pore sizes ranging from ~15 to ~62 nm and a planar Au electrode after deposition of Cu at a fixed potential of 0.15 V (vs Ag/AgCl in 3 M KCl) for 10 minutes in a solution of 5 µM Cu²⁺ in 0.1 M H₂SO₄. SWV was performed at an increment of 1 mV and an amplitude of 50 mV.

size of 15 and 42 nm were used respectively. Also noteworthy is that all tested NPG electrodes showed higher responses when compared to planar Au electrodes.

4.3.1.3 Comparison MPS and BDS modification

As already demonstrated in section 3.3.1.3, MPS and BDS modified NPG electrodes display negatively charged sulfonate groups, which can serve the purpose of attracting positively charged trace metal ions. To verify to what extent the gold surfaces were covered with BDS, the surface coverages were calculated according to equation 4.1, while $\Gamma$ is the surface coverage, $Q$ represents the charge of the reductive peak, $n$ comprises the number of electrons involved in the reduction process, with $F$ as the Faraday constant, $A$ as the geometric surface area of the individual electrodes and $q$ as the roughness factor.

$$\Gamma = \frac{Q}{n\cdot F \cdot A \cdot q}$$  \hspace{1cm} (4.1)

The surface coverages determined can be seen in Table 4.2. The planar Au electrodes possess an easily accessible surface which results in the highest surface coverages of BDS of 36.4 ± 0.7 pmol cm⁻². Electrodes with average pore sizes below 10 nm display
the lowest surface coverages of $9.4 \pm 1.9 \text{ pmol cm}^{-2}$, which can be attributed to the reduced accessibility of the diazonium species to the gold surface. This effect might be enhanced by the formation of the film that can narrow down the NPG channels and therefore limiting the access of subsequently following molecules. Upon increasing the average pore size of the NPG electrodes the surface coverage gradually increased from $24.3 \pm 1.9$ to $31.8 \pm 0.6 \text{ pmol cm}^{-2}$ for 15 and 62 nm electrodes respectively. The results suggest that the NPG surface becomes more accessible with increasing pore size, which results in a more dense coverage of the BDS film. A schematic representation of the BDS film electrochemically reduced to a NPG surface is displayed in Scheme 4.5 A (not to scale). The strongly negative charged and bulky sulfonate group of the BDS molecule likely hinders the dense film formation on roughened surfaces as they repel each other during electrochemical reduction.

The functioning principle of the BDS modified NPG based $\text{Cu}^{2+}$ sensor is shown in Scheme 4.5 B. The negative charged sulfonate groups of the BDS film constitute two main functions. They attract positively charged free $\text{Cu}^{2+}$ ions, for improved UPD and subsequent stripping of the Cu monolayer. Also the blocked NPG surface is protected from any biofouling species that might cling to the surface therefore blocking access for $\text{Cu}^{2+}$ deposition and subsequently hinder detection [22]. In addition to repelling biofouling materials, the sulfonate groups may also hinder access of large molecules into the pores of nanostructure therefore increasing the biofouling resistance of the electrodes (as indicated in Scheme 4.5 B) [81].

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Average pore size [nm]</th>
<th>Surface coverage $\Gamma$ [pmol cm$^{-2}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planar</td>
<td>-</td>
<td>$36.4 \pm 0.7$</td>
</tr>
<tr>
<td>NPG 10</td>
<td>$8.7 \pm 1.2$</td>
<td>$9.4 \pm 1.9$</td>
</tr>
<tr>
<td>NPG 15</td>
<td>$15.2 \pm 2.2$</td>
<td>$24.3 \pm 1.9$</td>
</tr>
<tr>
<td>NPG 40</td>
<td>$41.7 \pm 3.4$</td>
<td>$31.0 \pm 0.6$</td>
</tr>
<tr>
<td>NPG 60</td>
<td>$61.8 \pm 3.6$</td>
<td>$31.8 \pm 0.6$</td>
</tr>
</tbody>
</table>

Table 4.2: Surface coverages of electrochemically reduced BDS on planar and NPG electrodes of differing pore sizes.
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Figure 4.5: (A) schematic representation of BDS electrochemically reduced onto a NPG surface and (B) proposed functioning principle of the Cu$^{2+}$ sensor (not to scale).

Sample SWV stripping voltammograms obtained from MPS and BDS modified NPG electrodes are displayed in Figure 4.6 A/C. The corresponding peak current densities are shown in the calibration plots Figure 4.6 B/D respectively. The SWV voltammograms show stripping of the deposited Cu monolayer on the NPG electrodes. In the case of MPS modified NPG electrodes (~15 nm average pore size), a linear concentration range of 0.05 - 5 µM for the detection of Cu$^{2+}$ was obtained (Figure 4.6 B). The BDS modified NPG electrode (~42 nm average pore size) displayed a linear range from 0.2 to at least 5 µM free Cu$^{2+}$ concentration (Figure 4.6 D). The reduced

Figure 4.6: Baseline corrected square wave voltammograms of (A) MPS modified NPG (~15 nm average pore size) and (C) BDS modified NPG (~42 nm average pore size). (B) and (C) display the peak current densities of MPS and BDS modified NPG electrodes of the added Cu$^{2+}$ concentrations, respectively. Signals were recorded in (A)/(B) in 0.1 M H$_2$SO$_4$ and (C)/(D) in 0.1 M Na$_2$SO$_4$ supporting electrolyte versus Ag/AgCl in 3 M KCl.
detection in the lower range (i.e. 0.05 µM from MPS modified NPG and 0.2 µM from BDS modified NPG) might be due to the longer distance between the sulfonate functional group and the electroactive surface. The Cu$^{2+}$ ions are not as in close proximity as in the case of MPS modified NPG therefore leading to reduced responses [22]. Both modification techniques (MPS and BDS) showed very good linear response indicating the suitability of the approach for the detection of use for Cu$^{2+}$. In the case of BDS modified NPG the supporting electrolyte was changed from 0.1 M sulphuric acid to 0.1 M Na$_2$SO$_4$. The proposed trace metal sensor is intended for use in near neutral pH solution (artificial human serum pH 7.4) and therefore testing at low pH is not suitable. It should be noted however that Cu$^{2+}$ can precipitate from Na$_2$SO$_4$ solutions and it was necessary to sonicate the test solution extensively before testing.

The main advantage of the BDS modified NPG surfaces is the higher stability of the gold-carbon bond opposed to the sulphur-gold bond of the thiol modified NPG [23]. The stripping of any underpotential deposited Cu may also lead to stripping of the thiol from the surface. This would have an impact on the available surface area for Cu depositioning, therefore changing the obtained signals. To verify to what extent this may happen, NPG electrodes (NPG-42) were modified with MPS and utilized for multiple depositioning and stripping cycles. Figure 4.7 A shows the steady increase of the SWV signal after multiple depositioning and stripping cycles. The increase was found to be approximately ~6 % from one run to the next. After the five consecutive runs the total increase comprised ~27 %. The increase in the signal shows that MPS modified NPG electrodes cannot be used for multiple Cu$^{2+}$ determinations. The sixth run displayed in Figure 4.7 A was performed after leaving the electrodes in the test solution for 24 hours, which results again in an increase in signal, which can be related

![Figure 4.7: Baseline corrected SWVs of (A) MPS modified NPG and (B) BDS modified NPG after a series of repeated deposition and stripping cycles in 0.1 M H$_2$SO$_4$ supporting electrolyte at a Cu$^{2+}$ concentration of 5 µM (vs Ag/AgCl in 3 M KCl).](image-url)
to the detachment of the thiol film from the electrode leading to more available electroactive surface area, as well as increased in the amount Cu\(^{2+}\) at the negative charged sulfonate group resulting in higher readings. In comparison to MPS modified NPG, BDS modified NPG electrodes did not show any changes in signal response after multiple depositioning and stripping cycles as can be seen in Figure 4.7 B. Even after 24 hours (5th cycle) in the Cu\(^{2+}\) solution, no changes can be observed. This demonstrates the main advantage of the modification of electrode surfaces with diazonium compounds opposed to thiol compounds.

### 4.3.1.4 BDS modified NPG sensor characteristics

The BDS modified NPG (NPG-42) Cu\(^{2+}\) sensor was evaluated for use as accurate, inexpensive and disposable device. The WHO (World Health Organization) suggest that copper concentrations in drinking water can vary between \(\leq 0.005\) and \(>30\) ppm [82], with the primary source of Cu most often being arising from the corrosion of copper plumbing. In contrast levels of copper in running water tend to be low. The Environmental Protection Agency (EPA) of the United States has therefore set a limit of safe copper concentrations in drinking water of 1300 ppb (20.5 µM) [83]. As can be seen in Figure 4.8 the linear range of the sensor was between 0.2 to 25 µM in 0.1 M Na\(_2\)SO\(_4\) supporting electrolyte. The sensor therefore operates in the range needed to determine copper contaminations in drinking water. In comparison to previously reported Cu\(^{2+}\) sensors, the reported sensor operates favorably. In general Cu\(^{2+}\) sensors reported in the literature focus on achieving high sensitivity and low LOD. This however might not always be practical when the goal is to achieve commerciable devices. As pointed out in Table 4.3 the linear ranges of most of the sensors based on anodic square wave (ASV) stripping techniques are not capable of operating in the actual range of copper concentrations in samples that lie in the range 0 - 20.5 µM. Orozco et al. achieved a linear range of 0 to 10 µM [8], Huang et al. achieved 0.002 to 0.08 µM [80], Beltagi et al. achieved 0.04 to 2.0 µM [84] and Ding et al. achieved 0.06 to 2.2 µM [85].

The sensitivity was 8.18 µA cm\(^{-2}\) µM\(^{-1}\) (calculated using the geometric surface area). The LOD was calculated by dividing the standard deviation of the background signal by the sensitivity and multiplying with a signal to noise (S/N) ratio of 3. The LOD was found to be 18.9 nM (~1.2 ppb) which is higher than that obtained with MPS modified NPG electrodes [80]. This is likely due to the difference in NPG film
thickness. Here NPG electrodes (NPG-42) with roughness factors ranging from 6-8 were utilized, while Huang et al. used electrodes with roughnesses >50 [80]. It is therefore likely that the LOD can be decreased and the sensitivity can be increased upon increasing the NPG film thickness.

As the sensor would only be used as a disposable device there is no need for the preparation of sensor strips that are capable of multiple testing tasks. However multiple testing of this device showed the stability of the system. In general Cu$^{2+}$ sensor are not tested for long term stability. For the development of commerçiable devices it is important that the sensor exhibits the same sensing capabilities at the time of preparation as at the time of use. It is anticipated that diazonium modified NPG displays a more stable system than thiol modified NPG. To test how this modification technique performs in real samples, Cu$^{2+}$ concentrations were measured in water samples (tap, rain and river water) and in artificial human serum that was spiked with varying Cu$^{2+}$ concentrations.

**Figure 4.8:** Peak current densities of SWVs obtained from a BDS modified NPG electrode (~42 nm average pore size) at differing Cu$^{2+}$ concentrations in 0.1 M Na$_2$SO$_4$ supporting electrolyte (vs Ag/AgCl in 3 M KCl).
Table 4.3: Comparison of free Cu$^{2+}$ ion sensors reported in the literature utilizing square wave stripping techniques in a wide range of tested media.

<table>
<thead>
<tr>
<th>Electrode/ Modification</th>
<th>Media</th>
<th>Linear range [µM]</th>
<th>Sensitivity</th>
<th>LOD Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycrystalline gold modified with short chained thiols MES$^a$</td>
<td>10 mM H$_2$SO$_4$</td>
<td>1 - 80</td>
<td>0.071 ±0.002 µA µM$^{-1}$</td>
<td>1 µM (67 ppb) [22]</td>
</tr>
<tr>
<td>Gold nanoparticle-modified ultramicroelectrode arrays</td>
<td>10 mM H$_2$SO$_4$/HNO$_3$ soil samples</td>
<td>0 - 10</td>
<td>25.9 ± 1.3 nC·µM$^{-1}$</td>
<td>0.12 µM (7.4 ppb) [8]</td>
</tr>
<tr>
<td>MPS$^b$ modified NPG electrode</td>
<td>0.1 M NaNO$_3$</td>
<td>0.002 - 0.08</td>
<td>58.76 µA µM$^{-1}$</td>
<td>0.031 nM (0.002 ppb) [80]</td>
</tr>
<tr>
<td>Montmorillonite-calcium modified carbon paste electrode</td>
<td>0.1M HCL Pre-treated water samples</td>
<td>0.04 – 2.0</td>
<td>0.129 µA µM$^{-1}$</td>
<td>11.1 nM (0.75 ppb) [84]</td>
</tr>
<tr>
<td>Reduced Graphene Oxide + Polyvinyl Butyral Nanofibers</td>
<td>Acetate buffer pH 4.4</td>
<td>0.06 - 2.2</td>
<td>103.51 µA·µM$^{-1}$·cm$^{-2}$</td>
<td>4.10 nM (0.21 ppb) [85]</td>
</tr>
<tr>
<td>BDS modified NPG electrodes</td>
<td>100 mM H$_2$SO$_4$ 100 mM Na$_2$SO$_4$ Untreated water samples Blood serum</td>
<td>0.2 - 25</td>
<td>8.18 µA µM$^{-1}$·cm$^{-2}$</td>
<td>18.9 nM (1.2 ppb) This work</td>
</tr>
</tbody>
</table>

$^a$ MES: Mercaptoethanesulfonate $^b$ MPS: (3-mercaptopropyl)sulfonate

4.3.1.5 Test in water samples

The copper concentrations of water samples which flow through interior copper plumbing are significantly higher than those that can be found in the environment such as in rivers or lakes. Therefore a variety of water samples were chosen for testing. These were tap water, rain water and river water samples. Tap water samples were taken from a tap with copper plumbing. Rain water samples were collected from drainage water from a copper coated roof, while river water samples were collected on the shore of the river Shannon in Limerick, Ireland. Due to the protective layer of the sulfonate groups on the sensor, the samples could be tested without any pre-treatment. The response of the sensor was then verified using AAS. The results are displayed in Table 4.4. The concentration of the copper concentration of the tap water was significantly higher than that of the environmental samples, as expected. The results obtained by the BDS-NPG sensor (561 ± 4 ppb) compared well to those of the AAS (599 ± 3 ppb). The AAS
Table 4.4: Comparison of obtained results from the Cu\textsuperscript{2+} sensor to results obtained from AAS.

<table>
<thead>
<tr>
<th>Sample</th>
<th>AAS [Cu\textsuperscript{2+}] [ppb]</th>
<th>BDS-NPG sensor [Cu\textsuperscript{2+}] [ppb]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water</td>
<td>599 ± 3</td>
<td>561 ± 4</td>
</tr>
<tr>
<td>Rain water</td>
<td>13 ± 1\textsuperscript[a]</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>River water</td>
<td>3 ± 3\textsuperscript[a]</td>
<td>1 ± 0\textsuperscript[b]</td>
</tr>
</tbody>
</table>

\textsuperscript[a] Concentration is too low to rely on AAS measurements.
\textsuperscript[b] Concentrations were found to be below the LOD of the sensor and are therefore not reliable.

results obtained from the environmental samples (i.e. rain and river water) were found to be below the detection limit of the AAS and could therefore not be used for quantification, but was a good benchmark to determine the scope of the concentration. The response of the BDS-NPG sensor showed a good correlation with that obtained by AAS. The slight decrease in response in all cases might be due to the fact that the BDS-NPG sensor cannot detect complexed copper present in the sample, while the AAS detects copper in every form.

4.3.1.6 Test in artificial human serum

To test the capabilities of the BDS-NPG sensor to block biofouling reagents the sensor was tested in complex organic media. For this purpose artificial human serum with the average salt and sugar concentrations of a healthy human body was prepared \cite{11}. A key component of artificial human serum is the protein albumin, which keeps fluid from leaking out of blood vessels, nourishes tissues and is responsible for the transport of vitamins, hormones, \textit{etc.} throughout the body. Therefore 7\%(w/v) bovine serum albumin (BSA) was added. It is important to note that the appropriate copper concentration that was added to the test solution had to be added before the BSA, as the resulting increase in viscosity lead to a problematic homogenous blending of the copper throughout the test solution. A sample calibration plot is shown in Figure 4.9. The lowest detectable concentration of free Cu\textsuperscript{2+} in artificial human serum was 0.8 µM. The reduction in detectability was likely due to the partial complexation of Cu\textsuperscript{2+} in the artificial human serum solution. Complexed copper cannot be detected by the sensor and therefore a reduction in detectability would be anticipated. It is important to note
that the sensor introduced here cannot be used as a copper sensing device in human serum, due to the fact that copper in serum is bound to the protein ceruloplasmin [11] and is not available as free Cu^{2+} in the body. However the sensor can be successfully used to detect free Cu^{2+} in a complex media such as artificial serum, with high biofouling and electrode surface poisoning capabilities.

In addition to the detection of Cu^{2+} on BDS modified NPG, NPG can be utilized as substrate for the enzymatic detection of D-fructose in solution and is discussed below.

### 4.3.2 Results and Discussion of the fructose biosensor

#### 4.3.2.1 Surface modification of NPG and the effect of pore size on the catalytic response

After cleaning in acid, surface modification of the NPG electrodes was performed via two methods. In both cases the surface was functionalized with carboxylic acid groups. In the first approach, functionalization was achieved by
Chapter 4  Mod. NPG electr. and their appl. as sensor and biosensor

modification with mercaptopropionic acid (MPA). MPA spontaneously forms a relatively uniform distributed network of molecules on the gold surface leaving the carboxylic acid group exposed to the liquid interface. The negative charge of the carboxylic acid group then favors the physical adsorption of FDH [61]. After adsorption of FDH, carbodiimide (in the form of CMC) was added to facilitate amide bond formation resulting in crosslinking of the enzyme to itself and to the thiol bound carboxylic acid groups, thus forming a more stable film on the electrode surface (MPA-FDH) [86]. In the second approach, an aryldiazonium salt (2-carboxy-6- naphtoyl diazonium salt, NA-DS), was electrochemically reduced on the electrode surface [66,75]. A typical reduction scan of NA-DS on NPG can be seen in Figure 3.9 A in section 3.3.1.2. The void spaces resulting from the reduction procedure were subsequently filled with MPA (NA-DS-FDH). Crosslinking was achieved as in MPA-FDH modified NPG electrodes.

NA-DS-FDH modified NPG electrodes with average pore sizes ranging from ca. 9 – 62 nm were prepared to evaluate the influence of pore size on the catalytic current densities. The conditions used for preparation can be found in section 3.2.3.2. A number of factors need to be considered in evaluating the most efficient system: i) The average pore size needs to be sufficiently large for the FDH enzyme to penetrate through and access all the available area. ii) The length of the binding group (NA-DS in this case) also plays a considerable role. If the chain length is too long, the distance between the enzyme and the electroactive surface area becomes too far for efficient DET, which inevitably leads to a reduced current density. iii) The average pore size should not be too large, as this would lead to low loadings of enzyme on the porous structure again.

Figure 4.10: (A) Cyclic voltammograms of NA-DS-FDH modified NPG electrodes with different average pore sizes in the presence of 50 mM fructose and (B) current density as a function of average pore size (n = 5) at 0.5 V. Conditions: pH 5.5, 25 °C at a scan rate of 5 mV s⁻¹.
leading to reduced current densities. iv) The nanoporous structure can favor enzyme orientation in such a way that enables improved DET. The influence of the average pore size of NA-DS-FDH modified NPG on the current density is shown in Figure 4.10. While the blank sample in each case show no catalytic current in 100 mM McIlvain buffer (pH 5.5, 25 °C), the addition of 50 mM D-fructose triggers the catalytic process on scanning from -0.2 to 0.6 V (vs Ag/AgCl). The highest current densities were obtained with NPG electrodes with an average pore size of 42 nm. The current density steadily increased when the average pore size was increased from 9 to 42 nm, with a significant decrease upon increasing the average pore size to over 62 nm. This steep decrease in current density is associated with the decrease in available electroactive surface area, thus decreasing the enzyme loading which in return leads to lower current densities. Electrodes with an average pore size of ca. 42 nm were therefore utilized for all subsequent experiments.

### 4.3.2.2 Storage stability

The two types of modified NPG electrodes (MPA-FDH and NA-DS-FDH) were evaluated for their storage stability. Stability tests were conducted by performing cyclic voltammograms after designated period of times (example for NA-DS-FDH and MPA-FDH are shown in Figure 4.11 A-B). In both cases the normalized current density decreased by approximately 50 % after the first day (Figure 4.11 C). This rapid decrease can be associated with the exposure of the enzyme to the bulk buffer solution during storage. In contrast to the immobilization of enzyme on a diazonium or thiol film, the encapsulation of FDH in a polymer matrix minimizes the enzymes ability to unfold and therefore leads to more stable biosensors [54] (Table 4.5). However the encapsulation of enzymes can bring upon new challenges in terms of mass transport. After 6 days both FDH modified NPG electrodes showed a stable response with NA-DS-FDH and MPA-FDH retaining ca. 40 % and 25 % residual current density, respectively. The higher residual current density of NA-DS-FDH modified NPG electrodes can be associated with the more stable carbon-gold bond in comparison to the sulphur-gold bond generated in the MPA-FDH modified electrodes [87]. NA-DS-FDH modified NPG electrodes showed the best stability, and were therefore utilized in the preparation of fructose biosensors.
4.3.2.3 Effect of temperature and pH

The effect of pH and temperature on the response of the biosensor was examined. The optimum pH was found to be between 5.0 and 5.5, with significantly reduced current densities below pH 5.0 and above pH 5.5 (Figure 4.12 A). At a pH of 7.0 the catalytic activity of FDH was barely detectable. The optimum pH of the free enzyme reported in the literature for the oxidation of D-fructose is 4.0 and FDH was reported to be stable between pH 4.5 and 6.0 [47]. The observed shift in optimum pH may arise from the negatively charged carboxylic acid groups on the electrode surface as previously described for negatively charged acetate membranes [57] and carbon nanoparticles [55]. At a fixed pH of 5.5 the current density increased from 20 to 35 °C, in good agreement with the reported optimum temperature of 37 °C (Sorachim SA) [88]. When the temperature was increased above 37 °C, a drastic decrease in current density was observed (Figure 4.12 B).
Figure 4.12: Plot of current density obtained at NA-DS-FDH modified NPG electrodes as a function of (A) temperature (at a pH 5.5) and (B) pH (at a temperature of 25 °C). Conditions: 100 mM fructose, 100 mM McIlvain buffer, scan rate of 5 mV s⁻¹ (n≥3). Measurements obtained at a potential of 0.5 V with baseline subtraction.

4.3.2.4 Calibration of the biosensor

A sample calibration curve of the NA-DS-FDH modified NPG biosensor is displayed in Figure 4.13 A. The arrows indicate the absolute concentration of fructose added to the test solution (100 mM McIlvain buffer, 25 °C, pH 5.5) at a defined time. A

Table 4.5: Comparison of fructose biosensors reported in the literature

<table>
<thead>
<tr>
<th>Substrate and immobilization</th>
<th>Mediator</th>
<th>Applied potential [V] (vs Ag/AgCl)</th>
<th>Linear range [mM]</th>
<th>LOD [μM]</th>
<th>K_M [mM]</th>
<th>sensitivity [mA mM⁻¹ cm⁻²]</th>
<th>stability</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adsorption and crosslinked on MPA modified Au electrode</td>
<td>TTF[a]</td>
<td>0.2</td>
<td>0.01-1.0</td>
<td>2.4</td>
<td>5.4</td>
<td>29.1 mA mM⁻¹ cm⁻²</td>
<td>~ 30 days</td>
<td>[89]</td>
</tr>
<tr>
<td>Immobilization in a membrane mimetic layer on a gold electrode</td>
<td>Co-enzyme Q6</td>
<td>0.5</td>
<td>0.01-0.5</td>
<td>10</td>
<td>-</td>
<td>15 mA mM⁻¹ cm⁻²</td>
<td>10 % decrease in 4 days</td>
<td>[90]</td>
</tr>
<tr>
<td>Adsorbed and crosslinked on PAMAM[b] mod. Au disc electrode</td>
<td>HCF[c]</td>
<td>0.3</td>
<td>0.25-5.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>30 % decrease after 5 h</td>
<td>[91]</td>
</tr>
<tr>
<td>Polymer entrapment in SPE/MWCNT</td>
<td>Os polymer</td>
<td>0.15</td>
<td>0.1-5</td>
<td>2</td>
<td>4.2</td>
<td>1.6 μA mM⁻¹ cm⁻²</td>
<td>10 % decrease after 1 month</td>
<td>[54]</td>
</tr>
<tr>
<td>Incorporation in a carbon paste matrix</td>
<td>DET</td>
<td>0.4</td>
<td>0.5-10</td>
<td>75</td>
<td>35</td>
<td>3 nA mM⁻¹</td>
<td>Operation stability 10 h</td>
<td>[92]</td>
</tr>
<tr>
<td>Adsorption and crosslinking on diazonium mod. NPG electrodes</td>
<td>DET</td>
<td>0.15</td>
<td>0.05-0.3</td>
<td>1.2</td>
<td>0.68</td>
<td>3.7 μA mM⁻¹ cm⁻²</td>
<td>40 % remaining after ~6 days</td>
<td>This work</td>
</tr>
</tbody>
</table>

[a] Tetrathiafulvalene  
[b] Poly(amideamine) dendrimer  
[c] Hexacyanoferrate
Figure 4.13: (A) Plot of current density obtained at NA-DS-FDH modified NPG electrodes as a function of fructose concentration. The arrows indicate the fructose concentration at different times. (B) Calibration plots obtained at 18 (●) and 42 nm (■) pore size electrodes. The inset shows the linear range of the biosensor (25 °C at pH 5.5). Conditions: applied potential of 0.15 V.

stable reading was typically obtained within 5 seconds. The linear range of the biosensor was 0.05 – 0.30 mM with a $K_M$ (Michaelis-Menten constant) value of 0.68 ± 0.04 mM. The sensitivity was $3.7 \pm 0.2 \, \mu\text{A cm}^{-2} \, \mu\text{M}^{-1}$ and the LOD was 1.2 μM. When compared to results from other fructose biosensors, the LOD obtained here was the lowest (e.g. 2.4 μM was the next lowest value [89]). When compared to a DET based fructose biosensor where FDH was incorporated into a carbon paste matrix [92], the biosensor described here compares very favorably in terms of LOD and sensitivity (Table 4.5) despite operating at a considerably reduced potential. This may be due to favorable orientation and loading of FDH throughout the nanoporous network, decreasing the LOD and improving the sensitivity.

4.3.2.5 Interference study

Table 4.6: Current density obtained on addition of a range of sugars and ascorbic acid (all at a concentration of 250 μM, n = 4).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Current density [nA cm$^{-2}$]</th>
<th>% response [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-fructose</td>
<td>194.0 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>D-glucose</td>
<td>9.6 ± 0.4</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>D-galactose</td>
<td>2.4 ± 1.2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>D-mannitol</td>
<td>3.2 ± 0.4</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>36.8 ± 1.2</td>
<td>19 ± 3</td>
</tr>
</tbody>
</table>
Figure 4.14: Current response obtained on addition of 1: D-fructose, 2: D-glucose, 3: D-galactose, 4: D-mannitol and 5: ascorbic acid at concentrations of 250 μM (E = 0.15 V vs Ag/AgCl).

The response to potential interferences (D-glucose, D-galactose, D-mannitol, and ascorbic acid) was examined (at concentrations of 250 μM, Table 4.6). After addition of D-fructose a characteristic increase in current density was observed (Figure 4.14 (1)). Upon addition of the sugars (Figure 4.14 (2-4)) minimal changes in current density were obtained, indicating that the sugars do not interfere with the response of the sensor (the highest response of 5 % was obtained with D-glucose). On addition of ascorbic acid, a 19 ± 3 % increase in current was observed (Figure 4.14 (5)), which likely arises from the oxidation of ascorbic acid at the bare electrode, despite the relatively low applied potential of 0.15 V (vs Ag/AgCl).

4.3.2.6 Analysis of food samples

Natural sweeteners (agave, honey and maple syrup) and beverages (cola and a sports drink) with high and low D-fructose concentrations were used. Agave and sports drinks are of particular interest as they possess a large excess concentration of either D-fructose or D-glucose. An enzymatic spectrophotometric kit was used as a reference method. A summary of the results obtained can be seen in Table 4.7. The response of the biosensor compares very well with that of the enzymatic kit. The response of the biosensor in the sports drink was 8 % higher than that of the enzymatic kit. This response likely arises from the large excess of D-glucose which has been reported to lead to imprecise readings from the enzymatic kit (Figure 4.15) [93]. This may also account for the slightly lower values obtained by the biosensor with the Agave and
honey samples. For the maple syrup sample the response obtained with the biosensor was in excellent agreement with the kit. Maple syrup consists of approximately 80% sucrose which is not detected by the enzymatic kit or the biosensor. Therefore high concentrations of sucrose do not affect the biosensor. In the cola sample, the response of the biosensor was 87% of that of the kit, the reasons for this are unclear and may arise from a component in the drink sample. Given that other samples contain high levels of glucose, fructose and sucrose, the lowered response does not arise from the presence of these sugars.

Table 4.7: Summary of results obtained from a commercially available fructose enzymatic kit and the fructose biosensor.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[Glucose][a] [mmol L⁻¹]</th>
<th>[Fructose][a] [mmol L⁻¹]</th>
<th>[Fructose][b] [mmol L⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agave</td>
<td>1060 ± 9</td>
<td>3385 ± 7</td>
<td>3303 ± 16</td>
</tr>
<tr>
<td>Cola</td>
<td>152 ± 2</td>
<td>165 ± 8</td>
<td>144 ± 2</td>
</tr>
<tr>
<td>Honey</td>
<td>1642 ± 60</td>
<td>2198 ± 41</td>
<td>2174 ± 19</td>
</tr>
<tr>
<td>Maple syrup</td>
<td>15 ± 0</td>
<td>16 ± 1</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>Sports drink</td>
<td>196 ± 5</td>
<td>62 ± 1</td>
<td>67 ± 2</td>
</tr>
</tbody>
</table>

[a] Determined using enzymatic kit  
[b] Determined by the fructose biosensor
4.4 Conclusions

Surface modified NPG electrodes were utilized for use as a trace metal sensor (shown in the case of Cu$^{2+}$) and as an enzymatic biosensor for the detection of D-fructose.

Surface modified NPG electrodes, which exhibit an intrinsic biofouling resistance, is a suitable material for the detection of trace metal concentrations of copper. The average pore size of the prepared NPG was found to impact on the sensitivity of the sensor. By altering the available surface area for the underpotential deposition of Cu, the detection range could be adjusted. Surface modification of NPG with sulfonate functional groups was achieved by utilizing two different strategies, i.e. SAM formation of MPS and by electro-grafting of freshly synthesized BDS layers through electrochemical reduction. MPS modified NPG electrodes displayed high sensitivity and low limits of detection for free Cu$^{2+}$ in solution. However the gold-thiol bond is less stable than carbon-gold bonds, which was observed for multiple depositioning and stripping cycles. In contrast BDS modified NPG showed no change after multiple deposition and stripping cycles. The longer distance of sulfonate groups in the BDS film was found to lower the detection limit in contrast to MPS modified NPG sensors. The detection range from 0.2 to at least 25 µM was well capable to detect the legal concentrations (United States, EPA) of Cu$^{2+}$ in drinking water of 20.5 µM (1300 ppb). The sensor was tested in a range of water samples (being tap, rain and river water). The results obtained compared well with results obtained from AAS experiments, while the copper concentrations in rain and river water were too low to be determined by AAS. To test the combined biofouling protection of the BDS surface modification and the nanoporous structure, the sensor was tested in artificial human serum. The sensor was capable to precisely determine the free Cu$^{2+}$ concentration in this complex media.

NPG electrodes were also functionalized with carboxylic acid groups using thiol terminated SAMs and an electrochemically reduced 2-carboxy-6-naphtoyl diazonium salt. The adsorption of FDH on these modified electrodes followed by crosslinking occurs in a manner that enables direct electron transfer to occur. The response of the biosensor correlated very well with the results obtained with a commercially available enzymatic kit. The biosensor demonstrated rapid response times (less than 5 seconds), with a linear range of 0.05 - 0.3 mM D-fructose concentration, a sensitivity of $3.7 \pm 0.2 \mu A \text{ cm}^{-2} \text{ mM}^{-1}$ and a LOD of 1.2 µM. The addition of a range of sugars did not affect
the response. The biosensor is a promising alternative to established analytical measurements for the detection of D-fructose concentrations. The modified bioelectrode could also be useful in the development of biofuel cells, which will be discussed in the following chapter.
4.5 References


Chapter 5:

NPG electrodes and their application in biofuel cells
Chapter 5  NPG electrodes and their application in biofuel cells.

5.1 Introduction

The immobilization of enzymes on electrodes is of significant interest in the development of enzymatic fuel cells (EFC) and biosensors (as already shown in chapter 4) [1,2]. EFCs have significant potential as a source of clean, renewable energy for low-power devices; however they are still at an early stage of development [3]. The mode of operation of EFCs is similar to that of conventional fuel cells, where the fuel is oxidized at the anode side and providing electrons at the cathode to reduce oxygen to water (see Figure 5.1) [4]. In EFCs, enzymes are employed for the oxidation and reduction steps. The most extensively studied systems consist of a bioanode using the enzymes: glucose oxidase (GOx), cellobiose dehydrogenase (CDH) or glucose dehydrogenase (GDH); and a biocathode utilizing $O_2$ reducing bilirubin oxidase (BOD) or laccase [5-8]. The development of EFCs faces two significant barriers; low power output and stability of the response. These barriers arise from the stability of the enzyme and the rate of electron transfer between the enzyme and the electrode [9]. To overcome these difficulties, a range of approaches have been utilized to improve the efficiency of enzyme immobilization that enables direct (mediatorless) electron transfer (DET) between the enzyme and the electrode. These approaches entail using novel enzymes, a range of electrode materials and wide range of modified electrodes [10]. Two types of materials are often used as electrodes for EFCs. Carbon based nanostructures (CBNs) in a wide range of types e.g.: carbon cloth (CC), toray paper (TP), and morphologies such as nanoparticles, nanotubes, nanofibers are easy to fabricate and of low cost. An advantage of using carbon materials in electrochemical applications lies in the wide potential window that it can be utilized in. Graphene based materials, were reported to cause an activation of the immune system, most likely by the response of leukocytes in the system [11]. They have been evaluated as immunotherapy tools, vaccine carriers and drug delivery systems [11]. Intracellularly localized functionalized multi-walled carbon nanotubes (MWCNT) were also reported to partially degrade in primary microglia cells [12]. MWCNT were capable of crossing the blood brain barrier in mouse brain, a property of interest for therapeutic and diagnostic applications [13]. However these nanomaterials possess cytotoxicity effects which raise issues with biocompatibility [14-16]. Gold, as a noble metal, is biocompatible and is utilized in a wide range of applications where good biocompatibility is required [17]. Nanoporous gold (NPG) can be prepared by dealloying bimetallic alloys, where the less noble element (Ag, Ni, etc.) is removed to prepare a gold material with a three-dimensional network of tunable pore
sizes and channels that can be used to immobilize enzymes (as demonstrated in chapter 2) [18,19]. The encapsulation of biocatalysts has been extensively studied [20-22]. Storage, thermal and operational stability of enzymes such as xylanase, laccase and lipase could be significantly improved upon immobilization in NPG structures; therefore the physical confinement of enzymes in the porous structures is a significant factor in enzyme stabilization [23,24].

In this chapter, NPG electrodes of high surface area are used as supports for four redox enzymes: FAD-dependent glucose dehydrogenase (GDH) and D-fructose dehydrogenase (FDH), *Myrothecium verrucaria* bilirubin oxidase (*Mv*BOD) and *Bacillus pumilus* bilirubin oxidase (*Bp*BOD). *Glomorella cingulata* FAD-GDH overexpressed in *Pichia pastoris* (GDH) is an extracellular, glycosylated enzyme that is specific for just two substrates: β-D-glucose and D-xylose. The catalytic oxidation of β-D-glucose by GDH is unaffected by oxygen unlike with GOx where turnover of oxygen reduces the current and produces hydrogen peroxide which can degrade the enzyme [25]. GDH has good stability and a high turnover rate, making it a good candidate in place of GOx which is widely used in glucose sensors and EFCs [26,27]. BOD is a ‘blue copper’ oxidase that reduces oxygen to water and has been extensively used as the cathodic enzyme in EFCs. The enzyme (*Myrothecium verrucaria*) is a monomeric redox protein with a molecular mass of 66 kDa and an isoelectric point of 4.2 [28]. Different approaches have been undertaken to immobilize *Mv*BOD on NPG electrodes [29-31]. Mediated electron transfer (MET) using osmium redox polymers are utilized frequently; however this introduces mediators that decrease the redox potential of cathode. Direct (mediatorless) electron transfer (DET) is desirable but it is difficult to obtain systems that possess high faradaic activity and long-term stability. To help overcome these difficulties, NPG electrodes can be chemically modified. Alkane thiol self-assembled monolayers (SAMs) although widely used, are sensitive to changes in pH and ionic strength. Changes in the applied potential or temperature can result in desorption of SAMs from the electrode surface, while their chemical stability is also limited due to the relative ease of oxidation of the monolayer [32]. Pinson and co-workers pioneered the modification of electrodes with diazonium species [33]. Diazonium salts can be electrochemically reduced on gold, resulting in monolayer or multilayer coverage depending on the chemical composition of the salt [34]. After activation of carboxylic acid functional groups on the surface modified layer with carbodiimide, nucleophilic addition of primary amine groups on the surface of the enzyme can result in immobilization in a manner that enables DET [35]. If DET is not feasible, MET can be
Figure 5.1: Schematic diagram of a biofuel cell (A) that utilizes FAD-GDH on the anode and MvBOD on the cathode and (B) a conventional fuel cell that utilizes hydrogen and oxygen from air to generate electricity.

achieved by immobilizing the enzyme in a film of redox polymer which shuttle electrons between the active site of the enzyme and the surface of the electrode [3].

In this study two EFCs were prepared, which utilized glucose (GDH) and fructose (FDH) as biofuels for the oxidation on the respective bioanodes. For the biocathodes two differing modification techniques, resulting in DET (MvBOD) and MET (BpBOD), of BOD were utilized for the catalytic reduction of O₂. The four enzymes were immobilized on NPG supports with a range of pore diameters and film thicknesses. Using the optimal pore sizes for each enzyme, EFCs were prepared which had significantly improved stability when compared with the stability of EFCs prepared on planar gold surfaces.
5.2 Experimental

5.2.1 Reagents and Materials

Nitric acid (70%), sulfuric acid (95-98%), potassium phosphate monobasic and dibasic, sodium phosphate dibasic, potassium chloride, citric acid (anhydrous), 4-morpholineethanesulfonic acid (MES), 6-amino-2-naphthoic acid (NA), 3-mercaptopropionic acid (MPA), sodium nitrite, N-cyclohexyl-N'- (2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate (CMC), poly(ethylene glycol) diglycidyl ether (PEGDGE), uric acid, L-ascorbic acid, D-(−)-fructose, D-(+)-glucose, D-lactose, urea, L-cystine, calcium sulphate dehydrate, magnesium sulphate and albumin from bovine serum were obtained from Sigma- Aldrich Ireland, Ltd.. Acetonitrile anhydrous >99.8%, sodium chloride and sodium bicarbonat were purchased from Fischer Scientific. All chemicals were used as received unless stated otherwise. The osmium redox polymer [Os(2,2´-bipyridine)$_2$(poly-vinylimidazole)$_{10}$Cl]Cl with an $E_0$ of +220 mV vs. Ag/AgCl was prepared using a published procedure and was kindly provided by NUIG (Galway) [36]. PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{+2+}$ redox polymer ($E_0$ = +350 mV vs Ag/AgCl) [37] and Bacillus pumilus bilirubin oxidase (BpBOD) [38] was kindly provided by CNRS, Bordeaux. D-Fructose dehydrogenase from Gluconobacter sp. (FDH) was purchased from Sorachim SA and used at a concentration of 4 mg ml$^{-1}$. Myrothecium verrucaria bilirubin oxidase (MvBOD) was kindly provided Novozymes. GDH was prepared as previously described and was kindly provided by BOKU Vienna [39]. An Elgastat maxima-HPLC (Elga, UK) was used to obtain deionized water with a resistivity of 18.2 MΩ cm.

5.2.2 Preparation and characterization of NPG electrodes

Commercially available microscope glass slides (J. Melvin Freed Brand, USA) were utilized as the substrate for magnetron sputtering in an ultra-high vacuum chamber (ORION-5-UHV) at room temperature. The sputtering chamber was equipped with 3 metal targets for deposition, Au (AJA International Inc., USA), Ag and Ti (Kurt J. Lesker Company Ltd., UK) with 99.99% purity. Sputtering was achieved as previously described in section 2.2.2. Prior to deposition, the glass sheets were exposed to Ar plasma under vacuum to ensure a clean surface for improved adhesion and homogenous metal deposition. A 10 nm Ti adhesion layer was first deposited, followed by a pure Au layer of a thickness of one third of subsequent layer. This pure Au layer was required to suppress any possible electrochemical response from the Ti layer. An Ag$_{70}$/Au$_{30}$ layer
with thickness varying from 100 to 1000 nm was then deposited. The glass sheets were cut into squares of approximately 0.5 - 0.7 cm$^2$ using a circular saw. The sputtered sheets were dealloyed in 70% nitric acid at varying temperatures and dealloying times. After the formation of the nanopores a silver wire (Farnell Components Ltd. Ireland) was soldered to the surface using a 99.99% pure indium wire (Sigma-Aldrich Ltd.). The soldering point was supported by a two component epoxy glue (EVO-STIK, Bostik Industries Ltd) to ensure adhesion to the surface. Dielectric paste (Gwent Group, UK) was used to insulate the electrode components and to define an electroactive surface area. After drying in a vacuum chamber, the electrodes were ready for use. NPG electrodes were characterized as previously shown in section 2.2.3.

5.2.3 Surface modification of NPG and enzyme immobilization

After preparation of the NPG electrodes they were cleaned in 0.5 M H$_2$SO$_4$ by cycling the potential between -0.2 and 1.6 V at 0.1 V s$^{-1}$ vs Ag/AgCl (3 M KCl). To minimize changes to the structure of the nanopores this step was limited to 2 cycles [35]. The electrodes were rinsed with deionized water and dried in a vacuum chamber. For the covalent attachment of MvBOD a fresh solution of 2-carboxy-6-naphtoyl diazonium salt (NA-DS) was synthesized by mixing a 2 mL solution (20 mM in acetonitrile) of 6-amino-2-napthoic acid (NA) with 2 mL of a solution of NaNO$_2$ (2 mM) in 1 M HCl in an ice bath as already demonstrated in section 3.2.3.2 [40,41]. A single potential scan from 0 to -0.5 V at 200 mV s$^{-1}$ was performed to electrochemically reduce the NA-DS to the surface (see Figure 5.2). After rinsing with deionized water 3-mercaptopropionic acid (MPA) was attached to the surface by immersion of the electrodes 1 mM MPA solution over night. Prior to enzyme attachment the NA-DS/MPA-NPG electrodes were thoroughly rinsed with deionized water and dried in a vacuum chamber. Afterwards a solution of 20 μL MvBOD (0.36 mg ml$^{-1}$) diluted in 0.01 M MES buffer pH 6.0 and was drop-cast on to the surface of the electrode. Alternatively the electrodes were modified by dropping a 20 μL aliquot of 4 mg mL$^{-1}$ FDH solution, as already described in section 4.2.5. To improve the level of penetration of the enzyme into the pores, the electrodes were exposed to a vacuum of maximum 0.098 MPa for approximately 3 min and stored at 4 °C for 1 h. This procedure was found to improve the loading of the enzyme throughout the porous structure leading to higher current densities, while not apparently changing the enzyme activity [29]. Crosslinking was achieved by immersing the modified electrodes in 5 mM CMC.
solution at 4 °C for 2 h. The electrodes were then tested in 0.1 M PBS (K⁺) with 0.2 M KCl as supporting electrolyte by scanning the potential from 0.7 - 0 V at 5 mV s⁻¹. GDH modified electrodes were prepared by preparing a solution with a ratio of 8 μL of the redox polymer Os(bpy)₂(PVI)₁₀Cl²⁺/³⁺ with a concentration of 6 mg ml⁻¹ with 4.8 μL 10 mg mL⁻¹ GDH solution and a crosslinking solution of 1.9 μL of 15 mg mL⁻¹ PEGDGE. 20 μL of this freshly prepared enzyme/ polymer/ crosslinker solution were then drop-casted on the electrodes. After exposure to a vacuum of maximum 0.098 MPa for approximately 3 min they were allowed to dry at 4 °C over night. Electrodeposition of PAA-PVI-[Os(dcl-bpy)₂Cl]¹⁺/²⁺ redox polymer and BpBOD was achieved following a published procedure as already demonstrated in section 3.2.4 [37]. In short, 30 μL solution containing 1 mg mL⁻¹ PAA-PVI-[Os(dcl-bpy)₂Cl]¹⁺/²⁺ redox polymer and 1 mg mL⁻¹ BpBOD enzyme solution in McIlvain buffer 0.15 M at pH 5.0 was drop-casted on each NPG electrode. Pulsed deposition was performed by pulsing the potential from 0.9 V (2 s) to -0.4 V (3 s) vs Ag/AgCl in 3 M KCl for 1-10 minutes.
5.2.4 Electrochemical measurements

All electrochemical measurements were performed using a CHI620A or CHI802A potentiostat operating in a standard three electrode configuration with NPG, Ag/AgCl (in 3 M KCl) and Pt wire as working, reference and counter electrodes, respectively. The experiments for GDH/MvBOD EFCs were conducted in 50 mM PBS at pH 7.4 and in artificial serum which was mixed by average quantities of the following compounds in a healthy human body: uric acid (0.36 mM), L-ascorbic acid (0.054 mM), D-(+)-glucose (5 mM), D-(-)-fructose (0.2 mM), D-lactose (15 μM), urea (4.45 mM), L-cystine (0.075 mM), calcium sulphate dehydrate (2.38 mM), magnesium sulphate (0.87 mM), sodium chloride (103 mM), sodium bicarbonate (25.5 mM) and 7% of bovine serum albumin (BSA) [42]. The experiments for FDH/BpBOD EFCs were conducted in 150 mM McIlvain buffer at pH 5.0.
5.3 Results and Discussion

As described previously [43] the confinement of enzymes can improve their long term stability and activity by protecting the enzyme from biofouling agents or by diminishing the protein's ability to unfold. NPG electrodes with a range of pore diameters and surface morphology were prepared and examined for use in EFC applications.

5.3.1 Characterization of NPG

As already demonstrated in section 2.3.1.3 the structure and morphology of a metal surface produced by chemical etching of a bimetallic alloy can vary substantially depending on the etching conditions [44]. NPG electrodes with a wide range of pore and

Figure 5.3: SEM images of NPG prepared by dealloying at 20°C and 15 min using substrate thicknesses of 100 nm (A,B), 300 nm (C,D) and 500 nm (E,F).
crack sizes as well as substrate thicknesses (Table 5.1) were utilized to evaluate the effects on current density and for stability. Figure 5.3 shows examples of the electrode surfaces prepared using the same dealloying conditions (20 °C for 15 minutes). On varying the dealloying conditions, pores ranging from 9 to 62 nm in diameter can be prepared (Table 5.1) while the cracks on the surface range from 220 to 3180 nm in length. This range of electrodes was used as supports for BOD, GDH and FDH for use as cathodes and anodes in EFCs.

Table 5.1: List of alloy thicknesses and the average pore and crack sizes obtained at different dealloying conditions.

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<tr>
<td>100</td>
<td>20</td>
<td>1</td>
<td>9 ± 1</td>
<td>280 ± 50</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>15</td>
<td>18 ± 4</td>
<td>220 ± 30</td>
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<td>100</td>
<td>40</td>
<td>5</td>
<td>42 ± 3</td>
<td>N/A[a]</td>
</tr>
<tr>
<td>100</td>
<td>60</td>
<td>5</td>
<td>62 ± 3</td>
<td>N/A[a]</td>
</tr>
<tr>
<td>300</td>
<td>20</td>
<td>5</td>
<td>16 ± 2</td>
<td>1500 ± 240</td>
</tr>
<tr>
<td>300</td>
<td>20</td>
<td>15</td>
<td>24 ± 3</td>
<td>1400 ± 400</td>
</tr>
<tr>
<td>300</td>
<td>40</td>
<td>5</td>
<td>32 ± 2</td>
<td>1000 ± 300</td>
</tr>
<tr>
<td>300</td>
<td>60</td>
<td>5</td>
<td>34 ± 3</td>
<td>900 ± 200</td>
</tr>
<tr>
<td>500</td>
<td>20</td>
<td>5</td>
<td>17 ± 2</td>
<td>2100 ± 440</td>
</tr>
<tr>
<td>500</td>
<td>20</td>
<td>15</td>
<td>20 ± 2</td>
<td>3200 ± 800</td>
</tr>
<tr>
<td>500</td>
<td>40</td>
<td>5</td>
<td>27 ± 3</td>
<td>3400 ± 680</td>
</tr>
<tr>
<td>500</td>
<td>60</td>
<td>5</td>
<td>35 ± 3</td>
<td>3800 ± 400</td>
</tr>
</tbody>
</table>

[a] No cracks were observable

5.3.2 Influence of surface morphology and nanopore layer thickness on catalytic responses

MvBOD was covalently attached (Figure 5.2) to the NPG electrodes [44], while GDH was immobilized by drop-casting the enzyme with Os redox polymer (Os(bpy)$_2$(PVI)$_{10}$Cl) in the presence of a cross linking polymer, PEGDGE [8]. Due to
the nature of the two different coupling methods the observed catalytic responses of the enzymes may be significantly altered. Using a single potential cycle the electrochemical reduction of the in-situ synthesized 2-carboxy-6-naphtoyl diazonium salt (NA-DS) at the surface of the electrode enables close to monolayer modification as previously reported on gold nanoparticle modified gold disk electrodes [40,41]. After attachment of NA-DS to the gold surface a short chained thiol (MPA) was used to completely block the surface and avoid rapid denaturation [45,46] of the enzyme at the bare gold surface. MPA is also capable of promoting DET between MvBOD and gold electrodes, potentially improving the catalytic current [40]. Provided the entire surfaces of the pores are accessible to the enzymes, such modification can enable high loadings of MvBOD.

In contrast, due to the viscous nature of the solution combined with the surface wettability, the accessible electrode area may be reduced when preparing GDH modified electrodes [47].

The highest current densities (using the geometric surface area) were achieved for NPG electrodes that had a thickness of 500 nm and an approximate average pore size of 17 nm (Figure 5.4 A). Current densities of ~800 μA cm$^{-2}$ were obtained in O$_2$ saturated PBS buffer, slightly lower than the diffusion controlled limiting current of ~1200 μA cm$^{-2}$ predicted by the Randles- Sevcik equation (D$_{O_2}$ of 2.6x10$^{-5}$ cm$^2$ s$^{-1}$ [48] and [O$_2$] of 1.23 mM [49]). In comparison, electrodes with approximately the same average pore sizes but with thinner layers, i.e. 300 and 100 nm, had current densities (geometric) of ca. 180 and 60 μA cm$^{-2}$ respectively. The higher current density of the 500 nm electrodes arises in part from the increased thickness of the electrode. Based on the thickness alone the response is predicted to be 300 μA cm$^{-2}$; clearly additional
Chapter 5  

NPG electrodes and their application in biofuel cells.

**Figure 5.5:** Plot of current density versus pore size for GDH/ Os polymer modified electrode with different electrode thickness based on the geometric (A) and electrochemically addressable (B) surface areas at 5 mV s\(^{-1}\) and 10 mM glucose concentration.

Factors, such as crack formation on the NPG surface, need to be taken into account. The crack sizes that develop on the NPG during the dealloying process need to be considered. As silver is etched from the alloy the rapid rearrangement of gold atoms at the interface can cause cracks to appear on the surface which are subsequently refilled upon continuous etching [18,25]. These cracks can increase the catalytic current in two ways. The channels could bind \(Mv\) \(BOD\) in a manner that may result in a more favorable orientation of the enzyme. In addition, the supply of \(O_2\) to the enzyme in the cracks may be improved, due to improved access to the electrode surface. In general, optimal current densities (geometric) were achieved for electrodes with average pore sizes ranging between 10 and 25 nm for all electrodes, while the 500 nm thick nanopore layer gave the highest response in each case. The increase in layer thickness showed a corresponding increase in current density (geometric) for each electrode thickness, indicating that the response was not limited by access to the full length of the pores nor by the rate of diffusion of oxygen through the nanoporous structures. When utilizing the current densities based on the electrochemically active surface areas (as determined by the gold oxide stripping technique [50]), slightly different behavior was observed (Figure 5.4 B). While the 100 and 300 nm thick nanopore electrodes show similar trends, the 500 nm electrodes had a much higher response of up to 18.0 \(\mu A\) cm\(^{-2}\). The highest current density, as determined by the geometric surface area, did not correspond to the maximum current density determined using the electrochemically addressable surface area, indicating that the surfaces of the electrodes are not fully available to the enzyme. This is likely due to the fact that electrodes with an average pore size of ca. \(\sim 16\) nm possess a structure which hinders the immobilization of \(Mv\) \(BOD\), (dimensions of A: 4.0 nm B: 5.0 nm and C: 6.0 nm) [51], deep in the porous structure. On increasing
the average pore size above 20 nm, the electrochemically addressable surface area was
decreased resulting in a decrease in the response due to the lower amounts of
catalytically active enzyme, an effect that was also previously observed for glucose
oxidase [52]. No catalytic response was obtained in control experiments using N₂
saturated PBS (Figure 5.6 A).

Electrodes prepared using GDH and osmium redox polymer show significant
differences with the geometric current density being independent of the average pore
size (Figure 5.5 A) for both the 100 and 300 nm thick nanopore layers. Note that in
control experiments, the ability of an unmodified NPG electrode to oxidize glucose was
examined, no response was observed. The 100 nm thick electrodes had responses in the
same range as planar gold electrodes, at ca. 100 μA cm⁻² (Figure 5.5 A). This response
is in agreement with previous work with MvBOD and an osmium polymer matrix that
showed near identical responses when utilizing planar and NPG electrodes [18], where
the response was limited by planar diffusion of the substrate.

The 300 nm thick electrodes however had current densities that were a factor of
two times higher, i.e. ca. 250 μA cm⁻² (Figure 5.5 A). This increase is also probably
due to the larger cracks on the electrode surface that are more likely to be accessible by
the relative viscous drop-casting mixture of osmium redox polymer, GDH and
crosslinker PEGDGE than the pores itself. As previously reported [53], this improved
accessibility results in an increased surface coverage of the polymer matrix on the gold
surface leading to enhanced current densities. When considering the current density
calculated using the electrochemically addressable surface area, there was no significant
difference between the 100 and 300 nm thick electrodes, indicating that the accessible
enzyme loadings were similar. It is also evident that by increasing the average pore size
of the NPG, the current densities approached those of planar electrodes. On the 500 nm
thick electrodes the polymer film was not mechanically stable and became detached
upon swelling of the polymer film. This instability is probably due to the wide cracks
present on these electrodes (Figure 5.3 E) which impedes the stable attachment of the
polymer film to the surface. The results indicate that the polymer/enzyme matrix is
unable to fully penetrate into the porous network of the NPG electrodes. Upon
increasing the average pore size the current density (based on the electroactive surface
area) increased and approached the values achieved at planar gold electrodes (Figure
5.5 B).
From these optimization studies, the electrodes with the highest current densities (geometric) were examined for use in an enzymatic fuel cell. For the cathode, a 500 nm thick NPG electrode with an average pore size of ~17 nm was utilized, while a 300 nm thick NPG electrode with an average pore size of ~16 nm was utilized for the anode. This combination is labelled EFC1. As a comparison, an EFC that possesses lower current densities on both electrodes was prepared. This consisted of a 500 nm thick cathode with average pore size of ~20 nm and a 300 nm anode with an average pore size of ~24 nm (EFC2).

5.3.3 Stability of enzyme modified NPG electrodes

MvBOD was covalently attached to gold surfaces (i.e., planar and NPG), while GDH was encapsulated in an osmium polymer matrix and drop-casted on the electrode surface. The catalytic response for a biocathode with a 300 nm film thickness and an average pore size of ~24 nm was compared with the response obtained at a planar gold electrode (Figure 5.6 C). The response at the NPG modified electrode was more stable

![Example cyclic voltammograms of (A) MvBOD modified NPG (300 nm film thickness and ~32 nm average pore size) and (B) osmium polymer/GDH modified NPG (300 nm film thickness and ~16 nm average pore size) after defined period of times. The arrow indicates the development of the catalytic response after enzymatic decay. Plot of catalytic activity versus time for (C) MvBOD modified NPG and (D) GDH modified electrodes. Measurements were executed after storage in PBS buffer (pH 7.4) at 4 °C.](image-url)
than that of the planar gold electrode. For the NPG electrode catalytic responses where obtained for up to 200 hours, in contrast planar gold electrodes only showed a response for up to 50 hours. The half-life of the biocathode was 20 and 4 hours for NPG and planar gold, respectively. It should also be noted that coupling of $MvBOD$ via diazonium substantially improved the stability of the electrodes. In section 2.3.5 it was already demonstrated that physically adsorbed $MvBOD$ on NPG only showed catalytic activity for 4 hours [44]. In addition to the higher electrochemically active surface area, NPG electrodes clearly provide a more stable environment for the immobilized $MvBOD$ enzyme. In contrast to the biocathode, the bioanode did not demonstrate an increase in stability (Figure 5.6 D). This is likely due to the relatively higher viscosity of the solution, which makes it more difficult to penetrate through the porous structure and adsorb onto the electrode surface, in essence the porous structure is not as available.

5.3.4 Application as a glucose EFC

EFC1 (Figure 5.7 A) produced a maximum power density of 17.5 $\mu$W cm$^{-2}$ (at a potential of 0.193 V) in a 50 mM phosphate buffer at a glucose concentration of 5 mM. On testing in artificial serum [42], a power density of 7.0 $\mu$A cm$^{-2}$ was obtained (at a potential of 0.166 V). Such a decrease likely arises from the increased solution viscosity together with fouling of the electrode surface by BSA. The power density of EFC2 (Figure 5.7 B) in 50 mM phosphate buffer was lower than that of EFC1 with a maximum value of 10.4 $\mu$W cm$^{-2}$ (at a potential of 0.197 V) and also decreased in artificial serum to 6.5 $\mu$W cm$^{-2}$ at a potential of 0.156 V). It should be noted that while the power densities of EFC1 and EFC2 differed, the responses in artificial serum were similar, indicating that the response was limited by the solution viscosity and biofouling effects [2].

![Figure 5.7: Plot of current and power density versus potential for EFC1 (A) and EFC2 (B), in PBS and artificial serum at pH 7.4.](image-url)
Figure 5.8: Decrease in power density under continuous operation of EFC’s at a potential of -0.21 V (vs Ag/AgCl 3 M KCl) of planar Au and NPG electrodes modified with MvBOD and GDH as bioanode an biocathode respectively in PBS at pH 7.4.

Based on the 2-3 fold difference in the response (Figure 5.4 A and Figure 5.5 A), the response of the biofuel cell in buffer is limited by the anode, the same limitation is likely to pertain in artificial serum. Both cells had an open circuit voltage (OCV) of ~0.45 V. The response of the EFCs compares well with the responses described in recent reports EFC based on gold electrodes (Table 5.2). A biofuel cell based on 1-[bis(2-naphthoquinonyl) aminomethyl]pyrene (pyr-(NQ)$_2$) modified multi walled carbon nanotubes (MWCNT) with immobilized MvBOD and PQQ-GDH, generated a power density of ~14 μW cm$^{-2}$ in 10 mM glucose. In comparison to this report, the biofuel cell described here has a high power density and improved stability. The highest power density was achieved on an ordered macroporous gold electrode with an output of 178 μW cm$^{-2}$. However this response was based on a high glucose concentration (30 mM) under non-physiological conditions (acetate buffer at pH 6.0) [54]. Stability data has been reported on two of the cells (Table 5.2). The stability of an EFC based on Au nanoparticles/polycrystalline Au [55] had a half-life of 8 hours. An EFC based on Au nanoparticles on an Au microwire showed a decrease in response of 10 % in 2 hours [56].

By comparison the EFC described here have improved half-lives demonstrating that NPG provides a stable support for applications in EFC (Figure 5.8). NPG based EFC’s modified with MvBOD as cathode and GDH as anode could retain ~60 % of its
Table 5.2: List for the comparison of recently reported EFCs based on gold electrodes in regard to power densities, OCV and operation medium.

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<tr>
<td>Au nanoparticles on polycrystalline gold</td>
<td>CtCDH 5 mM glucose</td>
<td>MvBOD air saturated</td>
<td>3.0</td>
<td>0.63</td>
<td>Human plasma pH 7.4</td>
<td>[55]</td>
</tr>
<tr>
<td>Gold leaf NPG</td>
<td>AnGOx 5 mM glucose</td>
<td>MvBOD O₂ saturated</td>
<td>3.4</td>
<td>0.56</td>
<td>PBS pH 7.0</td>
<td>[57]</td>
</tr>
<tr>
<td>Au nanoparticles on Au micro wire</td>
<td>CtCDH 2.5 mM glucose</td>
<td>MvBOD air saturated</td>
<td>~4</td>
<td>0.66</td>
<td>PBS pH 7.4</td>
<td>[56]</td>
</tr>
<tr>
<td>3D ordered Macroporous Au</td>
<td>EcGDH 30 mM glucose</td>
<td>Laccase air saturated</td>
<td>178</td>
<td>0.52</td>
<td>Acetate buffer pH 6.0</td>
<td>[54]</td>
</tr>
<tr>
<td>Highly ordered macroporous Au</td>
<td>AnGOx 10 mM glucose</td>
<td>Laccase O₂ saturated</td>
<td>38</td>
<td>0.52</td>
<td>PBS at pH 7.4</td>
<td>[53]</td>
</tr>
<tr>
<td>Pyr-(NQ)₂ [a] modified MWCNT</td>
<td>PQQ-GDH Pyr(NQ)₂/MWCNT 10 mM glucose</td>
<td>MvBOD Pyr(NQ)₂/MWCNT air saturated</td>
<td>~14</td>
<td>0.62</td>
<td>MOPS buffer pH 6.5</td>
<td>[58]</td>
</tr>
<tr>
<td>This work</td>
<td>GcGDH 5mM glucose</td>
<td>MvBOD O₂ saturated</td>
<td>17.5 7.0</td>
<td>0.45 0.45</td>
<td>PBS, Serum pH7.4</td>
<td>-</td>
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[a] Pyr(NQ)₂:1-[(Bis(2-naphthoquinyonyl)aminomethyl]pyrene

initial power density after 8 hours of continuous operation. In contrast the planar Au modified EFC’s retain less than 40 % after continuous operation. This increase in operation stability is associated with the increased stability of the biocathode, which is protected from accelerated deactivation due to the embedding of the enzyme in the porous structure.

5.3.5 Application as a fructose EFC

In addition to the use of glucose as biofuel for EFC applications, D-fructose also poses great potential as biofuel due to the high content of D-fructose e.g. in fruits. D-
fructose oxidizing bioanodes were prepared by covalent attachment and crosslinking of FDH surface modified NPG electrodes as already demonstrated in section 4.2.5. To achieve high loadings of the enzyme on the NPG surface, electrodes with 1000 nm film thickness (ca. 20 nm average pore size) were utilized. A characteristic cyclic voltammogram of FDH modified NPG can be seen in Figure 5.9 A with a concentration of 10 mM D-fructose at 25 °C in 150 mM McIlvain buffer (pH 5.0). For the biocathode, polymer matrix encapsulated BpBOD modified NPG electrodes were utilized. As already discussed in section 3.3.2.2, electrodeposition of PAA-PVI-[Os(dcl-bpy)_2Cl]^+/2+ redox polymer solution with an $E_0$ of +350 mV vs Ag/AgCl containing 1 mg mL$^{-1}$ of enzyme solution (BpBOD), can easily be achieved upon pulsing the potential from 0.9 V (for 2 s) to -0.4 V (for 3 s) vs an Ag/AgCl in 3 M KCl reference electrode. Figure 5.9 A (red line) shows the catalytic response of redox polymer/BpBOD modified NPG electrode (42 nm average pore size and 270 s deposition time) in 150 mM O$_2$ saturated McIlvain buffer solution at 25 °C. The two modified NPG electrodes were connected and utilized as a fructose biofuel cell. Current densities of the bioanode and biocathode

**Figure 5.9:** (A) Cyclic voltammograms of an FDH based bioanode in 10 mM D-fructose (black line) and BpBOD (red line) modified biocathode (O$_2$ saturated 150 mM McIlvain buffer pH 5.0) at a scan rate of 5 mV s$^{-1}$. (B) LSV of the two connected bioelectrodes at a scan rate of 1 mV s$^{-1}$ (10 mM D-fructose, O$_2$ saturated conditions in 150 mM McIlvain buffer pH 5.0) and the resulting power curve. (C) Amperometry performed at 0.2 V in 10 mM D-fructose solution under air saturated conditions over a period of time (150 mM McIlvain buffer pH 5.0). All experiments were performed at a controlled temperature of 25 °C.
were found to be 220 $\mu$A cm$^{-2}$ (at a potential of 0.6 V vs Ag/AgCl) and -240 $\mu$A cm$^{-2}$ (at a potential of 0.0 V vs Ag/AgCl), respectively. Therefore in a biofuel cell setup the limiting electrode is expected to be the bioanode. A typical LSV and calculated power curve of the resulting biofuel cell can be seen in Figure 5.9 B. Despite the low concentration of D-fructose (10 mM) in solution high power densities of up to 13 $\mu$W cm$^{-2}$ could be achieved at an operation potential of 0.18 V vs Ag/AgCl. The OCV was found to be 0.64 V. The half-life time of the FDH/BpBOD biofuel cell was found to be $ca.$ 19 h Figure 5.9 C at an operation potential of 0.2 V.

It is important however to note that the experiments conducted resulted in very initial results. The used NPG electrodes and the immobilization techniques require further study to show the capabilities of this system.
5.4 Conclusions

NPG electrodes were prepared with average pore sizes ranging from 9 to 60 nm and average crack sizes ranging from 220 to 3770 nm and then evaluated for the use as anode and cathode electrodes in enzymatic fuel cells. MvBOD was covalently attached to the electrode using carbodimide coupling to a diazonium modified NPG surface. Anodes were prepared by crosslinking an osmium redox polymer with GDH on NPG. The optimum responses of 800 and 250 μA cm$^{-2}$ were obtained with a MvBOD modified electrode (500 nm film thickness with an average pore size of 15 nm) and a GDH modified electrode (300 nm film thickness with an average pore size of ~15 nm), respectively. While the average pore size had no effect on the response of the anode, the catalytic response of the cathode electrode increased linearly with the thickness of the layer. MvBOD modified NPG electrodes demonstrated improved half-life’s (factor of four) when compared to planar modified electrodes, while GDH modified electrodes showed no improvement in stability. Enzymatic fuel cells were prepared, with the optimal response occurring with a 500 nm thick MvBOD modified NPG cathode and a 300 nm GDH modified anode, which generated power densities of 17.5 and 7.0 μW cm$^{-2}$ in PBS and artificial serum, respectively, at an OCV of ~0.45 V. These EFC’s retained over 60 % of their initial power density after 8 hours of continuous operation.

In addition to the oxidation of glucose as biofuel, alternative sugars such as D-fructose could be used in EFC applications. Herein, NPG electrodes were utilized as substrate for FDH and BpBOD immobilization which was achieved by covalent attachment to diazonium modified films and through encapsulation in electrodeposited PAA-PVI-[Os(dcl-bpy)$_2$Cl]$^{4/2+}$ redox polymer matrixes, respectively. Despite the low concentration of biofuel (D-fructose) of 10 mM a power density of up to 13 μW cm$^{-2}$ at an operating potential of 0.18 V vs Ag/AgCl was very high. The half-life of ca. 19 h, at an operation potential of 0.2 V, further indicates the potential of FDH/BpBOD EFCs and serves as a proof of concept.
Chapter 5  NPG electrodes and their application in biofuel cells.

5.5 References

Chapter 5  NPG electrodes and their application in biofuel cells.


Chapter 5  
NPG electrodes and their application in biofuel cells.


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Chapter 6:

Conclusions and Recommendations
6.1 Conclusions

The main objective in this work was to prepare and characterize NPG as a substrate for high surface area electrodes. The applications of the NPG electrodes include trace metal sensing, bio-sensing and energy harvesting via bio fuel cells.

Silver/gold alloys were prepared using a magnetron sputtering technique. Glass sheets were first sputtered with an adhesion layer composed of titanium, followed by a pure gold layer and the subsequent alloy layer. The sputtered sheets were dealloyed using concentrated HNO$_3$. The etching conditions chosen were found to have a significant impact on the generated porous structure, making it possible to tune the pore morphology to specific needs. The following conditions were altered to tune the resulting morphology and pore sizes: i) atomic alloy composition, ii) dealloying temperature, iii) dealloying time and iv) the film thickness. The alloy composition was found to be crucial for the formation of a nanoporous structure. If the silver content is too low only surface pitting occurs, while if it is too high, the resulting nanoporous network is mechanically instable and may collapse. The dealloying temperature and time were found to have the highest impact on the roughness and therefore the pore sizes generated. By applying short dealloying times at low temperatures, the pores were found to be as small as <5 nm, which resulted in surface roughnesses that were 45 times higher than planar gold for 100 nm thick NPG films, with the drawback of a high residual silver content. A method was developed to select the etching conditions needed to receive desired morphologies for specific purposes such as enzyme immobilization, sensing or capacitor applications. Pore size determination throughout the differing pore structures was achieved by manual readout, which was found to be the most reliable for heterogeneous porous structures. The pores were fully addressable for thicknesses up to at least 500 nm. This was evident through the linear relationship displayed by the results received for the calculation of the $R_f$ values. The influence of the pore size on the bioelectrochemical response of cyt c and $MvBOD$ was evaluated. Maximal current densities of ca. 30 $\mu$A cm$^{-2}$ were observed at cyt c modified NPG electrodes with an average pore size of ~9 nm. High current densities of ca. 65 $\mu$A cm$^{-2}$ were achieved for the reduction of O$_2$ to H$_2$O on $MvBOD$ modified NPG electrodes, with the response likely arising from surface adsorption of the enzyme.

A wide variety of surface modifications have been utilized throughout this work. For the covalent attachment of enzymes on surface functionalized NPG electrodes the focus was directed on innovative diazonium compound in contrast to thiol compounds.
Chapter 6  Conclusions and Recommendations

While the thiol-gold bonds form spontaneously in a highly orientated manner dependent on the functional groups and chain length of the spacer groups, diazonium compounds can be electrochemically reduced to an electroactive surface following subsequent synthesis. The carbon-gold bond is more stable than the thiol-gold bond making it an interesting alternative for a variety of applications. A variety of diazonium compounds with differing functional groups were utilized. Near monolayer AQ functionalized diazonium films showed that NPG electrodes with sufficient pore sizes of ~10 nm had similar surface coverages as planar modified Au electrodes, utilizing the same surface modification strategy. Following this conclusion a wide variety of synthesis strategies were adapted and developed for NPG substrate. Amine and hydroxylamine functionalized NPG was utilized to covalently attach laccase to the surface.

A method to synthesize and covalently attach BDS (a sulfonate terminated diazonium salt) to the surface of NPG substrate, which exhibit an intrinsic biofouling resistance, has been developed and evaluated for use as a heavy metal sensor (Cu). The average pore size of the prepared NPG was found to impact on the sensitivity of the sensor. By altering the available surface area for the underpotential deposition of Cu, the detection range could be adjusted. To compare the performance of BDS modified copper sensors, NPG was also surface functionalized using SAMs composed of a short chained thiol with a sulfonate functional group (MPS). MPS modified NPG electrodes displayed high sensitivity and low limits of detection for free Cu\(^{2+}\) in solution. However the gold-thiol bond was found to be less stable than carbon-gold bonds, when multiple measurements were conducted. In contrast BDS modified NPG showed no change after multiple deposition and stripping cycles. The longer modifying group in the BDS film was found to lower the detection limit in contrast to MPS modified NPG sensors. The detection range from 0.2 to at least 25 \(\mu\text{M}\) was within the legal concentration limit of 20.5 \(\mu\text{M}\) (1300 ppb) in drinking water (United States, EPA). The sensor was tested in a range of water samples (being tap, rain and river water). The results obtained compared well with results obtained from AAS experiments. To test the combined biofouling protection of the BDS surface modification and the nanoporous structure, the sensor was tested in artificial human serum. The sensor was capable of precisely determining the free Cu\(^{2+}\) concentration in this complex media.

In addition carboxylic acid terminated diazonium compounds were synthesized and electro-grafted to NPG electrodes. Enzymes such as FDH could be covalently attached to the diazonium film in a way that enables DET of the active center of the enzyme to the electrode to occur. FDH modified NPG electrodes were utilized to
prepare D-fructose biosensors and the response tested in a variety of natural sweeteners and sugar containing beverages. The response of the biosensor correlated very well with the results obtained with a commercially available enzymatic kit. The biosensor demonstrated rapid response times (less than 5 seconds), with a linear range of 0.05 - 0.3 mM D-fructose concentration, a sensitivity of $3.7 \pm 0.2 \, \text{μA cm}^{-2} \text{mM}^{-1}$ and a LOD of 1.2 μM. The addition of a range of sugars such as D-glucose, D-galactose and D-mannitol did not affect the response. The biosensor is a promising alternative to established analytical measurements for the detection of D-fructose concentrations.

In addition to FDH, $Mv$BOD could also be covalently attached by crosslinking on carboxylic acid terminated diazonium films. $Mv$BOD modified NPG electrodes were utilized as biocathodes in a biofuel cell setup. For the bioanode NPG electrodes were drop-casted with $\text{Os(bpy)}_2(\text{PVI})_{10}\text{Cl}^{2+/3+}$ redox polymer that encapsulated FAD-GDH enzyme and crosslinking agent. For the study NPG electrodes with average pore sizes ranging from 9 to 60 nm and average crack sizes ranging from 220 to 3770 nm were utilized. The optimum responses of 800 and 250 μA cm$^{-2}$ were obtained with a $Mv$BOD modified electrode (500 nm film thickness with an average pore size of 15 nm) and a GDH modified electrode (300 nm film thickness with an average pore size of ~15 nm), respectively. While the average pore size had no effect on the response of the anode, the catalytic response of the cathode electrode increased linearly with the thickness of the layer. $Mv$BOD modified NPG electrodes demonstrated improved half-life’s (factor of four) when compared to planar modified electrodes, while GDH modified electrodes showed no improvement in stability. The electrodes with the highest current densities were utilized as an EFC and generated power densities of up to 17.5 and 7.0 μW cm$^{-2}$ in PBS and artificial serum, respectively, at an OCV of ~0.45 V. These EFC’s retained over 60 % of their initial power density after 8 hours of continuous operation.

EFCs could also be prepared by utilizing D-fructose as biofuel. FDH modified NPG electrodes, as used for the D-fructose biosensor, were utilized as the anode, while the cathodes were prepared by encapsulating $Bp$BOD in an electrodeposited film of PAA-PVI-$[\text{Os(dcl-bpy)}_2\text{Cl}]^{t+/2+}$ redox polymer. The modified electrodes showed high current densities of ~515 μA cm$^{-2}$ towards the reduction of O$_2$ to H$_2$O, even for thin NPG films of 100 nm (as shown in chapter 3). Despite low concentrations of the fuel (D-fructose) of 10 mM, a high power density of up to 13 μW cm$^{-2}$ at an operating potential of 0.18 V vs Ag/AgCl was found. The half-life of ca. 19 h, at an operation potential of 0.2 V, further indicates the potential of FDH/$Bp$BOD EFCs and serves as a proof of concept.
6.2 Recommendations

As a result drawn from the conclusions discussed above, following recommendations for future work, in the field of bioelectronics on NPG substrate, are made:

- Further research is necessary to evaluate the effect the nanoporous structure has on immobilized enzymes. To evaluate if there is an enhanced stability effect due to the protection from unfolding of the protein structure, it is recommended to utilize BpBOD due to the favorable dipole moment that the enzyme exhibits by pH changes in the solution [1]. In addition to the stability effect, the orientation of the enzyme in the nanoporous network also needs to be evaluated. It is anticipated that the orientation of the enzyme in the nanoporous becomes negligible, as the channels become narrow enough for DET to occur on either side of the channel. This might be indicated by screening the DET/MET ratios of differing pore sizes.

- The use of BDS modified NPG electrodes for the detection of Cu\(^{2+}\) in such complex solutions such as artificial serum is a promising result that needs to be further investigated. To analyze the full potential of the system, multi heavy metal sensing experiments should be performed [3]. Heavy metals such as cadmium, lead, copper and mercury can be simultaneous measured as they possess differing stripping potentials of -0.6, -0.3, 0.0 and 0.2 vs Ag/AgCl, respectively [2].

- A wide variety of diazonium film modifications, including amine and carboxylic acid functionalization, was presented throughout this work. CtCDH has been reported to undergo DET on a mixed thiol film of 4-aminothiophenol and 4-mercaptobenzoic acid [4]. Biosensors and biofuel cells based on CtCDH modified NPG electrodes could be used for stable lactose biosensors or lactose/glucose based biofuel cells.

- FDH based biofuel cells have been described throughout this work. These were tested with low concentrations of D-fructose, but still produced considerable power output. The testing of this system should be performed with real samples such as in fruits or juices. It could be imagined to connect a series of fuel cells, to improve the power output, to a DC/DC converter to boost the operational voltage to where it can actually power a real device, such as a LED or a small electromotor. Fruits such as grapes could be used as they possess high fructose concentrations of up to 8 % [5].
6.3 References


Chapter 7:

Appendix: Publications
1 Introduction

The use of gold, has been the focus of significant attention over the past 15 years, and in particular in catalytic applications, which has been referred to as a modern “gold rush” [1]. Hundreds of papers are published annually on innovative uses of nanoparticulate gold [2]. Traditionally gold in its bulk form had always been regarded as an inert and inactive metal. However, the discovery that nanoparticulate gold can act as a catalyst for heterogeneous reactions in both the gas and liquid phases, [2] acted as a trigger for the wide spread use of gold in a range of catalytic reactions.

Nanoporous gold (NPG) has been the focus of many electrochemical investigations, such as the effect of residual Ag in the alloy on the electrocatalytic properties [3], the electrochemical properties of NPG in biofouling solutions [4] or more recently the potentiometric responses of carbon based materials, such as graphite, graphene, carbon nanoparticles, carbon nanotubes (single- or multi-walled SWCNT/MWCNT), carbon fibres (such as carbon cloth (CC) and Toray paper (TP)) or carbon composite materials, have been used in a range of electrochemical applications. The advantages of carbon based electrodes include low cost, ease of fabrication and wide potential windows [14]. Nevertheless, despite these advantages carbon based nanostructures (CBNs) possess a decisive disadvantage. For example, Magrez et al. tested the toxicity effects of CBNs in lung tumour cells and concluded that CBNs are not only toxic, with the hazardous effect being size-dependent, the cytotoxicity of CBNs is enhanced when the surface of the particles is functionalized after acid treatment [15].

The aim of this work is the preparation and evaluation of NPG electrodes for potential applications in biosensors and biofuel cells [16]. Due to the potentially toxic nature of CBNs, carbon based materials are not suitable for this purpose highlighting an advantage of gold based electrodes. While gold nanoparticles can have toxic effects, these have been limited to mechanical effects in the human body due to the small size of nanoparticles. Small nanoparticles (12 nm diameter) can cross the blood brain barrier [17], while objects of 30 nm or while objects smaller...
than 30 nm in size can be adsorbed by living cells [18]. However, to date there have been no reported toxicity effects observed with bulk gold, indicating that NPG is an attractive substrate material for use in implanted biodevices. For such applications, a range of obstacles need to be overcome. The pore sizes of the manufactured NPG electrodes need to be suitable for the intended application. If the pore sizes of the NPG material are smaller than the diameter of the biomolecule, the pores will be inaccessible for immobilization; if the pore sizes exceed the diameter of the biomolecule, the resultant lower active surface areas can lead to lower responses. The wettability and surface addressability of these nanostructures are also crucial.

In this report we describe how the pore diameter of NPG can be adjusted to produce surfaces with pore diameters ranging from ~5 to ~80 nm with differing pore morphologies. The surface addressability of the material was extensively studied and the limitations are demonstrated. The use of these electrodes as supports for the redox proteins cytochrome c and bilirubin oxidase for electrochemical applications is described, focussing on the effect of pore and crack size of NPG on the faradaic response of immobilized cytochrome c and *Myrothecium verrucaria* bilirubin oxidase.

2 Experimental

2.1 Reagents and Materials

Nitric acid (70%), sulfuric acid (95–98%), potassium chloride, potassium phosphate monobasic and dibasic, poly(ethylene glycol) diglycidyl ether (PEGDGE), ethanol 96% (v/v), 6-mercaptop-1-hexanol, 11-mercaptoundecanoic acid, N-cyclohexyl-N-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMC), sodium fluoride (NaF) and cytochrome c from equine heart were obtained from Sigma-Aldrich Ireland, Ltd. All chemicals were used as received unless stated otherwise. *Myrothecium verrucaria* cytochrome c and *MvBOD* was kindly provided by Novozymes. Deionized water with a resistivity of 18.2 MΩcm was obtained from an Elgastat maxima-HPLC (Elga, UK).

2.2 Electrode Preparation

Nanoporous gold electrodes were fabricated on commercially available microscope glass slides (J. Melvin Freed Brand, USA) using thermal evaporation in an ultrahigh vacuum chamber (ORION-5-UHV) at room temperature. The chamber was equipped with 3 metal targets for substrate deposition, Au (AJA International Inc., USA), Ag and Ti (Kurt J. Lesker Company Ltd., UK) with 99.99% purity. Prior to deposition, the glass sheets were exposed to Ar plasma under vacuum to ensure a clean surface for improved adhesion and homogenous metal deposition. A 10 nm Ti adhesion layer was deposited followed by a pure Au layer with one third of the thickness of the following alloy layer. An Ag$_{x}$/Au$_{1-x}$ alloy layer (50 to 1000 nm) was deposited on top of this layer. The sputtered glass sheets were then cut into squares of approximately 0.5–0.7 cm$^2$ using a circular saw. The samples were dealloyed using 70% nitric acid at temperatures ranging from 0°C to 60°C for periods of 1 to 60 minutes. The chemical etching procedure leads to the formation of a nanoporous structure through the continuous dissolution of the Ag content from the alloy.

After the formation of nanopores a silver wire was soldered to the surface using a 99.99% pure indium wire (Sigma-Aldrich Ltd.). The soldering point was supported by epoxy glue (EVO-STIK, Bostik Industries Ltd) to ensure adhesion to the surface. After drying overnight, dielectric paste (Gwent Group, UK) was used to insulate the electrode components and to define an electroactive surface area. The paint was afterwards dried under vacuum.

2.3 Characterization of NPG Electrodes

2.3.1 Electrochemical Characterization

Electrochemical measurements were performed using a CHI1030C multichannel potentiostat or CHI620A/CHI820C potentiostat. NPG electrodes were used as the working electrode in a three electrode setup, with Pt wire (ALS Co. Ltd., Tokyo, Japan) and Ag/AgCl in 3 M KCl (JJ Cambria Scientific Ltd., UK) as the counter and reference electrodes, respectively. The NPG electrodes were cleaned by cycling over the potential range −0.2 to 1.65 V at a scan rate of 0.1 V s$^{-1}$ for 2 cycles in 0.5 M H$_2$SO$_4$ followed by rinsing with deionized water for approximately 30 s. Differential pulse voltammograms (DPV) of cytochrome c were obtained in the potential range from −0.2 to 0.3 V, using a potential increment of 1 mV, pulse amplitude of 50 mV and a pulse period and
width of 0.2 s and 0.016 s, respectively. Linear sweep voltammetry (LSV) of MvBOD was performed in the potential range from 0.7 to 0.1 V with the sweep rate of 2 mV s⁻¹.

2.3.2 Determination of \(A_{\text{real}}\) and Roughness Factor \(R_f\)

\(A_{\text{real}}\), the actual surface area, was determined using an Au oxide stripping technique. The potential of the NPG electrodes was cycled from −0.2 to 1.65 V at 0.1 V s⁻¹ for 2 cycles in 0.5 M H₂SO₄. \(A_{\text{real}}\) was calculated using the capacitance of the electrode and the conversion factor of 390 \(\mu\text{Ccm}^{-2}[20]\). Values of \(R_f\) values were calculated using the ratio of \(A_{\text{real}}\) to \(A_{\text{geo}}\), geometric area. The latter was determined using ImageJ software from a high resolution photograph.

2.3.3 Morphology Characterization

A scanning electron microscope (SEM, Hitachi SU-70) was used to determine the morphology of the nanoporous gold electrodes. SEM images were taken at three different magnifications: 25 k, 80 k and 200 k magnification at a working distance of 2 mm and 10 kV. SEM images, converted into a black and white image with the ISODATA function of ImageJ software, were used to determine the pore size distribution of NPG. The pore sizes were determined by manually measuring the distances between the pores, with at least 35 different measurements performed for each electrode, at least 3 different regions of the electrodes.

2.3.4 Silver Content Determination

The silver content was determined using energy-dispersive x-ray spectroscopy (EDX) performed using the following conditions: 15 mm working distance, 20 kV and ca. 25–30% dead-time. To ensure comparable results the same specifications were used on all sample sheets.

2.4 Enzymes Immobilization Procedures

2.4.1 Cytochrome C Immobilization on NPG

NPG electrodes were exposed to a solution of 6-mercaptop-1-hexanol (1 mM) and 11-mercapto-undecanoic acid (1 mM) in a 60% ethanol and 40% water (v/v) solution. The ethanol concentration was chosen to ensure the thiols were soluble while not affecting the dielectric paste. After 24 hours the gold electrodes were rinsed with ethanol and a 4.4 mM phosphate buffer solution pH 7.0 and then dried in a stream of \(N_2\) to remove loosely attached thiol molecules. The dried electrodes were placed in a 5 mM solution of CMC in 100 mM phosphate buffer at pH 7.0 for 30 minutes at 4 °C. The electrodes were immediately placed in a solution of buffer (4.4 mM phosphate buffer, pH 7.0) containing cytochrome \(c\) (50 \(\mu\text{M}\)), for 1 hour at 4 °C. The modified electrodes were then rinsed carefully with buffer (4.4 mM phosphate, pH 7.0).

Any electrodes which were not used immediately were stored dry at 4 °C.

2.4.2 Immobilization of Myrothecium verrucaria Bilirubin Oxidase (MvBOD) on NPG

MvBOD were adsorbed on nanoporous gold electrodes. A 10 \(\mu\text{l}\) aliquot of the enzyme solution (0.35 mgml⁻¹) was placed on a clean, dry nanoporous gold electrode and allowed to adsorb in the vacuum chamber under pressure (10⁻² mbar) for ~3 min. The electrode was removed from the vacuum chamber, covered with 10 \(\mu\text{l}\) of 15 mgml⁻¹ poly (ethylene glycol) diglycidyl ether (PEGDGE) solution and allowed to dry in air at room temperature for 15 min.

3 Results and Discussion

3.1 Morphology and Pore Size Tuning of NPG

The alloy composition and thicknesses were varied to clarify the effects of etching conditions (dealloying temperature and dealloying time) on the resulting porous structure.

3.1.1 Influence of Alloy Composition

The porous structure may differ significantly as a result of altering the alloy composition during the sputtering process. The alloy compositions used were \(\text{Ag}_{35}/\text{Au}_{65}\), \(\text{Ag}_{50}/\text{Au}_{50}\) and \(\text{Ag}_{65}/\text{Au}_{35}\) in atomic % (at. %). After dealloying of the differing alloy compositions at 40 °C for 15 minutes [21], electrodes were prepared and characterised. As observed by SEM the resultant structures differed substantially from each another. On dealloying the \(\text{Ag}_{35}/\text{Au}_{65}\) alloy (Figure 1A), the pores were evenly distributed across the sample. In comparison with a \(\text{Ag}_{50}/\text{Au}_{50}\) alloy (Figure 1B), the pores of the \(\text{Ag}_{50}/\text{Au}_{50}\) alloy were much larger. The \(\text{Ag}_{65}/\text{Au}_{35}\) alloy however does not display a continuous distribution of pores, with islands of material surrounded by pores. The size of these pores was much smaller than those in Figure 1A. The \(R_f\) value for the \(\text{Ag}_{65}/\text{Au}_{35}\) alloy under these dealloying conditions were ca. 2 times higher than those of the \(\text{Ag}_{50}/\text{Au}_{50}\), which make them appear to be the most suited substrate for high area surfaces. However the level of reproducibility in preparing these electrodes was low.

If the silver content in the alloy is too low no nanoporous structure could be obtained upon dealloying as can be seen with an \(\text{Ag}_{35}/\text{Au}_{65}\) alloy (Figure 1C). At this low concentration of silver, only surface pitting occurs. These results obtained are in good agreement with literature reports [22]. Based on these results, the \(\text{Ag}_{65}/\text{Au}_{35}\) alloy was used in all subsequent experiments.
3.1.2 Influence of Dealloying Time and Temperature

The pore sizes, distribution and general morphology of the generated nanoporous structure depend significantly on the dealloying conditions used. As a basis for dealloying, sputtered Ag\textsubscript{70}/Au\textsubscript{30} alloys with an alloy thickness of 100 nm were prepared. The addressable surface area of these electrodes was varied by altering the etching conditions in terms of the dealloying temperature and time period used. A representative sample of the surface structures is shown in Figure 2. The SEM images were obtained from dealloying 100 nm Ag\textsubscript{70}/Au\textsubscript{30} alloy sheets at differing conditions (at A: 1 min/0.5°C, B: 1 min/20.8°C, C: 5 min/0.5°C, D: 5 min/20.8°C, E: 1 min/40.2°C, F: 1 min/59.9°C, G: 5 min/40.2°C and H: 15 min/60.5°C) respectively. The pore sizes increased from Figure 2A to Figure 2H, ranging from 4.4 nm (Figure 2A) to 78 nm (Figure 2H) as displayed in Table 1. This increase in pore size entailed a decrease in roughness ($R_f$) from A: 44 ± 1 to H: 4 ± 1. This decrease in $R_f$ is due to the fact that the larger pores provide less surface area than the smaller pores, as they possess more void space.

The mechanism for dealloying involves the acid etching of a defined alloy, which entails the removal of the less noble metal, silver, from the alloy [8a]. The amount of silver left in the alloy after dealloying can provide insight into the pore size and the surface roughness (Table 1). The lower the remaining amount of silver in the alloy, the bigger the pores and the lower the roughness. When examining the samples shown in Figure 2, the silver content decreased from A (40.5 ± 0.5%) to G (0.6 ± 0.5%). The results obtained were very reproducible, as evidenced by the low values for the standard deviation.

As already demonstrated by Erlebacher et al., the pore sizes increase by increasing the grade of dealloying. The average pore size change increased from 4.4 (Figure 2A) to 78 nm (Figure 2H). These plots can be used as a model to determine the dealloying conditions needed to generate electrodes with specific roughnesses or pore sizes for defined applications. Figure 2 also gives insight to the morphological transformations during continuous dissolution of silver and rearrangement of the gold structure on the surface interface. Due to the rigid structure of the sputtered alloy on the glass sheets, the structure loses its ability to flexibly move during the dissolution process, leading to fracturing of the surface, as seen in Figure 2A-D. This is in contrast to gold-silver alloy leaves which can...
be stabilized on glassy carbon (GC) electrodes after dealloying. The leaves, which are floated on nitric acid for dealloying [23], possess the flexibility needed to contract during dealloying, showing no cracks or ligaments post dealloying [24]. It can also be observed that these cracks, which are generated from the instant the sheets come in contact with the oxidizing agent, grow and extend (Figure 2A-D) by ongoing etching, to the point when they are no longer distinguishable from pores (Figure 2E-H). These results are summarized in Table 1 with the average crack lengths ranging from 120 nm for sheets dealloyed at 0.5°C and 1 min to 754 nm for sheets dealloyed at 20.8°C and 60 min. Under the latter conditions the cracks form pores by continuous rearrangement of the gold atoms on the surface interface [8c].

The influence of time and temperature is clearly evident from the 3-dimensional plots (Figure 3). An exponential decrease in $R_t$ value was observed with small increases in dealloying time and temperature (Figure 3A). The opposite effect was observed for the average pore size (Figure 3B). Due to the high level of reproducibility of the preparation procedure, these electrodes provide the opportunity to prepare enzymatic biofuel cells and biosensors where the optimum loading of enzyme and efficient usage of electroactive area can be achieved by tuning the surface roughness and the pore size of the support.

### 3.1.3 Surface Addressability of NPG

Surface addressability is a common problem in regard to nanostructured electrodes. These issues are commonly caused by molecules that are too bulky to be able to penetrate through the pores, thus making the generated area inaccessible for the intended applications. These surface addressability issues can also be caused by the inability of the surface area to become fully wet due to diffusion, mass transport issues, capillary forces or pressure differences. For this reason it was necessary to test the NPG electrodes for surface addressability by eliminating the main causes for incomplete wetting. To minimize these issues the NPG electrodes were dried under vacuum in a desiccator to ensure that the pores are as free from water as possible. The electrodes were then used directly, so that capillary forces that would otherwise draw water from the air, by condensation of water (dependant on air humidity) from the air and subsequent capillary pull, are used in a constructive manner to wet the electrodes with the sulphuric acid solution. This capillary pull has been referred to the adjustment of a capillary equilibrium that porous solids occupy, as previously demonstrated with sand in the early 1940’s [25]. A range of pore structures at different alloy thicknesses were prepared to examine the influence of thickness on surface addressability. Under the assumption that surface addressability should be most difficult for the smallest pores, NPG electrodes were manufactured with pore sizes of ~5 nm. A sample plot of NPG electrodes at different thicknesses (50–1000 nm) can be seen in Figure 4. The experiments showed that $R_t$ increases linearly up to a thickness of 500 nm. An example can be seen with an approximate 3 fold $R_t$ increase from 44±1 to 131±12 for electrodes dealloyed at 0°C for 1 min at 100 and 300 nm alloy sheets. Therefore the issues of wettability can be assumed to play no major role for NPG which has been dried in a vacuum chamber before testing. This preparation method was used for subsequent applications with an alloy thickness of 100 nm.

### 3.2 The Effect of Pore Size to Cytochrome C Immobilization

Cytochrome c was chosen as a model redox probe to monitor the influence of pore size on the electrochemical response as it has very well defined electrochemical prop-

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**Table 1. Summary of results obtained during tailoring of nanoporous gold electrodes from 100 nm alloy thick sputtered sheets toward roughnesses and pore sizes for electrochemical applications**

<table>
<thead>
<tr>
<th>Dealloying time [min]</th>
<th>Dealloying temp. [°C]</th>
<th>$R_t$ values</th>
<th>Ag content [at. %]</th>
<th>Average pore size [nm]</th>
<th>Average crack length [nm]</th>
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</thead>
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<td>1</td>
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<td>44±1</td>
<td>40.5±0.5</td>
<td>4.4±0.9</td>
<td>120±36</td>
</tr>
<tr>
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<td>12±1</td>
<td>7.5±0.3</td>
<td>8.7±1.2</td>
<td>277±46</td>
</tr>
<tr>
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<td>40.2</td>
<td>4±1</td>
<td>2.8±0.4</td>
<td>24.2±3.4</td>
<td>477±72</td>
</tr>
<tr>
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<td>3±0</td>
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<td>51.0±3.8</td>
<td>N/A [a]</td>
</tr>
<tr>
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<td>8.1±1.6</td>
<td>209±38</td>
</tr>
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<td>15.2±2.2</td>
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</tr>
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<td>1.6±0.5</td>
<td>41.7±3.4</td>
<td>N/A [a]</td>
</tr>
<tr>
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<td>0.8±0.4</td>
<td>61.8±3.4</td>
<td>N/A [a]</td>
</tr>
<tr>
<td>15</td>
<td>0.6</td>
<td>8±1</td>
<td>6.4±0.4</td>
<td>14.4±2.0</td>
<td>150±23</td>
</tr>
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<td>15</td>
<td>19.7</td>
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<td>2.4±0.3</td>
<td>17.9±3.8</td>
<td>221±27</td>
</tr>
<tr>
<td>15</td>
<td>40.2</td>
<td>4±0</td>
<td>1.8±0.4</td>
<td>45.7±3.7</td>
<td>N/A [a]</td>
</tr>
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<td>1.0±0.5</td>
<td>77.9±4.6</td>
<td>N/A [a]</td>
</tr>
<tr>
<td>60</td>
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<td>5±0</td>
<td>3.5±0.5</td>
<td>28.7±1.8</td>
<td>599±72</td>
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<tr>
<td>60</td>
<td>20.8</td>
<td>3±0</td>
<td>1.9±0.3</td>
<td>43.2±3.8</td>
<td>754±76</td>
</tr>
<tr>
<td>60</td>
<td>40.2</td>
<td>3±0</td>
<td>0.6±0.5</td>
<td>63.7±3.3</td>
<td>N/A [a]</td>
</tr>
</tbody>
</table>

[a] The cracks have been filled through continuous rearrangement of gold, leading to no measurable average crack size for these samples.
erties and can be readily attached to the electrode surface. It can clearly be observed that the response achieved by DPV is highly dependent on the dealloying conditions used in the electrode manufacturing process (Figure 5A). The responses range from approximately 30 to less than 3 μA cm⁻².

The highest current densities were obtained for electrodes with an average pore size of ≈9 nm, where the pores are sufficiently large to be accessible to the redox probe while also possessing a large electroactive surface area. When the pore size was decreased to ca. 5 nm, the current density decreased substantially. While this electrode provides the largest electroactive surface area, the average pore size is close to the actual size of the redox protein and the surface area in the pores is not accessible.

When the average pore size of the NPG electrodes was...
increased, a steady decrease in current density was observed. As cytochrome c should be able to penetrate through this porous structure without difficulty, the response will be limited by the electroactive surface area. This area is gradually decreased by coarsening of the generated pores, leading to the loss of electrochemical response due to lower loadings of the protein. To further clarify the pore size effect, the direct faradaic response of \( \text{MvBOD} \) was examined.

### 3.3 DET of \( \text{MvBOD} \) on Pore Size Tuned NPG Electrodes

\( \text{MvBOD} \) is a ‘blue copper’ protein that reduces oxygen to water. The enzyme possesses high catalytic activity at neutral pH while utilising relatively low overpotentials for the reduction of \( \text{O}_2 \). DET of BOD adsorbed on flat and also unmodified, bare gold electrode is difficult with an unstable faradaic response reported previously [26]. However, the high roughness factor of the support enables significant improvement in the faradaic response and stability of \( \text{MvBOD} \) when compared to a flat gold electrode. As previously described, NPG of roughness factor 26 was used as a support for \( \text{MvBOD} \). The high response arises from the porous morphology of NPG and the high loading of enzyme obtained under the conditions used [27]. Current densities of ca. 65 \( \mu \text{A cm}^{-2} \) were observed at \( \text{MvBOD} \) NPG electrodes prepared by dealloying at 0.5 ℃ for 5 min (Figure 6A). The size of the pores of 8 ± 2 nm for this surface is similar to the size of \( \text{MvBOD} \) (A: 5.3 nm B: 8.4 nm and C: 14.3 nm) [28], but clearly the enzyme is too large to penetrate into the pores.

On plotting the current densities (using the real electroactive surface area rather than the geometric area) against the applied potential, it can be observed that higher enzyme loadings could be achieved with smaller pore sizes, i.e. electrodes prepared at 0 ℃/5 min and 40 ℃/1 min (Figure 7). This change is indicative of a more favourable orientation of \( \text{MvBOD} \) on the surface of these electrodes. The other preparation conditions lead to similar faradaic responses for each system, indicating that a more favourable orientation of \( \text{MvBOD} \) occurred on such surfaces. Similar results have previously been reported by Shleev \textit{et al.} on the influence of Au nanoparticle size on the \( \text{MvBOD} \) response after immobilization [29].

On increasing the temperature of dealloying to 20 ℃ (Figure 6A) (and therefore increasing the pore sizes), the current density decreased. Further increases in the temperature of the process using the same dealloying time of 5 min resulted in a slight decrease in the response. The average pore sizes of NPG fabricated on dealloying at 40 ℃ and 60 ℃ are 42 nm and 62 nm respectively, smaller than the size of the enzyme.

When the dealloying temperature was maintained at 40 ℃ and the time varied from 1 to 5 and to 15 min (pore diameters of 24, 42 and 78 nm, respectively), a similar trend was observed (Figure 6B). Current densities of ca. 23 \( \mu \text{A cm}^{-2} \) were obtained for NPG dealloyed for 1 min.
These conditions of dealloying enable the fabrication of NPG electrodes with an average pore size of 24 nm which is larger than the size of the enzyme. On increasing the time of dealloying to 5 and 15 min, the average pore size doubled to 42 nm and 46 nm, respectively, again resulting in a decrease in the current density (Figure 6B).

From the results obtained with MvBOD, it appears that the faradaic response occurs from enzyme molecules adsorbed at the surface of the electrode. With cyt c in contrast, the optimum response was obtained with electrodes of pore sizes that were larger than the protein, where the protein was incorporated in the pores [21]. To stabilize the response, the electrodes were coated with PEGDGE-epoxy polymer. This cross-linking agent stabilizes the adsorbed MvBOD by creating covalent bonds with nucleophilic groups such as amine, thiol and hydroxyl groups on the surface of the enzyme [30]. However a decrease in the response was observed, with a 20% response obtained after 4 hours (Figure 8).

4 Conclusions

The differences in morphology of NPG electrodes fabricated by sputtering were observed on changing the composition of Au and Ag in the alloy and the temperature and time of the dealloying process. An alloy composition of Ag85/Au15 was the optimal material with a high surface area and roughness factor (Rq) and evenly distributed pores A significant increase in the average pore size from 4.4 to 78 nm was observed on increasing the time from 1 to 60 min and the temperature of dealloying from 0.5 to 60.5 °C. Simultaneously, a decrease in roughness factor (Rq) arising from lower surface areas was observed. The surface area of NPG was fully addressable regardless of the pore size and Rq, increasing linearly up to an alloy thickness of 500 nm. The influence of the pore size on the bioelectrochemical response of cyt c and Myrothecium verrucaria bilirubin oxidase was evaluated. Maximal current densities of ca. 30 μA cm⁻² were observed at cyt c modified NPG electrodes with an average pore size of ca. 10 nm. With bilirubin oxidase, high current densities of ca. 65 μA cm⁻² were detected at MvBOD modified NPG electrodes with the response likely arising from surface adsorption of the enzyme.

Acknowledgements

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Nanoporous Gold Electrodes with Tuneable Pore Sizes for Bioelectrochemical Applications
The Immobilization of Fructose Dehydrogenase on Nanoporous Gold Electrodes for the Detection of Fructose

Till Siepenkoetter,[a] Urszula Salaj-Kosla,*[a] and Edmond Magner*[a]

Nanoporous gold electrodes were utilized as a support for the detection of D-fructose. The immobilization of fructose dehydrogenase was achieved by adsorption on thiol- and diazonium-bound carboxylic acid functional groups and subsequent cross-linking through carbodiimide-initiated amide bond formation. The biosensor showed a linear response in the range 0.05–0.3 mM of D-fructose with a sensitivity of 3.7 ± 0.2 µA cm⁻² mM⁻¹ and a limit of detection of 1.2 µM. The response of the biosensor in a range of natural sweeteners and beverages compared very favorably to the results obtained by a commercially available kit. Accurate readings were obtainable after a very fast response time of less than 5 seconds. The biosensor showed high specificity towards D-fructose in the presence of interfering sugars.

1. Introduction

Fructose is a monosaccharide that does not occur naturally in human diet, but is produced by digestive enzymes in intestinal mucosa. As the initial step of its metabolism in the human body is insulin independent, fructose has been proposed as a “healthier” alternative in comparison to natural sugars and is widely used in artificial sweeteners.[2] Due to the inexpensive production of sweeteners such as high-fructose corn syrup (HFCS), via the enzymatic isomerization of fructose and glucose in starch isolated corn,[3] sweeteners have gradually replaced natural sugars in the production of foods and beverages.[2] These sugar replacements have been widely linked with the epidemic of obesity.[4] Large intakes of fructose have been linked to hepatic steatosis, impaired insulin resistance and glucose tolerance, increased blood pressure and enhanced plasma triglyceride concentrations.[2, 5] In addition to these metabolic effects, high levels of fructose intake have also been reported to have substantial effects on the metabolism of minerals.[2, 4]

Through the ubiquitous presence of sweeteners (in form of fructose), there is a need for the development of rapid and accurate analytical methods to monitor daily sugar intake. Established methods for the quantitative and qualitative detection of D-fructose include chromatographic (such as TLC,[7] GC coupled with MS[8] and HPLC[9]) electrophoretic,[10] titration,[11] gravimetric,[12] calorimetric,[13] fluorimetric[14] and spectrophotometric[15] methods. While these methods are sensitive they are time consuming and require trained laboratory staff. Biosensors pose an alternative method of analysis that can enable rapid and accurate analysis with high levels of sensitivity and substrate specificity.[16] For the enzymatic detection of fructose in solutions, biosensors have often utilized the redox enzyme D-fructose dehydrogenase from Gluconobacter species (FDH).[17] FDH was first described by Adachi et al.[18] The enzyme (EC Nr.: 1.1.99.11) has an approximate molecular weight of ~140 kDa FDH and is comprised of 3 subunits; a flavin adenine dinucleotide (FAD) subunit (MW: 67,000 Da), a heme C (cytochrome c) subunit (MW: 50,800 Da) and a peptide domain of unknown function (MW: 19,700 Da).[19–20] The FAD subunit of the enzyme is linked to the catalytic conversion of D-fructose to 5-dehydro- D-fructose. The reduced form of FAD is then oxidized in an intramolecular electron transfer step to the heme subunit.[19–20] Direct electron transfer (DET) of the heme subunit can then occur at an electrode.[21] Various fructose biosensors based on DET have been reported in the literature. A wide variety of substrate materials based on carbon (carbon paste,[22] screen printed graphene electrodes (SPGE),[23] graphite,[24] glassy carbon (GC),[25] porous carbon[26] multi and single walled nanotubes (MWCNT[27] & SWCNT[28]Pt[22b] and colloidal Au[29] have been used for enzymatic fructose biosensors.

Nanoporous gold (NPG) has been used as an electrode in a number of biosensors and biofuel cells.[30] NPG can be prepared by dealloying an Au alloy through the continuous etching of a less noble metal (such as Ni or Ag) from the alloy.[31] This etching procedure results in the formation of a well-defined three-dimensional nanostructure that has shown to be a beneficial substrate for the immobilization of redox enzymes.[32, 33] Besides being conductive, mechanically stable, and free of surface contaminants (through the etching procedure), NPG was found to increase the storage, thermal and operational stability of redox enzymes such as xylanase,[33] laccase,[34] lipase[35] and bilirubin oxidase.[36] NPG electrodes can be easily modified using alkanethiol based self-assembled monolayers (SAMs). However the chemical stability of thiol based SAMs is easily affected by physiological changes in the test solution (such as pH, temperature, ionic strength and applied potentials).[36] A more suitable method has been introduced by Pissen
In this report, we examine the use of nanoporous gold (NPG) as an electrode support for FDH in the development of a biosensor for fructose. The sensor displayed a linear response over the range 0.05–0.3 mM and was used to accurately determine the fructose concentration in a range of food samples.

2. Results and Discussion

2.1. Preparation and Characterization of NPG

NPG electrodes were prepared by dealloying a silver/gold alloy (Ag 70 at.%, Au 30 at.%) in concentrated nitric acid (70%). The kinetics and mechanism involved in the dealloying process have been extensively described by Erlebacher et al. During the etching process Ag atoms are constantly removed from the alloy, while Au atoms rapidly rearrange on the alloy liquid interface. This process leads to the formation of Au islands that are enriched with Ag atoms on the inside; leading to the characteristic gold bubbles in NPG structures. Through continuous etching, more silver is dissolved at the liquid interface resulting in coarsened gold bubbles. As a result the pore sizes can be tuned by altering the dealloying conditions in terms of temperature, time, concentration of acid, etc. In this study NPG electrodes with average pore sizes ranging from 9–62 nm were prepared using the experimental conditions listed in Table 1. The NPG electrodes displayed uniform distributed bicontinuous structures (Figure 1), that enables the preparation of electrodes, in a reproducible manner.

![Figure 1. SEM images of NPG electrodes dealloyed at A) 20 °C and 1 min, B) 20 °C and 5 min, C) 40 °C and 5 min, and D) 60 °C and 5 min. The average pore sizes are ca. 9, 18, 42 and 62 nm, respectively.](image)

| Table 1. Dealloying conditions utilized and the resulting average pore sizes. |
|-----------------------------|-----------------------------|-----------------------------|
| Dealloying temperature [°C] | Dealloying time [min] | Average pore size [nm]     |
| 20                          | 1                          | 9 ± 1                       |
| 20                          | 5                          | 18 ± 4                      |
| 40                          | 5                          | 42 ± 3                      |
| 60                          | 5                          | 62 ± 3                      |

[a] Determined manually from SEM images (n > 100 data points).

2.2. Surface Modification of NPG and the Effect of Pore Size on the Catalytic Response

After cleaning in acid, surface modification of the electrodes was performed via two methods. In both cases the surface was functionalized with carboxylic acid groups. In the first approach, functionalization was achieved by modification with mercapto-propionic acid (MPA). MPA spontaneously forms a relatively uniform distributed network of molecules on the gold surface leaving the carboxylic acid group exposed to the liquid interface. The negative charge of the carboxylic acid group then favors the physical adsorption of FDH. After adsorption of FDH, carbodiimide (in the form of CMC) was added to facilitate amide bond formation resulting in crosslinking of the enzyme to itself and to the thiol bound carboxylic acid groups, thus forming a more stable film on the electrode surface (MPA-FDH). In the second approach, an aryldiazonium salt (2-carboxy-6-naphtoyle diazonium salt, ND), was electrochemically reduced on the electrode surface. A typical reduction scan of ND on NPG can be seen in Figure S1. The void spaces resulting from the reduction procedure were subsequently filled with MPA (ND-FDH). Crosslinking was achieved as in MPA-FDH modified NPG electrodes.

ND-FDH modified NPG electrodes with average pore sizes ranging from ca. 9–62 nm were prepared to evaluate the influence of pore size on the catalytic current densities. A number of factors need to be considered in evaluating the most efficient system: i) The average pore size needs to be sufficiently large for the FDH enzyme to penetrate through and access all the available area. ii) The length of the binding group (ND in this case) also plays a considerable role. If the chain length is too long, the distance between the enzyme and the electroactive surface area becomes too far for efficient DET, which inevitably leads to a reduced current density. iii) The average pore size should not be too large, as this would lead to low loadings of enzyme on the porous structure again leading to reduced current densities. iv) The nanoporous structure can favor enzyme orientation in such a way that enables improved DET. The influence of the average pore size of ND-FDH modified NPG on the current density is shown in Figure 2. While the blank sample in each case show no catalytic current in 100 mM McIlvain buffer (pH 5.5, 25 °C), the addition of 50 mM D-fructose triggers the catalytic process on scanning from −0.2 to 0.6 V (vs Ag/AgCl). The highest current densities were obtained with NPG electrodes with an average pore size of 42 nm. The current density steadily increased when the average pore size was increased from 9 to 42 nm, with a significant decrease upon increasing the average pore size to over 62 nm. This steep
decrease in current density is associated with the decrease in available electroactive surface area, thus decreasing the enzyme loading which leads to lower current densities. Electrodes with an average pore size of ca. 42 nm were therefore utilized for all subsequent experiments.

2.3. Storage Stability

The two types of modified NPG electrodes (MPA-FDH and ND-FDH) were evaluated for their storage stability. Stability tests were conducted by performing cyclic voltammograms after designated period of times (example shown in Figure S2). In both cases the normalized current density decreased by approximately 50% after the first day (Figure 3). This rapid decrease can be associated with the exposure of the enzyme to the bulk buffer solution during storage. In contrast to the immobilization of enzyme on a diazonium or thiol film the encapsulation of FDH in a polymer matrix minimizes the ability of the enzyme to unfold and therefore leads to more stable biosensors.\textsuperscript{[23]} ND-FDH modified NPG electrodes showed the best stability, and were therefore utilized in the preparation of fructose biosensors.

2.4. Effect of Temperature and pH

The effect of pH and temperature on the response of the biosensor was examined. The optimum pH was found to be between 5.0 and 5.5, with significantly reduced current densities below pH 5.0 and above pH 5.5 (Figure 4 A). At a pH of 7.0 the catalytic activity of FDH was barely detectable. The optimum pH of the free enzyme reported in the literature for the oxidation of d-fructose is 4.0 and FDH was reported to be stable between pH 4.5 and 6.0.\textsuperscript{[18]} The observed shift in optimum pH may arise from the negatively charged carboxylic acid groups on the electrode surface as previously described for negatively charged acetate membranes\textsuperscript{[25]} and carbon nanoparticles.\textsuperscript{[24a]} At a fixed pH of 5.5 the current density increased from 20 to 35 \( \mu \text{A} \cdot \text{cm}^{-2} \) at a scan rate of 5 mV s\(^{-1}\). When the temperature was increased above 37 °C, a drastic decrease in current density was observed (Figure 4B).

2.5. Calibration of the Biosensor

A sample calibration curve of the ND-FDH modified NPG biosensor is displayed in Figure 5 A. The arrows indicate the
absolute concentration of fructose added to the test solution (100 mM McIlvain buffer, 25 °C, pH 5.5) at a defined time. A stable reading was typically obtained within 5 seconds. The linear range of the biosensor was 0.05–0.30 mM with a $K_M$ (Michaelis-Menten constant) value of $0.68 \pm 0.04$ mM. The sensitivity was $3.7 \pm 0.2 \mu A cm^{-2} mM^{-1}$ and the limit of detection was 1.2 μM. When compared to results from other fructose biosensors, the LOD obtained here was the lowest (e.g. 2.4 μM was the next lowest value[43]). When compared to a DET based fructose biosensor where FDH was incorporated into a carbon paste matrix,[44] the biosensor described here compares very favorably in terms of LOD and sensitivity (Table 3) despite operating at a considerably reduced potential. This may be due to favorable orientation and loading of FDH throughout the nanoporous network, decreasing the LOD and improving the sensitivity.

2.6. Interference Study

The response to potential interferences (d-glucose, d-galactose, d-mannitol, and ascorbic acid) was examined (at concentrations of 250 μM, Table 2). After addition of d-fructose a characteristic increase in current density was observed (Figure 6(1)). Upon addition of the sugars (Figure 6 (2–4)) minimal changes in current density were obtained, indicating that the sugars do not interfere with the response of the sensor (the highest response of 5% was obtained with glucose). On addition of ascorbic acid, a $19 \pm 3\%$ increase in current was observed (Figure 6(5)), which likely arises from the oxidation of ascorbic acid.

Table 2. Current density obtained on addition of a range of sugars and ascorbic acid (all at a concentration of 250 μM, n = 4).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Current density [nA cm$^{-2}$]</th>
<th>% response [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-fructose</td>
<td>194.0 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>d-glucose</td>
<td>9.6 ± 0.4</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>d-galactose</td>
<td>2.4 ± 1.2</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>d-mannitol</td>
<td>3.2 ± 0.4</td>
<td>2 ± 0</td>
</tr>
<tr>
<td>ascorbic acid</td>
<td>36.8 ± 1.2</td>
<td>19 ± 3</td>
</tr>
</tbody>
</table>
acid at the bare electrode, despite the relatively low applied potential of 0.15 V (vs Ag/AgCl).

2.7. Analysis of Food Samples

Natural sweeteners (agave, honey and maple syrup) and beverages (cola and a sports drink) with high and low d-fructose concentrations were used. Agave and sports drink are of particular interest as they possess a large excess concentration of either d-fructose or d-glucose. An enzymatic spectrophotometric kit was used as a reference method. A summary of the results obtained can be seen in Table 4. The response of the biosensor compares very well with that of the enzymatic kit. The response of the biosensor in the sports drink was 8% higher than that of the enzymatic kit. This response likely arises from the large excess of d-glucose which has been reported to lead to imprecise readings from the enzymatic kit (Figure S3).

This may also account for the slightly lower values obtained by the biosensor with the Agave and honey samples. For the maple syrup sample, the response obtained with the biosensor was in excellent agreement with the kit. Maple syrup consists of approximately 80% sucrose which is not detected by the enzymatic kit and or the biosensor. Therefore high concentrations of sucrose do not affect the biosensor. In the cola sample, the response of the biosensor was 87% of that of the kit, the reasons for this are unclear and may arise from components in the drink sample. Given that other samples contain high levels of glucose, fructose and sucrose, the lowered response does not arise from the presence of these sugars.

| Table 3. Comparison of fructose biosensors reported in the literature |
|--------------------------|-----------------|-----------------|-----------------|-----------------|
| Adsorption and crosslinked on MPA modified Au electrode | TTF[
| Adsorption and crosslinked on a gold electrode | Coenzyme Q6 | 0.2 (vs Ag/AgCl) | 0.01–1.0 | 2.4 | 5.4 | 29.1 | mA mM⁻¹ cm⁻² | – 30 days | [43] |
| Immobilization in a membrane mimetic layer | HCF[45] | 0.5 (vs Ag/AgCl) | 0.01–0.5 | 10 | - | 15 mAM M⁻¹ cm⁻² | 10% decrease in 4 days | [45] |
| Adsorbed and crosslinked on PAMAM[46] modified Au disc electrode | Os polymer | 0.3 (vs Ag/AgCl) | 0.25–5.0 | - | - | - | 30% decrease after 5 h | [46] |
| Polymer entrapment in SPMWCNTE | 0.15 (vs Ag pseudodo ref.) | 0.1–5 | 2 | 4.2 | 1.6 μA mM⁻¹ cm⁻² | 10% decrease after 1 month | [23] |
| Incorporation of in a carbon paste matrix | DET | 0.4 (vs Ag/AgCl) | 0.5–10 | 75 | 35 | 3 nAMM⁻¹ | Operation stability | 10 h | [44] |
| Adsorption and crosslinking on diazonium modified NPG electrodes | DET | 0.15 (vs Ag/AgCl) | 0.05–0.3 | 1.2 | 0.68 | 3.7 μAM M⁻¹ cm⁻² | 40% remaining after 6 days | this work |


3. Conclusions

The preparation of NPG electrodes with a wide range of average pore sizes as substrates for d-fructose biosensors is described. The NPG electrodes were functionalized with carboxylic acid groups using thiol terminated SAMs and an electrochemically reduced diazonium salt. The adsorption of FDH on these modified electrodes followed by crosslinking occurs in a manner that enables direct electron transfer to occur. The response of the biosensor correlated very well with the results obtained with a commercially available enzymatic kit. The biosensor demonstrated rapid response times (less than...
5 seconds), with a linear range of 0.05–0.3 mM D- fructose concentration, a sensitivity of 3.7±0.2 µA cm⁻²·mM⁻¹ and a limit of detection of 1.2 µM. The addition of a range of sugars did not affect the response. The biosensor is a promising alternative to established analytical measurements for the detection of D-fructose concentrations. The modified bioelectrode could also be useful in the development of biofuel cells.

Experimental Section

Reagents and Materials

Sodium phosphate dibasic, citric acid (anhydrous), nitric acid (70%), sulfuric acid (95–98%), hydrochloric acid (35–37%) 6-aminonaphthoic acid (NA), 3-mercaptopropionic acid (MPA), sodium nitrite, N-cyclohexyl-N-((2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMC), D(-)-fructose, D(+)-glucose, D-galactose, D-mannitol and ascorbic acid were obtained from Sigma-Aldrich Ireland, Ltd. Acetonitrile anhydrous >99.8%, was purchased from Fischer Scientific. All chemicals were used as received unless stated otherwise. Food samples i.e. honey, agave, maple syrup, cola and sports drink were purchased from a local supermarket and were used after appropriate dilution. D-Fructose dehydrogenase from Gluconobacter sp. (FDH) was purchased from Sorachim SA and used at a concentration of 4 mg mL⁻¹. Deionized water with a resistivity of 18.2 MΩ cm was generated by an Elgastat Purelab Pulse system (Elga, UK).

Preparation of NPG Electrodes

Nanoporous gold (NPG) electrodes were prepared using microscope slides (J. Melvin Freed Brand, USA) as the support material. The glass slides were cleaned in an ultra-high vacuum chamber (ORION-5-UHV) at room temperature utilizing an Ar plasma to ensure homogeneous metal deposition and improved adhesion. The slides were sputtered in the vacuum chamber equipped with 3 metal targets (99.99% pure), i.e. Au (AJA International Inc., USA), Ag and Ti (Kurt J. Lesker Company Ltd., UK). A 10 nm thick layer of Ag and Ti (Kurt J. Lesker Company Ltd., UK). A 10 nm thick layer of Ti was deposited to improve adhesion of the subsequent layers. The Ti layer was covered by a gold layer (35 nm) to suppress any electrochemical response from the Ti layer and to improve adhesion of the alloy layer. The latter layer (100 nm) was composed of 70% Ag and 30% Au (at. %) (Scheme 1A). After sputtering the slides were cut into pieces of approximately 0.5–0.7 cm² in area using a circular saw. Dealloying was performed in concentrated nitric acid at a defined temperature and for a defined period of time, depending on the desired average pore size.}\(^\text{[32a,b]}\) NPG with average pore sizes of 9, 18, 42 and 62 nm were dealloyed at 20 °C and 1 min, 20 °C and 5 min, 40 °C and 5 min and 60 °C and 5 min, respectively\(^\text{[32a]}\) (Scheme 1B). The dealloyed sheets were rinsed extensively with deionized water and dried in a vacuum chamber. A silver wire (Farnell Components Ltd. Ireland) was then soldered to the NPG surface using a 99.99% pure indium wire (Sigma-Aldrich Ltd.). The soldering point was mechanically strengthened by the addition of a two component epoxy glue (EVO-STIK, Bostik Industries Ltd) that also suppressed any electrochemical response from the indium wire. The electroactive surface area of the NPG electrode was defined using a dielectric paste (Gwent Group, UK). The resulting NPG electrodes had a geometric area of 0.2–0.4 cm². After drying of the paint the electrodes were ready for use.

NPG Electrode Characterization

The electrochemical stripping of gold oxide by cyclic voltammetry from −0.2 to 1.6 V at 0.1 V s⁻¹ in 0.5 M H₂SO₄ was used to calculate the electrochemically addressable surface area of each NPG electrode by applying a conversion factor of 390 µcm² μF⁻¹. This method also cleans the Au surface. However, continuous scanning also promotes successive etching of the NPG surface resulting in increased average pore sizes, which is not desirable in this case. To avoid substantial changes to the nanopores this step was limited to 2 full cycles. The geometric surface areas (\(A_{\text{geo}}\)) of the NPG electrodes were determined by recording a high resolution photograph of each electrode on a millimeter grid and measuring the area using ImageJ software.\(^\text{[48]}\) The average pore sizes and surface morphology of the NPG electrodes were determined as previously described\(^\text{[32a,b]}\) by taking high resolution SEM images (Hitachi SU-70) of the electrodes. SEM images were taken at a magnification of \(\times 2017\) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

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**Scheme 1.** A) Sputtered glass sheet with layers of titanium, pure gold and gold/silver alloy (bottom to top). B) NPG electrode surface post-dealloying by concentrated nitric acid. C) Electrochemical reduction of ND on the NPG surface utilizing a single scan and subsequent filling of the void spaces with MPA. D) Preparation of a MPA SAM on NPG substrate by immersion over night at 4°C. E) After adsorption of FDH on the two modified electrodes the enzyme was crosslinked using CMC (sizes not to scale).
120,000 for each sample and were afterwards converted into black and white images with the ISODATA function of ImageJ software. These images were used to determine the average pore sizes distribution of NPG. The pore sizes were determined by manually measuring the distances between the pores, with at least 35 different measurements performed for each electrode, on at least 3 SEM images.

Surface Modification of NPG Electrodes and Enzyme Immobilization

Functionalization of the NPG electrodes with carboxylic acid group was achieved by electrochemical reduction of 2-carboxy-6-naphtoyl diazonium salt (ND). ND was prepared in situ by mixing a 2 mL solution (20 mM in acetonitrile) of 6-amino-2-naphthoic acid (NA) with 2 mL of a solution of NaNO₂ (2 mM) in 1 M HCl in an ice bath.[40,45] After deoxygenation of the reaction solution, the potential was scanned from 0.6 to −0.5 V at 200 mV/s vs Ag/AgCl (3 M KCl). To avoid multilayer formation of ND on the NPG surface, this step was limited to one single potential scan.[46,47] Due to the single scan, full coverage of the ND on the NPG surface cannot be achieved. Therefore the void spaces were used to be filled by a short chain thiol, i.e., MPA (1 mM) to reduce substrate oxidation at the bare electrode by immersion in thiol solution overnight at 4°C (Scheme 1C). Thiol modified NPG electrodes were prepared by immersing NPG electrodes in a 2 mM MPA solution overnight at 4°C (Scheme 1D). Prior to enzyme modificiation the surface modified NPG electrodes were thoroughly rinsed and dried in a vacuum chamber. A 20 µl aliquot of 4 mg mL⁻¹ FDH solution was drop cast on to the electrode surface and left in a vacuum chamber (maximum 0.098 MPa) for approximately 3 minutes to enhance the loading of the enzyme.[51] It is important that the enzyme solution is not allowed to dry during this process in order to avoid loss of activity. The electrodes were left at 4°C for 1 h. Crosslinking of FDH was achieved by immersing the electrodes in a solution of CMC (5 mM) at 4°C for 2 h. The response of the NPG electrodes was examined in 100 mM McIlvain buffer at different temperatures and pH’s.

Electrochemical Measurements

Electrochemical measurements were conducted using CHI potentiostat’s (models CHI620 A, CHI802C and CHI1030C). All measurements were performed using a standard three electrode configuration with NPG, Ag/AgCl (in 3 M KCl) and Pt wire as working, reference and counter electrodes, respectively. All current densities were calculated utilizing the geometric surface area. Stability measurements were performed at defined periods of time after storage in 100 mM McIlvain buffer pH 5.5 at 4°C. Calibrations were performed at a potential of 0.15 V vs Ag/AgCl in 3 M KCl, a potential just above the onset potential of ca. 0.0 V for the oxidation of FDH immobilized on the electrode, but not too high to oxidize interfering compounds at the bare electrode.

Measurements of Real Samples

Samples of honey, agave and maple syrup were heated to 60°C under continuous stirring before preparing stock solutions of 1% (w/v)%. Cola and sports drink were used without prior pretreatment. For the determination of the fructose content using a commercial available enzymatic kit (R-biopharm, Cat. No. 10139 106035) the samples were diluted to yield a glucose + fructose concentration of between 0.15−1.0 g L⁻¹. Samples were diluted in McIlvain buffer[50] in the range 0.1−0.3 mM to reduce the concentration of fructose to that of the linear range of the fructose biosensor and then tested directly. The limit of detection (LOD) was determined by multiplying the absolute standard deviation of three blank samples by 3 and dividing by the sensitivity of the biosensor.[53]

Acknowledgements

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: Nanoporous gold · Fructose · Dehydrogenase · Biosensor · Electrochemistry


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Immobilization of Redox Enzymes on Nanoporous Gold Electrodes: Applications in Biofuel Cells

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Immobilization of Redox Enzymes on Nanoporous Gold Electrodes: Applications in Biofuel Cells


Nanoporous gold (NPG) electrodes were prepared by dealloying sputtered gold:silver alloys. Electrodes of different thicknesses and pore sizes were prepared by varying the temperature and duration of the dealloying procedure; these were then used as supports for FAD-dependent glucose dehydrogenase (GDH) (Glomrella cingulata) and bilirubin oxidase (BOx) (Myrothecium verrucaria). Glucose dehydrogenase was immobilized by drop-casting a solution of the enzyme with an osmium redox polymer together with a crosslinked polymer, whereas bilirubin oxidase was attached covalently through carbodiimide coupling to a diazonium-modified NPG electrode. The stability of the bilirubin-oxidase-modified NPG electrode was significantly improved in comparison with that of a planar gold electrode. Enzyme fuel cells were also prepared; the optimal response was obtained with a BOx-modified NPG cathode (500 nm thickness) and a GDH-modified anode (300 nm), which generated power densities of 17.5 and 7.0 μW cm⁻² in phosphate-buffered saline and artificial serum, respectively.

Introduction

The immobilization of enzymes on electrodes is of significant interest in the development of enzymatic fuel cells (EFCs) and biosensors.[1] EFCs have significant potential as a source of clean, renewable energy for low-power devices; however, they are still at an early stage of development.[2] The mode of operation of EFCs is similar to that of conventional fuel cells: the fuel is oxidized at the anode side and the electrons are transferred to the cathode to reduce oxygen to water.[3] In EFCs, enzymes are employed for the oxidation and reduction steps. The most extensively studied systems consist of a bioanode using enzymes including glucose oxidase (GOx), cellobiose dehydrogenase (CDH), or glucose dehydrogenase (GDH), and a biocathode utilizing O₂-reducing bilirubin oxidase (BOx) or lactase.[4] The development of EFCs faces two significant barriers of low power output and reduced lifetimes, which are related to the stability of the enzyme and the rate of electron transfer between the enzyme and the electrode.[5] A range of approaches to overcome these difficulties have been utilized to improve the efficiency of enzyme immobilization and enable direct (mediatorless) electron transfer (DET) between the enzyme and the electrode. These approaches entail using novel enzymes, a range of electrode materials, and a wide range of modified electrodes.[6]

Two main types of materials are used as electrodes for EFCs. Carbon-based nanostructures (CBNs) in a wide range of types, for example, carbon cloth (CC), Toray paper (TP), and morphologies such as nanoparticles, nanotubes, and nanofibers, are easy to fabricate with low cost. A significant advantage of using carbon materials in electrochemical applications lies in the wide potential window that can be used. Graphene-based materials have been reported to cause an activation of the immune system, probably through the response of leukocytes in the system.[7] They have been evaluated as immunotherapy tools, vaccine carriers, and drug delivery systems.[8] Intracellularly localized functionalized multivalved carbon nanotubes (MWCNTs) have also been reported to undergo partial degradation in primary microglia cells.[9] MWCNTs were capable of crossing the blood brain barrier in mouse brain, a property of interest for therapeutic and diagnostic applications.[10] However, these nanomaterials display cytotoxicity effects, which raises issues with biocompatibility.[10] Gold, as a noble metal, is biocompatible and is utilized in a wide range of applications for which good biocompatibility is required.[11] Nanoporous gold (NPG) can be prepared by dealloying bimetallic alloys, that is, the less noble element (Ag, Ni, etc.) is removed to produce a gold material with a 3D network of tunable pore sizes and channels that can be used to immobilize enzymes.[12] NPG is conductive, chemically and mechanically stable, free of surface issues with biocompatibility.
contaminations, and readily functionalized; such properties are attractive for applications in biosensors and biofuel cells.[14] The encapsulation of biocatalysts has been studied extensively.[14] The storage and thermal and operational stability of enzymes such as xylanase, laccase, and lipase could be improved significantly upon immobilization in nanoporous gold structures. In other words, the physical confinement of enzymes in porous structures is a significant factor in enzyme stabilization.[15]

In this report, NPG electrodes of high surface area are used as supports for two redox enzymes: FAD-dependent GDH and Myrothecium verrucaria BOX. Glomarella circumdata FAD-GDH overexpressed in Pichia pastoris (GDH) is an extracellular, glycosylated enzyme that is specific for just two substrates: β-D-glucose and o-xylose. The catalytic oxidation of β-D-glucose by GDH is unaffected by oxygen, unlike that with GOx in which turnover of oxygen reduces the current and produces hydrogen peroxide, which can degrade the enzyme.[16] GDH has good stability and a high turnover rate, making it a good candidate in place of glucose oxidase (GOx), which is widely used in glucose sensors and EFCs.[17] BOX is a “blue copper” oxidase that reduces oxygen to water, and has been used extensively as the cathodic enzyme in EFCs. The enzyme is a monomeric redox protein with a molecular mass of 66 kDa and an isoelectric point of 4.2.[18] Different approaches have been undertaken to immobilize BOX on NPG electrodes.[19] Mediated electron transfer (MET) using osmium redox polymers is utilized frequently; however, this introduces mediators that decrease the redox potential of the cathode. Direct (mediatorless) electron transfer (DET) is desirable, but it is difficult to obtain systems that possess high faradaic activity and long-term stability. NPG electrodes can be chemically modified to help overcome these difficulties. Alkanethiol self-assembled monolayers (SAMs), although widely used, are difficult to obtain systems that possess high faradaic activity and long-term stability. NPG electrodes can be chemically modified to help overcome these difficulties. Alkanethiol self-assembled monolayers (SAMs), although widely used, are difficult to obtain systems that possess high faradaic activity and long-term stability. NPG electrodes can be chemically modified to help overcome these difficulties. Alkanethiol self-assembled monolayers (SAMs), although widely used, are difficult to obtain systems that possess high faradaic activity and long-term stability. NPG electrodes can be chemically modified to help overcome these difficulties.

Results and Discussion

As described previously,[20] the confinement of enzymes can improve their long-term stability and activity by protecting them from biofouling agents or by diminishing the ability of the proteins to unfold. NPG electrodes with a range of pore diameters and surface morphologies were prepared and examined for use in EFCs.

Characterization of NPG

The structure and morphology of a metal surface produced by chemical etching of a bimetallic alloy can vary substantially depending on the etching conditions.[21] NPG electrodes with a wide range of pore and crack sizes and substrate thicknesses (Table 1) were utilized to evaluate the effects on current density and stability. Figure 1 shows examples of the electrode surfaces prepared using the same dealloying conditions (20 °C for 15 min). Upon varying the dealloying conditions, pores ranging from 9 to 60 nm in diameter could be prepared (Table 1), and the cracks on the surface ranged from 220 to 3180 nm in length. These electrodes were used as supports for BOX and GDH for use as cathodes and anodes in biofuel cells.

Table 1. List of alloy thicknesses and average pore and crack sizes obtained under different dealloying conditions.

<table>
<thead>
<tr>
<th>Alloy thickness (nm)</th>
<th>Dealloying temp. (°C)</th>
<th>Dealloying time [min]</th>
<th>Ave. pore size [nm]</th>
<th>Ave. crack length [nm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>20</td>
<td>1</td>
<td>9 ± 1</td>
<td>280 ± 50</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>15</td>
<td>18 ± 4</td>
<td>220 ± 30</td>
</tr>
<tr>
<td>100</td>
<td>40</td>
<td>5</td>
<td>42 ± 3</td>
<td>NA[4]</td>
</tr>
<tr>
<td>100</td>
<td>60</td>
<td>5</td>
<td>62 ± 3</td>
<td>NA[4]</td>
</tr>
<tr>
<td>300</td>
<td>20</td>
<td>5</td>
<td>16 ± 2</td>
<td>1500 ± 240</td>
</tr>
<tr>
<td>300</td>
<td>20</td>
<td>15</td>
<td>24 ± 3</td>
<td>1400 ± 400</td>
</tr>
<tr>
<td>300</td>
<td>40</td>
<td>5</td>
<td>32 ± 2</td>
<td>1000 ± 300</td>
</tr>
<tr>
<td>300</td>
<td>60</td>
<td>5</td>
<td>34 ± 3</td>
<td>900 ± 200</td>
</tr>
<tr>
<td>500</td>
<td>20</td>
<td>5</td>
<td>17 ± 2</td>
<td>2100 ± 440</td>
</tr>
<tr>
<td>500</td>
<td>20</td>
<td>15</td>
<td>20 ± 2</td>
<td>3200 ± 800</td>
</tr>
<tr>
<td>500</td>
<td>40</td>
<td>5</td>
<td>27 ± 3</td>
<td>3400 ± 680</td>
</tr>
<tr>
<td>500</td>
<td>60</td>
<td>5</td>
<td>35 ± 3</td>
<td>3800 ± 400</td>
</tr>
</tbody>
</table>

[a] No cracks were observed.

Influence of surface morphology and nanopore layer thickness on catalytic responses

BOX was attached covalently (Scheme 1) to the NPG electrodes,[22] whereas GDH was immobilized by drop-casting the enzyme with an Os redox polymer in the presence of a cross-linking polymer, PEGDGE.[46] Because of the nature of the two different coupling methods, the observed catalytic responses of the enzymes may be altered significantly. Using a single potential cycle, the electrochemical reduction of the 6-amino-2-napthoic acid diazonium salt (NA) synthesized in situ at the surface of the electrode enables close to monolayer modification, as previously reported on gold-nanoparticle-modified gold disk electrodes.[20] After attachment of diazonium to the gold surface, a short-chained, thiol mercapto-propionic acid
with the surface wettability, the accessible electrode area may be reduced in the preparation of GDH-modified electrodes.\[26a\]

The highest current densities (using the geometric surface area) were achieved for NPG electrodes with a thickness of 500 nm and an average pore size of approximately 17 nm (Figure 2A). Current densities of around 800 \(\mu\text{Acm}^{-2}\) were ob-

(MPA), was used to block the surface completely and avoid rapid denaturation\[23\] of the enzyme at the bare gold surface. MPA is also capable of promoting DET between BOx and gold electrodes, potentially improving the catalytic current.\[26a\] Provided the entire surfaces of the pores are accessible to the en-

Figure 1. SEM images of NPG prepared by dealloying at 20 °C for 15 min using substrate thicknesses of A,B) 100 nm, C,D) 300 nm, and E,F) 500 nm.

Scheme 1. Reactions used for the covalent attachment of BOx on gold.

Figure 2. Plots of current density for BOx/diazonium-modified gold electro-
des of different thicknesses as a function of average pore diameter using the A) geometric and B) electrochemically addressable surface areas. Current densities were calculated at a potential of 0 V vs. Ag/AgCl after subtraction of the background density in saturated \(\text{N}_2\).

obtained in \(\text{O}_2\)-saturated PBS buffer, which are slightly lower than the diffusion-controlled limiting current of approximately 1200 \(\mu\text{Acm}^{-2}\) predicted by the Randles–Sevcik equation \(\left[D_{\text{O}_2}\right.\) of

\(2.6 \times 10^{-5} \text{ cm}^2\text{s}^{-1}\.[29]\) and \([\text{O}_2]\) of 1.23 mM].\[30\] In comparison, electrodes with approximately the same average pore sizes but with thinner layers, that is 300 and 100 nm, had current densities (geometric) of about 180 and 60 \(\mu\text{Acm}^{-2}\), respective-

lys. The higher current density of the 500 nm electrodes arises in part from the increased thickness of the electrode. Based on the thickness alone, the response is predicted to be 300 \(\mu\text{Acm}^{-2}\); clearly, additional factors such as crack formation on the NPG surface need to be taken into account. The sizes of the cracks that develop on the NPG during the dealloying process need to be considered. As silver is etched from the alloy, the rapid rearrangement of gold atoms at the interface
can cause cracks to appear on the surface, which are subsequently refilled upon continuous etching. These cracks can increase the catalytic current in two ways. First, the channels could bind Box in a manner that may result in a more favorable orientation of the enzyme. In addition, the supply of O₂ to the enzyme in the cracks may be enhanced owing to the improved access to the electrode surface. In general, optimal current densities (geometric) were achieved for electrodes with average pore sizes ranging between 10 and 25 nm for all electrodes, and the nanopore layer of 500 nm thickness gave the highest response in each case. The increase in layer thickness gave rise to a corresponding increase in current density (geometric) for each electrode thickness, indicating that the response was limited neither by access to the full length of the pores, nor by the rate of diffusion of oxygen through the nanoporous structures. Slightly different behavior was observed upon utilization of the current densities based on the electrochemically active surface areas as determined by the gold oxide stripping technique (Figure 2b). Whereas the nanopore electrodes of 100 and 300 nm thickness show similar trends, the 500 nm electrodes had a much higher response of up to 18.0 μA cm⁻². The highest current density, as determined by the geometric surface area, did not correspond to the maximum current density determined using the electrochemically addressable surface area, indicating that the surfaces of the electrodes are not fully available to the enzyme. This is probably because electrodes with an average pore size of about 16 nm possess a structure that hinders the immobilization of Box (dimensions: A = 4.0, B = 5.0, and C = 6.0 nm) deep in the porous structure. On increasing the average pore size above 20 nm, the electrochemically addressable surface area decreased, resulting in a lower response owing to the smaller amounts of catalytically active enzyme; such an effect was also observed previously for glucose oxidase. No catalytic response was obtained in control experiments using N₂-saturated PBS (data not shown).

Electrodes prepared using GDH and osmium redox polymer show significant differences, with the geometric current density independent of the average pore size (Figure 3A) for both the 100 and 300 nm nanopore layers. Note that in control experiments, the ability of an unmodified NPG electrode to oxidize glucose was examined, and no response was observed. The electrodes of 100 nm thickness exhibited responses in the same range as planar gold electrodes, that is, approximately 100 μA cm⁻² (Figure 3A). This response is in agreement with previous work with Box and an osmium polymer matrix, which showed near identical responses for planar and NPG electrodes for which the response was limited by planar diffusion of the substrate.

The electrodes of 300 nm thickness, however, had current densities two times higher, that is, around 250 μA cm⁻² (Figure 3A). This increase is also probably caused by the larger cracks on the electrode surface, which are likely to be more accessible to the relatively viscous drop-casting mixture of osmium redox polymer, GDH, and crosslinker PEGDGE than the pores themselves. As previously reported, this improved accessibility results in an increased surface coverage of the polymer matrix on the gold surface, leading to enhanced current densities. Upon consideration of the current density calculated using the electrochemically addressable surface area, there was no significant difference found between the electrodes of 100 and 300 nm thickness, indicating that the accessible enzyme loadings were similar. It was also evident that upon increasing the average pore size of the NPG, the current densities approached those of planar electrodes. On the 500 nm electrodes, the polymer film was not mechanically stable and became detached upon swelling of the polymer film. This instability is probably caused by the wide cracks present on these electrodes (Figure 1E), which impede the stable attachment of the polymer film to the surface. The results indicate that the polymer/enzyme matrix is unable to penetrate fully into the porous network of the NPG electrodes. Upon increasing the average pore size, the current density (based on the electroactive surface area) increased and approached the values achieved at planar gold electrodes (Figure 3B).

From these optimization studies, the electrodes with the highest geometric current densities were examined for use in an enzymatic fuel cell. For the cathode, a 500 nm-thick NPG electrode with an average pore size of approximately 17 nm was utilized, and for the anode, a 300 nm-thick NPG electrode with an average pore size of around 16 nm was employed.

Figure 3. Plots of current density versus pore size for GDH/Os polymer-modified electrode with different electrode thicknesses based on the A) geometric and B) electrochemically addressable surface areas at 5 mV s⁻¹ and 10 mM glucose concentration.
This combination is labeled EFC1. As a comparison, an EFC that possesses lower current densities on both electrodes was prepared. This consisted of a 500 nm-thick cathode with an average pore size of approximately 20 nm and a 300 nm anode with an average pore size of around 24 nm (EFC2).

**Stability of enzyme-modified NPG electrodes**

Box was attached covalently to the gold surfaces (planar and nanoporous gold), whereas GDH was encapsulated in an osmium polymer matrix and drop-cast on the electrode surface. The catalytic response for a biocathode with a 300 nm alloy layer and an average pore size of approximately 24 nm was compared with that obtained at a planar gold electrode (Figure 4A). The response at the NPG-modified electrode was more stable than that at the planar gold electrode. For the NPG electrode, catalytic responses were obtained for up to 200 h; in contrast, planar gold electrodes only showed a response for up to 50 h. The half-lives of the biocathodes were 20 and 4 h for NPG and planar gold, respectively. Note also that coupling of BOX through diazonium improved the stability of the electrodes substantially. In a previous report, we demonstrated that physically adsorbed BOX on NPG only showed catalytic activity for 4 h. In addition to the higher electrochemically active surface area, NPG electrodes clearly provide a more stable environment for the immobilized BOX enzyme. In contrast to the biocathode, the bioanode did not demonstrate an increase in stability (Figure 4B). This is probably because of the relatively higher viscosity of the solution, which makes it more difficult to penetrate through the porous structure and adsorb onto the electrode surface; in essence, the porous structure is not as available.

**Application as a BFC**

The EFC1 fuel cell (Figure 5A) produced a maximum power density of 17.5 \( \mu \text{Wcm}^{-2} \) (at a potential of 0.193 V) in a 50 mM phosphate buffer at a glucose concentration of 5 mM. On testing in artificial serum, a power density of 7.0 \( \mu \text{Acm}^{-2} \) was obtained (at a potential of 0.166 V). Such a decrease probably arises from the increased solution viscosity together with fouling of the electrode surface by bovine serum albumin (BSA). The power density of EFC2 (Figure 5B) in 50 mM phosphate buffer was lower than that of EFC1, with a maximum value of 10.4 \( \mu \text{Wcm}^{-2} \) (at a potential of 0.197 V), and also decreased in artificial serum to 6.5 \( \mu \text{Wcm}^{-2} \) (at a potential of 0.156 V). Note that although the power densities of EFC1 and EFC2 differed,
the responses in artificial serum were similar, indicating that the response was limited by the solution viscosity and the biofouling effects.\[34\]

On the basis of the two- to threefold difference in the response (Figures 3 and 4), the response of the biofuel cell in buffer is limited by the anode; the same limitation is likely to pertain in artificial serum. Both cells had open-circuit voltage (OCV) of approximately 0.45 V. The response of the EFCs compares well with the responses described in recent reports of EFCs based on gold electrodes (Table 2). A biofuel cell based on 1-[bis(2-naphthoquinonyl) aminomethyl]pyrene (pyr-(NQ))\[34\]-modified multiwalled carbon nanotubes (MWCNTs), with immobilized Box and POQ-GDH, generated a power density of around 14 μW cm$^{-2}$ in 10 mM glucose. In comparison to this report, the biofuel cell described here has a high power density and improved stability. The highest power density was achieved on an ordered macroporous gold electrode with an output of 178 μW cm$^{-2}$. However, this response was based on a high glucose concentration (30 mM) under non-physiological conditions (acetate buffer at pH 6.0).\[38\] Stability data have been reported on two of the cells. The stability of an EFC based on Au nanoparticles/polycrystalline Au\[36\] had a half-life of 8 h. An EFC based on Au nanoparticles on a Au microwire showed a 10% decrease in response in 2 h.\[38\]

By comparison, the EFCs described here have improved half-lives, demonstrating that NPG provides a stable support for applications in EFCs (Figure 6). NPG-based EFCs modified with Box as the cathode and GDH as the anode could retain approximately 60% of the initial power density after 8 h continuous operation. In contrast, the planar Au modified EFCs retained less than 40% of the initial power density after continuous operation. This increase in operation stability is associated with the increased stability of the biocathode, which is protected from accelerated deactivation owing to the embedding of the enzyme in the porous structure.

### Table 2. Power densities and OCVs of EFCs based on gold electrodes.

<table>
<thead>
<tr>
<th>Substrate material</th>
<th>Anode</th>
<th>Cathode</th>
<th>Power density [μW cm$^{-2}$]</th>
<th>OCV [V]</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Au nanoparticles on polycrystalline gold[36]</td>
<td>CitCDH, 5 mM glucose</td>
<td>MvBox, air saturated</td>
<td>3.0</td>
<td>0.63</td>
<td>human plasma pH 7.4</td>
</tr>
<tr>
<td>Au leaf NPG[37]</td>
<td>AngGOx, 5 mM glucose</td>
<td>MvBox, O$_2$, saturated</td>
<td>3.4</td>
<td>0.56</td>
<td>PBS pH 7.0</td>
</tr>
<tr>
<td>Au nanoparticles on gold microwires[36]</td>
<td>CitCDH, 2.5 mM glucose</td>
<td>MvBox, air saturated</td>
<td>ca. 4</td>
<td>0.66</td>
<td>PBS pH 7.4</td>
</tr>
<tr>
<td>3D ordered macroporous Au[36]</td>
<td>EGDH, 30 mM glucose</td>
<td>laccase, air saturated</td>
<td>178</td>
<td>0.52</td>
<td>acetate buffer pH 6.0</td>
</tr>
<tr>
<td>Highly ordered macroporous Au[36]</td>
<td>AngGOx, 10 mM glucose</td>
<td>laccase, O$_2$, saturated</td>
<td>38</td>
<td>0.52</td>
<td>PBS pH 7.4</td>
</tr>
<tr>
<td>Pyr-(NQ)$_2$/MWCNT, POQ-GDH, 10 mM glucose</td>
<td>Pyr-(NQ)$_2$/MWCNT, BOx, air saturated</td>
<td>ca. 14</td>
<td>0.62</td>
<td>MOPS buffer pH 6.5</td>
<td></td>
</tr>
<tr>
<td>This study</td>
<td>GcGDH, 5 mM glucose</td>
<td>MvBox, O$_2$, saturated</td>
<td>17.5</td>
<td>0.45</td>
<td>PBS pH 7.4</td>
</tr>
</tbody>
</table>

**Conclusion**

NPG electrodes were prepared with average pore sizes ranging from 9 to 60 nm and average crack sizes ranging from 220 to 3770 nm, and then evaluated for use as the anode and cathode electrodes in enzymatic fuel cells. Box was attached covalently to the electrode through carbodiimide coupling to a diazonium-modified NPG surface. Anodes were prepared by crosslinking an osmium redox polymer with GDH on NPG. The optimal responses of 800 and 250 μA cm$^{-2}$ were obtained with a Box-modified electrode of 500 nm in thickness with an average pore size of 15 nm, and a GDH-modified electrode of 300 nm in thickness with an average pore size of approximately 15 nm, respectively. Although the average pore size had no effect on the response of the anode, the catalytic response of the cathode electrode increased linearly with the thickness of the layer. Box-modified NPG electrodes demonstrated improved half-lives (factor of four) compared with planar modified electrodes, whereas GDH-modified electrodes showed no improvement in stability. Enzymatic fuel cells were prepared; the optimal response occurred with a 500 nm Box-modified NPG cathode and a 300 nm GDH-modified anode, which generated power densities of 17.5 and 7.0 μW cm$^{-2}$ in PBS and artificial serum, respectively, at an OCV of approximately 0.45 V. These EFCs retained over 60%
of their initial power densities after 8 h of continuous operation.

**Experimental Section**

**Reagents and materials**

Nitric acid (70%), sulfuric acid (95–98%), potassium phosphate monobasic and dibasic, potassium chloride, 4-morpholineethanesulfonic acid (MES), 6-amino-2-naphthoic acid (NA), 3-mercaptopropionic acid (MPA), sodium nitrite, N-cyclohexyl-N’-(2-morpholinoethyl) carbodiimide metho-p-toluenesulfonate (CMC), polyethylene glycol (diglycidyl ether (PEGDEG), uric acid, l-ascorbic acid, α-(-)-fructose, α-(+)-glucose, α-lactose, urea, l-cystine, calcium sulfate dehydrate, magnesium sulfate, and albumin from bovine serum were obtained from Sigma-Aldrich, Ireland, Ltd. Acetonitrile anhydrous (> 99.8%), sodium chloride, and sodium bicarbonate were purchased from Fischer Scientific. All chemicals were used as received unless stated otherwise. The osmium red polymer [Os[2(2’-bipyridine)_2]Cl] with an E° potential of 220 mV versus Ag/AgCl was prepared according to a published procedure.[41] Myrothecium verrucaria bilirubin oxidase (BOx) was kindly provided by Novozymes. GDH was prepared as described previously.[42] An Elgastat maxima-HPLC (Elga, UK) was used to obtain deionized water with a resistivity of 18.2 MΩ cm.

**Preparation of NPG electrodes**

Commercially available microscope glass slides (J. Melvin Freed Brand, USA) were utilized as the substrate for magnetron sputtering in an ultra-high-vacuum chamber (ORION-5-UVH) at room temperature. The sputtering chamber was equipped with three metal targets for deposition: Au (AJA International Inc., USA), Ag, and Ti (Kurt J. Lesker Company Ltd., UK) with 99.99% purity. Prior to deposition, the glass sheets were exposed to Ar plasma under vacuum to ensure a clean surface for improved adhesion and homogenous metal deposition. A 10 nm Ti adhesion layer was first deposited, followed by a pure Au layer with a thickness of one third that of the subsequent layer. This pure Au layer was required to suppress any possible electrochemical response from the Ti layer. A Au/Ag/Au layer with thickness varying from 100 to 500 nm was then deposited. The glass sheets were cut into squares of approximately 0.5–0.7 cm. After preparation of the NPG electrodes, they were cleaned in 0.5 M H_2SO_4 by cycling the potential between −0.2 and 1.6 V at 0.1 V s⁻¹ versus Ag/AgCl (3 M KCl). This step was limited to two cycles to minimize changes to the structure of the nanopores.[23] The electrodes were rinsed with deionized water and dried in a vacuum chamber. For the covalent attachment of BOx, a fresh solution of 2-carboxy-6-naphthoyl diazonium salt (NA-DS) was synthesized by mixing a 2 mL solution (20 mM in acetoniitrile) of 6-amino-2-naphthoic acid (NA) with 2 mL of a solution of NaNO₂ (2 mM) in 1 M HCl in an ice bath.[26] A single potential scan from 0 to −0.5 V at 200 mV s⁻¹ was performed for electrochemical reduction of the NA diazonium salt to the surface (see Scheme 1). After rinsing with deionized water, MPA was attached to the surface by immersion of the electrodes in 1 mM MPA solution overnight. Prior to enzyme attachment, the NA-MPA-NPG electrodes were rinsed thoroughly with deionized water and dried in a vacuum chamber. Subsequently, a solution of BOx (20 μL, 0.36 mg mL⁻¹) diluted with MES buffer (0.01 M, pH 6.0) was drop-casted onto the surface of the electrode. The electrodes were exposed to vacuum (maximum 0.098 MPa) for approximately 3 min to improve the penetration depth of the enzyme, and were stored at 4 °C for 1 h. This procedure was found to improve the loading of the enzyme throughout the porous structure, leading to higher current densities, but did not appear to change the enzyme activity.[19a] Crosslinking was achieved by immersing the modified electrodes in 5 mM CMC solution at 4 °C for 2 h. The electrodes were then tested in 0.1 M PBS (K⁺) with 0.2 M KCl as supporting electrolyte, by scanning the potential from 0.7 to 0 V at 10 mV s⁻¹. For the GDH-modified electrodes, a solution of the redox polymer Osg(bpy)₃(PVIL)₂(Cl (8 μL, 6 mg mL⁻¹), GDH solution (4.8 μL, 10 mg mL⁻¹) and the PEGDEG crosslinker (1.9 μL, 15 mg mL⁻¹) was prepared. This freshly prepared enzyme/polymer/crosslinker solution (20 μL) was then drop-cast on the electrodes. After exposure to a vacuum of maximum 0.098 MPa for approximately 3 min, the electrodes were allowed to dry at 4 °C overnight.

**Electrochemical measurements**

All electrochemical measurements were performed using a CHI660A potentiostat operating in a standard three-electrode configuration with NPG, Ag/AgCl (in 3 M KCl), and Pt wire as working, reference, and counter electrodes, respectively. The experiments were conducted in 50 mM PBS (pH 7.4) and in artificial serum, which was mixed according to the average quantities of the following compounds in a healthy human body: uric acid (0.36 mM), l-ascorbic acid (0.054 mM), α-(+)-glucose (5 mM), α-(−)-fructose (0.2 mM), α-lactose (15 μM), urea (4.45 mM), l-cystine (0.075 mM), calcium sulfate dehydrate (2.38 mM), magnesium sulfate (0.87 mM), sodium chloride (103 mM), sodium bicarbonate (25.5 mM), and 7% BSA.[31]

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