Nanoporous gold electrodes as matrices for enzyme immobilization for application in biosensors and biofuel cells

Thesis presented for the award of
Doctor of Philosophy (Ph.D.)

by

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Under the Supervision of Professor Edmond Magner

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DECLARATION

The work presented here, unless otherwise stated, is the original work of the author and was carried at the University of Limerick. No part of this thesis has been previously submitted to this or any other university.

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Urszula Sałaj-Kośla
September 2012
ABSTRACT

Robust nanoporous gold electrodes were fabricated by sputtering a gold-silver alloy onto a glass support and subsequent dealloying of the silver component. Alloys were prepared with either a non-uniform or uniform distribution of silver alloy which showed clear differences in morphology on characterization with scanning electron microscopy. The surface area of these electrodes was up to 28 times that of the geometric surface area. The surface area accessible to modification by redox proteins was determined using cyt c as a model system. Covalent immobilization of cyt c at SAMs modified planar and nanoporous gold electrodes resulted in ca. 9 and 11 times higher surface coverages at uniform and non-uniform nanoporous gold, respectively, than at planar gold electrodes.

Well defined mediatorless bioelectrocatalytic reduction of oxygen was obtained on nanoporous gold electrodes prepared using a vacuum method and subsequently modified with Myrtocadium verrucaria bilirubin oxidase (MvBOD). Diffusion limited faradaic response, with current densities of 0.8 mA/cm², was observed when the enzyme modified electrode was stabilized with a layer the P017-epoxy polymer. The enzyme, Trametes hirsuta laccase (ThLc) also displays direct electron transfer at unmodified nanoporous gold electrodes. The observed current densities of ca. 0.03 mA/cm² were 10 times higher than the current densities at the ThLc modified electrode made by drop-casting and are in contrast to the absence of a response at unmodified polycrystalline gold electrodes.

Nanoporous and planar gold electrodes modified with Aspergillus niger glucose oxidase (GOx) and Corynascus thermophilus cellobiose dehydrogenase (CtCDH) together with Os redox mediators and PEGDGE as a cross-linking agent resulted in glucose and lactose detecting biosensors. The sensors had \((I_{\text{max,app}}, K_{M,app}, \text{sensitivity})\), limits of detection (LOD). GOx and CtCDH modified electrodes were utilized as anodes with MvBOD and Melanocarpus albomyces Le (rMaLc) modified cathodes in biofuel cells. A maximal power density of 41 µA/cm² for the CtCDH/MvBOD biofuel cell in 5 mM lactose and O₂ saturated buffer was obtained. The power densities of the biofuel cells were also evaluated in artificial plasma, where decreased values were observed.
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‘Now this is not the end. It is not even the beginning of the end.

But it is, perhaps, the end of the beginning’

- Winston Churchill
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- Bioelectrochemistry of Redox Enzymes Immobilized on Mesoporous Gold Electrodes, 8th – 12th May, 2011, XXI International Symposium on Bioelectrochemistry and Bioenergetics in Cracow, Poland

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LITERATURE REVIEW
1.1 Proteins in electrochemistry

Electrochemical methods allow the characterization of biological charge transfer systems. Furthermore, they enable the redox properties of enzymatic proteins and an understanding of biological electron transfer processes to be obtained. However, examining the electrochemical properties of redox enzymes is difficult. For example, the native state of a protein can be compromised when immobilized on the electrode surface [1]. Enzymes are functionalized proteins containing a redox center within a complex polypeptide structure. They possess some remarkable properties e.g. high catalytic efficiency. Their selectivity and specificity to particular substrates often allow them to separate even racemic mixtures [2-3]. There are two approaches to achieving effective coupling between an enzyme and an electrode, the approach used is determined by the distance between the redox center and the electrode surface, the orientation of the redox centre and the conformation of the protein. Direct or unmediated electron transfer (DET) is possible when the protein redox center is sufficiently close to the electrode surface, properly orientated and the reasonably rapid electron exchange between the redox centre and the electrode is possible. Otherwise mediated or indirect electron transfer (MET) may be required.

A number of metal electrodes are used as supports for redox enzymes, e.g. Au [4], Ag [5], Bi [6], Hg [7], Al [8], Pt [9]. The reduction of cytochrome c (cyt c) on Hg was first described in 1972 by Betso et al. [10]. However, because of the material of the electrode, that resulted in protein denaturation, the reversible electron transfer was not obtained [10]. Similar effects were also observed on bare electrode surfaces for other redox proteins. Bare metal electrodes are generally recognized to be largely incompatible with biological molecules.

Cyt c (from equine heart) is a basic redox protein with a molecular weight of 12300 g mol$^{-1}$ and an approximately spherical shape of diameter 34 Å [11]. It participates in photosynthesis and in biological respiration as an electron shuttle. Cyt c consists of a single polypeptide chain of 104 amino acids covalently attached by two thioether bonds at Cys14 and Cys17 to a heme group. The polypeptide chain is organised into five α-helical segments and six β-turns. The amino acids of cyt c are...
classified according to the degree of exposure of their side chain into three groups: exposed, partially exposed and buried. Naturally, most of the hydrophobic residues are buried and most of the charged residues are exposed. Approximately 4% of the heme is exposed at the surface representing about 0.06% of the total solvent accessible surface area in the cyt c structure [12]. Cyt c has an isoelectric point of 10 (the isoelectric point (pI) is the pH at which the number of positive charges and the number of negative charges of a compound are equal).

1.2 Protein redox potentials

The reduction-oxidation or “redox” formal potential, $E^{\circ'}$, is a parameter characteristic to each chemical species. It indicates the tendency of a chemical species to accept one or more electrons and thereby be reduced. The redox formal potential is related to the change in free energy and the change in enthalpy and entropy by the following thermodynamic equation (equation 1):

$$E^{\circ'} = -\frac{\Delta G^{\circ'}_{rc}}{nF} = -\frac{\Delta H^{\circ'}_{rc}}{nF} + \frac{T\Delta S^{\circ'}_{rc}}{nF}$$

where $E^{\circ'}$ is the redox formal potential, $n$ the number of electrons transferred per molecule, $F$ the Faraday constant, $\Delta G^{\circ'}_{rc}$ the Gibbs free energy, $\Delta H^{\circ'}_{rc}$ the enthalpy change, $\Delta S^{\circ'}_{rc}$ the entropy change and $T$ the temperature.

Enthalpy and entropy changes are measured by analysing the temperature dependence of the chemical species reduction potential. There are several factors which determine $\Delta H^{\circ'}_{rc}$ and $\Delta S^{\circ'}_{rc}$ and, thus, $E^{\circ'}$. It has to be mentioned that those factors have an influence on enthalpy as well as entropy and change both, but to various extents.
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The change in enthalpy is primarily influenced by metal – ligand binding interactions and the electrostatic forces at the interface between the metal in the protein environment and the solvent. Entropy is dependent on the conformation of polypeptide chains and their organization in solvents [13]. Furthermore, the reduction potential of proteins can be influenced by the nature of the ligands, the type of binding of the ligands to the heme groups, polarity of heme environment, accessibility of the heme to the solvents and electrostatic interactions between heme and its propionates [14].

1.2.1 Factors affecting $\Delta H^{\circ}_{rc}$

Enthalpy is a state function. The value of enthalpy is influenced by the structural properties of redox proteins. $\Delta H^{\circ}_{rc}$ mainly depends on the type of bonds between metal and ligands and the electrostatics between metal and protein environment and the solvent [13].

The ligand – protein interaction and its influence on the redox potential was presented by Battistuzzi et al. [13]. They exchanged Met80 in cyt c for N-donor ligands (imidazole or azide) in water solution causing a considerable decrease in $E^{\circ'}$ of the protein. This change in $E^{\circ'}$, resulting from stabilization of the ferric state, is a result of the strong electron donating properties of N-donor ligands. This is an almost totally enthalpic process in origin. The same ligands were also bound to microperoxidase-11 (MP-11) what gave an entropic contribution to the change of redox potential. This result was explained by the partial water exclusion from the heme environment. Moreover, the higher value of redox potential of cyt c relative to MP-11 is primarily enthalpic in nature and arises from the $\pi$-electron-acceptor character of the thioether sulphur atom of the axially bound Met80 to iron [15].

Another factor which affects the value of $\Delta H^{\circ}_{rc}$ is the environment surrounding the heme. The negative value of $\Delta H^{\circ}_{rc}$ for cyt c and attributed it partially to the hydrophobic heme surroundings [16]. On the other hand, positive enthalpy of HRP can be explained by the polar nature of heme pocket that stabilizes the ferriheme [13]. Moreover, the ability of ligands to exclude or replace water molecule from the heme environment and thus to increase the hydrophobicity can also stabilize the ferroheme [15].
The type of solvent used is an important factor which determines the redox properties of proteins and enzymes. Non-polar solvents favor the reduced form of a protein. Again, ferroheme would be expected to be more stable in hydrophobic solvents. This can be explained by the decrease in enthalpy when cytochrome c was transferred from water to the less polar solvent such as glycerol [14]. An opposite effect was presented by Borsari et al. for cyt c dissolved in a mixture of aqueous solution [17] as well as by O’Donoghue et al. for immobilized MP-11 in glycerol [18], which can not be explained by hydrophobic stabilization of ferroheme. The reason is not very well understood. However, each protein should be examined individually due to the changes in the structure and different interactions with the surrounding environment.

Finally, another factor affecting the enthalpy is the formation of and breakage of hydrogen bonds. As described by Battistuzzi and co-workers, the strong H-bond interactions between His170 and Asp235 residues in HPR confer the anionic character of the His170 imidazole group which coordinates the heme. This would cause the stabilization of the oxidized state of heme and an increase of $\Delta H^\circ_{\text{rc}}$ [13].

1.2.2 Factors affecting $\Delta S^\circ_{\text{rc}}$

Entropy is a thermodynamic function that indicates the degree of disorder of a system containing the protein and solvent in each oxidation state. Changes in entropy depend on solvent induced reorganization effects, the alteration of the solvents dielectric about the metal redox centers and the effect of ligands [18].

The positive entropy changes observed for the reduction of MP-11 and HRP [13-15] can be explained by a decrease in the solvent ordering arising from electrostatic interactions between the reduced form of the heme and the surrounding water molecules. Electrostatic heme-solvent and heme-protein interaction will be weakened on reduction, while the hydrophobic interaction will be strengthened. In addition, interactions between the reduced form of the heme and a polar solvent such as water, increases the degree of ordering of the solvent, with a concomitant decrease in entropy [13]. The opposite effect can be observed when the water is replaced by less polar glycerol creating a less extensive H-bonding net. The decrease in the ordering of solvent occurs and leads to an increase in entropy [14]. Another important parameter is
the structure and the type of ligands in the protein molecule. Cyt c has all six coordination positions filled and is not as susceptible to solvent organization on reduction as MP-11 which has a more exposed heme. Ligation of MP-11 in the 6th coordination position results in a significant decrease in the change in entropy caused by suppression of the electrostatic interactions between the polar solvent and the heme [15].

1.3 Protein electron transfer

In 1977 two groups independently reported reversible electron transfer between cyt c and a modified electrode. Yeh and Kuwana showed that the protein exhibits a diffusion controlled direct electron transfer response at a tin-doped indium oxide electrode [19]. Eddowes and Hill demonstrated the direct electrochemical response of cyt c on a gold electrode surface modified with 4,4’-bipyridyl. 4,4’-bipyridyl provided a hydrophilic surface, which could transiently bind the protein. They proposed that hydrogen bonding between lysine residues of the protein and nitrogens of the bipyridyl layer on the electrode surface stabilizes the electrode-protein complex [1].

Modification of the surface of the electrode is generally required to promote interactions between the protein and the electrode to enable rapid and reversible electron transfer. Modification of the electrode surface can promote binding of the redox protein in a manner, which enables electron transfer to occur. Surface modification of the electrode can prevent irreversible and degradative adsorption of the protein at the metal surface. Li and co-workers used imidazole to modify a silver electrode, which can bind cyt c resulting in a Faradaic response without prior purification of the enzyme. They showed that the surface of silver is oxidised, and N-H bonds in the imidazole molecule are broken under mild condition. These results indicate that silver was not only modified but also reacts with imidazole [20]. The most frequently used modifiers are thiols which form self-assembled monolayers (SAMs) at metal electrodes.
SAMs are an important tool for studying interfacial ET, as well as the adsorption and immobilization processes of proteins. Functionalization of the surface of metal electrodes with low molecular weight compounds facilitates the electrochemical communication at the electrode-protein interface. These functional groups are attached to the electrode surface, such as thiols or disulfide, to create a layer which controls the surface environment and, thus, surface characteristics exposed to the incoming biomolecule. This potentially protective layer may prevent irreversible unfolding of the protein. Moreover, the appropriate layer can build a structure where the protein becomes oriented in a manner that provides a short distance between the redox centre and the electrode surface allowing efficient electrochemical communication. The monolayers are optically transparent, stable over time and introduce chemical and functional diversity at the interface. Characterisation of SAMs can be readily performed with a variety of techniques including electrochemistry, ellipsometry, contact angle goniometry, IR spectroscopy, x-ray photoelectron spectroscopy and mass spectroscopy [21].

The structure of each component of the SAMs consists of a terminal group (endgroup), an alkane chain and a headgroup (Figure 1.1). SAMs are usually bifunctional compounds of the type X~Y. The X part binds to the electrode surface through nitrogen, phosphorus or sulphur. The Y part interacts with the electron transfer domain of the protein [22]. The process of self-assembly starts when the headgroups (X) of the compounds are chemisorbed on the metal surface. The layer is stabilized by van der Waals interactions between alkyl chains. The chains re-arrange into the most energetically favourable structure, to reduce contact with the polar solution. This is achieved by tilting of the molecule to angles of 28-40° at the surface [23].

The properties of SAMs depend on the length of the alkane chain. Longer alkane chains (n > 6) result in a more ordered thiol structure, with more crystallinity and a capability to act as a more effective interfacial barrier. However, such layer area is also more impermeable to solvents and electrolyte solutions [24]. Thiols with more than 6 methylene groups create thick layers that can passivate the electrode surface. In effect, they create a barrier and prevent redox species making electroactive contact with the electrode surface. This phenomenon can also decrease the background signal or charging current of the electrode [23].
The nature of the endgroup of SAMs has an influence on the overall properties of the monolayer. There are many different terminal groups available to functionalize alkanethiols. The most frequently utilized possess functional groups such as \(-\text{CH}_3\), \(-\text{CF}_3\), \(-\text{OH}\), \(-\text{COOH}\) and \(-\text{CN}\). There are a few factors, which are related to the choice of endgroup, that influence the permeability, defect density, capacitance, crystallinity, surface energy and resistance of gold oxide of SAMs (Figure 1.2).

Alcohols and methyl terminated SAMs have a high degree of crystallinity but possess a more significant tilt angle in packed SAMs. On the other hand, self-assembled monolayers with carboxylic acid endgroups have a liquid-like structure indicative of a porous barrier which reduces the blocking properties in comparison to other endgroups. To achieve the same blocking characteristics, SAMs with \(-\text{COOH}\) groups require an increase in the number of methylene groups in the alkane chain [23, 26].

Figure 1.1 Diagram of one component of SAM, modified from ref. [25].
The optimal electrochemical response can sometimes be achieved with mixed SAMs. Mixed SAMs can contain both alcohol and carboxylic acid terminated thiols and are effective in promoting DET for cyt c. Their charge (–COOH groups in solution at pH 7 create a negatively charged or acidic surface) and the shape of the layer can provide a surface that enhances binding of the protein in a manner that promotes fast electron transport. Moreover, it was shown previously that mixed self-assembled monolayers provide faster reversible kinetics for which protein when compared with single-thiol SAMs [27].

SAMs are stable in the potential range -1.4 to +0.8 V vs. SCE [28]. Mechler and co-workers formed SAMs using negatively charged short chains or long chained mercaptocarboxylic acid and hydrophobic octanethiol [29]. Groot et al. presented results of electron transfer for cyt c immobilized on a gold electrode modified with layers of carboxylic acid-terminated thiols. Their data proved conclusively that the rate of electron transfer depended on the thiol length. The Laviron method [30] was used to determine the rate constant for electron transfer as a function of thiol length. For short

![Figure 1.2 Endgroups influence on SAM properties, modified from ref. [25].](image)
thiols no changes were observed for $k_{et}$ of both proteins, but for long thiols the electron-transfer rate constant decreased with increasing length of the thiol [31].

The morphology of the electrode can also be important in facilitating electron transfer between an enzyme active center and the electrode surface. Brown et al. showed that direct, reversible electron transfer occurred at the submonolayers coverage of cyt $c$ on 12 nm-diameter colloidal gold particles on SnO$_2$. On aggregation and increasing the size of the particles up to 22 and 36 nm, the electrochemical response of cyt $c$ became irreversible or quasireversible. The colloidal nanoparticles of 12 nm in diameter created an ensemble of closely-spaced isolated elements and behaved as microelectrodes providing an example of how nanoscale morphology can be important protein voltammetry [32].

Electrodes can also be modified via the attachment of different materials. Wang et al. described the direct electrochemical response of cyt $c$ at glassy carbon electrode modified with single walled carbon nanotubes (SWCNTs) [33]. The use of CNTs as the electrode modifiers improves the current density by generating a large surface area. In addition, CNTs can act as wires and facilitate connection with the active site of biological molecule. Tasca et al. adsorbed SWCNTs on glassy carbon electrodes and subsequently modified the SWCNTs with aryl diazonium salts to attach Phanerochaete sordid CDH. This resulted in a stable, lactose biosensor based on direct electron transfer between the enzyme and the electrode surface. This biosensor had a current density of 500 $\mu$A cm$^{-2}$ at 200 mV versus normal hydrogen electrode (NHE) in 5 mM lactose solution at pH 3.5, which is the highest reported DET value to date [34].

Another way to achieve direct electron transfer is by minimising the distance between the redox centre in the biomolecule and the surface of the electrode. If the protein – electrode surface distance is large, the electron transfer process will require the use of mediators to facilitate the connection with the electrode [35]. There are two types of mediators. Mediators can be free in solution or attached to the electrode or enzyme surface. The latter category of mediators could be trapped in conducting polymers [22]. Hale and co-workers built a biosensor by applying ferrocene-containing siloxanes polymers as mediators, which enabled electron transfer between the reduced form of glucose oxidase (GOx-FADH$_2$) and carbon paste electrode. The optimization of the sensor and, thus the GOx response depended on the structure of the polymeric
backbone [36]. Osmium containing redox polymers are another example of mediators which have attracted much attention. A wide range of complexes have been described in the literature [37-38]. The stability of the complexes can be tailored by altering their structure while the potential can be altered by changing the ligands [39-40]. Gregg and Heller described the characteristics of such polymers which include fast rates of diffusion of substrate and product through the polymer, rapid electron self-exchange and high permeability to solution species. In addition, redox polymers containing osmium can bind strongly to gold, carbon, graphite and other electrode surfaces. Osmium polymers are able to carry large catalytic currents for redox reactions, enable facile electrochemical communication with the active site of enzymes and promote the stability of the biological molecules. GOx was covalently immobilized at the electrodes in 1 µm thick film containing osmium redox polymers. The effects of varying factors such as the cross-linker and oxygen concentrations, film thickness and temperature, on the electrochemical response of GOx containing redox polymers films were examined. In addition, the kinetic limitation observed derived from the low driving forces between the enzyme and the polymer [41].

The first enzyme electrode was built by Clark and Lyons in 1962. The biosensor was constructed from GOx trapped between a semi-permeable membrane placed on the surface of a platinum electrode [42]. The flavin adenine dinucleotide group (FAD) is reduced by glucose to the FADH₂ form. The FADH₂ form is re-oxidized to the FAD form by molecular oxygen [43]. This is an example of the first generation of biosensors, which are based on the electrochemical activity of substrates or products and not of the enzyme. Their main disadvantages are that (1) oxygen based biosensors are dependent on the oxygen concentration which is difficult to keep at a constant level and (2) they can have high background currents. Moreover, the high applied potential leads to significant electrochemical interference. To overcome the problems a permselective membrane was applied which excludes interfering compounds.

The second generation of biosensors utilizes various types of mediators whose key role is to facilitate fast electron transport between a proteins redox center and an electrode. Mediators should have a formal potential close to the formal potential of prosthetic group of the enzyme in order to decrease interference from the electroactive species and be able to diffuse rapidly between the enzyme and the electrode. Importantly, the properties of mediators should not be affected by variations in the
oxygen partial pressure and possess desirable electrochemical properties such as good stability in both oxidation and reduction state and a low redox potential that is independent of pH. Commonly used mediators include ferrocene and ferrocyanide. A range of derivatives such as mono- and dicarboxylic ferrocenes [44] are available [45]. They are utilized in their soluble form, but other types of mediators can also be covalently attached to the electrode or the enzyme surface or entrapped in a hydrogel or in conductive polymers. An example was described by Kajiya et al., who described the use of nicotinamide adenine dinucleotide electrodeposited with glucose dehydrogenase (GDH) and entrapped in a polypyrrole film with hydroquinone sulfonate [46]. These mediators create “reagentless” biosensors [43].

The most advanced biosensor configuration, known as a third generation biosensor, is based on DET. These kinds of biosensors indicate excellent selectivity with low operating potentials which are close to the redox potential of the enzyme. Moreover, they allow the possibility of modulating the desired properties of the analytical devices by using novel interface technologies and chemical engineering techniques and they do not need any mediators [47]. The most widely used enzymes are O$_2$-dependent oxidases, NAD$^+$-dependent dehydrogenases, PQQ-dependent dehydrogenases, peroxidases and multi cofactor enzymes containing a primary redox site (FAD, PQQ) internally coupled with electron acceptors such as heme-moieties [28].
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1.4 Enzyme Immobilization

1.4.1 Introduction

Almost all chemical reactions connected with the functioning of living organisms require the participation of enzymes. Enzymes are biochemical compounds that catalyse specific chemical reactions. As is the case for all catalysts, enzymes reduce the activation energy of reactions. They are not used up during the reaction and do not affect the equilibrium. [48].

Enzymes can be divided into six classes. Every class has specific properties and takes part in a different type of reaction [49]. If these reactions are run under optimal conditions the enzymes will exhibit excellent catalytic activity. However, when conditions are changed, enzymes will lose their catalytic activity. Enzyme denaturation can be caused by a change of pH, high temperature, mechanical or heat treatment. Furthermore, enzymes are unstable in organic solvents. Unfortunately, all these disadvantages present barriers for the use of enzymes in industrial applications. Another reason that limits the use of enzymes in industrial processes are their high cost of isolation and purification [50-51].

The best way to use and retain the catalytic properties of an enzyme is to immobilize it onto a solid structure. Immobilization, in effect, entails the confinement of a molecule in a liquid phase onto a suitable inert support. This process offers many benefits including ease of recovery, the possibility of reuse, improved stability and the prevention of protein contamination of the final product [52-53].

The first immobilization of an enzyme was carried out by J. M. Nelson and E. G. Griffin in 1916. They immobilized invertase on a solid like charcoal. It was also demonstrated that the immobilized enzyme had the same activity as the native enzyme. [54]. In this process the main aim of the immobilization was achieved - produce a more stable enzyme system.

The immobilization process can be treated as an interaction between two species: the phase which acts as the carrier and the adsorbing molecule (i.e. a protein or enzyme) [55]. These interactions can be built using a range methods broadly classified
as physical or chemical. Physical methods, where weak effects between enzymes and support exist, include:

- containment of an enzyme within a membrane reactor
- adsorption and deposition on to carrier
- inclusion or gel entrapment
- encapsulation with a liquid membrane
- encapsulation with a solid membrane
- formation of enzymatic Langmuir-Blodgett films

Covalent immobilization methods, where covalent bonds are formed with the enzyme, include:

- covalent attachment to a water-insoluble matrix
- crosslinking with use of a multifunctional, low molecular weight reagent
- co-crosslinking with other neutral substances
- immobilization via ionic interaction [50, 56]

Of these methods five are in frequent use (Figure 1.3):

- adsorption and deposition (noncovalent adsorption):
  - van der Waals forces, hydrogen bonds and hydrophobic interactions
  - immobilization via ionic interaction
- covalent attachment
- gel entrapment and encapsulation
- crosslinking [56].
Each of the specified methods possesses numerous advantages and disadvantages. However, a general method that can be applied to the immobilization of any enzyme is not yet available. This is due to the widely varying chemical characteristics of enzymes, the different properties of substrates and products, and different applications of obtained products.

Figure 1.3 The methods of immobilization, modified from ref. [56].
1.4.2 Noncovalent adsorption

Enzymes may bind to the surface via electrostatic interactions, such as van der Waals forces (dispersion forces and dipole interaction), hydrogen bonds, hydrophobic or ionic interactions. These forces are weak and are characterized by a low value of enthalpy (about 20 kJ mol\(^{-1}\)). Embedding a molecule onto an inert solid phase in this manner is called physical adsorption [57]. Immobilization via noncovalent adsorption techniques is advantageous in so far as it is simple, cheap and quick. Noncovalent adsorption does not require chemical modification of enzymes [58]. It has to be also mentioned that physical adsorption can occur via a number of interactions.

Gupta et al. described the immobilization of lipase from *Burkholderia multivorans* on a hydrophobic membrane via van der Waals interactions between the lipophilic surface areas of the carrier and the protein. After immobilization, during which the activity of the enzyme was retained, the enzyme was easily washed away from the membrane and purified. This shows the tendency of enzymes to leach from the surface in the desorption process [59]. Unfortunately, this is a crucial drawback of noncovalent adsorption which can be caused by higher temperature, a change in pH or the ionic strength.

Hydrogen bonding is the most common form of intermolecular bonding. This is a weak interaction which relies on the electrostatic attraction between hydrogen and the electronegative atom containing free couples of electrons. Hydrogen bonds may be formed easily by proteins which can be immobilized on hydrophilic phases, e.g. SiO\(_2\), TiO\(_2\) [60]. Oliva et al. have shown hydrogen bonds, next to electrostatic, are possibly the main form of interaction between TiO\(_2\) and human serum albumin HSA [61].

Hydrophobic interactions are a phenomenon where nonpolar compounds bind to minimize their contact with polar solvents and their contact with polar solvents. Alkyl, vinyl or phenyl groups can be added to the surface of a solid support phase in order to increase the hydrophobicity of the surface [59].

Immobilization of enzymes via ionic interactions, another form of noncovalent adsorption, depends on the charges of both the carrier and the enzyme. The overall charge of the enzyme is determined by the type and density of the charged amino acids residue on its surface and the pH of the solution. The \(pI\) is the pH at which a particular
molecule or surface carries no net electrical charge. At a pH below the isoelectric point the protein carries a net positive charge, above it has a net negative charge [62]. Therefore, if a protein is positively charged the carrier surface should be negatively charged and vice versa. Ionic immobilization is strongly dependent not only on pH, but also on the ionic strength of the immobilization solution. High salt concentrations can lead to ion exchange and the washing (leaching) out of enzymes bonded via ionic interactions [63]. As an example, electrostatic interactions dominate the bonding process between SBA-15 and xylanase [50].

1.4.3 Covalent attachment

Chemical adsorption is an immobilization process where atoms or molecules are attached to the surface through the formation of covalent bonds. The enthalpy of covalent bond formation is higher than that for the physical adsorption process, typically 200 kJ/mol [56]. Formation of covalent bonds ensures strong binding and negligible leaching of the enzyme. The bonds are normally formed between functional groups present on the surface of the support and functional groups of amino acids on the surface of the enzyme [53, 64]. For protein and enzyme immobilization the most useful surface functional groups are thiols, carboxylic acids, alkyl chlorides and amines [61]. Immobilization via covalent bonds mainly occurs in aqueous solutions. However, covalent techniques are time consuming methods because the process of attachment requires several chemical steps [65].

Bolivar et al. described the immobilization of enzymes on a glyoxyl agarose phase. The strategy of immobilization used enables the multipoint covalent attachment of the enzyme. The amino groups at the enzymatic surface are predominantly used in the immobilization process to the carrier since they react with many different functional groups. It is important to immobilize enzymes at mild pH values where only the amino groups are reactive. However, under mild conditions glyoxyl agarose based support does not immobilize most enzymes. This can be overcome by the stabilization of imino bonds of the enzyme using a compound (e.g. thiols) that does not affect the aldehyde groups permitting the multipoint covalent attachment. Obtaining the multipoint covalent attachment required long immobilization times and correct alignment of functional groups between the enzyme and support surface [66].
1.4.4 Gel encapsulation

In this type of immobilization, enzymes are free in solution (sol), but restricted in movement by the lattice of a gel. There are several methods for the entrapment of enzymes into a gel:

- ionotropi gelation of macromolecules with multivalent cations (e.g. alginate)
- temperature induced gelation (e.g. agarose gelatin)
- organic polymerization by chemical or photochemical reaction (e.g. poliacrylamide)
- precipitation from an immiscible solvent [56].

Sol-gel materials have desirable properties for use as supports in the field of enzyme immobilization. They can be prepared under mild conditions, have high thermal stability, chemical inertness, and abilities to form films. They show negligible swelling in aqueous and non-aqueous solutions and, moreover, the sol-gel materials exhibit tunable porosity [67]. Sol-gel matrices can immobilize enzymes and proteins whilst maintaining their native properties and reactive character, which make them a potential tool for the development of biosensors. The pore sizes in a sol-gel have to be controlled to allow the diffusion of analyte to the redox active sites and prevent enzymes escaping at the same time [68].

The sol-gel synthesis starts with hydrolysis of siloxanes (Si(OR)₄). This leads to condensation and polymerization, ultimately yielding the solid sol-gel. The chain of reactions mentioned above creates the low molecular weight of μ-oxo-bridges siloxane oligomers. During condensation the viscosity of the sol increases by crosslinking between oligomers forming the solid porous material [69].

A significant disadvantage of sol-gel methods is the mass transfer barrier imposed by the narrow pore network. That has an adverse influence on biochemical applications involving large substrates. However, control over the pore size distribution is difficult. Aggregation of enzymes can still occur in porous matrices [70].
The other way to prevent enzymes leaching from porous supports is to encapsulate them inside the pore spaces. Encapsulation is used in nature, e.g. by liposomes. Liposomes are composed of a phospholipids bilayer and may be utilized to trap drugs and pharmaceutical products to transport them inside the organism. Currently it is possible to manufacture liposomes and use them in the cosmetics industries [71]. Liposomes create a barrier for hydrophilic molecules protecting them against pH and ionic strength changes [72].

1.4.5 Cross-linking

The first protein cross-linking technique was development by Doscher and Richards in 1963. The reactive amine residues on the protein surface were cross-linked via the reaction of glutaraldehyde [73]. It was also the first method applied to stabilize enzymes crystals for X-ray diffraction studies. However, this method had several drawbacks, such as often low mechanical stability and poor reproducibility [74].

Cross-linking is the only type of immobilization which does not require any support. It relies on the molecules connecting together in order to make large 3-dimensional complex structures. Cross-linking can be achieved by two methods: physical and chemical. Physical method of cross-linking leads to aggregation resulting in the high density aggregates due to e.g. the evaporation of solution. This technique is well known in the biotechnology industry. The chemical method of cross-linking involves the formation of covalent bonds between molecules by means of multifunctional reagents [56].

Cross-linked enzymes aggregates (CLEAs) are cross-linked physical aggregates of enzymes. For example, penicillin G acylase (penicillin amidohydrolase) was immobilized by physical aggregation, using precipitants such as ammonium sulfate, poly(ethylene glycol) and organic solvents, followed by chemical cross-linking to form insoluble CLEAs. Penicillin G acylase catalyzes the synthesis of ampicillin. The enzyme in the CLEAs form is highly active and stable biocatalysts in the synthesis then penicillin cross-linked crystals (CLECs) [75].
The cross-linking method is seldom applied, because of the relatively low stability of the obtained systems. Nevertheless, it is combined with other techniques of immobilization in order to enhance the stability of formed systems.

1.5 Enzymes immobilization and characterization in biosensor applications

A biosensor is a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals [76]. The transducer detects specific interactions with the analyte material and converts this chemical data into an electronic signal for further analysis. For in vivo applications a biosensor should be small for ease of implantation. It should provide selective and accurate (±5%) analysis in real time. A biosensor should be a low-cost to enable efficient and quick production. Moreover, the analytical signal generated by the device should be sufficiently large and stable [77].

The biosensors market is dominated by glucose sensors for diabetics. The major companies such La Roche, LifeScan, Abbott and Bayer have produced over 40 different blood glucose meters available for consumers [78-79]. Biochemical devices for self-monitoring of the glucose level use a 0.3 – 1 µL sample of blood and require 5 – 20 seconds to perform the analysis [79-80]. Glucose oxidise (GOx) and FAD-dependent glucose dehydrogenase (FAD-GDH) are the most commonly used enzymes in glucose biosensors. The chosen enzyme covers the screen-printed strip-shaped electrodes [80]. Currently the screen-printing suspension can contain carbon and many metals widely available for low temperature application [79, 81]. However, there are also different sensors for detection of heavy metals such as copper or lead [82-84], pesticides [85], phenols [86], lactate [87] or environmentally heavy gasses like carbon monoxide [88] or nitrous oxide [89] developed.
Various methods have been described for the immobilization of enzymes (section 1.3). Current trends dominating in biosensors research try to lead to an ideal situation where enzymes are attached to the modified electrode surface in the way to obtain DET. Such a system allows the biocatalytic reaction through the electron tunneling to be the only limiting step [90]. Nevertheless, DET of enzymes is difficult to achieve. Thus, this procedure alone besides its simplicity is rarely used in biosensor construction [91].

Direct attachment of enzymes at the electrode surface eliminates the need for mediators. However, due to the instability of DET, the combination of physical adsorption with crosslinking in a sandwich type of structures is common. Sandwich type of immobilization increases the stability of the biosensor. It is a simple procedure strongly binding enzyme molecules. Unfortunately, the process of crosslinking decreases the catalytic activity of the enzymes and limits the mass transport of the substrates and products [92]. Safina et al. described lactose biosensors based on cellobiose dehydrogenase adsorption on commercially available screen-printed carbon electrodes. To stabilize the enzyme layer a mixture of glutaraldehyde and poly(ethyleneglycol)diglycidyl ether was used. Such a biosensor showed good working characteristic with high reproducibility (RSD between 1.5 – 2.2 %) and sensitivity (limit of detection of ca. 250 nM for 50 µL and ca. 125 nM for 100 µL sample volume). Detection of lactose in dairy products took 65 s. The stability of the biosensors remained unchanged in working buffer and milk products during 8 hours of analysis [93].

The use of redox hydrogel to wire enzymes to the electrode surface is a promising approach. The enzymes are chemically bound through covalent or ionic interactions in a way that there is an electron transfer pathway to the electrode (e.g. electron hopping mechanism). Heller et al. covalently linked enzyme molecules embedded within osmium based redox complexes facilitating and stabilizing electron transfer from its active sites [94]. Such a method requires mild condition and is universal for many enzymes. However, the redox hydrogels form large diffusion barrier blocking the mass transport to the enzymes [91]. In addition, systems based on mediated electron transfers are complicated and it is difficult to characterize them.
Chapter 1  

**Introduction and literature review**

Several techniques based on spectroscopy and combined with direct electrochemistry can be used to characterize electrodes with immobilized enzymes. Surface enhanced infrared absorption spectroscopy (SEIRA) and surface enhanced raman spectroscopy (SERS) rely on enhancement effects at the metal surfaces such as Au, Ag and Pt. SEIRA is used to monitor the proteins at the electrode surface and observe the reactions taking place during the cyclic voltammetry measurement inside the specially designed cell [95]. Ataka et al. used SEIRA to investigate the functionality of cyt c monolayer adsorbed to the carboxyl and hydroxyl terminated SAMs at the gold electrodes by enhancing the adsorbed signal magnitude of ca. 2 orders [95]. It was found that the surface enhancement effect of SEIRA depends on the enzymes linkage to the electrode and decays rapidly over the distance of ca. 8 nm. This property eliminates the signals from the bulk and allows the detection of minute spectral changes of the analyzed molecules [95-96].

SERS can be used to investigate the vibrational properties of immobilized proteins yielding structural information on the molecule and its local interactions. Thus, SERS can be used to analyze single protein and recognize it. Zuo et al. attached cyt b_{562} to the electrochemically roughened silver electrode modified with amino and carboxyl terminated alkanethiols. Such a system was excited at 413 nm to achieve the heme-based electronic transition resonance and coupling with an Ag surface. The resulting spectra were selectively sensitive to vibrations of the heme cofactor of the immobilized protein [97].

Fluorescence and UV-Vis spectroelectrochemistry can also be used to characterize enzymes at the electrodes. Krzeminski et al. monitored the oxidation state of a Cu type 1 redox centre inside an enzyme nitrate reductase. Fluorescently marked enzyme molecules were attached to SAMs modified gold electrode. Its catalytic activity was determined electrochemically, whereas the redox state of Cu type 1 was determined from the changes in the fluorescence intensity between the attached fluorophore and Cu site [98]. Marritt et al. reported potential controlled UV spectra for multiheme nitrite reductase immobilized on mesoporous SnO$_2$ electrode. The addition of nitrite substrate confirms the catalytic activity of the immobilized enzyme. The UV-Vis analysis shows that the enzyme can be reduced electrochemically and then reoxidized by the substrate turnover. That confirms the catalytic activity of the majority of the film [99].
1.6 Biofuel cells

![Scheme of simple biofuel cell, modified from ref. [100].](image.png)

Another application of the electrochemical properties of enzymes immobilized at electrode surfaces is in biofuel cells. The first biofuel cell was described by Potter in 1912 where a potential was generated at a platinum electrode placed in *Escherichia coli* bacteria (*E.coli*) or yeast cultures [100]. Biofuel cells are defined as fuel cells that rely on enzymatic catalysis [101] and their main role is to convert chemical energy to electrical current. In biofuel cells the source of the chemical energy can be a small compound which comes from living organisms or plants [102]. The first biological fuel was glucose, used in the first enzyme-based biofuel cell reported by Yahiro et al. in 1964, where glucose oxidase (GOx) served as an anodic catalyst [103]. In biofuel cells the biological fuel is oxidized at the anode with reduction of oxygen at the cathode to generate water (Figure 1.4). Thus, the fundamental mechanism is electron transfer. Typically, the electrodes are separated from each other by a semi-permeable membrane and are connected via external circuitry [100].
There are two types of biofuel cells, (1) primary or direct biofuel cells and (2) secondary or indirect biofuel cells. The first type of biofuel cell requires a biocatalyst which participates in the redox reaction to generate electricity. The role of the biocatalyst is to transform the substrates in the metabolic process to generate the fuel for the cell. The efficient electron conduction between the biocatalyst and the electrodes constituted a critical challenge in their development. In the second type the biocatalyst functions as a promoter to help produce fuels such as hydrogen or methane from sugar. The role of the biocatalyst here is to facilitate electron transfer chain between the fuel substrates and the electrode surfaces. The greatest challenge in indirect biofuel cells is to find the appropriate operating conditions for the biocatalyst. Enzymes typically prefer ambient temperatures, whereas biofuel cells can require higher temperatures. Due to the discrepancy in operating condition, only H₂–O₂ biofuel cells have been examined as indirect cells to date [104-105].

The performance of biofuel cell is characterized by power output which depends on the current density obtained at different cell voltage. The maximal power of biofuel cells is achieved at a current density and potential value within the range of the operating voltage of the cell. An open circuit potential (OCP) is defined as the difference of the electrical voltage at the terminals of a device when no net current flows is called. It can be also termed as resting potential or zero-current potential because the device is disconnected from any circuit and, hence, no work is done. Ideally, the difference in the thermodynamic potentials of the fuel/oxidized product redox couple and the oxidant/reduced product redox couple determined the OCP [106].

For most enzyme fuel cells, O₂ is the oxidant of choice due to the high reduction potential maximizing the potential output of the cell and because it is easy available. The extensively studied enzymes for O₂ reduction are: cytochrome oxidase, laccases and bilirubin oxidase. Cytochrome oxidase in vivo is a integral membrane protein known as complex IV in the electron transport chain which pumps protons to ATP synthase. However, cytochrome oxidase is less useful considering the electrochemical efficiency because of its high overpotential [107]. In addition, this enzyme is a poor fuel cell catalyst reducing O₂ at low potentials below about 0.1 V [108].

The enzymes laccase and bilirubin oxidase are called blue Cu oxidase. Similarities in the structure of both enzymes are derived from the redox centre for the
reduction of $O_2$ with three Cu atoms coordinated by histidine ligands (the type 2/3 cluster). Electrons are transported to the active site, one at a time, from a fourth Cu site (type 1 or blue Cu centre), located at the top of the hydrophobic pocket [109]. Laccases that have been widely used in biofuel cells are produced by fungi because these are able to reduce $O_2$ at higher potential than plant laccases [110]. Nevertheless, their pH optimum is between 3 – 5 which makes them less useful for biofuel cells at physiological pH. Moreover, laccases are inhibited by the presence of chloride ions [110-111]. Thus, as an alternative bilirubin oxidase was studied [112-113]. The operating potential of bilirubin oxidase is similar to that of laccases, but it is active at pH 7 and tolerant to Cl$^-$. The application of bilirubin oxidase in the cathode of biofuel cell was first described by Ikeda et al. in 2001, who showed that it reduces $O_2$ to $H_2O$ at potentials higher than 0.7 V at pH 7 in the presence of 2,2’-azinobis(3-ethylbenzothiazoline-6-sulfonate (ABTS) mediator [115].

Enzymatic anodes in biofuel cells utilize sugars such as glucose as common fuels. Thus, glucose oxidase and cellobiose dehydrogenase are obvious choices for anodic enzymes. Fungal glucose oxidase catalyzes the oxidation of glucose at a flavin adenine dinucleotide centre (FAD) which is buried in a cleft in the protein and, hence, DET is unlikely. It has to be mentioned that many authors reported DET using native glucose oxidase and different nanomaterials. Guiseppi-Elie et al. described spontaneous adsorption of FAD of glucose oxidase at carbon nanotubes (CNTs) displaying quasireversible electron transfer and an increased response in 25 mM glucose solution [116]. There is ongoing debate if truly DET based glucose oxidation was obtained. Nevertheless, the mediated electron transfer strategies are used extensively for glucose oxidation at the anode.

Cellobiose dehydrogenase is another enzyme that uses glucose as a fuel. However, glucose is the substrate of the highest affinity to cellobiose dehydrogenase and, the oxidation of glucose is performed with lower efficiency than by glucose oxidase. The native substrate for cellobiose dehydrogenase is cellobiose, but the enzyme oxidases also lactose with high efficiency [117].

Laccase or bilirubin oxidase modified cathodes together with glucose oxidase and cellobiose dehydrogenase modified anodes are widely used in many types of biofuel cells based either on direct or mediated electron transfer. Electrodes in biofuel
cells have been prepared using materials with a range of morphologies, which increase the surface area of the electrodes and enable a higher loading of enzymes. The first membrane-, mediator- and cofactor-less glucose/O$_2$ biofuel cell was reported by Coman et al. Cellobiose dehydrogenase and laccase were adsorbed at spectrographic graphite electrodes. An open circuit potential of 0.73 V, a maximal power density of 5 $\mu$W/cm$^2$ at 0.5 V and a half-life time of 38 hours in air-saturated 0.1 M citrate-phosphate buffer, pH 4.5 containing 5 mM glucose was obtained [118]. This approach was repeated for a biofuel cell working in physiological condition. Laccase was replaced by bilirubin oxidase to obtain an OCP of 0.62 V and 0.58V, a maximal power density of ca. 3 and 4 $\mu$W/cm$^2$ at 0.37 V and 0.19 V in phosphate buffer pH 7.4 and human serum, respectively [119]. Flat electrodes can be modified with metal and carbon nanoparticles (NPs) resulting in surfaces with high roughness factors. Wang et al. described a sugar/O$_2$ DET based biofuel cell that utilized cellobiose dehydrogenase immobilized on thiol-modified gold nanoparticles on a gold electrode with glutaraldehyde as a cross linker. On the cathodic site, bilirubin oxidase was adsorbed on the unmodified gold nanoparticles at a gold electrode. The biofuel cell was tested in air-saturated 50 mM phosphate buffer saline containing 100 mM glucose and 5 mM lactose and in human blood and plasma. An OCP of 0.68 V and power densities of 15 $\mu$W/cm$^2$ and 10 $\mu$W/cm$^2$ at 0.52 V in 5 mM lactose and 100 mM glucose containing phosphate buffer were obtained. In human blood, an OCP of 0.65 V and maximal power density of 3 $\mu$W/cm$^2$ at 0.45 V was achieved. Half-lives of 12, 8 and 2 hours in 5 mM glucose-containing phosphate buffer saline, human plasma and human blood respectively were obtained [120]. Chen et al. reported an MET based biofuel cell. Glucose oxidase and laccase were incorporated in Os-containing redox polymers which then were used to modify 7 $\mu$m diameter carbon fibers. An OCP of 0.8 V and maximal current density of 137 $\mu$W/cm$^2$ on the insertion of the biofuel cell into a still, aerated citrate buffer pH 5 containing 15 mM glucose at 37°C was obtained [121].

Biofuel cells have a number of advantageous properties. They produce fewer emissions and reduce the demand for nonrenewable fuel sources [122]. These advantages indicate that biofuel cells can have a range of applications in sensors, micro-chips, implantable devices, portable power supplies and in drug delivery.
The study of biofuel cells is currently of significant interest with many research groups active in this field presently. Ramanavicius et al. described basic, mediator free biofuel cells based on the enzymes quino-hemoprotein-alkohol dehydrogense QH-ADH, alcohol oxidase AOx, microperoxidase MP-8 immobilized on the anode and cathode. These biofuel cells can convert the chemical energy stored in ethanol to electrical energy. [122]. Katz and co-workers presented “smart” biofuel cells, which can be selectively turned on and off by chemical signals from the appropriate composition of enzymes and pH. This investigation lead to the possibility of developing simple AND/OR logical gates, which in turn leads to more complicated uses of biocatalyst modified electrodes as well as providing possible applications in bioelectronics and biocomputing [63]. However, there are still limitations in biofuel cell e.g. long-term stability and efficiency which have to be improved.

1.7 High surface area materials

1.7.1 Introduction

The choice of appropriate immobilization method should initially be preceded by a choice of a suitable support. Porous materials of high surface area are of particular interest. The increasing importance of nanostructure materials is not only due to the high surface area, but also because of the variety of their forms and shapes. Suitable examples include carbon fibers, nanotubes and fullerenes from carbon, silicates, aluminosilicates, and also nanostructures of metals and metal oxides with controllable interior “nanospace”. Porous materials of high surface area may also be developed through a combination of various chemical moieties. Examples of such materials may be classed as composites, intercalation materials, or doped materials [123].

There are two possible strategies to pursue when developing high surface area materials. The first is to fabricate small particles or clusters with high surface to volume ratios, e.g. highly supported metal catalysts. The second is to create materials with
voids or pores highly compared to the amount of bulk support material, like zeolites or amorphous silicas.

The synthesis and fabrication of nanomaterials of high surface area can be divided into two approaches:

- The ‘bottom-up’ approach based on using single, individual atoms, molecules or clusters. Porous materials of high pore volume (void volume) or those required to form colloidal dispersions are fabricated in this way. The ‘bottom-up’ approach is widely used to obtain more homogenous structures in composition, with less defects, and possessing better short and long range ordering.

- The ‘top-down’ approach is the basis for techniques such as attrition and milling. It is the typical method of making nanoparticles by breaking up bulk material, despite the fact it can cause internal stress in addition to imperfection of the surface structure. These stresses and imperfections lead to a reduction in conductivity due to inelastic surface scattering and contamination [124].

Academic and commercial applications of high surface area materials are valued in the multibillion dollar range. Among others they are used as:

- Battery, capacitors and fuel cells elements for improvements and new operations
- Biochemical and chemical separations
- Product-specific catalysts for petrochemical process
- Thermal barrier materials for use in high temperature engines
- Air separation
- Porous materials for energy storage technologies.

1.7.2 Electrochemistry of porous materials of high surface area

Electrochemical techniques can give detailed information about the compositions of porous materials as well as their performances as electrochemical and electrooptical sensors. Among others, an electrosynthetic route can be used to modify
and prepare high surface area materials. Other electrochemical techniques may be used to characterize the photochemical and magnetochemical properties of particular high surface area materials. Moreover, electrochemical techniques can be utilized to design electrocatalysts for use in synthesis. These electrocatalysts may also be used for sensing and find applications as electrode materials and in fuel cells, solar cells, capacitors, electrooptical devices and more [124].

It is worth noting that electrosynthesis is used to fabricate a variety of types of porous materials. For example, Panella et al. reported the production of Cu-BTCs (BTC: benzene-1,3,5-tricarboxylate), which are a type of MOFs (metal-organic frameworks) [125], and Yarger et al. described a one-step electrodeposition process to grow film-type Zn-Al layered double hydroxide (LDH) directly from a conducting substrate [126].

Electrochemistry can also be a source of techniques for the hybridization of new materials with unique properties. Gao and co-workers described the electrochemical insertion of lithium into anatase nanorods with a large specific surface area and pore volume exhibiting a high initial capacity [127].

To characterize the resultant materials, cyclic voltammetry was used to monitor the presence of lithium inserted into anatase nanorods as discussed above. The adsorption of the lithium in the sample was demonstrated by the appearance of peaks located in the appropriate positions on the voltammogram [127]. Moreover, cyclic voltammetry was used by Long and co-workers to report the electroanalysis of high surface area catalysts and other nanoscale electroactive materials [128]. The charge-discharge performance can be used to evaluate the electrochemical behavior of materials Yang et al. simulate Li/MnO$_2$ cells to check the properties of 1-D MnO$_2$ nanorods. It was found that 1-D MnO$_2$ nanorods can be used as competitive cathode material for rechargeable lithium batteries [129].

Smooth, structurally perfect, strain free and crystal orientation ideal electrodes with known surface area serve are used in theoretical descriptions. However the electrodes with rough surface area are of more practical use. The need to present the maximum surface area to the reaching interface between electrode and electrolyte has resulted in the development of porous electrodes. Hampson and McNeil divided porous electrodes into five classes; (1) electrodes, in which the surface area is increased over
the projected geometric area, (2) porous, also called granular electrodes produced through electrodeposition processes, (3) hydrophobic gas electrodes, whose operations depends upon the establishment of solid-liquid gas interphases, (4) flow-through electrodes and (5) flooded, static, ‘natural’ porous electrodes [130].

Solid porous electrodes have large contact areas per unit volume between electrolyte, reactant and electronic conductor [131]. Thus, their use is widespread in sensors and fuel cells, where they provide the surface site for reactions.

1.8 Nanoporous gold

In recent years, metal nanostructured materials have been used to prepare electrochemical sensors and biosensors. Generally, highly porous materials can be categorized as (1) intrinsic, such as zeolites, which possess large molecular scaffolds with natural porosity and (2) synthetic, in which porosity comes from self-assembly processes during fabrication. The third class of porous materials is created by structures formed in a three-dimensional (3D) spinodal decomposition phase separation into a mesoscale bicontinuous structure to selective etch one phase. An example of the third class is the thin, metallic, mesoporous membrane nanoporous gold (NPG) [132].

Nanoporous gold material is a type of nanostructured gold with 3D sponge morphology which possesses tunable pores in the nanometer size range [133]. NPG has a series of unique characteristics. It is chemically and mechanically stable and conductive, possesses large surface area and pores with a size between few nanometers to several microns. NPG has been demonstrated to be a good carrier for the immobilization of biomacromolecules due to not only a high surface area but also well-ordered pore structure and high-specific pore volume. In addition, NPG has excellent catalytic activity [134].

NPG structures can be obtained by melting and mixing two metal precursors, annealing them and machining to fabricate desired dimensions. The base for this type
of nanoporous gold is an inexpensive 12-carat white gold leaf which contains 50 wt% of Au and Ag and is commercially available for decorative purpose. Leaf is a term to determine the thin foils made by hammering. The procedure is divided into a number of steps: (1) rolling the foil to a thickness between 50 – 100 µm, then (2) cutting it into pieces to build a sandwich structure and (3) beating the sandwich structure to increase the surface area. The steps are repeated to obtain the foil thickness in a range between 100 and 200 nm [132].

The method demonstrated above prevents the straight-forward integration of both alloy precursors and limits their versatility, thus ideally alloy should be deposited by electroplating, evaporating or sputtering which are directly compatible with conventional microfabrication. Ji and Searson described a two-step process for the fabrication of nanoporous nanowires by simultaneous electroplating gold and silver alloy from basic cyanide solutions into a nanoporous alumina template with subsequent etching of the less noble metal [135]. However, it is difficult to produce uniformly thick and crack-free NPG structures by electroplating. Magnetron sputtering is the most frequently used technique to deposit thin films [136-137]. This technique enables control of properties such as: composition, residual stress and grain size by adjusting gas composition, pressure, flow, substrate temperature, gun power and ions accelerations towards the substance [138]. The less commonly used technique to microfabricate an ideal alloy is (co-)evaporation of both constituents with an electron beam, which offers a control of film thickness and composition.

The porosity within metal film structure is obtained via a simple dealloying process. Erlebacher [139] described several characteristics of nanoporous metals. First of all, the composition of each alloy must contain at least two metals, one more noble and one less noble. The less noble metal is usually present at higher levels. The difference in standard potential of each component in the pure form has to be separated by a few hundred millivolts [139]. Roberson fabricated and characterized nanoporous gold thin films derived from an alloy with a Ag to Au ratio of 70% to 30% [140]. Moreover, the evolution of porosity during dissolution is a dynamic process. It is not a simply extraction of one phase from two-phase material, thus the alloy has to be homogenous. The rate of diffusion of the more noble metal should to be sufficiently fast [139].
The most often used alloy which shares the aforementioned statistics is Ag-Au alloy due to the solid solubility across the entire composition range for both components. In addition, Ag and Au have face-centered-cubic structure with similar lattice parameters [135]. The dealloying of Ag-Au alloy causes the selective dissolution of less noble Ag. As a result of the process the remaining gold will self-organize into an interconnected network of pores and ligaments to create a nanoporous sponge structure which leads to an increase in the surface area of the sample [141]. Dealloying can be carried out in concentrated nitric acid, which is simple and rapid. Unfortunately, this method does not allow control of the pore size. Alternatively, dealloying can be processed in electrochemical cell in perchloric acid under anodic potential. The application of this method results in a smaller porosity because electrochemical dealloying increases the rate of Ag dissolution compared to the Au diffusion rate [138, 141-142]. Musat et al. reported the formation of nanoporous gold from 62.5 wt% Ag and 37 wt% Au alloy through electrochemical leaching by applying a potential of 1.1 V in 1 M HClO$_4$ electrolyte versus KCl saturated Hg/Hg$_2$Cl$_2$ electrode [143]. Although the method has a number of controllable parameters, such as: potential, the time at which the less noble metal begins to dissolve, electrolyte composition, in some cases it is preferred to use dealloying in nitric acid.

However the porosity evolution process called a pattern formation during dealloying was first described in 1920s [144]. Erlebacher et al. clarified the mechanism of porosity evolution during dealloying using an ‘analytical atomistic model’ [139, 141].

The potential of nanoporous gold is enormous. The whole surface area of NPG is electrically accessible; hence they represent nearly ideal substrate materials or electrodes for electrocatalysis. Zhang et al reported high electrocatalytic activity toward methanol oxidation in alkaline media at NPG film. They suggested that the porous morphology of the gold surface which allows trapping OH$^-$ anions, facilitates methanol oxidation [145]. In addition, because NPG are mechanically rigid, often biocompatible and chemically stable, they can be used for applications as sensors in biofuel cells and catalysts. They can serve also as templates for the new porous materials fabrication. Xu et al. and Zielasek et al. independently described the unique properties of nanoporous gold for low-temperature CO oxidation with catalytic oxygen to the nonhazardous CO$_2$ [146-147]. Qui et al presented the immobilization of laccase on NPGL. Structures with
pore sizes of 40 - 50 nm were demonstrated to be suitable supports for the enzyme. A direct electrochemical response was achieved on a glassy carbon electrode covered with NPGL previously immersed in the laccase solution. The stability of the enzyme electrode was also examined with no changes in response on storage at 4°C for a month [148]. Yin et al. exploited the properties of NPG for the aerobic oxidation of D-glucose to D-gluconic acid under mild conditions [149]. Selective oxidation of alcohols and sugars is an important reaction in food, detergents and pharmaceutical industry, Nanoporous gold may also be prepared on substrate materials Roberson deposited a Ag_{70}Au_{30} alloy on a three-inch Si wafer. A porosity of 10 – 30 nm was obtained by selective leaching of silver from an alloy. NPG film served to check the pore and ligament (a band disconnecting pores and cracks at the surface) evolution during annealing at 450°C for 15 hours. The entire process of annealing resulted in a linear increase in the ligament width. Moreover, during the first 10 hours, the exponential growth in average pore width is observed. However, the average pore width during last 5 hours appeared to decrease. Roberson carried out the stress study as well. It was expected that etching would result in increased stress in NPG film. The opposite effect was found. After etching the tensile stress decreases and as a result of annealing the relaxation occurred [140]. In addition, NPG posses excellent optical properties in plasmonics and SERS. It can work in high-performance instrumentation application for chemical inspections and biomolecular diagnostics as active, stable and economically affordable substrates [150].
1.9 Scope of this project

The aim of the work presented in this thesis is to develop conductive nanoporous materials of high surface area for use as supports for enzyme immobilization in biosensors and biofuel cells. The main points investigated and the findings obtained will be discussed in the following chapters:

- Two types of porous gold: gold leaf and sputtered gold were prepared and characterized. Both materials were compared in terms of their electrochemically addressable surface area, ease of use and stability. The optimal materials were then used as a support for the immobilization of redox enzymes (Chapter 2).

- Two different types of sputtered nanoporous gold were examined to study the surface area accessible for redox enzymes using cyt c as a model system. Immobilization of Corynascus thermophilus cellobiose dehydrogenase on SAM modified nanoporous gold surfaces was used to enable direct electron transfer. Myrothecium verrucaria bilirubin oxidase and Trametes hirsuta laccase were also immobilized into the unmodified nanoporous gold by physical adsorption in the vacuum atmosphere (Chapter 3).

- Corynascus thermophilus cellobiose dehydrogenase, Aspergillus niger glucose oxidase, Myrothecium verrucaria bilirubin oxidase and Melanocarpus albomyces laccase were incorporated in hydrogels with Os redox polymers and a cross-linking agent and immobilized at nanoporous gold electrodes. These electrodes, based on mediated electron transfer were then used to detect lactose and glucose. The electrodes were also used as anodes and cathodes in biofuel cells which were characterised in buffer solutions and in solutions of artificial plasma (Chapter 4).
1.10 REFERENCES

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Introduction and literature review

CHAPTER 2:

NANOPOROUS GOLD. FABRICATION AND CHARACTERIZATION
2.1 Introduction

Predominantly, a biosensor comprises of a support with carefully selected molecules immobilized into its surface. The function of the conductive carrier is to create a structure for a reliable and mechanically stable biosensor which assesses a specific biological response. The morphology of the material chosen for the carrier should be rough with a high surface area-to-volume ratio which in contrast to flat surfaces improves the loading of the immobilized macromolecules increasing the sensitivity of the device. Moreover, rough surface with tunable pores (voids) allows the biomolecules to be enclosed and enables fast and efficient electron transfer with the redox centre of the enzyme.

Recent developments of electrodes in applications for biosensors are dominated by novel carbon and gold materials. Carbon materials enable the physical adsorption of biomolecules on the surface without any chemical modification. Thus, direct electron transfer can be achieved. The wide range of carbon-based materials, from planar graphene sheets, nanotubes and nanofibres to nanoparticles which are used to modify the flat surfaces, enable, in theory, construction of a biosensor for a range of applications [1]. Patolsly et al. described the covalent attachment of flavin adenine dinucleotide (FAD) cofactor to the ends of SWCNTs modified gold surface followed by reconstitution of GOx. It was shown that SWCNTs are able to work as connectors transporting electrons between gold electrodes and the redox centre of GOx over distances greater than 150 nm with the electron transfer rate constant depending on the length of the nanotubes. The electrode acts not only as an efficient glucose biosensor with turnover rates from the active site of GOx of 4100 s\(^{-1}\) but also shows the possible application of SWCNTs in hybrid systems [2]. Graphene which is a two-dimensional monolayer of carbon atoms packed into a honeycomb lattice, is also used to promote electron transfer due to its extraordinary conductivity and high surface area [3]. Wu et al. reported the direct electrochemistry of FAD unit and GOx on graphene. A biosensing platform with GOx assembled on graphene was also proposed. It was shown that the biosensor prepared in such a way enables detection of glucose in phosphate buffer with a high sensitivity (ca. 110 ±3 µA mM\(^{-1}\) cm\(^{-2}\)), a low detection limit (10 ± 2 µM) and in a wide linear range (0.1
This platform was also used to determine the glucose level in human serum. The results were in a good agreement with the values obtained by commercial blood glucose monitors with the relative deviation of ca. 4% [4]. The use of carbon nanomaterials in commercial devices offers many advantages. It is not only cheap, but also compact. However, graphene is a relatively new material and at this point it is difficult to ascertain how successful it will be as an electrode material for applications in bioelectrochemistry [5].

As carbon nanomaterials are widely used in nanotechnology, it is also important to establish if there are any possible health hazards related to these materials. Magrez et al. studied the cellular toxicity of nanomaterials based on carbon as a function of their surface modification and aspect ratio. Human lung tumour cells H596 as well as three types of carbon structures: multi-walled carbon nanotubes (MWCNTs), carbon nanofibres (CNFs) and carbon nanoparticles (CNPs) were chosen for in vitro toxicity tests. The results received by Magrez and co-workers clearly show that carbon black particles demonstrated the highest toxicity as evidenced by the number of viable cells at all concentrations and time points tested [6]. Moreover, MWCNTs always demonstrated lower toxicity than the other materials and, surprisingly, fibres were less toxic than particles. To explore the effect of surface properties, MWCNTs and CNFs were chemically modified with carbonyl (C=O), carboxyl (COOH) and hydroxyl (OH) groups. The toxicity of modified carbon based materials increased significantly when the following groups were used: C=O > COOH > OH. The mechanism of cell death induced by carbon based nanomaterials is still not clear. Cell death was observed either after contact of nanomaterials with the cell membrane or after internalization within the cells [6].

Different supports have been used in biosensors as alternatives to carbon based nanostructures. In recent years, gold has been frequently used. The interest in gold derive from its excellent properties as a noble metal. Gold is not affected by oxidation and does not create stable oxides under ambient conditions. It is also stable over a wide pH range, highly conductive and biocompatible [7-8]. Gold porous thin films are of great interest for use in diagnostics [9], sensors, catalysis and coatings for medical and MEMS-type [10] devices due to its increased surface area, biological compatibility and chemical inertness. Among many various alloy systems used to fabricate nanoporous gold surfaces,
Au–Ag alloy is the most attractive. Although both Au and Ag have an identical FCC lattice structure and similar atomic radii their electrochemical properties are significantly different [11-12]. Au (I) salts can cause hypersensitive skin reactions and much less frequently, blood dyscrasias (blood disorders which appear when one part of the blood is not present in the normal supply, e.g. the low levels of iron cause anaemia, thrombocytopenia is caused by low platelet count). Metallic Au (0) is not toxic. However, Cu, Ni and Pd are known to act as sensitizers. Preclinical work using Au (0) particles as delivery vehicles for gene therapy indicated that the material was both safe and efficient [13].

In this study, two types of nanoporous gold: gold leaf and sputtered gold were used. Both materials, with different morphologies and surface roughness were characterised in terms of the electrochemically addressable surface area, ease of use, stability. The most robust material was used as a support for the immobilization of enzymes with potential applications in biosensors and biofuel cells.
2.2 Experimental

2.2.1 Reagents

Nitric acid (70%), sulfuric acid (95 – 98%), copper sulphate (≥99%), perchloric acid, potassium chloride (≥99%), potassium phosphate monobasic (≥99%) and dibasic (≥98%), 1-hexadecanethiol (99%), 1-mercaptobutanol (95%), 6-(ferrocenyl)hexanethiol, potassium ferrocyanide (≥99%), ruthenium (III) hexamine chloride (98%), 1-(ferrocenyl)ethanol were obtained from Sigma-Aldrich Ireland, Ltd. Absolute ethanol was obtained from Lennox Ltd., Ireland. Deionised water (resistivity of 18.2 MΩ cm was obtained from an Elgastat maxima-HPLC (Elga, UK).

2.2.2 Electrodes fabrication

2.2.2.1 Nanoporous gold leaf electrodes preparation

12-carat Au-Ag leaf (50 wt % Au) was obtained from Wilhelm Wasner Blattgold, Germany. After dealloying in (70% w/v) nitric acid for 15 min at 30 °C, the free-standing nanoporous gold films were washed several times with ultrapure water and floated on water. The floating nanoporous gold films were attached to a polished glassy carbon (GC) electrode and allowed dry for 12 h at 4 °C to create a high surface area nanoporous gold leaf/GC electrode, as described previously [14].

2.2.2.2 Nanoporous and planar gold electrodes preparation

Metal targets for substrate deposition, Au (AJA International Inc., USA), Ag and Ti (Kurt J. Lesker Company Ltd., UK), were >99.99% pure. Magnetron sputtering was carried out at room temperature in an ultra-high vacuum chamber (ORION-5-UHV custom sputtering system) onto plain pre-cleaned glass microscope slides. Prior to metal deposition, the glass slides were cleaned by Ar plasma under vacuum. Sample substrates were tilted at ~70° from the surface normal and rotated at 20-40 rpm to ensure uniform
deposition. Sputter deposition rates were calibrated using a quartz crystal thickness monitor. The planar Au substrate was prepared by deposition of a ~10 nm thick Ti adhesion layer followed by a ~100 nm thick Au layer. A thinner Au substrate layer (35 nm) was used as a base for the deposition of a composite layer of Ag\textsubscript{x}Au\textsubscript{1-x} with a uniform distribution of Ag and Au. The ~35 nm thick Au layer improves adhesion and prevents delamination of the Ag\textsubscript{x}Au\textsubscript{1-x} alloy layer during dealloying [15]. In addition, any potential redox activity of the underlying Ti layer is suppressed. Each metal was sputtered at a constant sputtering rate to produce a ~100 nm thick Ag\textsubscript{67}Au\textsubscript{33} alloy layer with a uniform distribution of Ag throughout the alloy. Ag\textsubscript{x}Au\textsubscript{1-x} substrates with a nonuniform distribution of Ag were prepared by simultaneously depositing Au at a constant sputtering rate while Ag was deposited at a gradually increasing sputtering rate. This procedure produced a ~100 nm thick Ag\textsubscript{x}Au\textsubscript{1-x} layer with a higher distribution of Ag near the surface of the alloy layer. Both ‘uniform’ and ‘nonuniform’ substrates were dealloyed in concentrated (70% w/v) nitric acid for 15 min at 37 °C. On removal from the nitric acid bath, the substrates were thoroughly rinsed with deionised water and dried in a stream of nitrogen. The electrodes were electrically connected via a Ag wire attached to the Au substrate using indium and an epoxy glue. A circular electrode area (0.28 cm radius) was defined using an insulating paint (Gwent Electronic Materials Ltd., UK). The geometric surface area of each np-Au electrode was 0.246 cm\textsuperscript{2} (A\textsubscript{geo}).

The structure and chemical composition of nanoporous gold was characterized using a scanning electron microscope (SEM; Hitachi SU-70) equipped with an energy dispersive X-ray spectrometer (EDS, Oxford Instruments). In order to minimize charging effects, beam voltages were adjusted between 3 and 5 kV, and a corner of the sample was electrically connected to the sample holder with silver paint. The Au pore, ligament, and crack sizes were determined manually by identifying a minimum of 10 of each feature and making measurements across the shortest distance using ImageJ software.

### 2.2.3 Electrochemical measurements

All electrochemical measurement were performed using either a CHI832 bipotentiostat or CHI630A potentiostat (CHI Instruments, Austin, Texas, USA) in a three-electrode electrochemical cell with a Pt wire (ALS Co. Ltd., Tokyo, Japan) and
Ag/AgCl/3 M KCl (IJ Cambria Scientific Ltd., UK) as counter and reference electrodes, respectively. Prior to each measurement, the electrodes were cleaned by scanning (10 scans) from 0 to 1.5 V at 200 mV/s in 0.5 M H$_2$SO$_4$, followed by scanning at 50 mV/s between 0 and 0.8 V in 0.1 M PBS buffer at pH 7.0. Planar Au electrodes were cleaned by dipping in Piranha solution (1: 3 (v/v) mixture of 30% H$_2$O$_2$:98% H$_2$SO$_4$) for 30 s followed by rinsing in ultrapure water source.

### 2.2.4. Electrochemical characterization

Surface-confined and diffusion-controlled probes were used to determine the electrochemically addressable surface area ($A_{real}$) and roughness factor ($R_f$) of the nanoporous gold electrodes. Au oxide stripping, underpotential deposition of copper (Cu UPD) and the formation of monolayers of redox thiols were used to determine the active surface on each nanoporous gold electrode. Diffusion-controlled experiments using negatively and positively charged redox probes and the bulk Cu deposition were also performed.

The Au oxide stripping technique was used to determine the surface area of the nanoporous gold electrodes. Cyclic voltammetry in the potential range of 0 to 1.5 V in 0.5 M H$_2$SO$_4$ was used to coat nanoporous gold electrodes with a Au oxide monolayer which was subsequently removed. The charge integrated under the gold oxide reduction peak was used to calculate the electrochemically active surface area and roughness factor $R_f$ for each electrode. A theoretical charge density of 390 μC/cm$^2$ associated with reduction of the gold oxide was used to obtain the electrochemically active surface area of each type of the Au electrode ($A_{real}$) [16]. Cu UPD was performed using cyclic voltammetry in the potential range of 0.6 to -0.2 V in aqueous solution of either 1 mM or 2 mM CuSO$_4$ in 0.1 M H$_2$SO$_4$[17-18].

The electrochemical responses of the redox probes, potassium ferrocyanide (5 mM Fe(CN)$_6^{3-}$ in 100 mM KCl), ruthenium (III) hexamine chloride (5 mM Ru(NH$_3$)$_6^{3+}$ in 100 mM KCl) and 1-(ferrocenyl)ethanol (5 mM FcEtOH in 100 mM KCl) were investigated at each np-Au electrode design. Bulk deposition of Cu was completed in aqueous solutions of either 1 or 2 mM CuSO$_4$ in 0.1 M H$_2$SO$_4$ [17-18]. The $R_f$ values
from the Cu UPD results was calculated by comparison of either surface coverage \( (\Gamma, \text{ pmol/cm}^2) \) or the current density \( (I, \text{ mA/cm}^2) \) at a planar Au electrodes to that obtained at np-Au electrodes. Both parameters were normalised to the geometric surface area of the gold electrodes \( (A_{geo}, 0.246 \text{ cm}^2) \). The surface coverage for Cu was determined by integration of the anodic current and converted to \%\ monolayer coverage using a theoretical value for complete Cu monolayer on Au of 2000 pmol/cm\(^2\) \[19\]. \( A_{real} \) values were obtained by multiplying \( A_{geo} \) by \( R_f \).
Chapter 2 Nanoporous gold. Fabrication and characterization

2.3 Results and discussion

2.3.1 Characterization of nanoporous gold leaf electrodes

A free-standing 50 wt% Au-Ag sheet was used to fabricate nanoporous gold leaf electrodes [20]. Porous surfaces in the sample were obtained by dealloying for 15 min in concentrated HNO$_3$ at 30° C. Figure 2.1 shows a SEM image of the sample with pores and ligaments size of ca. 15 and 30 nm, respectively. The catalytic activity of nanoporous gold leaves with different pore sizes was investigated by Yin et al. who found that the nanoporous gold leaf sample with a ligament size of 30 nm exhibited the highest stability with good activity during the catalytic process [21]. Table 2.1 shows the distribution of the elements on the top on the nanoporous gold leaf after the dealloying step. The amount of Au in the sample is ca. 91% demonstrating that during dealloying Ag is not removed completely from the material.

![SEM image of the np-Au leaf dealloyed for 15 min at 30° C.](image)

Figure 2.1 SEM image of the np-Au leaf dealloyed for 15 min at 30° C.

The next step was to attach the porous sample to a stable and conductive carrier. Among others, carbon coated copper grids [22] and silicon wafers [23] had been used as possible carriers. In this study, a glassy carbon (GC) electrode was used as a support for the nanoporous gold leaves.
Table 2.1 EDS data of nanoporous gold leaf and uniform and non-uniform nanoporous gold samples dealloyed in 70% (w/v) nitric acid at 37° C for 15 min.

<table>
<thead>
<tr>
<th></th>
<th>Au</th>
<th>Ag</th>
<th>Ti</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanoporous gold leaf</td>
<td>91.16%</td>
<td>8.84%</td>
<td>-</td>
</tr>
<tr>
<td>Uniform nanoporous gold</td>
<td>98.19%</td>
<td>0.60%</td>
<td>1.21%</td>
</tr>
<tr>
<td>Non-uniform nanoporous gold</td>
<td>96.36%</td>
<td>2.88%</td>
<td>0.76%</td>
</tr>
</tbody>
</table>

Figure 2.2 Nanoporous Au leaf film attached to a GC electrode.

Attempts were made to modify nanoporous gold leaves on GC electrode (Figure 2.2) with thiols modifiers. However, it was found that thiol modification disrupts the weak attractive forces between the Au leaf and GC surface, leading to the detachment of the nanoporous gold leaf from the GC. Attempts to modify the nanoporous gold leaf with thiols prior to attachment to the GC were also not successful due to the failure of the thiol modified leaves to cover the GC or breaking of the leaf in the thiol solution. The attachment of the nanoporous gold leaf film to the GC electrode could be improved by dropping a Nafion suspension on dry nanoporous gold on GC electrode [14, 24]. However, this procedure could block the ability to subsequently modify the surface.
Qiu et al. have shown the successful immobilization of enzymes on nanoporous gold leaf electrodes was possible [24]. From the three strategies of laccase immobilization: covalent coupling, electrostatic adsorption and physical attachment, surprisingly the last turned out to be the most efficient due to the amount and specific activity of the immobilized enzyme as well as the lack of leakage [25]. Glucose oxidase was also immobilized on nanoporous gold leaves fabricated from a Ag-Au foil of 58-42 wt%. Yin et al. demonstrated the aerobic oxidation of glucose over nanoporous gold to D-gluconic acid under mild condition [21]. However, due to the lack of mechanical stability and the problems with attachment to the GC electrode reported previously, the use of nanoporous gold leaf made from 12 karat white gold sheets was abandoned. These electrodes were not sufficiently robust for use as supports for enzyme immobilization.

### 2.3.1 Characterization of nanoporous gold electrodes

Au-Ag sheets, the Au and Ag alloys can also be fabricated by magnetron sputtering system on Si wafers or different stable carriers under high vacuum (ca. $10^{-4}$ Pa) [26]. In magnetron sputtering the target is bombarded by energetic ions generated by collisions with free electrons inside a plasma. Ions are accelerated towards the target surface, transferring their kinetic energy to it. During this process atoms are ejected from the target material and condense into a layer on the surface of the material [27].

![Figure 2.3 Schematic diagram of layered structure of nanoporous gold electrodes (not to scale).](image-url)
This approach was used to fabricate nanoporous gold electrodes (section 2.2.2.2). Glass was chosen as a mechanically stable support for the films. The glass slide was then covered with a titanium adhesion layer to enable better attachment followed by a pure gold layer which insulates the electrochemically active titanium layer. A Au$_{33}$-Ag$_{67}$ layer was then sputtered on the gold layer (Figure 2.3). Two sputtering procedures uniform and non-uniform (section 2.2.2.2) were used to prepare gold films with different morphologies. Porous structures in both samples were obtained on dealloying. In this corrosion process, the less noble metal, silver is selectively dissolved and the remaining more noble metal, gold self-organises into an interconnected network of tunable pores, ligaments and cracks. The morphology and the composition of nanoporous gold electrodes were characterised after dealloying for 15 minutes at 37° C using SEM and EDS. Figure 2.4 A and B demonstrates the differences in morphology of both nanoporous gold samples. The pore, ligament and crack sizes in the nanoporous gold film sputtered non-uniformly are 12 – 20 nm, 15 – 18 nm and 55 – 65 nm, respectively. Uniformly sputtered nanoporous gold possess pore, ligament and crack sizes of 17 – 23 nm, 18 – 25 nm and 40 – 55 nm, respectively. Although, uniform nanoporous gold material has a relatively fine structure, the surface of non-uniform nanoporous gold has an increased number of cracks which arise from the stresses introduced to the samples during dealloying [28]. A cross-sectional SEM image (Figure 2.4 C and D) demonstrates the worm-like structure of interconnected ligaments and cracks distributed regularly throughout the entire volume of the nanoporous gold films. In addition, it is clear that the pure Au base layer remains intact after the dealloying process.
EDS data (Table 2.1) confirmed the removal of Ag in the dealloying process with only 0.6 and 2.88 wt % Ag remaining in uniform and non-uniform nanoporous gold samples, respectively.

The electrochemically addressable surface area of the nanoporous gold electrodes was examined by gold oxide stripping and copper underpotential deposition (Cu UPD). Redox probes and Cu bulk deposition were used to examine the surface accessibility and the diffusion profile at the electrode surfaces.
Cyclic voltammograms of planar and nanoporous gold electrodes in 0.5 M H$_2$SO$_4$ show peaks corresponding to the formation of Au oxides [16] at 1.2 V and 1.4 V and a sharp gold oxide reduction peak at 0.9 V (Figure 2.5). The absence of other peaks in the cyclic voltammogram confirmed that Ag was removed from the surface during the dealloying process in agreement with the EDS data (Table 2.1).

A second method of determining the electroactive surface area and the roughness factors ($R_f$) of the electrodes was Cu UPD. Figure 2.6 shows the voltammograms obtained for Cu UPD, with two pairs of peaks evident. The redox wave between approximately 0 V to 0.15 V is attributed to reduction of Cu$^{2+}$ to Cu and is followed by oxidation of the deposited Cu to freely diffusing Cu$^{2+}$. Bulk Cu deposition is controlled by the diffusion of aqueous Cu$^{2+}$ to the electrode surface. The second pair of broad peaks obtained in the region between 0.2 V and 0.4 V corresponds to the deposition of a Cu adlayer, followed by stripping of Cu on the gold surface. Cu UPD is to a surface-confined process. The cyclic voltammograms obtained are in a good agreement with the literature [29-30]. A significant enhancement of the current signal is observed for the Cu UPD at both nanoporous gold electrodes in comparison to planar Au electrode. In contrast, no enhancement in current was observed for bulk Cu deposition (Figure 2.6). This is due to
diffusion controlled response in solution with Cu\(^{2+}\) being reduced only at the surface and perhaps in the upper regions of the pores. The diffusion controlled response is caused by overlapping of the diffusion fields which occurs between each individual pore establishing a semi-infinite linear diffusion field [31]. The current density corresponding to bulk Cu deposition as a diffusion controlled process depends on the geometric surface area of the electrode \(A_{\text{geo}}\), while Cu UPD is a surface confined process with the observed current dependant on the electrochemically active surface area \(A_{\text{real}}\) [32]. A concentration of 1 mM CuSO\(_4\) was sufficient to achieve maximal coverage of the electrode surface. This was confirmed by the unchanged Cu UPD response when the concentration of Cu\(^{2+}\) was increased to 2 mM CuSO\(_4\). Simultaneously the magnitude of the current for the bulk deposition of Cu doubled (Figure 2.5 A, C). At a planar gold electrode, submonolayer coverage of Cu was observed.

**Figure 2.6** Cyclic voltammograms of (A, C) bulk in 1 mM and 2 mM CuSO\(_4\) electrolyte, respectively and (B) UPD of copper in 1 mM CuSO\(_4\) at (dashed line) non-uniform, (dotted line) uniform nanoporous and (full line) planar gold electrodes at scan rate 1 mV/s.
A comparison of the electrochemically active area and roughness factor \((R_f)\) of the electrodes is shown in Table 2.2. The highest electroactive surface area determined by both the Au stripping and bulk Cu methods was observed with non-uniformly sputtered nanoporous gold. However, at the non-uniform nanoporous gold the value of \(R_f\) obtained from the Cu UPD was lower than that measured by the Au oxide stripping technique. This can be explained by the fact that non-uniform nanoporous gold has smaller pores and ligaments, as demonstrated by SEM analysis, which may be inaccessible. The \(R_f\) factor for the uniform nanoporous gold obtained in the Au oxide stripping method is in a good agreement with the value determined in the Cu UPD.

Table 2.2 The comparison of calculated roughness factor \((R_f)\), electroactive area \((A_{real})\) and percent of monolayer formation at each Au electrode.

<table>
<thead>
<tr>
<th>Probe</th>
<th>(R_f)</th>
<th>(A_{real})</th>
<th>% monolayer formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>uniform</td>
<td>non-uniform</td>
<td>uniform</td>
</tr>
<tr>
<td></td>
<td>nanoporous gold</td>
<td>nanoporous gold</td>
<td>planar gold</td>
</tr>
<tr>
<td>Au Ox/Red</td>
<td>16.1</td>
<td>28.1</td>
<td>0.251</td>
</tr>
<tr>
<td>bulk Cu(^{2+/0})</td>
<td>1.05</td>
<td>1.35</td>
<td>0.246</td>
</tr>
<tr>
<td>UPD Cu(^{2+/0})</td>
<td>15.5</td>
<td>19.0</td>
<td>66</td>
</tr>
</tbody>
</table>

Nanoporous and planar gold electrodes were also characterised using the redox probes, ruthenium (III) hexamine chloride (Figure 2.7 A), potassium ferrocyanide (Figure 2.7 B) and 1-(ferrocenyl)ethanol as positively and negatively charged and neutral probes, respectively (Figure 2.7 C). No significant increase in current density between planar and nanoporous gold electrodes was observed. As explained for bulk Cu deposition, the current density is limited by overlapping of the diffusion fields between the individual pores leading to the establishment of a semi-infinite linear diffusion field.
Due to its robustness and the high surface area-to-volume ratio of nanoporous gold is an attractive material in biosensors and biofuel cells. However, bare metal surface may cause the denaturation of many redox enzymes. Thus, it was important to determine the ability to modify nanoporous gold with alkane thiols. Figure 2.8 shows the response observed with ferricyanide at full monolayer coverage state thiol at non-uniform and uniform nanoporous gold and planar gold electrode. It was found that a self-assembled monolayer of 1-hexadecanethiol suppressed the redox response of ferricyanide.
Figure 2.8 Suppression of the \( \text{Fe(CN)}_6^{3-/4-} \) redox response in 0.1 M KCl by the formation on the non-uniform nanoporous (dashed line), uniform nanoporous (dotted line) and planar (full line) gold electrodes of a SAM monolayer on immersion in a 10 mM 1-hexadecanethiol solution for 72 hours.
2.4 Conclusions

Two types of nanoporous gold materials, gold leaves and sputterd gold electrodes were fabricated and characterised. Comparison of the two materials demonstrated the advantage of using nanoporous gold electrodes over nanoporous gold leaf due to the mechanical stability of the former. The crucial step for the fabrication of the robust electrode is the sputtering of the titanium adhesion layer. The titanium layer strongly attached the next pure gold layer of the nanoporous gold, whereas the weak adhesion forces between nanoporous gold leaf film and the glassy carbon electrode were easily disrupted.

A number of methods were used to characterize the electrochemical addressable surface area $A_{\text{real}}$ and the surface area accessible for modification. Values for $A_{\text{real}}$ of 0.251 cm$^2$, 3.961 cm$^2$ and 6.931 cm$^2$ for planar, ‘uniform’ and ‘non-uniform’ nanoporous gold electrodes, respectively were determined from the Au oxide stripping method. In addition, bulk Cu deposition and UPD of Cu$^{2+}$ were identified as suitable and complementary methods to estimate $A_{\text{real}}$. It was also confirmed that the planar and nanoporous gold surfaces can be modified by the thiol molecules what allows to apply gold electrodes in redox enzymes immobilization.
2.5 REFERENCES

CHAPTER 3:

DIRECT ELECTRON TRANSFER BETWEEN REDOX ENZYMES AND NANOPOROUS GOLD ELECTRODES
3.1 Introduction

The very first biosensor was described by Leland C. Clark Jr. in 1962 and commercialised in 1974 by Yellow Springs Instruments Company. In this biosensor molecular oxygen was used as oxidising agent oxidises glucose to gluconic acid. The consumption of the co-substrate O₂ occurred at the platinum electrode, as in the Clark oxygen electrode [1]. Since then many glucose biosensors have been developed based on the detection of O₂ or produced H₂O₂ [2]. To make detection independent of concentration of O₂ the ‘artificial’ redox mediators have been used. Until now many of the commercialized biosensors produced by Yellow Springs Instruments Company, La Roche, Abbott or Bayer are based on mediated electron transfer (MET) [3]. In general, mediators are low-molecular weight compounds that are soluble and freely-diffusing e.g. ferrocene derivatives [4-5]. Cass et al. described the biosensor for the analysis of glucose. The ferrocene (1,1’-dimethylferrocene)/ferricinium redox couple was used as an effective mediator of electron transfer between immobilized glucose oxidase (GOx) and the electrode [6]. To integrate the sensors components by adsorption of the mediators with the enzymes on the electrode surface, soluble mediators were replaced by metal complexes, mainly of osmium and ruthenium, attached to flexible polymeric backbone.. In these systems mediators surround the enzyme molecules, and are able to transfer electrons to and from the enzyme and the electrode [7]. Mao et al. successfully used Os²⁺/³⁺ complexes (PVP-[Os(N,N'-dialkylated-2,2'-biimidazole)₃]²⁺/³⁺ and PVI-[Os(4,4'-diamino-2,2’bipyridine)₂Cl]⁺/²⁺ to ‘wire’ the FAD/FADH₂ redox centres of GOx to an electrode. The dialkylated bis-imidazole complex is a fast redox couple analogue of 2,2’-bipyridine. A limiting current density of 1.15 mA/cm² in 15 mM glucose buffer and slightly decreased stability of ca. 3.5% over 20 hours was obtained at the anode with glucose oxidase cross-linked in redox hydrogel with PVI-[Os(4,4’-diamino-2,2’bipyridine)₂Cl]⁺/²⁺ [8].

The response of a biosensor is directly determined by the rate of biocatalysis of enzymes which accomplishes the conversion of reactant to product. The class of enzymes which can be used to fabricate biosensors are oxidases that consume dissolved oxygen and produce hydrogen peroxide. Within oxidases, glucose oxidase from
Aspergillus niger was the first enzyme commonly used in biosensors. GOx (from Aspergillus niger) is a dimeric glycosylated protein composed of two identical monomers. Each monomer has two domains: one binds to the substrate and the second to a co-factor, flavin adenine dinucleotide (FAD), which is a powerful oxidising agent. The two molecules of FAD are tightly bound together but non-covalently attached to the apoenzyme. GOx catalyses the oxidation of β-D-glucose into glucono-δ-lactone, which then hydrolyses into gluconic acid [9]. Simultaneously molecular oxygen is reduced to hydrogen peroxide (equations 1 – 3) [10].

\[
\begin{align*}
 GOx(FAD) + \beta-D-glucose & \rightarrow GOx(FADH_2) + glucono-\delta-lactone \\
 GOx(FADH_2) + O_2 & \rightarrow GOx(FAD) + H_2O_2 \\
 glucono-\delta-lactone + H_2O & \rightarrow D-gluconic\ acid
\end{align*}
\]

Due to the non-covalent attachment, FAD molecules can be easily removed from the holoenzyme without denaturing of apoenzyme [11]. Willner et al. reported the removal of FAD redox centre form GOx and then reconstitution the apo-protein on a PQQ linked FAD monolayer on at gold electrode [12]. The same group showed that GOx could be reconstituted using FAD modified Au crystals. This approach shows that glucose is still oxidised by the enzyme when the electrons are relayed to the electrode via the FAD-Au-nanoparticles unit [13].

Cellobiose dehydrogenase (CDH) also catalyses the oxidation of glucose to glucono-δ-lactone. Moreover, unlike GOx, CDH catalyses the oxidation of lactose, cellobiose, cellodextrins, maltose, diphenolic compounds [14]. CDH is an extracellular oxidoreductase produced by phytopathogenic and wood-degrading fungi [15]. CDHs are divided into three subgroups: class I, produced by basidiomycetes and class II and III, produced by ascomycetes. Although class I CDHs have shorter sequences and work
better in acidic pH, they exhibit a poor oxidising ability for monosaccharides. In contrast, some of the CDHs from class II are more complex in sequence, work better in neutral and basic pH as well as have high turnover number with monosaccharides and oligosaccharides. Class III CDHs was also found in the ascomycetes. However, no CDH from class III has been characterized or actively expressed so far [16]. CDH is a monomeric protein consisting of two co-factors: a larger flavin adenine dinucleotide (FAD) domain and a smaller heme b domain connected via protease cleavable linker region [17]. Electron transfer (IET) takes place between two domains. Two possible models of the catalytic role of heme b and the heterogeneous electron transfer between CDH and the electrode can occur. The first is ‘the electron-chain model’, where an electron is transferred from the flavin adenine dinucleotide unit to the heme and then finally to a one-electron acceptor. In the second model, ‘the electron sink’ model, the heme can accept and store electrons but the heme itself is not required for activity as the acceptor can directly interact with FAD unit [15]. The actual mechanism in operation depends on the nature of the electron acceptor. In the case of reduction of cyt c and DET to the electrodes, ‘the electron-chain model’ is the proposed route for electron transfer [18]. In contrast, the direct reduction of different one- or two-electron acceptors at the FAD unit favours ‘the sink model’ [19]. DET between CDH and planar gold or graphite electrodes has been described in detail. Stoica et al. connected CDHs from *P. sordida*, *Trametes villosa* and *Myriococcum thermophilum* to a gold electrode modified with thiols with the heme b domain [20]. It was found that thiols with alcohol end groups orient the enzymes in the most favourable position at the electrode surface [20]. Using graphite electrode, the DET has been observed. Larsson et al. reported the adsorption of CDH on graphite electrode. The contact between the enzyme and the graphite surface was obtained through the heme b domain with the direct bioelectrocatalysis of cellobiose and lactose [21-22].

Blue multi-copper oxidases constitute an important class of enzymes that reduces molecular oxygen to water in a four-electron reduction process with concomitant one-electron oxidation of the substrate [23]. The most studied to date members of the multi-copper oxidases family are: bilirubin oxidase (BOD) and laccase (Lc). However, the structure of all copper oxidases is similar. Cu-enzymes contain four copper ions classified historically due to their spectral characteristics into three types: T1 and T2 which is connected to the binuclear T3 into the T2/T3 cluster. Both Cu T1
and T2 are EPR-active in contrast to the T3 centre which is not EPR detectable [24]. The role of each copper redox centre is related to the catalytic properties of the enzyme. Thus, the T1 copper site acts as a primary electron acceptor, which then transfers electrons in an intramolecular electron transfer mechanism (IET) through a His-Cys-His bridge to T2/T3 cluster, where four-electron reduction of O₂ to H₂O occurs without formation of highly reactive oxygen species [25-26].

There are more than 60 types of Lcs with different thermodynamic and kinetic properties isolated from various type of plants, fungal and bacterial sources [27-28]. A single organism can be a source of multiple forms of Lcs with different primary structure and biochemical properties, as shown by Kojima et al. for basidiomycete Lc from *Coriolus hirsutus* [29-30]. Lcs catalyze the oxidation of para- and ortho-diphenols, aminophenols, aryl diamines, polyphenols, polyamines and lignins [31]. The key characteristic of Lc is the formal potential of its redox centres. In addition, the formal potential of the T1 copper site influences the catalytic efficiency of the enzyme [32-33]. The value of the formal potential of the T1 redox centre was determined for many Lcs using potentiometric titration and was found to vary between 430 and 790 mV vs. NHE [34-35]. The high redox potential Lcs are of great interest due to their efficiency in bioremediation, detoxification and especially in biofuel cells research.

BOD is another multi-copper oxidase which, unlike Lc, catalyses the reaction of bilirubin and other tetrpyroles [36]. It can also oxidase diphenols and aryl diamines [37]. The enzyme was discovered and characterised by Murao and Tanaka in 1986 [38]. There are two main ascomycetes sources of BOD: *Myrothecium verrucaria* and *Trachyderma tsunodae*. The structure of BOD is very similar to the Lc. The T1 copper site is a primary electron acceptor which communicates with a T2/T3 cluster. The formal potential of the T1 active site varies depending on the source of the enzyme. For *Myrothecium verrucaria* it is between 570 – 670 mV vs. NHE [37-38] and for *Trachyderma tsunodae* between 615 – 715 mV vs. NHE at pH 7 [39-40]. Due to the value of the redox potential BOD is also classified as high redox potential ‘blue’ copper oxidase. However, in contrast to fungal Lc which is active at acidic pH, BOD remains active at neutral pH. On the other hand BOD is rapidly inactive in human serum being affected by products from the reaction between urate and O₂ [41-42].

In this chapter different immobilization techniques based on direct electron transfer are used to obtain efficient communication between nanoporous gold electrodes
and redox enzymes. Cellobiose dehydrogenase modified nanoporous gold electrodes are used to detect lactose concentration in the buffer at pH 7.4. Bilirubin oxidase and laccase modified nanoporous gold detects the changes in O₂ concentrations.
3.2 Experimental

3.2.1 Reagents

Sulphuric acid (95 – 98%), potassium phosphate monobasic (≥99%) and dibasic (≥98%), sodium phosphate dibasic (≥99%), sodium chloride, sodium fluoride, 6-mercapto-1-hexanol (97%), 11-mercaptoundecanoic acid (95%) 16-mercaptohexadecanoic acid (90%), 11-mercaptoundecanol (97%), 9-mercapto-1-nonanol (96%), 8-mercapto-1-octanol (98%), 1-dodecanethiol (≥98%), sodium hydroxide (≥97%), 1-thioglycerol (≥97%), Nafion®, poly(ethylene glycol)diglycidyl ether, N-cyclohexyl-N’-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC; ≥99%), D- (+) -glucose (99.5%), D-lactose, citric acid anhydrous, glutaraldehyde (25% in H$_2$O) were obtained from Sigma-Aldrich Ireland, Ltd. Absolute ethanol was obtained from Lennox Ltd., Ireland. Deionised water (resistivity of 18.2 MΩ cm) was obtained from an Elgastat maxima-HPLC (Elga, UK).

3.2.2 Enzymes

Cytochrome c from equine heart was obtained from Sigma-Aldrich Ireland, Ltd. The concentrations of cytochrome c solutions were determined spectrochemically by using the molar absorption coefficient at 409 nm (106100 M$^{-1}$ cm$^{-1}$) [43]. The enzyme was purified according to a published procedure [44]. The preparation of homogeneous cellobiose dehydrogenase from Corynascus thermophilus (CtCDH) was kindly provided by Dr Roland Ludwig. The enzyme was purified to homogeneity according to a published procedure [45]. The concentration of CtCDH was 8.4 mg/ml and 22.85 mg/ml. The preparation of homogeneous laccase from the basidiomycete Trametes hirsute (ThLc) was kindly provided by Prof. Alexander I. Yaropolov. The enzyme was purified to homogeneity using a published procedure [46-47]. The concentration of ThLc stock solution of 2.83 mg/ml was determined spectrochemically by using the molar absorption coefficient at 280 nm (84739 M$^{-1}$ cm$^{-1}$) [48]. Myrothecium verrucaria bilirubin oxidase (MvBOD) was purchased from Amano Enzyme Inc. (Nagoya, Japan).
Chapter 3

DET based electrodes

The activity of MvBOD was 2.63 u/mg. The enzyme was purified to homogeneity using a published procedure [49].

3.2.3 Enzyme immobilization procedures

Different immobilization procedures based on thiols self-assembled monolayer (SAMs) and adsorption under vacuum were used to modify polycrystalline, planar and nanoporous gold electrodes.

Polycrystalline gold electrodes (CHI Instruments, Austin, Texas, USA) were exposed to 1 mM thiol solution in ethanol. After 24 hours the gold electrodes were removed from the thiol solution, rinsed gently with ethanol and a 4.4 mM phosphate buffer solution pH 7.0 and dried with a stream of N₂ to remove loosely attached thiol molecules. Then the electrodes were immediately placed in 50 µM cytochrome c solution in 4.4 mM phosphate buffer pH 7.0 for 1 hour at 4 °C. After 1 hour the modified electrodes were rinsed carefully in 4.4 mM phosphate buffer pH 7.0. The electrodes which were not used immediately were stored dry at 4 °C.

Planar gold as well as nanoporous gold electrodes were exposed to 1 mM of 6-mercapro-1-hexanol and 11-mercaptoundecanoic acid solution in ethanol. After 24 hours the gold electrodes were removed from the thiol solution, rinsed gently with ethanol and a 4.4 mM phosphate buffer solution pH 7.0 and dried with a stream of N₂ to remove loosely attached thiol molecules. The dried electrodes were placed in 5 mM solution of N-cyclohexyl-N’-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate (CMC) in 100 mM phosphate buffer at pH 7.0 for 30 minutes at 4 °C. Then the electrodes were immediately placed in 50 µM cytochrome c solution in 4.4 mM phosphate buffer pH 7.0 for 1 hour at 4 °C. After 1 hour the modified electrodes were rinsed carefully in 4.4 mM phosphate buffer pH 7.0. The electrodes which were not used immediately were stored dry at 4 °C.

CtCDH was immobilized on planar and nanoporous gold electrodes. Each electrode was exposed to 1 mM 1-thioglycerol for 2 hours. Then the electrodes were removed from the thiol solution, rinsed gently with ethanol and 50 mM phosphate buffer saline (150 mM NaCl) pH 7.4. 8 µL drop of 8.5 mg/ml CtCDH was placed on the
top of the electrodes. The drops were allowed to gently dry in the fridge for 3 hours. Nafion® (8 µL) was placed on the CdCDH-modified gold electrodes. Prior to use, Nafion® was neutralized to pH 5.5 with NH₄OH [50]. The electrodes were stored dry at 4 °C.

MvBOD and ThLc were adsorbed on nanoporous gold electrodes. 20 µL of the enzyme solution (40 µg/ml) 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 µL of P017-epoxy solution and allowed to dry in air at room temperature for 15 min. As a control experiment, electrodes were modified by drop-casting 20 µL of MvBOD or ThLc solution (40 µg/ml) which were then kept at 4°C for 2 hours prior to use.

P017-epoxy was kindly provided by Prof. Wolfgang Schuhmann. P017-epoxy was chosen as a cross-linking agent based on the short-time stability measurements. Three uniform gold electrodes became bases for BOD adsorption. Then each electrode was covered with a different stabilizer: 10 µL of 2.25 mg/ml P017-epoxy, 10 µL of Nafion® and 10 µL of glutaraldehyde and left to dry for 15 minutes. The modified electrodes were then scanned over 1 hour in oxygen saturated 0.1 M citrate-phosphate buffer, pH 7.0 at scan rate of 5 mV/s.

### 3.2.4 Electrochemical measurements

All electrochemical studies were performed using either a CHI832 bipotentiostat or CHI630A potentiostat (CHI Instruments, Austin, Texas, USA) in a standard three-electrode electrochemical cell with a 0.5 mm diameter Pt wire counters electrode (ALS Co. Ltd., Tokyo, Japan) and Ag/AgCl in 3 M KCl reference electrode (IJ Cambria Scientific Ltd., UK). Immediately prior the use, nanoporous gold electrodes were electrochemically cleaned by scanning (10 scans) from 0 to 1.5 V at 200 mV/s in 0.5 M H₂SO₄, followed by scanning at 50 mV/s between 0 and 0.8 V in the electrolyte used to experiments. The electrodes were then dried in a vacuum chamber at 10⁻² mbar for 10 min. All current densities were normalized to the electrode geometric surface area.
3.3 Results and discussion

3.3.1 Voltammetric characterization of cytochrome c immobilized on planar and nanoporous gold electrodes

Cytochrome c (cyt c, described in Chapter 1) was used as a model system to study the accessible surface area to examine the possible application of porous gold materials for the immobilization of enzymes in biosensors and biofuel cells. The redox behaviour of cyt c immobilized on the gold electrodes depends on the structure and properties of the self-assembled monolayer. Specific interactions between an enzyme and the surface are created by an appropriate self-assembled monolayer. If the interactions are too weak, the monolayer can not be created. The adsorption then is incomplete. If the interactions are too strong, it can cause the denaturation of the enzyme due to changes in conformation [51].

A set of thiols were examined to choose the most efficient SAMs for cyt c immobilized on polycrystalline gold electrodes by electrostatic interactions. Reproducible results with stable peaks current were obtained for SAMs containing 11-mercaptoundecanoic acid and 6-mercapto-1-hexanol (HS(CH₂)₁₀COOH/HS(CH₂)₆OH). In comparison to homogenous SAMs, mixed SAMs result in faster and more reversible electron transfer kinetics for immobilized cyt c [52]. Additionally, SAMs terminated in –COOH and –OH groups provide pH-dependent charged surfaces and, thus, surfaces capable of extensive hydrogen bonding over some ranges of potential [51]. HS(CH₂)₁₀COOH is deprotonated at neutral pH and form negatively charged monolayers to bind the positively charged cyt c. In addition the layer of longer chains of deprotonated carboxylic acids and shorter chains of alcohols creates the structure which helps to orientate the protein with the redox centre close to the electrode surface.
Figure 3.1 shows a cyclic voltammogram of cyt c immobilized on HS(CH$_2$)$_{10}$COOH/HS(CH$_2$)$_6$OH modified polycrystalline gold electrode. The peak-to-peak separation potential was 26 mV. An ideal one-electron reversible redox couple should give a symmetric voltammogram with a peak separation of 0 V ($\Delta E_p$) [53]. In this study, the voltammogram is nearly symmetric and the value of $\Delta E_p$ is indicative of quasireversible and efficient electron transfer. The mixture of HS(CH$_2$)$_{10}$COOH and HS(CH$_2$)$_6$OH is the most optimal self-assembled monolayer for cyt c immobilization, good agreement with literature reports. Mixed SAMs of HS(CH$_2$)$_{10}$COOH and HS(CH$_2$)$_6$OH provided substantial electroactive surface coverage (between 15 – 30 pmol/cm$^2$ for a theoretical coverage required for monolayer at planar electrode surface) [54]. This mixture was used to immobilize cyt c onto all modified gold electrodes.

According to Chen et al., no electrochemical response for SAMs containing 1-dodecanethiol was reported [51]. Such alkanethiols do not possess negatively charged endgroups to which cyt c can bind. In addition, it was demonstrated that the topography of the gold substrate influences the degree of interaction between a HOOC-SAM and cyt c. If the gold substrate becomes smoother, the extent of adsorption and magnitude of electrochemical response of the cyt c immobilized on the HOOC-SAM decreases.
significantly [51, 55-56]. Moreover, on hydrophobic surfaces the immobilized enzymes change the conformation. The heme is no longer well protected and, thus, the protein denature [51].

**Figure 3.2** Cyclic voltammogram of 50 µM cyt c covalently immobilized on a HS(CH$_2$)$_{10}$COOH/HS(CH$_2$)$_6$OH mixed SAMs at planar (solid line and inset), uniform (dashed line) and non-uniform (dotted line) nanoporous gold electrodes in 4.4 mM K$_2$HPO$_4$-KH$_2$PO$_4$ buffer, pH 7.0, at scan rate of 100 mV/s.

Cyt c was immobilized at planar and porous gold electrodes modified with mixed SAMs of HS(CH$_2$)$_{10}$COOH and HS(CH$_2$)$_6$OH (Figure 3.2). To stabilize the voltammetric response $N$-cyclohexyl-$N'$-(2-morpholinoethyl)carbodiimide metho-$p$-toluenesulfonate (CMC) was used. The role of CMC is to activate the surface carboxyl groups of SAMs so that they react with lysine residues on cyt c to form covalent amide bonds. The topography of the porous gold versus planar and polycrystalline gold electrodes with SAMs modification noticeable influence the voltammetry of cyt c (Figure 3.2). Rough electrode surfaces modified with mixed SAMs to generate monolayers with higher defect densities provide optimal faradaic response in contrast to the poor response obtained at smooth surfaces with minimal defects and long chain homogeneous carboxylic acid terminated SAMs [55]. Values of $E''$ values for cyt c
immobilized at both uniform and non-uniform nanoporous gold electrodes (Table 3.1) were constant over a wide range of scan rates (10 – 1000 mV/s) and in good agreement with previous reports [57]. The voltammograms are nearly symmetric with some deviations in the peak separations ($\Delta E_p$) and full width at half-maximum (fwhm) (Figure 3.2 and Table 3.2), indicative of a one-electron reversible transfer reaction. This deviation may arise from a heterogeneous distribution of the cyt c on the modified electrode surface. The response of cyt c (inset Figure 3.2) at planar gold electrode surfaces modified with mixed SAMs of HS(CH$_2$)$_{10}$COOH and HS(CH$_2$)$_6$OH displays asymmetric cyclic voltammograms ($\Delta E_p > 70$ mV) with broad, less defined anodic peak in comparison to identical immobilization conditions on nanoporous gold electrodes. This observation indicates that cyt c were adsorbed on SAMs modified planar gold surface, however not in an electroactive state due to the improper orientation [55].

**Table 3.1** Voltammetric characterization of cyt c/HS(CH$_2$)$_{10}$COOH and HS(CH$_2$)$_6$OH SAMs modified nanoporous gold electrodes.

<table>
<thead>
<tr>
<th>Electrode design</th>
<th>$E^{0r}$ [mV]$^a$</th>
<th>$\Delta E_p$ [mV]$^b$</th>
<th>fwhm [mV]$^c$</th>
<th>$\alpha$</th>
<th>$k_{et}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniform nanoporous gold</td>
<td>-12 (±2)</td>
<td>18 (±1)</td>
<td>115 (±1)</td>
<td>0.53</td>
<td>2.4 (±0.9)</td>
</tr>
<tr>
<td>Non-uniform nanoporous gold</td>
<td>-11 (±1)</td>
<td>18 (±1)</td>
<td>114 (±1)</td>
<td>0.50</td>
<td>3.3 (±0.9)</td>
</tr>
</tbody>
</table>

$^a$ data obtained from scan rates in the range 10 – 1000 mV/s

$^b$ $\Delta E_p = (E_{p,a} - E_{p,c})$

$^c$ $\Delta E_p$ and fwhm obtained at scan rate of 50 mV/s

Laviron’s method was used to calculate the electron transfer coefficient ($\alpha$) and heterogeneous rate constant ($k_{et}$) for cyt c at both uniform and non-uniform nanoporous gold electrodes (Table 3.1) [58]. An irreversible response (peak separation >200 mV/n, where n = 1) occurred for scan rates greater than 1.25 and 1.75 V/s for uniform and non-uniform nanoporous gold respectively. Values of $\alpha$ were determinate from the slopes of the linear regions of plots of peak potential versus scan rate (Figure 3.3 A and C), while $k_{et}$ was determined for scan rates in the range 1.25 – 4 V/s and 1.75 – 4 V/s for uniform and non-uniform nanoporous gold electrodes respectively. A range of rate values for $k_{et}$ of 2.4 (±0.9) s$^{-1}$ for uniform and 3.3 (±0.9) s$^{-1}$ for non-uniform nanoporous gold electrode (Table 3.1) was obtained. The deviation from the $k_{et}$ values may arises from a range of possible orientations of cyt c molecules on the negatively charged
mixed SAMs layer. In addition, the rate constant was ca. 2 orders of magnitude less than those achieved at similarly modified flat gold electrodes by Dolidze et al. [59]. The diversity in the values of $k_{et}$ possibly arises from differences in the immobilization procedure.

![Graphs A, B, and C showing plots of peak current densities and peak separations.](image)

**Figure 3.3** (A) Plots of the anodic and cathodic peak current densities for immobilized cyt c as a function of the scan rate at uniform (hollow circles) and non-uniform (solid circles) nanoporous gold electrodes; (B) dependence on the anodic and cathodic peak for adsorbed cyt c on the logarithm of the scan rate at uniform (hollow circles) and non-uniform (solid circles) nanoporous gold electrodes; (C) linear regions on the plots in (B) used to calculate α (peak separation >200 mV/n with n = 1).

The surface coverage of electrochemically active cyt c at uniform and non-uniform nanoporous gold respectively was ca. 9 and ca. 11 times that predicted for full monolayer coverage per geometric surface area at planar gold (Table 3.2). Considering that the use of porous substrate enables the immobilization of high proteins loadings. Increasing the quantity of immobilized enzyme molecules on substrates such as nanoporous gold has the potential to significantly increase the sensitivity of biosensors.
Table 3.2 Comparison of calculated surface coverage and % monolayer formation at uniform, non-uniform and planar gold electrode.

<table>
<thead>
<tr>
<th></th>
<th>Surface coverage [pmol/cm²]</th>
<th>% monolayer formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniform nanoporous gold</td>
<td>139 (±18)</td>
<td>930 (±120)</td>
</tr>
<tr>
<td>Non-uniform nanoporous gold</td>
<td>164 (±14)</td>
<td>1091 (±95)</td>
</tr>
<tr>
<td>Planar gold</td>
<td>15</td>
<td>100</td>
</tr>
</tbody>
</table>

3.3.2 Cellobiose dehydrogenase (*Corynascus thermophilus*) immobilization on nanoporous gold electrodes

![Figure 3.4](image1.png)

Figure 3.4 Cyclic voltammograms of *Ct*CDH entrapped under a Nafion® membrane at a thioglycerol modified planar (solid line), uniform (dashed line) and non-uniform (dotted line) nanoporous gold electrodes in (A) 50 mM K₂HPO₄-KH₂PO₄ buffer saline, pH 7.4 and (B) 20 mM lactose solution, at scan rate of 5 mV/s.

The bioelectrocatalytic oxidation of lactose by *Corynascus thermophilus* CDH at 1-thioglycerol modified planar and porous gold electrodes was examined (Figure 3.4 A and B). SAMs of 1-thioglycerol with hydroxyl terminated groups were chosen due to the stability of thioglycerol over a wide pH range [60]. In addition to 1-thioglycerol, thiols such as 4,4’-aldrithiol [20] and 6-mercapto-1-hexanol [15] have also been used.
CtCDH did not adsorb strongly or bind electrostatically to the thiol modified electrodes, hence a Nafion® layer was applied.

**Table 3.3** Voltammetric characterization of CtCDH at 1-thioglycerol SAMs modified planar and nanoporous gold electrodes.

<table>
<thead>
<tr>
<th>Electrode design</th>
<th>E°' [mV]</th>
<th>ΔE_p [mV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uniform np-Au</td>
<td>-85 (±3)</td>
<td>60 (±2)</td>
</tr>
<tr>
<td>Non-uniform np-Au</td>
<td>-85 (±1)</td>
<td>43 (±1)</td>
</tr>
<tr>
<td>Planar np-Au</td>
<td>-88 (±3)</td>
<td>48 (±2)</td>
</tr>
</tbody>
</table>

Cyclic voltammograms of CtCDH immobilized under a Nafion® membrane at 1-thioglycerol modified planar and porous gold electrodes show well-defined oxidation and reduction peaks (**Figure 3.4 A**). The relatively low value of ΔE_p (40 – 60 mV) indicates the quasireversible behaviour of CtCDH at the electrode surfaces (**Table 3.3**). Similar results were obtained for *Trametes villosa*, *Phanerochaete sordida*, *Myriococcum thermophilum* [20], *Humicola insolens* and *Phanetochaete chrysosporium* CDHs [17, 61]. The differences in the surface morphology of gold electrodes influence the voltammetric response of CtCDH, as was also noticed for cyt c (**Figure 3.1** and **Figure 3.2**). The smooth surface of a planar gold electrode with possibly a full and defect free monolayer of 1-thioglycerol provides a lower faradaic response in contrast to the higher response at uniform and non-uniform porous gold electrodes. Porous gold surfaces modified with thiols bind higher amounts of enzyme compared to that at a planar surface resulting in the higher current densities observed (**Figure 3.4 A**).
Table 3.4 Comparison of current densities for CtCDH and roughness factors at uniform, non-uniform and planar gold electrodes.

<table>
<thead>
<tr>
<th>Electrode design</th>
<th>Current density [μA/cm²]</th>
<th>Roughness factor, $R_f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-uniform nanoporous gold</td>
<td>9</td>
<td>28.1</td>
</tr>
<tr>
<td>Uniform nanoporous gold</td>
<td>6</td>
<td>16.1</td>
</tr>
<tr>
<td>Planar nanoporous gold</td>
<td>2.5</td>
<td>1</td>
</tr>
</tbody>
</table>

* data obtained in 20 mM lactose solution

An enzyme electrode with CtCDH can be used as a biosensor based either on DET or MET due to the catalytic properties of CtCDH in detection of sugars. As was reported by Harreiter et al., CtCDH is the enzyme that exhibits highest current density at alkaline pH [60]. However, CtCDH as also glucose recognition enzyme can not replace glucose oxidase and glucose dehydrogenase in glucose biosensors due to its low affinity to glucose substrate. Here, lactose was used as a substrate in CtCDH catalysis due to its importance in the determination in milk and dairy products especially for those who do not tolerate lactose. In addition, in contrast to cellobiose, high concentrations of lactose do not result in substrate inhibition [62]. The reaction for the oxidation of lactose by CtCDH is shown in equation 4.

\[
CDH(FAD) + \beta-D-lactose \rightarrow CDH(FADH_2) + lactono - \delta-lactone
\]  

(4)

Figure 3.4 B shows cyclic voltammograms of CtCDH entrapped under a Nafion® membrane at 1-thioglycerol modified gold electrodes in 20 mM lactose solution. The highest voltammetric response was obtained at non-uniform porous gold, followed by uniform and planar gold, respectively. Such results were expected and are in good agreement with the electroactive surface area of each electrode available for the
enzyme molecules investigated through the covalent immobilization of cyt c model protein. However, considering the ratio of the current densities of CtCDH-modified non-uniform and uniform electrodes to planar gold (4:2:1), the ratio the current densities of cyt c-modified non-uniform and uniform electrodes to planar gold (11:9:1) and finally the ratio of the roughness factors (28:16:1) of non-uniform, uniform and planar electrodes respectively (Table 3.4), the voltammetric response at both porous gold electrodes in 20 mM lactose (Figure 3.4 B) is lower than expected. These observations confirm that the smaller pores became inaccessible to CtCDH penetration. The catalytic current densities at non-uniform and uniform nanoporous gold electrodes are limited by the diffusion of the product of the oxidation of lactose by CtCDH (equation 4). In addition, mass transfer resistance is caused by the use of Nafion® membrane. However, the Nafion® membrane is necessary to for stability of the biosensor.

Figure 3.5 Comparison of the current densities in the presence of increasing concentrations of lactose at planar (triangles), uniform (diamonds) and non-uniform (circles) gold electrodes modified with CtCDH and 1-thioglycerol SAMs. Inset: current response at low concentration of lactose. The electrodes were tested in 50 mM K$_2$HPO$_4$-KH$_2$PO$_4$ buffer saline, pH 7.4, at scan rate of 5 mV/s.
Planar and nanoporous gold electrodes with CrCDH entrapped under a Nafion® membrane at thioglycerol SAMs were used as lactose biosensors. In Figure 3.5 calibration curves in the presence of different concentrations of lactose are shown. Although the shape of the calibration curves is unusual, the linear range for planar, non-uniform and uniform nanoporous gold electrodes is similar and reaches 20 mM (Figure 3.5 inset). The unusual shape of the calibration curves possibly arises from two factors; substrate inhibition and mass-transfer limitation. In the calibration curve (Figure 3.5) for planar gold electrode the reduction of the current density is ca. 50% at 50 mM lactose, in uniform nanoporous gold ca. 25% and in non-uniform ca. 15%. However, weaker apparent substrate inhibition in the high surface area electrodes is observed. Nevertheless, all electrodes show the maximal current density at the same substrate concentration. The decrease in the catalytic current density of the biosensors is possibly limited by the mass transfer what is observed in Figure 3.5. Unfortunately, the system was no longer investigated due to the lack of reproducibility.

3.3.3 Direct electron transfer of bilirubin oxidase (*Myrothecium verrucaria*) at unmodified nanoporous gold electrodes

The direct bioelectrocatalytic reduction of oxygen by *Myrothecium verrucaria* bilirubin oxidase (*MvBOD*) and *Trametes hirsuta* laccase (*ThLc*) was studied (Figure 3.6 and 3.10). Both enzymes were adsorbed at the unmodified surfaces of uniform porous gold electrodes. Uniform nanoporous gold electrodes were chosen due to their larger pore and ligament sizes compared to those of non-uniform nanoporous gold. To absorb the enzymes at the nanoporous gold, a vacuum of $10^{-2}$ mbar was applied first to dry and remove all liquids from the pores of the electrodes after the cleaning step and then to force the enzyme to penetrate into the empty voids.
Figure 3.6 MvBOD adsorbed at nanoporous gold electrodes in pH 7.0, 0.1 M citrate-phosphate buffer, scan rate: 5 mV \text{s}^{-1}. (A) Cyclic voltammogram of the BOD-nanoporous gold electrode made by drop-cast in presence of O$_2$ (full line) and in presence of Ar (dotted line). (B) Cyclic voltammogram of the BOD-nanoporous gold electrode without stabilizer in presence of O$_2$ (full line) first scan, (dashed line) second scan and in presence of Ar (dotted line). (C) Cyclic voltammogram of the BOD-nanoporous gold electrode with P017-epoxy as stabilizer in presence of O$_2$ (full line) first scan, (dashed line) second scan and in presence of Ar (dotted line). (D) The proposed structure of P017-epoxy.

Figure 3.6 A shows the low current densities of ca. 0.04 mA/cm$^2$ obtained at MvBOD modified electrodes prepared by placing a drop of enzyme solution onto the surface of an electrode that had been first dried under vacuum. On changing the deposition conditions by first drying the electrodes in vacuum, followed by deposition of the enzyme solution in vacuum, significantly higher current densities of ca. 0.3 mA/cm$^2$ (Figure 3.6 B) were obtained. The high catalytic responses observed arise from the high surface areas of the electrodes combined with the high loadings of enzyme which were achieved by using vacuum to dry the electrode and then adsorb the enzyme. The use of heating to evaporate the solution in the pores was avoided in order to prevent further morphological changes in the electrode arising from thermal stresses [63]. On repeated potential scans the response of the electrode was observed to be
unstable with a 50% decrease in the current density (Figure 3.6 B). This indicates that MvBOD is relatively loosely bound and easily removed from the surface of the electrode. To stabilize the voltammetric response at the electrode three possible cross-linking agents: Nafion®, glutaraldehyde and P017-epoxy polymer were tested (Figure 3.7). In short duration (1 hour) stability measurements electrodes stabilized with P017-epoxy polymer (Figure 3.6 D) lost 13% of the initial voltammetric response compared to losses of ca. 25% and 50% for Nafion® and glutaraldehyde, respectively. In conclusion, P017-epoxy was chosen as a cross-linking agent and was used for further analysis. The role of P017-epoxy is to stabilize the adsorbed MvBOD by creating covalent bonds with nucleophilic groups such as amine, thiol and hydroxyl groups on the surface of the enzyme [64]. Effectively, a cap-like structure was formed on the electrode which prevents leakage of the enzyme.

Figure 3.7 Cyclic voltammograms of BOD-nanoporous gold electrode with (A) P017-epoxy, (B) Nafion® and (C) glutaraldehyde as stabilizers in presence of O2 saturated 0.1 M citrate-phosphate buffer, pH 7.0; scan rate: 5 mV/s, scans were repeated for a period of one hour.
On modifying the electrode with P017-epoxy, current densities of 0.8 mA/cm$^2$ were observed (Figure 3.6 C). In oxygen saturated solution the onset potential for biocatalytic oxygen reduction at MvBOD-modified nanoporous gold electrodes was ca. 500 mV (Figure 3.6 C), in good agreement with the redox potential of the T1 site [36, 65]. A limiting current density of ca. 0.6 mA/cm$^2$ (calculated from the Randles-Sevcik equation and utilising values of $2.6 \times 10^{-5}$ cm$^2$/s and 420 µM for the diffusion coefficient and concentration of oxygen, respectively [66] and a roughness factor of 2.1 for the reduction of dioxygen [67]), indicating that the observed current density of 0.8 mA/cm$^2$ is controlled by diffusion of O$_2$ from the bulk solution. At high currents, the response was quite noisy over the potential range 0.35 – 0.1 V, which may have arisen from the highly porous and uneven nature of the electrode. Variations in the amount of oxygen (as well as the amount of adsorbed enzyme) may result in localised variations in the concentration of oxygen, disrupting the response observed. As expected electrodes made by drop-casting and vacuum method did not demonstrate a catalytic current in Ar saturated electrolyte (Figure 3.6).

![Figure 3.8](image-url)  
*Figure 3.8 Effect of F$^-$ inhibition on the biocatalytic oxygen reduction at Myrothecium verrucaria bilirubin oxidase modified nanoporous gold electrodes measured in oxygen saturated 0.1 M citrate-phosphate buffer in pH 7.0.*
Of the halides $F^-$ is the most efficient inhibitor of blue-copper oxidases [40]. The inhibitory effect of halogens is related to the atomic radius of the ions and, hence, $F^-$ with the smallest radius has the highest binding affinity [40, 68]. The inhibition mechanism of $F^-$ relies on binding to the T2/T3 copper site and then blocking the transfer of electrons from the T1 site [32, 40]. Surprisingly, the addition of $F^-$ had little influence on the biocatalytic current of $Mv$BOD immobilized at nanoporous gold electrodes under aerobic conditions. The maximal loss of activity of an electrode stabilized with P017-epoxy was ca. 3.5%. (Figure 3.8) Such a low inhibitory effect indicates that the normal deleterious effect of $F^-$ on the response was not observed. The significantly reduced inhibitory effect may arise from exclusion of $F^-$ from the pores by the polymer and from confinement of the enzyme in the pores of the electrode in a manner which precludes binding of $F^-$ to the T2/T3 site.

![Graph](image.png)

**Figure 3.9** Stability of the $O_2$ reduction current for *Myrothecium verrucaria* bilirubin oxidase adsorbed on uniform nanoporous gold electrodes under P017-epoxy film in 0.1 citrate-phosphate buffer, pH 7.0.

The stability of the reduction current observed for $Mv$BOD-modified nanoporous gold was examined in $O_2$ saturated 0.1 M citrate-phosphate buffer (pH 7.0) for 12 hours (Figure 3.9). The half life of the electrodes was ca. 7 hours. However, the
plot of current density versus time can be divided into three regions. Within the first six hours of the experiment, the current decreased in a linear manner and may be ascribed to the leaching of the cross-linking agent and the enzyme. Within next 3 hours there is a strong decrease in the current density and thus, the fast drop in the stability of the $Mv$BOD-adsorbed electrode up to its complete loss. The decrease in stability in the middle region of the Figure 3.9 may be explained by the weak physical forces which do not hold the enzyme molecules inside the electrode structure. At first the enzyme molecules slowly flow out from the pores, but they are blocked by the cross-linking agent P017-epoxy and the surface tension, which is higher than the weight force. However, in time the weight of the forming drop of the enzyme and P017-epoxy increases, and thus the surface tension is not able to hold it on. In conclusion, the stability of the electrode is lost after ca. 10 hours. The noise in the Figure 3.9 may have arisen from the variation of the oxygen amount in highly porous and uneven nature of the electrode.

3.3.4 Direct electron transfer of laccase (*Trametes hirsuta*) at unmodified nanoporous gold electrodes

![Cyclic voltammogram](image)

**Figure 3.10** Cyclic voltammogram of (A) $Th$Lc/nanoporous gold electrode made by drop-cast in presence of $O_2$ (full line) and in presence of $N_2$ (dotted line); (B) $Th$Lc/nanoporous gold electrode with P017-epoxy as stabilizer in presence of $O_2$ (full line), air (dashed line) and $N_2$ (dotted line). Electrodes were tested in 100 mM phosphate-citrate buffer, pH 4.0; scan rate: 5 mV/s.
Vacuum methods to absorb the enzymes into the voids of unmodified uniform nanoporous gold electrodes were also applied in *Trametes hirsuta* laccase immobilization. Bioelectrocatalytic currents of ca. 2.5 µA/cm$^2$ at ThLc-modified electrodes prepared by placing a drop of enzyme solution onto the surface of vacuum dried electrodes (Figure 3.10 A) were obtained. On changing the immobilization conditions to vacuum deposition of the ThLc solution on a bare vacuum dried nanoporous gold electrode followed by covering the ThLc-modified electrode with a P017-epoxy cross-linking agent, the bioelectrocatalytic reduction of O$_2$ was observed with significantly higher currents of ca. 30 µA/cm$^2$ (Figure 3.10 B). As reported previously, chemisorption of the -NH$_2$ groups of the lysine residues of ThLc on nanoporous gold are as strong as those for –SH groups on gold [69]. However, to prevent any possible leaching of ThLc from the gold surface, a P017-epoxy cap was applied [70]. The role of P017-epoxy cap-like structure is to stabilise the adsorbed enzyme by creating covalent bonds with amine, thiol, or hydroxyl nucleophilic groups on the extended surface of the enzyme [71].

In an O$_2$ saturated solution, the onset potential for the biocatalytic reduction of O$_2$ at ThLc-modified nanoporous gold electrode of ca. 650 mV (Figure 3.10 B) is in good agreement with the redox potential of the T1 site of the enzyme [72]. Such a well-defined biocatalytic response for ThLc is in contrast to the complete absence of this process on Lc-modified planar polycrystalline Au electrodes [73]. Preferential orientation of Lc in nanocavities appear to enable efficient electron transfer via the T1 redox site of the enzyme, while reversible unfolding in the nanopores of fabricated electrodes possibly explain observed phenomenon [74]. The lower catalytic current observed in air arises from the low amount of O$_2$ in solution (Figure 3.10 B). As expected ThLc biocathodes do not demonstrate a catalytic response in N$_2$ saturated solution (Figure 3.10 A and B).

Although the mechanism of inhibition is different, it is established that halide ions influence the biocatalytic response for both direct and mediated electron reactions of Lcs. F$^-$ acts as a non-competitive inhibitor by blocking the electron transfer pathway from the T1 site to the T2/T3 cluster by binding to the T2/T3 copper [32, 75-77]. This is confirmed by the complete abolishment of the biocatalytic activity of ThLc-modified nanoporous gold electrodes by low concentration of F$^-$ (2 mM) (Figure 3.11 A). Contrary to F$^-$, Cl$^-$ is competitive inhibitor [68]. As reported by Vaz-Dominguez et al.,
Cl⁻ should not suppress the O₂ reduction in direct electron communication between Lcs molecules and the electrode surface through the restricted access to the T2/T3 copper cluster [76]. The opposite phenomenon is proposed for mediated electron communication. In presence of the bulkier Cl⁻, the access of redox mediators to the T1 site is blocked and the catalytic response suppressed [76]. Here, the addition of amounts of Cl⁻ causes a reduction in the bioelectrocatalytic current. Although the direct electron communication between ThLc and bare nanoporous gold electrode surface takes place, the biocatalytic response of ThLc biocathode decreases by ca. 50% (Figure 3.11 B).

**Figure 3.11** Effect of (A) F⁻ and (B) Cl⁻ inhibition on the biocatalytic reduction of oxygen at ThLc modified nanoporous gold electrodes in 0.1 M citrate-phosphate buffer, pH 4.0.
3.4 Conclusions

The accessibility of nanoporous gold surface to the redox proteins modification was successfully investigated by use cyt c as a model system. Three different groups of thiols were tested as possible SAMs layers covering the gold surfaces. The mixture of 11-mercaptoundecanoic acid and 6-mercapto-1-hexanol (HS(CH₂)₁₀COOH and HS(CH₂)₆OH) was found to create the most optimal SAMs layer for cyt c immobilization on gold. The covalent immobilization of cyt c at HS(CH₂)₁₀COOH and HS(CH₂)₆OH modified planar and nanoporous gold electrodes under the stabilization of CMC demonstrated of ca. 9 and ca. 11 times higher current density at uniform and non-uniform nanoporous gold, respectively, than at planar gold electrodes. The roughness factors (Rf) of uniform and non-uniform nanoporous gold were ca. 16 and ca. 28, respectively, compared to ca. 1 of planar gold electrode. Thus, the surface coverage of electrochemically active cyt c was still, however, multiples of that possible at planar gold electrode.

A similar strategy was applied to immobilize CtCDH at planar and nanoporous gold electrodes under Nafion® stabilizing membrane. A modifying layer of 1-thioglycerol created the most efficient surface on gold for the CtCDH immobilization. Lactose was used as a substrate to investigate the catalytic efficiency of the system. The calibration curves in different concentration of lactose solution were obtained. The unusual shape of the plots possibly aroused from both the substrate inhibition and the resistance in the mass transport. However, further investigation of the system was impossible due to the lack of reproducibility.

An immobilization procedure incorporating the use of vacuum was successfully applied for adsorption of ‘blue’ copper oxidases at unmodified uniform nanoporous gold electrodes. Vacuum of 10⁻² mbar was introduced first to dry the electrodes after the cleaning step and then to force the enzymes penetration into the voids of gold surfaces. Significantly higher current density of ca. 0.8 mA/cm² was observed when MvBOD was absorbed into nanoporous gold covered with the chosen cross-linking agent P017-epoxy compared the current densities of ca. 0.04 mA/cm² and ca. 0.3 mA/cm² obtained for the electrode prepared by drop-casting of MvBOD and in vacuum but without the cross-linking agent, respectively. A minimal inhibition of F⁻ of such a system was observed.
The stability of O$_2$ reduction current for the MvBOD-modified nanoporous gold under P107-epoxy cap-like structure was also investigated. It was found that the half-lifetime is of ca. 7 hours.

*ThLc* is another multi-copper oxidase adsorbed into voids of porous gold electrodes in vacuum under P017-epoxy cap-like layer. The current densities obtained are of ca. 0.03 mA/cm$^2$ which is 10 times higher than the current densities at the *ThLc* electrode made by drop-casting. *ThLc* immobilised on nanoporous gold was strongly dependent on the presence of halide ions. Fluoride ions completely inhibited the enzymatic response, whereas addition of Cl$^-$ had lower influence on *ThLc* bioelectrocatalytic activity. However, 200 mM Cl$^-$ caused a loss in activity of *ThLc* of 50%.
3.5 REFERENCES

Chapter 3

DET based electrodes

Chapter 3

DET based electrodes

CHAPTER 4:

MEDIATED ELECTRON TRANSFER BASED BIOELECTRODES FOR APPLICATIONS IN BIOSENSORS AND BIOFUEL CELLS
4.1 Introduction

A fuel cell is an electrochemical energy conversion device that converts the chemical energy into electrical through the chemical reaction. The concept of a fuel cell was discovered in the middle of 19th century by Christian Friedrich Schönbein, a Professor at the University of Basel in collaboration with Sir William Grove who invented the first fuel cell [1]. Each fuel cell consists of two electrodes connected in an external circuit. At an anode the oxidation of a fuel occurs, whereas at the cathode the reduction of an oxidizing agent takes place. Both electrodes are separated by an ion-selective membrane in two-compartment fuel cells where the mixing of fuel and oxidizing agent is prevented. In one-compartment (non-compartmentalized) fuel cells, the anode and cathode are in the same electrolyte. The power output of a fuel cell is the product of the current and voltage of the cell. Limitations of both parameters limit the power output. The size of the electrode, the loading of biocatalysts and limitations in electron transfer and mass transport (e.g. if a membrane is used) can all influence the cell current [2]. The cell voltage is the difference between the standard potential of the cathode and anode. Three types of overpotential limit the voltage of the fuel cell: ohmic (resistance), concentration and activation. The activation overpotential depends on the rate of the electron transfer. The lower the exchange current and the slower kinetics of the reaction, the larger the activation overpotential is observed. The concentration overpotential is related to changes in substrate concentration, limitations in mass transport cause an increase in the concentration overpotential. The ohmic overpotential is given by the resistance of the cell which is independent of the current and potential and follows Ohm’s law. The ohmic overpotential is generated by the resistance of the electrical connection, the electrolyte and the membrane used in the system [3].

The aim of the use of fuel cells is to replace fossil sources in the production of electricity and to reduce emissions to the atmosphere [1]. In addition, fuel cells, especially those utilizing biological species such as enzymes and biological fuels are the subject of increasingly intensive research efforts due to possible applications in self-powered sensors, miniaturized devices and portable electronics [4].
The first enzyme-based biofuel cell was reported in 1962 by Davis and Yarbrough. In a test of a glucose/glucose oxidase system with methylene blue, on open circuit potential of 180 mV was obtained. The reduction of O₂ on a platinum electrode was used as a cathodic reaction [5]. Enzymatic biofuel cells belong to the class of fuel cells where catalysts such as enzymes are assembled on an electrode surface and, thus, are employed to generate power from various substrates. Moreover, the biocatalysts may also promote the production of other simple substrates (e.g. hydrogen or methane) from more complicated fuels (e.g. sugars) [2].

Enzymes are attractive biocatalysts in biofuel cells application due to their high specificity, selectivity and relatively low cost [6]. The selectivity of enzymes allows single compartment cells (called also non-compartmentalized) to be used eliminating the need for a membrane. The first one-compartment biofuel cell was reported by Katz et al. in 1999. The oxidation of glucose by glucose oxidase took place at the anode, whereas oxygen was reduced by cyt c/cytochrome oxidase at the cathode. The maximal power generated was low, ca. 4 µW [7]. The use of membrane-less biofuel cells has gained significant interest due to its simplicity of construction, the lack of membrane and, hence, possible further miniaturization.

Another important aspect in biofuel cells research is the choice of bioelements and their connection to the anode and cathode. Most enzymatic fuel cells use O₂ and glucose as oxidizing and reducing agents. Both O₂ and glucose, are easily available, renewable and presented in the human fluids. The most frequently used bioelements in biofuel cells are blue multicopper oxidases (bilirubin oxidase [8] and laccase [9]) on the cathodic side and glucose oxidase [8], glucose dehydrogenase [10] or recently cellobiose dehydrogenase [11] at the anode. The methods of chemical or physical attachments of bioelements and the pathways of electron transfer between those enzymes and electrodes have been discussed in Chapter 1. However, problems such the low magnitude of the power output or low stability, prevents the efficient use of enzymatic biofuel cells. A glucose/O₂ multi-stacked biofuel cell generating a power output of 1.25 mW/mL has been described which could power a radio-controlled car of 16.5 g [12]. Quite high stability of a mediatorless glucose/O₂ gold nanoparticles-based biofuel cell was reported by Wang et al. The continuous operation of the device for 12 hours in physiological buffer with the loss of 20% in initial power density was
demonstrated [13]. A glucose/O\textsubscript{2} biofuel cell was successfully implanted into a grape by Mano et al. It was found that the power output of the cell depends on the cathode position inside the fruit. When the cathodic carbon fibre was situated near the centre of the grape the power density was 0.47 µW/mm\textsuperscript{2}. The higher power density of 2.4 µW/mm\textsuperscript{2} was obtained when the cathode was moved near the skin possibly reflecting limitations in substrate concentrations at the centre of the grape. A biofuel cell implanted into a grape after 24 hours of continuous operation maintained 78% of its initial power [8].

Instead of O\textsubscript{2}, hydrogen peroxide can be used as a substrate with a suitable enzyme e.g. microperoxidase-11 (MP-11) for the biocatalytic reduction of H\textsubscript{2}O\textsubscript{2} at the cathode. On the anodic site, instead of sugars, alcohol substrates have been used. Alcohol dehydrogenases are the main enzymes recommended used in such biofuel cells. Willner et al. proposed two biofuel cells with MP-11 reducing H\textsubscript{2}O\textsubscript{2} to H\textsubscript{2}O at a gold cathode and pyrroloquinoline quinine (PQQ) or PQQ-FAD/GOx at the gold anode. Biofuel cells using H\textsubscript{2}O\textsubscript{2} and NADH or glucose as fuels, respectively, had power outputs of 32 µW and 8 µW [14-15]. An example of non-compartmentalized and mediator-less biofuel cell able to convert chemical energy from oxidation and reduction of ethanol on both anode and cathode was described by Ramanavicius et al. Quinonohemoprotein-alcohol dehydrogenase from \textit{Gluconobacter} (QH-ADH) and alcohol oxidase with microperoxidase-8 (AOx and MP-8) were immobilized on the anode and cathode, respectively. The power density for such a biofuel cell was of ca. 1.5 µW/cm\textsuperscript{2} [16].

In this chapter Os redox polymers are used to obtain mediated electric connection between four redox enzymes: \textit{Aspergillus niger} glucose oxidase, cellobiose dehydrogenase from \textit{Corynascus thermophilus}, \textit{Melanocarpus albomyces} laccase and \textit{Myrothecium verrucaria} bilirubin oxidase and nanoporous and planar gold electrodes. The anodic enzymes, GOx and CDH, were examined in buffer containing different concentration of glucose and lactose substrate. In addition, the vacuum adsorption method introduced in the previous chapter is applied here to immobilize the mixture of the chosen Os-mediator and redox enzyme into the nanoporous gold surface. In the second part of this chapter, the bioelectrodes were incorporated into biofuel cells operating in physiological buffer saline, pH 7.4 at 37 °C and artificial plasma buffer.
4.2 Experimental

4.2.1 Reagents

Sulfuric acid (95 – 98%), potassium phosphate monobasic (≥99%) and dibasic (≥98%), sodium phosphate dibasic (≥99%), sodium chloride, D-(+)-glucose (99.5%), D-lactose, poly(ethylene glycol)diglycidyl ether (PEGDGE) were obtained from Sigma-Aldrich Ireland, Ltd. Absolute ethanol was obtained from Lennox Ltd., Ireland. Deionised water (resistivity of 18.2 MΩ cm) was obtained from an Elgastat maxima-HPLC (Elga, UK).

4.2.2 Enzymes

Purified preparations of Aspergillus niger glucose oxidase (GOx) was purchased from Sigma-Aldrich Ireland, Ltd.. The activity of GOx of 158.8 U/ml was determined using the activity indirect assay [17]. The preparation of homogeneous cellobiose dehydrogenase from Corynascus thermophilus (CtCDH) was kindly provided by Dr Roland Ludwig. The concentration of CtCDH was 22.85 mg/ml. Laccase from Melanocarpus albomycetes (rMaLc) produced in Trichoderma reesei was obtained from VIT Technical Research Centre of Finland. The concentration of the enzyme was 8.1 mg/ml. Myrothecium verrucaria bilirubin oxidase (MvBOD) was purchased from Amano Enzyme Inc. (Nagoya, Japan). The activity of MvBOD was 2.63 U/mg. The enzyme was purified to homogeneity using a published procedure [18].

4.2.3 Redox Os-polymers

[Os(2,2’-bipyridine)\textsubscript{2}(polyvinylimidazole)Cl\textsuperscript{+2+} (E\textsubscript{0h} = 0.22 V vs. Ag/AgCl; Os(bpy)\textsubscript{2}PVI) and [Os(4,4’-dimethyl-2,2’-bipyridine)\textsubscript{2}(polyvinylimidazole)Cl\textsuperscript{+2+} (E\textsubscript{0h} = 0.12 vs. Ag/AgCl; Os(dmmbpy)\textsubscript{2}PVI] [19] were kindly provided by Dr Donal Leech. Another group of redox mediators presented of Figure 4.1: P006-P71 (A; 11.1 % w/w in MeOH), P004-P71 (B; 2.2 % w/w in MeOH), P004-P91 (C; 6.5 % w/w in MeOH/H\textsubscript{2}O), P002-P91 (D; 8.6 % w/w in MeOH/H\textsubscript{2}O), P002-P110 (E; 3.7 % w/w in
MeOH); and P017-epoxy (0.9% w/w in MeOH) was kindly provided by Prof. Wolfgang Schuhmann.

**Figure 4.1** Proposed structures Os redox complexes.

### 3.2.4 Enzyme immobilization procedures

A solution was prepared using 8 µL of a 6 mg/ml suspension in water containing the Os-based redox polymer (Os(bpy)$_2$PVI or Os(dmbpy)$_2$PVI), 1.9 µL of a 15 mg/ml solution in water of PEGDGE and the enzyme 4.8 µL of a 10 mg/ml solution in water of either GOx, MvBOD or rMaLc or 2 µL of a 22.85 mg/ml solution of CrCDH was prepared in 200 µL eppendorf tube and gently mixed. The solution was carefully drop-coated onto the gold surface, ensuring all areas of the electrodes are covered. The deposited film was allowed to dry for 24 hours in darkness in room temperature. The modified electrodes were immersed in 50 mM phosphate buffer, 150 mM NaCl, pH 7.4 at 37 °C for at least 20 minutes prior to electrochemical measurements to allow for film swelling. Both, bioanodes and biocathodes obtained, were connected into biofuel cells.
The power density of biofuel cell was measured in oxygen saturated 50 mM phosphate buffer saline pH 7.4 at 37 °C in addition of 5 mM and 100 mM glucose and lactose.

30 µL of the mixture containing 40 µg/ml MvBOD and 200 µg/ml of P006-P71, P004-P71, P004-P91, P002-P91, P002-P110 and Os(bpy)$_2$ PVI polymer in 1:1 v/v ratio was divided in two equal portions. The first portion was placed on a clean, dry nanoporous gold electrode. The electrode was then left in the vacuum chamber under pressure for ca. 3 min to allow the enzyme to adsorb. The MvBOD-modified nanoporous gold was then removed from the chamber, covered with the second aliquot of enzyme and polymer solution and allowed to dry in air at room temperature for 2 hours.

3.2.5 Electrochemical measurements

All electrochemical studies were performed using either a CHI832 bipotentiostat or CHI630A potentiostat (CHI Instruments, Austin, Texas, USA) in a standard three-electrode electrochemical cell with a 0.5 mm diameter Pt wire counters electrode (ALS Co. Ltd., Tokyo, Japan) and Ag/AgCl in 3 M KCl reference electrode (IJ Cambria Scientific Ltd., UK). Immediately prior the use, nanoporous gold electrodes were electrochemically cleaned by scanning (10 scans) from 0 to 1.5 V at 200 mV/s in 0.5 M H$_2$SO$_4$, followed by scanning at 50 mV/s between 0 and 0.8 V in the electrolyte used to experiments. The electrodes were then placed in a vacuum chamber at 10$^{-2}$ mbar for 10 min. All current densities were normalized to the electrode geometric surface area.
4.3 Results and discussion

4.3.1 Bioelectrodes

The incorporation of enzymes into a three-dimensional redox hydrogel enables efficient electrical communication between the enzymatic active site and the electrode surface. The use of the redox hydrogel eliminates the need to orient the enzyme molecules at the electrode in a manner that promotes electron transfer. In addition, the hydrogel enables multiple layers of enzymes to be immobilized while also providing for efficient rates of electron transfer [20]. Two redox Os-complexes: Os(bpy)$_2$PVI and Os(dmbpy)$_2$PVI with standard redox potentials of 0.22 V and 0.12 V vs. Ag/AgCl,
respectively, were used to immobilize the redox enzymes: *Aspergillus niger* glucose oxidase (GOx), cellobiose dehydrogenase from *Corynascus thermophilus* (CtCDH), laccase from *Melanocarpus albomyces* (rMaLc) and *Myrothecium verrucaria* bilirubin oxidase (MvBOD) at planar and non-uniformly sputtered nanoporous gold electrodes. Non-uniform nanoporous gold electrodes were chosen due to their larger crack sizes compared to those of uniform nanoporous gold. Larger cracks will be better able to accommodate the hydrogel. A bifunctional cross-linking agent PEGDGE was also used as recommended by Heller and co-workers [21]. However, cross-linking reduces the segmental mobility of electrons in the redox polymers causing lower electron diffusivities [22]. Nevertheless, in the absence of the cross-linking agent the films dissolve [23-24].

**Figure 4.2 A** and **B** shows cyclic voltammograms of Os(bpy)₂PVI on planar and nanoporous gold, and **Figure 4.2 C** and **D** cyclic voltammograms of Os(dmmbpy)₂PVI on planar and nanoporous gold electrodes. The voltammograms are typical of polymer-bound Os complexes with redox potentials of 0.12 V and 0.22 V vs. Ag/AgCl for Os(dmmbpy)₂PVI and Os(bpy)₂PVI, respectively. At a scan rate of 5 mV/s, the voltammograms of both polymers are symmetrical with ca. 10 mV peak-to-peak separation at planar as well as nanoporous gold electrodes (**Figure 4.2**). The voltammogram of Os(bpy)₂PVI on nanoporous gold (**Figure 4.2 B**) has a slightly higher current density and capacitive current compared to the voltammograms of Os(bpy)₂PVI on planar gold (**Figure 4.2 A**) indicating that the nanoporous electrode has a higher surface area-to-volume ratio. No difference was observed for Os(dmmbpy)₂PVI deposited on modified planar (**Figure 4.2 C**) and nanoporous gold electrodes (**Figure 4.2 D**). The surface coverage of Os(bpy)₂PVI at both nanoporous and planar gold was ca. 58 pmol/cm². Lower surface coverages of 24 pmol/cm² and 37 pmol/cm² were obtained for Os(dmmbpy)₂PVI at nanoporous and planar gold, respectively. The difference in the surface coverage of Os(dmmbpy)₂PVI on gold electrodes arises from the non-uniform deposition of the Os polymer on the electrodes. Os(dmmbpy)₂PVI is less soluble in aqueous solution compared to Os(bpy)₂PVI. Non-uniform deposition of Os(dmmbpy)₂PVI could be observed via the presence of darker regions on the electrode surface even after stirring and sonicating. Such deposition did not occur with Os(bpy)₂PVI. In addition, blocking of the pores by the non-uniform deposition of Os(dmmbpy)₂PVI may have reduced the surface area available.
Figure 4.3 Optimization of the Os(bpy)$_2$PVI polymer-to-enzyme wt% ratio for a CtCDH-bioanode at planar gold electrode: (◊) 1 : 4, (●) 1 : 3, (Δ) 1 : 2 and 1 : 1 (■). Electrodes were tested in 50 mM phosphate buffer saline, 37 °C, pH 7.4; scan rate: 5 mV/s.

Optimization studies were carried at a CtCDH modified anode. Figure 4.3 shows the data obtained at planar gold electrodes. Four different Os(bpy)$_2$PVI polymer-to-enzyme wt% ratios: 1:4, 1:3, 1:2 and 1:1 were tested in the presence of 0, 2.5, 5, 10, 20, 35 and 50 mM lactose. Optimal current densities were obtained for Os(bpy)$_2$PVI polymer-to-CtCDH wt% 1:1 ratio (Figure 4.3), in agreement with previous reports [21, 25]. The GOx immobilization procedure has been described previously and used on different electrodes materials [26], thus, the optimization experiment was not repeated. Due to the deposition based immobilization procedure, individual electrodes may vary in the observed current density of ca. 25%. However, the same electrode showed reproducible results with the deviation of ca. 5% in the current density.
Figure 4.4 Cyclic voltammograms of (A, C) planar and (B, D) nanoporous gold electrodes modified with solutions consisting of (A, B) Os(bpy)$_2$PVI and (C, D) Os(dmbpy)$_2$PVI with GOx and PEGDGE in the presence of 0 (dashed line), 2.5, 5, 10, 20, 35 (full lines) and 50 (dotted line) mM glucose. (E) Comparison of the current density (mA/cm$^2$) in the presence of increasing concentrations of glucose at planar (solid symbols) and nanoporous (hollow symbols) gold electrodes modified using GOx and either Os(bpy)$_2$PVI (circles) or Os(dmbpy)$_2$PVI (diamonds) as the mediator. Electrodes were tested in 50 mM phosphate buffer saline, 37 °C, pH 7.4; scan rate: 5 mV/s.
Chapter 4  

MET based bioelectrodes

The response of the optimized GOx and CtCDH modified anodes was tested in solutions of substrate at physiological conditions (pH 7.4 and at 37 °C). At slow scan rates (5 mV/s) and in the presence of the appropriate substrate a sigmoidal-shaped cyclic voltammograms (Figure 4.4 A, B, C, D, Figure 4.5 and Figure 4.6) were obtained. Such cyclic voltammograms are characteristic of the bioelectrocatalytic oxidation of glucose at GOx, and lactose and glucose at CtCDH bioanodes. The maximal current densities generated depend on several factors including not only the type of the enzyme and the mediator but also the concentrations and ratios of enzymes to mediators which were optimized previously.

**Table 4.1** Comparison of the current densities obtained on GOx and CtCDH planar and nanoporous gold bioelectrodes modified with Os(bpy)$_2$PVI and Os(dmbpy)$_2$PVI in 50 mM substrate buffer.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mediator</th>
<th>Substrate</th>
<th><strong>Current density [mA/cm$^2$]</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td><strong>Planar gold electrode</strong></td>
</tr>
<tr>
<td>GOx</td>
<td>Os(bpy)$_2$PVI</td>
<td>Glucose</td>
<td>1.75</td>
</tr>
<tr>
<td>GOx</td>
<td>Os(dmbpy)$_2$PVI</td>
<td>Glucose</td>
<td>0.60</td>
</tr>
<tr>
<td>CtCDH</td>
<td>Os(bpy)$_2$PVI</td>
<td>Lactose</td>
<td>1.30</td>
</tr>
<tr>
<td>CtCDH</td>
<td>Os(dmbpy)$_2$PVI</td>
<td>Lactose</td>
<td>0.45</td>
</tr>
<tr>
<td>CtCDH</td>
<td>Os(bpy)$_2$PVI</td>
<td>Glucose</td>
<td>0.23</td>
</tr>
<tr>
<td>CtCDH</td>
<td>Os(dmbpy)$_2$PVI</td>
<td>Glucose</td>
<td>0.11</td>
</tr>
</tbody>
</table>

On comparing the choice of redox mediator, lower current densities were obtained at Os(dmbpy)$_2$PVI modified electrodes. This is likely a consequence of a lower solubility of Os(dmbpy)$_2$PVI compared to Os(bpy)$_2$PVI and, hence, non-uniform deposition of Os(dmbpy)$_2$PVI at the gold electrodes as described earlier. In addition, the electron transfer process between the enzyme and Os(dmbpy)$_2$PVI is possibly slower than between the enzyme and Os(bpy)$_2$PVI. Thus, Os(dmbpy)$_2$PVI was found to be a less effective mediator than Os(bpy)$_2$PVI, in agreement with previous reports [19]. In addition, blockage of the pores of nanoporous gold electrodes can reduce the surface
area available, as described earlier. **Table 4.1** shows the current densities at 50 mM substrate concentrations for planar and nanoporous gold electrodes modified with CtCDH and GOx, Os(bpy)₃PVI and Os(dmmbpy)₂PVI. Nearly identical current densities were observed at both gold electrodes at low concentrations of substrate. At glucose concentrations greater than 35 mM and lactose concentration greater than 20 mM, slightly higher current densities on GOx and CtCDH modified nanoporous gold, respectively, in comparison to planar gold electrodes are observed (**Figure 4.4 E** and **4.7 A**). The GOx modified nanoporous and planar gold electrodes responses are approximately linear to *ca.* 25 mM glucose when Os(bpy)₃PVI was used as a mediator and to *ca.* 12 mM with Os(dmmbpy)₂PVI. Identical sensitivity values of *ca.* 58 µA cm⁻² mM⁻¹ for planar and nanoporous gold electrodes modified with Os(bpy)₃PVI and GOx were obtained (**Figure 4.4 E**). On changing mediator to Os(dmmbpy)₂PVI sensitivities of 41 µA cm⁻² mM⁻¹ and 36 µA cm⁻² mM⁻¹ for nanoporous and planar gold electrodes, respectively, were achieved. The linear range of detection of lactose at CtCDH and Os(bpy)₃PVI modified electrodes was similar at both types of electrodes. On using Os(dmmbpy)₂PVI the linear range of up to *ca.* 10 mM versus 24 mM for Os(bpy)₃PVI, respectively, were obtained. The sensitivity of the CtCDH mediated nanoporous and planar electrodes were of 45 µA cm⁻² mM⁻¹ and 36 µA cm⁻² mM⁻¹ (Os(bpy)₃PVI) and of 49 µA cm⁻² mM⁻¹ and 33 for µA cm⁻² mM⁻¹ (Os(dmmbpy)₂PVI), respectively (**Figure 4.7**). The small differences in sensitivity between enzymes modified nanoporous and planar gold electrodes may be a result of slight diffusional changes as outlined previously for the diffusion-controlled electrode processes (**Chapter 3**). Low current densities were obtained for CtCDH modified nanoporous and planar gold electrodes in glucose solutions (**Figure 4.6** and **Figure 4.7 B**). Such low responses are possibly a consequence of the lower affinity of CtCDH for glucose in comparison to lactose [27].
Figure 4.5 Cyclic voltammograms of (A, C) planar and (B, D) nanoporous gold electrodes modified with a drop-cast solution consisting of (A, B) Os(bpy)$_2$PVI and (C, D) Os(dmbpy)$_2$PVI, with $Cr$CDH and PEGDGE in the presence of 0 (dashed line), 2.5, 5, 10, 20, 35 (full lines) and 50 (dotted line) mM lactose (A, B, C, D). Electrodes were tested in 50 mM phosphate buffer saline, 37° C, pH 7.4; scan rate: 5 mV/s.
Figure 4.6 Cyclic voltammograms of (E, G) planar and (F, H) nanoporous gold electrodes modified with a drop-cast solution consisting of (E, F) Os(bpy)$_2$PVI and (G, H) Os(dmbpy)$_2$PVI, with CitCDH and PEGDGE in the presence of 0 (dashed line), 2.5, 5, 10, 20, 35 (full lines) and 50 (dotted line) mM glucose. Electrodes were tested in 50 mM phosphate buffer saline, 37° C, pH 7.4; scan rate: 5 mV/s.
Figure 4.7 Comparison of the current density (mA cm$^{-2}$) in the presence of increasing concentration (A) lactose and (B) glucose at planar (solid symbols) and nanoporous (hollow symbols) gold electrodes modified using $Ct$CDH and either Os(bpy)$_2$PVI (circles) or Os(dmbpy)$_2$PVI (diamonds) as the mediator. PEGDGE was used as the cross-linker for all experiments. Electrodes were tested in 50 mM phosphate buffer saline, 37 °C, pH 7.4; scan rate: 5 mV/s.
Calibration plots of the current densities versus substrates concentration are shown in Figure 4.4E and 4.7. Values of $I_{\text{max,app}}$ (maximal current density) and $K_{M,\text{app}}$ (substrates concentration at the half-maximal velocity of the enzymatic reaction) were determined from Lineweaver-Burk and Eadie-Hofstee equations [28]. Table 4.2 lists the data obtained from $I_{\text{max,app}}$ and $K_{M,\text{app}}$ parameters for the tested systems. The $K_{M,\text{app}}$ values for nanoporous gold modified with $Ct$CDH and Os(bpy)$_2$PVI are lower than at planar gold electrodes using lactose and glucose substrates. A high value $K_{M,\text{app}}$ can indicate that the enzyme at the nanoporous surface has a higher affinity for the substrate [29]. A higher $K_{M,\text{app}}$ value from Lineweaver-Burk equation, 18.8 mM versus 16.2 mM was obtained for GOx and Os(bpy)$_2$PVI modified planar and nanoporous gold electrodes, respectively (Table 4.2). From Eadie-Hofstee analysis a higher $K_{M,\text{app}}$ value of 23.7 mM was calculated for a GOx and Os(bpy)$_2$PVI modified nanoporous gold electrode compared to 21.6 mM for planar gold electrodes in glucose. On the change of the Os mediator to Os(dmbpy)$_2$PVI, a higher $K_{M,\text{app}}$ values for a GOx modified planar gold were obtained. On the comparison of $Ct$CDH modified electrodes in different substrates, a higher $K_{M,\text{app}}$ values from both Lineweaver-Burk and Eadie-Hofstee equations on nanoporous gold surfaces versus planar gold in lactose were obtained. Differently, the higher $K_{M,\text{app}}$ values in glucose solution on planar gold were obtained. In comparison, Zhang et al. reported an electrode modified with gold nanoparticles covalently bound with GOx displayed a low $K_{M,\text{app}}$ of 4.3 mM [29]. Another glucose biosensors prepared using ferrocene-modified polymers had $K_{M,\text{app}}$ in the range 16–71 mM. Such a wide range of $K_{M,\text{app}}$ parameters depend on the length of the side alkyl chain of the polymers used [30]. The high $K_{M,\text{app}}$ value obtained at GOx modified nanoporous gold, provides a wide linear range for the biosensor.

For electrodes modified with $Ct$CDH and GOx and Os(dmbpy)$_2$PVI, the $I_{\text{max,app}}$ values for glucose are similar with no appreciable difference between the nanoporous and planar electrodes (Table 4.2). In the presence of lactose, the reduced (50%) values of $I_{\text{max,app}}$ were obtained for $Ct$CDH and Os(dmbpy)$_2$PVI on planar gold compared to nanoporous gold electrodes. On the replacement of the Os mediator with Os(bpy)$_2$PVI, identical $I_{\text{max,app}}$ values were obtained from Lineweaver-Burk analysis and slightly higher of 2.9 mA/cm$^2$ versus 2.5 mA/cm$^2$ from Eadie-Hofstee analysis for GOx modified nanoporous gold and planar surfaces, respectively, were calculated (Table 4.2). For $Ct$CDH and Os(bpy)$_2$PVI modified nanoporous and planar gold electrodes in
glucose solution, both $I_{\text{max,app}}$ values of 0.3 mA/cm$^2$ and 0.2 mA/cm$^2$ were obtained (Table 4.1). In lactose solutions, the higher $I_{\text{max,app}}$ values were obtained at planar gold electrodes. As an comparison, the $I_{\text{max}}$ parameter for $Ct$CDH adsorbed on graphite electrode in lactose solution of 4.1 µA/cm$^2$ and in glucose solution of 2.6 µA/cm$^2$ at pH 8.5 was determined [27].

Table 4.2 Comparison of $I_{\text{max,app}}$ and $K_{\text{M,app}}$ for GOx and $Ct$CDH immobilized at planar and nanoporous gold electrodes modified with Os(bpy)$_2$PVI or Os(dmppy)$_2$PVI.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Enzyme</th>
<th>Polymer</th>
<th>Substrate</th>
<th>Lineweaver-Burk</th>
<th>Eadie-Hofstee</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$I_{\text{max,app}}$ [mA/cm$^2$]</td>
<td>$K_{\text{M,app}}$ [mM]</td>
</tr>
<tr>
<td>Nanoporous gold</td>
<td>$Ct$CDH</td>
<td>Os(bpy)$_2$PVI</td>
<td>Lactose</td>
<td>1.5</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Os(dmppy)$_2$PVI</td>
<td></td>
<td>0.9</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Os(bpy)$_2$PVI</td>
<td></td>
<td>2.9</td>
<td>55.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Os(dmppy)$_2$PVI</td>
<td></td>
<td>0.5</td>
<td>7.1</td>
</tr>
<tr>
<td>Planar gold</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nanoporous gold</td>
<td>$Ct$CDH</td>
<td>Os(bpy)$_2$PVI</td>
<td>Glucose</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Os(dmppy)$_2$PVI</td>
<td></td>
<td>0.2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Os(bpy)$_2$PVI</td>
<td></td>
<td>0.2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Os(dmppy)$_2$PVI</td>
<td></td>
<td>0.2</td>
<td>9.4</td>
</tr>
<tr>
<td>Planar gold</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nanoporous gold</td>
<td>GOx</td>
<td>Os(bpy)$_2$PVI</td>
<td></td>
<td>2.2</td>
<td>16.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Os(dmppy)$_2$PVI</td>
<td></td>
<td>1.1</td>
<td>19.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Os(bpy)$_2$PVI</td>
<td></td>
<td>2.2</td>
<td>18.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Os(dmppy)$_2$PVI</td>
<td></td>
<td>1.2</td>
<td>28.9</td>
</tr>
<tr>
<td>Planar gold</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Limits of detection of 6 (± 0.4) µM and 7 (± 0.5) µM were obtained for the determination of lactose at $Ct$CDH modified nanoporous and planar gold electrodes, respectively. These results are in good agreement with previous reports of 4 µM for *Trametes villosa* and *Phanerochaete sordida* CDHs at spectrographic graphite electrodes [31]. Limits of detection of 16 (± 0.1) µM and 20 (± 0.1) µM were obtained for the determination of glucose at $Ct$CDH modified nanoporous and planar gold electrodes, respectively. In contrast, the glucose sensor based on GOx immobilized at nanoporous and planar gold electrodes demonstrated lower limits of detection of 2 (± 0.03) µM and 6 (± 0.05) µM, respectively. As described in the literature, the detection range for the determination of glucose concentrations should be between 0.2 – 20 mM.
to detect the amount in the normal and diabetic patients systems. However, it is preferable to have an operating range of 0.1 – 50 mM [32].

Figure 4.8 Cyclic voltammograms in the absence (full line) and presence (dotted line) of O$_2$ at (A, C) non-uniform nanoporous and (B, D) planar gold electrodes modified with (A, B) $Mv$BOD and (C, D) $rMa$Lc, Os(bpy)$_2$PVI and PEGDGE in 50 mM phosphate buffer saline at 37 °C, pH 7.4; scan rate: 5 mV/s.

Nanoporous and planar gold electrodes were also used as cathodes for immobilization of reducing enzymes. For both cathodes a drop-coating consisting of 38.6 wt% $Mv$BOD or $rMa$Lc, 38.6 wt% Os(bpy)$_2$PVI and 22.8 wt% PEGDGE was utilized. A enzyme-to-polymer weight ratio of 1 : 1 was used previously as optimized for the mediator [Os(4,4’-dichloro-2,2’-bipyridine)$_2$Cl]$^{3+/2+}$ in combination with $Mv$BOD
Due to difficulties with solubility and low surface coverage, it was not possible to use Os(dmbpy)$_2$PVI polymer. Limitations of the catalytic reduction if too little enzyme is available or by the increased resistance in the film if too much nonconductive enzyme is present can be expected [23]. Figure 4.8 shows the cyclic voltammograms of MvBOD and rMaLc at nanoporous and planar gold electrodes in the absence and presence of O$_2$. Near identical peak current densities of 0.52 mA/cm$^2$ and 0.49 mA/cm$^2$ at nanoporous and planar gold modified with MvBOD (Figure 4.8 A and B) and of 0.24 mA/cm$^2$ and 0.27 mA/cm$^2$ at planar and nanoporous gold modified with rMaLc (Figure 4.8 C and D) were obtained. At each electrode design, the onset of the O$_2$ reduction current began at a potential slightly positive of the standard potential of the mediator. A limiting peak current was observed at 0.22 V (vs. Ag/AgCl/KCl) the standard potential of the mediator, which decayed gradually at potentials less than ca. 0.15 V after which the current attained a potential-independent plateau value. There are two possible steps in the cathodic process (equations 2 and 3) that can give rise to the limitations in the current observed in Figure 4.7. The first step (equation 2) is the diffusion-limited reduction of enzymes by Os$^{2+/3+}$ and the second (equation 3) the diffusion limited flux of O$_2$ from solution. It is presumed that the rate of O$_2$ reduction catalyzed by either MvBOD or rMaLc and mediated by Os(bpy)$_2$PVI is high when compared to the rate of O$_2$ diffusion in the electrolyte resulting in a decrease in the amount of O$_2$ at the electrode surface. Nevertheless, it is difficult to determine the exact limiting step, because the cross-linking epoxy layer may limit the electrons diffusion and also influences the voltammetric response obtained.

\[ \text{Os}^{3+} \rightarrow \text{Os}^{2+} \quad (1) \]
\[ \text{Os}^{2+} + \text{Enzyme}^{0x} \rightarrow \text{Os}^{3+} + \text{Enzyme}^{\text{Red}} \quad (2) \]
\[ \text{Enzyme}^{\text{Red}} + \text{O}_2 \rightarrow \text{Enzyme}^{0x} + \text{H}_2\text{O} \quad (3) \]
Figure 4.9 Time-dependence of the charge density of the nanoporous gold electrodes modified with (A) GOx and (B) CtCDH, Os(bpy)$_2$PVI and PEGDGE in glucose saturated 50 mM phosphate buffer saline at 37 °C, pH 7.4; scan rate: 5 mV/s.

A major problem in the development of enzyme modified electrodes is their operational stability [6]. Stability studies at an applied potential of 0.5 V for 70 hours using GOx and CtCDH, Os(bpy)$_2$PVI and PEGDGE modified bioanodes in glucose saturated buffer (Figure 4.9 A and B). Both electrodes displayed immediate decreases in response, in contrast to previous reports where CtCDH bioanodes maintained their
stability for up to 11 hours [33-34]. Half lif es of ca. 20 hours were obtained for both anodes (Figure 4.9 A and B), shorter in comparison to the half life of ca. 2 days of laccase and BOD-‘wired’ carbon cloth electrodes modified with [Os(tpy)(dme-bpy)]^{2+/3+} and PAA-PVI-[Os(4,4’-dichloro-2,2’-bipyridine)_2Cl]^{1+/2+} (rotated at 500 and 300 rpm respectively under 1 atm O_2) [20, 23]. The possible cause of the lower stability of the bioelectrodes is the loss of the film from the electrode after 3 days described previously [20]. As proof the voltammetric response of Os^{2+/3+} at a modified electrode (in the absence of glucose was obtained just after immersion of the electrode in solution and 3 days later (Figure 4.10). If the deactivation of the enzyme took place the initial surface coverage of Os^{2+/3+} of 56 pmol/cm^2 would stay similar. After 3 days asurface coverage of Os^{2+/3+} of 17 pmol/cm^2 was observed, a loss of ca. 70 %. In addition, the observed decrease in response for GOx and CtCDH bioelectrodes occurred differently. The response of GOx modified electrodes decayed exponentially (Figure 4.9 A) whereas the decrease in charge density for CtCDH (Figure 4.9 B) was biphasic. The first phase (up to 17 hours) observed with CtCDH may arise from leakage of the hydrogel from the electrode surface, with the second slower phase possibly arising from a slower inactivation of CtCDH at the electrode surface.

![Figure 4.10](image-url)  
**Figure 4.10** Cyclic voltammograms (initial: full line and after 3 days: dotted line) of the nanoporous gold electrode modified with GOx, Os(bpy)_2PVI and PEGDGE in 50 mM phosphate buffer saline at 37 °C, pH 7.4; scan rate: 5 mV/s.
**Section 3.3.3** in Chapter 3 described the successful use of vacuum to prepare the MvBOD modified electrode based on direct electron transfer at unmodified nanoporous gold electrodes. A vacuum of $10^{-2}$ mbar was used first to dry and remove all liquids from the pores of the electrodes after the cleaning step and then to force the enzyme solution to penetrate into the pores of the nanoporous gold structures. This approach was also used here. Solutions containing Os polymer and MvBOD were deposited on a clean uniform nanoporous gold electrode. The modified electrodes were placed under vacuum for 3 min. Uniform nanoporous gold electrodes were used due to the larger pores and ligaments size compared to non-uniform electrodes. It was presumed that vacuum of $10^{-2}$ mbar will force the polymer and enzyme into pores and ligaments, as occurred with MvBOD [35]. Drop-casting of a solution of the polymer and MvBOD was also performed and the results are compared to the vacuum method.
Figure 4.12 Cyclic voltammograms of MvBOD and Os polymers: P004-P71 (A), P004-P91 (B), P006-P91 (C), P002-P91 (D), Os(bpy)$_2$PVI (E) and P002-P110 (F) modified nanoporous gold electrodes by a drop-cast in the presence of O$_2$ (full line), air (dashed line) and N$_2$ (dotted line) in 0.1 M citrate-phosphate buffer, pH 7.0; scan rate: 5 mV/s.
Figure 4.13 Cyclic voltammograms of MvBOD and Os polymers: P004-P71 (A), P004-P91 (B), P006-P91 (C), P002-P91 (D), Os(bpy)$_2$PVI (E) and P002-P110 (F) modified nanoporous gold electrodes by a vacuum in the presence of O$_2$ (full line), air (dashed line) and N$_2$ (dotted line) in 0.1 M citrate-phosphate buffer, pH 7.0; scan rate: 5 mV/s.
Table 4.3 Comparison of the current densities obtained at MvBOD and Os polymer modified uniform nanoporous gold electrodes, prepared using vacuum and drop-casting methods.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Drop-cast I / mA cm⁻²</th>
<th>Vacuum procedure I / mA cm⁻²</th>
</tr>
</thead>
<tbody>
<tr>
<td>P006-P71</td>
<td>0.035</td>
<td>0.036</td>
</tr>
<tr>
<td>P004-P71</td>
<td>0.280</td>
<td>0.071</td>
</tr>
<tr>
<td>P004-P91</td>
<td>0.080</td>
<td>0.052</td>
</tr>
<tr>
<td>P002-P91</td>
<td>0.016</td>
<td>0.016</td>
</tr>
<tr>
<td>P002-P110</td>
<td>0.020</td>
<td>0.032</td>
</tr>
<tr>
<td>Os(bpy)₂PVI</td>
<td>0.032</td>
<td>0.040</td>
</tr>
</tbody>
</table>

Figure 4.12 and Figure 4.13 shows cyclic voltammograms of MvBOD and Os polymer modified nanoporous gold electrodes prepared by the vacuum based method and by drop-casting of the solution. The Os polymers: P004-P71, P004-P91, P006-P91, P002-P91 and P002-P110 (Figure 4.11) were synthesized using covalent binding approach described in previous report [36]. In contrast, Os(bpy)₂PVI was synthesized using ligand exchange reaction. Current densities obtained in the immobilization method based on drop-casting were between 0.016 mA/cm² for P002-P91 and 0.280 mA/cm² when P004-P71 as a mediator was used (Table 4.3 and Figure 4.12). The use of vacuum improved the current densities obtained for P002-P110 and Os(bpy)₂PVI. The same current densities of 0.016 mA/cm² and 0.036 mA/cm² for P006-P71 and P002-P91 was obtained, respectively. In the case of P004-P71 and P004-P91, the current densities of 0.071 mA/cm² and 0.052 mA/cm² obtained by the vacuum method were lower than those from the drop-cast based immobilization. The changes in current densities observed with different Os polymers likely arise from the different structures of the Os mediators. P004 did not appear to penetrate the pores. Higher voltammetric responses ca. 3- and 2-fold with P004-P71 and P004-P91 Os complexes were observed on comparing the drop-casting with the vacuum method. However, P004-P71 was found to be the most effective and gives the highest current densities in both immobilization methods. Nevertheless, to protect the modified electrode surface and stabilize the redox response after the use of vacuum, PEGDGE as a cross-linking agent
was deposited. The comparison of the data shows slightly lower voltammetric response with PEGDGE (Figure 4.14 A) than without (Figure 4.14 B). This result can be explained by the lower diffusion kinetics effect that occur with the cross-linking film.

![Cyclic voltammograms of MvBOD and P004-P71 Os polymers modified nanoporous gold electrodes by a vacuum with (A) and without (B) a cross-linking agent PEGDGE in the presence of O$_2$ (full line), air (dashed line) and N$_2$ (dotted line) in 0.1 M citrate-phosphate buffer, pH 7.0; scan rate: 5 mV/s.](image)

**Figure 4.14** Cyclic voltammograms of MvBOD and P004-P71 Os polymers modified nanoporous gold electrodes by a vacuum with (A) and without (B) a cross-linking agent PEGDGE in the presence of O$_2$ (full line), air (dashed line) and N$_2$ (dotted line) in 0.1 M citrate-phosphate buffer, pH 7.0; scan rate: 5 mV/s.

As shown in the cyclic voltammograms (Figure 4.13) and in Table 4.3, no electrode prepared using the vacuum procedure demonstrates substantially higher current densities. Such results possibly indicate that the vacuum of $10^{-2}$ mbar is not able to force the Os polymers with MvBOD molecules incorporated to penetrate the pores. Moreover, the pore diameters could be also too small to accomodate the Os complex. To conclude, the results clearly show that the vacuum method did not improve the response as it was observed in section 3.3.3 in Chapter 3 for the direct electron transfer of MvBOD and ThLc at unmodified nanoporous gold electrodes [35].
4.3.3 Biofuel cells

Figure 4.15 Dependence of the power density on the operational voltage of BFC with CtCDH on the anode and (A, B, C, D) MvBOD and (E, F, G, H) rMaLaC on the cathodes in oxygen saturated 50 mM phosphate buffer saline at 37 °C, pH 7.4 containing 5 mM (full line) and 100 mM (dotted line) of (A, B, E, F) lactose and (C, D, G, H) glucose at (A, C, E, G) nanoporous and (B, D, F, H) planar gold electrodes; scan rate: 1 mV/s.
Figure 4.16 Dependence of the power density on the operational voltage of BFC with GOx on the anode and (A, B) MvBOD and (C, D) rMaLc on the cathodes in oxygen saturated 50 mM phosphate buffer saline at 37 °C, pH 7.4 containing 5 mM (full line) and 100 mM (dotted line) of glucose at (A, C) nanoporous and (B, D) planar gold electrodes; scan rate: 1 mV/s.

The modified electrodes with immobilized enzymes prepared by drop-cast were incorporated into one-compartment enzymatic fuel cells. The biofuel cell consists of bioanodes modified with CtCDH or GOx and biocathodes with modified MvBOD or rMaLc. Os(dmbpy)$_2$PVI with a standard redox potential of 0.12 V was used to modify the bioanodes whereas Os(bpy)$_2$ PVI with a standard redox potential of 0.22 V was used on the cathodes. In both electrodes PEGDGE as a membrane was used to stabilize water-soluble mediators.

The power density curves obtained for BFCs in 50 mM phosphate buffer saline at pH 7.4 are shown in Figure 4.15 and Figure 4.16. The plots display power density vs. cell potential curves [37]. The power densities obtained range between 8 – 18 µW/cm$^2$ and 12 – 21 µW/cm$^2$ at planar gold and 8 – 41 µW/cm$^2$ and 25 – 37 µW/cm$^2$ at nanoporous gold in buffer containing 5 mM and 100 mM fuel concentrations, respectively. The power density of biofuel cells depends directly on the current density
achieved at a specified cell voltage. However, the current density and, thus, the power density depend on many other factors e.g. the nature and amount of the enzymes used. The available electrode surface area has a strong effect on the power output. In contrast to planar electrodes, nanoporous gold electrodes enable higher amounts of redox enzyme to be immobilized in a manner that enables efficient rate of electron transfer. As shown in Figure 4.15 and 4.16 and in Table 4.4 significantly higher power densities were obtained at nanoporous BFCs in comparison to planar BFCs.

### Table 4.4 Comparison of power output and open circuit potentials obtained at mediated electron transfer based biofuel cells.

<table>
<thead>
<tr>
<th>Biofuel cell</th>
<th>OCP [V]</th>
<th>Fuel</th>
<th>Power density [µW/cm²]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Planar gold</td>
<td>Nanoporous gold</td>
</tr>
<tr>
<td></td>
<td></td>
<td>electrode</td>
<td>electrode</td>
</tr>
<tr>
<td>CtdCDH</td>
<td>0.54</td>
<td>5 mM glucose</td>
<td>8 at 0.25 V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM glucose</td>
<td>21 at 0.20 V</td>
</tr>
<tr>
<td></td>
<td>0.55</td>
<td>5 mM lactose</td>
<td>13 at 0.20 V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM lactose</td>
<td>12 at 0.20 V</td>
</tr>
<tr>
<td>rMalLc</td>
<td>0.55</td>
<td>5 mM glucose</td>
<td>11 at 0.18 V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM glucose</td>
<td>19 at 0.15 V</td>
</tr>
<tr>
<td></td>
<td>0.56</td>
<td>5 mM lactose</td>
<td>18 at 0.15 V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM lactose</td>
<td>17 at 0.15 V</td>
</tr>
<tr>
<td>GOx</td>
<td>0.51</td>
<td>5 mM glucose</td>
<td>9 at 0.15 V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM glucose</td>
<td>11 at 0.15 V</td>
</tr>
<tr>
<td></td>
<td>0.51</td>
<td>5 mM lactose</td>
<td>13 at 0.15 V</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100 mM lactose</td>
<td>14 at 0.13 V</td>
</tr>
</tbody>
</table>

A biofuel cell with MvBOD and CtdCDH as cathode and anode, respectively exhibited the highest power densities of 41 µW/cm² and 30 µW/cm² at a potential of 0.25 V in 5 mM and 100 mM lactose (Figure 4.15 A). At planar gold electrode the power density was (Figure 4.15 B) ca. 3 times less with values of 13 µW/cm² and 12 µW/cm² at an operational voltage of 0.20 V in 5 mM and 100 mM lactose, respectively. When lactose was replaced with glucose solution, lower power densities of 8 µW/cm² at 0.30 V and 25 µW/cm² at 0.25 V in 5 mM and 100 mM glucose were obtained at nanoporous gold surfaces (Figure 4.15 C and Table 4.4). Similar power densities of 8
µW/cm² at 0.25 V and 21 µW/cm² at 0.20 V in 5 mM and 100 mM glucose, respectively, at planar gold electrodes were obtained (Figure 4.15 D and Table 4.4). In addition, a decrease of 50 mV in the operational potential in 100 mM glucose solution was observed. On a rMaLc modified cathode, power densities of 24 µW/cm² and 28 µW/cm² versus 18 µW/cm² and 17 µW/cm² at 0.15 V in 5 mM and 100 mM lactose at nanoporous and planar gold, respectively, were observed (Figure 4.15 E and F and Table 4.4). In 5 mM and 100 mM glucose, the power densities of 20 µW/cm² and 36 µW/cm² at nanoporous gold and 11 µW/cm² and 19 µW/cm² at planar gold electrodes were obtained (Figure 4.15 G and H and Table 4.4). Slight decrease in the operational potential on nanoporous gold and planar gold of 20 mV and 30 mV were observed. The open circuit potential (OCP) of CtCDH/MvBOD biofuel cell in lactose and glucose solution differed by 10 mV. Such a slight difference was also observed for CtCDH/rMaLc biofuel cell in lactose and glucose (Table 4.4). In addition, using a CtCDH/MvBOD biofuel cell in lactose, the power output in the presence of 100 mM substrate was lower than at 5 mM. That may derive from the substrate inhibition at nanoporous gold electrodes.

A biofuel cell with GOx and MvBOD on nanoporous anode and cathode, respectively exhibited the maximal power densities of 29 µW/cm² at 0.19 V and 35 µW/cm² at 0.20 V in 5 mM and 100 mM glucose, respectively (Figure 4.16 A and B and Table 4.4). On changing to planar gold, ca. 3 times lower power density of 9 µW/cm² and 11 µW/cm² at 0.15 V in 5 mM and 100 mM glucose were obtained. The replacement of MvBOD by rMaLc resulted in similar power densities of 27 µW/cm² and 37 µW/cm² at nanoporous gold electrodes and 13 µW/cm² and 14 µW/cm² at planar gold in 5 mM and 100 mM glucose, respectively (Figure 4.16 C and D and Table 4.4). As a comparison, Boland et al. reported glucose/O₂ BFC based on Os redox mediators at highly-ordered macroporous gold electrodes with pore diameters of 500 nm. The incorporation of rMaLc at the cathode with GOx at the anode resulted in the power density of 38 µW/cm² at 0.300 V in 10 mM glucose [26]. The small difference in power output of the device at 10 and 5 mM of glucose possibly arises from the different mediators used to the electrodes modifications, the use of stirring during measurements, but also from the larger spherical pores diameter. Each of the changes can affect kinetics and diffusion. A shift in the operational voltage of 10 mV and 20 mV for maximal power densities of the GOx/rMaLc biofuel cell at nanoporous and planar gold
surfaces, respectively, was observed. An OCP of 0.51 V for both biofuel cells incorporating GOx at the anode was obtained (Table 4.4).

Figure 4.17 Dependence of the power density on the operational voltage of (A) GOx and rMaLc, (B) GOx and MvBOD, (C) CtCDH and rMaLc, (D) CtCDH and MvBOD biofuel cell on nanoporous gold electrodes in 5 mM glucose and air-saturated 50 mM phosphate buffer, pH 7.4; scan rate: 1 mV/s.

Biofuel cells prepared using enzyme immobilization at nanoporous gold surfaces were also tested in 5 mM glucose and air-saturated buffer (Figure 4.17). The power density obtained was up to four times lower than for the BFCs tested in the oxygen saturated buffer (Figure 4.15 and Figure 4.16), which is caused by the lower amounts of oxygen in the electrolyte indicates response limited by O$_2$ at cathodes. However, slightly higher power density of ca. 7 µW/cm$^2$ and 8 µW/cm$^2$ (Figure 4.17 A and B) versus 5.5 µW/cm$^2$ and 6 µW/cm$^2$ (Figure 4.17 C and D) were obtained when GOx bioanodes instead of CtCDH bioanodes were combined with rMaLc and MvBOD biocathodes, respectively. Such a difference may derive from the lower affinity of
CtCDH compared to GOx for glucose as a substrate [27, 38]. In addition, the power curves contained two peaks at ca. 0.10 V and 0.30 V (Figure 4.17). Origin of the additional peak is not known.

Figure 4.18 shows plot of current densities for the CtCDH anode and MvBOD cathode versus applied potentials used in the biofuel cells which provided the highest power of ca. 41 µW/cm² at 0.25 V (Figure 4.15 A). The electrocatalytic current at the bioanode occurs over the range of 0 V to 0.35 V and 0.55 V and 0.15 V for the biocathode. Due to the use of Os-polymers which are close in the value of redox potentials, the electrocatalytic current densities for both electrodes partially overlap. The anodic catalytic current densities measured at a potential of 0.25 V are significantly lower compared to the cathodic values, indicating that the CtCDH bioanode the limiting electrode of the BFC.

The stability of enzymatic fuel cells was tested for the representative system with CtCDH at the anode and MvBOD at the cathode. The measurements were
performed for 300 minutes in O₂ saturated and 100 mM glucose phosphate buffer saline, pH 7.4 at a applied potential of 0.25 V. After 100 minutes of operation, a 50% decrease (Figure 4.19) in power density was observed. Such an immediate loss in power density likely arises from the leakage of the film from the surfaces of both electrodes as described earlier (section 4.3.1). The power density stabilized with no change visible after 300 minutes (Figure 4.19). Willner et al. reported a similar loss of 50% in stability of glucose/H₂O₂ biofuel cell with glucose oxidase reconstituted onto a pyrroloquinoline quinine and FAD (PQQ-FAD) monolayer at the anode and microperoxidase-11 at the cathode over 180 min [14]. A stable glucose/O₂ DET based biofuel cell with CDH and BOD modified gold anode and cathode, respectively, was reported by Wang et al. Such a device maintained 90% of the original stability after 5-hours of measurement and lost only 20% after 12 hours of constant operation [13].

![Figure 4.19 Variation of the power output with time in O₂ saturated and 100 mM glucose 50 mM saline buffer, pH 7.4.](image)

The operation of the one-compartment biofuel cell was also evaluated in artificial plasma buffer, in which glucose and oxygen act as biofuel and biooxidant. The glucose concentration in human blood ranges between 3.9 mM and 5.8 mM with the average of 5 mM. The concentration of the molecular oxygen is significantly lower than
the value of 200 µM in aqueous solution under air, and is ca. 45 µM [39]. Figure 4.20 shows the power curves of the biofuel cells in the artificial plasma buffer. The power density of the GOx/rMaLc and GOx/MvBOD (Figure 4.20) systems decreased up to 3 µW/cm² and 2 µW/cm², respectively. A significant decrease in the power densities of 0.85 µW/cm² and 0.6 µW/cm² for biofuel cells with a CtCDH anode and rMaLc or MvBOD biocathodes, respectively, were reported. In addition, a shift toward lower cell voltage at the maximal power was observed. According to the detailed studies performed by Coman et al., the peak appearing at 0.19 V can be ascribed to the non-enzymatic electrochemical reactions with the compounds in the artificial plasma buffer, e.g. uric and ascorbic acids [11].

Figure 4.20 Dependence of the power density on the operational voltage of (A) GOx and rMaLc, (B) GOx and MvBOD, (C) CtCDH and rMaLc, (D) CtCDH and MvBOD biofuel cell in the artificial plasma buffer; scan rate: 1 mV/s.
4.4 Conclusions

The modification of nanoporous and planar gold electrodes with *Aspergillus niger* GOx and *Corynascus thermophilus* CtCDH connected to the electrode surface by Os redox mediators and PEGDGE as a cross-linking agent resulted in glucose and lactose detecting biosensors. The kinetic characteristics determining $I_{\text{max, app}}$, $K_{M, \text{app}}$ and sensitivity were evaluated. The optimization studies determined the limit of detection of 6 (± 0.4) µM and 16 (± 0.1) µM for CtCDH modified nanoporous gold to recognize lactose and glucose, respectively and 2 (± 0.03) µM for GOx bioelectrode detecting glucose. The stability studies in the glucose saturation buffer for CtCDH and GOx based biosensors showed the half lifetime of both bioelectrodes of *ca.* 20 hours.

The developed bioanodes were connected with *Mv*BOD and *rMa*Lc biocathodes and used to build one-compartment biofuel cells operating in lactose or glucose fuel and biooxidant. The highest power density of 41 µW/cm$^2$ was obtained by CtCDH/MvBOD BFC operating in 5 mM lactose buffer under O$_2$ saturation. In 5 mM glucose buffer GOx/MvBOD and GOx/rMaLc biofuel cells showed higher power density than upon replacement with CtCDH anodes. Such a behavior was also observed in air-saturated buffer. In addition, the power density of 3 µW/cm$^2$ and 2 µW/cm$^2$ for GOx/rMaLc and GOx/MvBOD compared to 0.85 µW/cm$^2$ and 0.6 µW/cm$^2$ for CtCDH/rMaLc and CtCDH/MvBOD was obtained in artificial plasma buffer simulating human blood. The stability studies of BFCs showed the loss of 50% of the power output during 5-hour test. The improvement in power output and stability of biofuel cells provides to the adaptation of the pore sizes in the material surface to the type of immobilization used [6, 40]. Thus, hydrogel with the enzyme, Os redox polymer and cross-linking agent would penetrate easier pores with bigger diameter and result in higher power density and stability of the device.

A vacuum method of electrodes preparation was also applied to the mixture of different Os-polymer and MvBOD to obtain mediated electron connection. It was found that any of the tested systems using vacuum procedure demonstrate compared to the electrodes made by drop-cast substantially high current density. Hence, vacuum procedure is not recommended for application to the electrodes with used morphology for mediated redox enzymes immobilization.
4.5 References

Chapter 4

MET based bioelectrodes

CHAPTER 5:

CONCLUSIONS
AND
RECOMMENDATIONS
5.1 Conclusions

The objective of this thesis was to develop a conductive gold material of high surface area which should serve as a support for the immobilization of enzymes and enable electrochemical communication to occur between the enzymes and the support. The results include detailed characteristics of the materials, the use of methods based on direct and mediated electric communication do immobilize the redox enzymes on nanoporous gold and a comparison of the response to that obtained using planar gold surfaces. Subsequently, nanoporous gold electrodes were used for the development of glucose and lactose biosensors and of biofuel cells.

Two types of nanoporous gold materials: nanoporous gold leaves and nanoporous gold electrodes were described in Chapter 2. A comparison of the two materials demonstrated the advantage of the mechanical stability of nanoporous gold electrodes over nanoporous gold leaf. The use of a titanium adhesion layer enables strong adhesion of the gold layers to the support whereas the weak adhesion forces between nanoporous gold leaf film and the glassy carbon electrode were easily disrupted. A number of methods were used to characterize the electrochemically addressable surface area \( A_{\text{real}} \) and the surface area accessible for modification of the electrodes. The gold oxide stripping method was the most reliable method to determine \( A_{\text{real}} \). The roughness factors \( R_f \) of uniform and non-uniform nanoporous gold were ca. 16 and ca. 28, respectively, compared to ca. 1 of planar gold electrode. Bulk Cu deposition and underpotential deposition of Cu confirmed that the nanoporous gold surface was accessible to the thiol molecules enabling the immobilization of redox enzymes.

The accessibility of nanoporous gold surface to redox proteins was successfully investigated using cyt c as a model system (Chapter 3). A mixture of 11-mercaptoundecanoic acid and 6-mercapto-1-hexanol (HS(CH\(_2\))\(_{10}\)COOH and HS(CH\(_2\))\(_6\)OH) was used to create the most optimal SAM layers for cyt c immobilization on gold. On covalent immobilization of cyt c at HS(CH\(_2\))\(_{10}\)COOH and HS(CH\(_2\))\(_6\)OH modified planar and nanoporous gold electrodes current densities were observed that were 9 and 11 times higher, respectively, than at planar gold electrodes.
A similar strategy was used to immobilize CtCDH at planar and nanoporous gold electrodes under Nafion® stabilizing membrane. A modifying layer of 1-thioglycerol created the most efficient surface on gold for the CtCDH immobilization. Lactose was used as a substrate to investigate the catalytic efficiency of the system. The unusual shape of the calibration curves possibly arises from two factors; substrate inhibition and mass-transfer limitation. However, further investigation of the system was not possible due to the lack of reproducibility.

An immobilization procedure utilising vacuum was successfully applied for adsorption of ‘blue’ copper oxidases at unmodified uniform nanoporous gold electrodes. Vacuum of $10^{-2}$ mbar was introduced first to dry the electrodes after the cleaning step and then to force the enzymes to penetrate into the voids of gold surfaces. Significantly higher current density of $ca. 0.8 \text{ mA/cm}^2$ was observed when $MvBOD$ was absorbed into nanoporous gold covered with the chosen cross-linking agent P017-epoxy compared with current densities of $ca. 0.04 \text{ mA/cm}^2$ and $ca. 0.3 \text{ mA/cm}^2$ obtained for the electrode prepared by drop-casting of $MvBOD$ and in vacuum but without the cross-linking agent, respectively. A minimal inhibition of $F^-$ of such a system was observed. The $O_2$ reduction current at $MvBOD$-modified nanoporous gold with P107-epoxy was decreased over time with a half life of $ca. 7 \text{ hours}$.

$ThLc$, another multi-copper oxidase, was also adsorbed into the voids of porous gold electrodes on application of vacuum. The current densities obtained, $ca. 0.03 \text{ mA/cm}^2$ were 10 times higher than the current densities at $ThLc$ electrodes prepared by drop-casting. The activity of $ThLc$ immobilised on nanoporous gold was strongly dependent on the presence of halide ions. Fluoride completely inhibited the enzymatic response, whereas the addition of $Cl^-$ had a less significant influence on the bioelectrocatalytic activity of the enzyme, with 50% loss in activity in the presence of 200 mM $Cl^-$.

The development of lactose and glucose sensors based on mediated electric connection between the hydrogel of redox enzymes GOx and CtCDH and Os polymers and gold surfaces was described in Chapter 4. The Os-redox complexes, Os(bpy)$_2$PVI and Os(dm bpy)$_2$PVI were used as mediators. In addition, PEGDGE was applied as a cross-linking agent providing a stabilizing film on the surface of the electrodes. Detailed studies showed a higher electrocatalytic response obtained with Os(bpy)$_2$PVI
as a mediator in comparison to \( \text{Os(dmbpy)}_2 \text{PVI} \). The limits of detection were 6 (± 0.4) µM and 16 (± 0.1) µM for \( \text{CtCDH} \) nanoporous gold electrodes for the detection of lactose and glucose, respectively and 2 (± 0.03) µM for \( \text{GOx} \) nanoporous gold bioelectrode detecting glucose. The half life of both bioelectrodes was \textit{ca.} 20 hours with the loss of the stability caused by the film leaching from the electrode surface. A similar approach was applied to the immobilization of \( \text{rMaLc} \) and \( \text{MvBOD} \) at nanoporous and planar gold electrodes using \( \text{Os(bpy)}_2 \text{PVI} \) as mediator. All modified electrodes were used to prepare one-compartment biofuel cells operating in solutions of either lactose or glucose and using \( \text{O}_2 \) as oxidant. The highest power density of 41 µW/cm\(^2\) was obtained with a \( \text{CtCDH/MvBOD} \) BFC operating in 5 mM lactose buffer under saturated \( \text{O}_2 \). However, due to possible application of these cells to operate under physiological conditions and in human blood, BFCs operating in glucose were used for further analysis. In 5 mM glucose and air-saturated buffer \( \text{GOx/MvBOD} \) and \( \text{GOx/rMaLc} \) biofuel cells showed higher power density than that observed with \( \text{CtCDH} \) modified anodes. Similar behavior was observed in artificial plasma buffer simulating human blood. The power densities of 3 µW/cm\(^2\) and 2 µW/cm\(^2\) for \( \text{GOx/rMaLc} \) and \( \text{GOx/MvBOD} \) compared to 0.85 µW/cm\(^2\) and 0.6 µW/cm\(^2\) for \( \text{CtCDH/rMaLc} \) and \( \text{CtCDH/MvBOD} \) were obtained. Stability studies of the BFCs showed a loss of 50% of the original power density during a 5-hour test. In addition, the anode was recognized as a limiting electrode. Vacuum deposition was also used to prepare mediated electrodes, however any of the tested systems using vacuum procedure demonstrated substantially lower current densities compared to control electrodes prepared by drop-casting. Hence, the vacuum procedure is not recommended for the electrodes.
5.2 Recommendations

As a result of the conclusions drawn above, the following issues are recommended for further investigation:

- The pore, ligament and crack sizes obtained in dealloying at 37 °C are not large enough to be penetrated by the hydrogel. By changing the dealloying parameters such as temperature (higher than 37 °C) and time (longer than 15 minutes), electrode morphologies with larger pores, ligaments and cracks can be achieved. That would allow the enzyme molecules or polymer-enzyme complexes to penetrate the porous material.

- The loading of the PEGDGE cross-linker was not optimised. High loadings of PEGDGE on the modified electrode reduces the segmental mobility of electrons [1]. Glutaraldehyde could be used as a replacement for PEGDGE.

- Control of lactose concentrations is important for people with lactose intolerance. CtCDH modified nanoporous gold electrodes could be utilised to determine the concentration of lactose in dairy products [2].

- The use of vacuum deposition with Os polymer hydrogels did not show significantly high current densities in comparison to immobilization based on the drop-casting. Larger pore, ligament and crack sizes obtained in changes in the dealloying process could result, on application of vacuum, in the hydrogel being able to penetrate into the pores of the electrodes.
5.3 REFERENCES

Characterization of Nanoporous Gold Electrodes for Bioelectrochemical Applications

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Supporting Information

ABSTRACT: The high surface areas of nanostructured electrodes can provide for significantly enhanced surface loadings of electroactive materials. The fabrication and characterization of nanoporous gold (np-Au) substrates as electrodes for bioelectrochemical applications is described. Robust np-Au electrodes were prepared by sputtering a gold—silver alloy onto a glass support and subsequent dealloying of the silver component. Alloy layers were prepared with either a uniform or nonuniform distribution of silver and, post dealloying, showed clear differences in morphology on characterization with scanning electron microscopy. Redox reactions under kinetic control, in particular measurement of the charge required to strip a gold oxide layer, provided the most accurate measurements of the total electrochemically addressable electrode surface area, \( A_{\text{real}} \). Values of \( A_{\text{real}} \) up to 28 times that of the geometric electrode surface area, \( A_{\text{geo}} \), were obtained. For diffusion-controlled reactions, overlapping diffusion zones between adjacent nanopores established limiting semi-infinite linear diffusion fields where the maximum current density was dependent on \( A_{\text{geo}} \). The importance of measuring the surface area available for the immobilization was determined using the redox protein, cyt c. The area accessible to modification by a biological macromolecule, \( A_{\text{macro}} \), such as cyt c was reduced by up to 40% compared to \( A_{\text{real}} \), demonstrating that the confines of some nanopores were inaccessible to large macromolecules due to steric hindrances. Preliminary studies on the preparation of np-Au electrodes modified with osmium redox polymer hydrogels and Myrothecium verrucaria bilirubin oxidase (MvBOD) as a biocathode were performed; current densities of 500 \( \mu A \) cm\(^{-2} \) were obtained in unstirred solutions.

INTRODUCTION

Nanoporous gold (np-Au) is a material of considerable versatility and diverse application.\(^1\)–\(^3\) np-Au is conductive, chemically and mechanically stable, free of surface contaminants (arising from preparation in nitric or perchloric acid), biocompatible, and easily functionalized.\(^1\)–\(^3\) It has a high surface-to-volume ratio and large surface area with pore sizes that are tunable from the nanometer to micrometer scale. These properties make np-Au attractive for applications in the immobilization of enzymes or antibodies,\(^4\)–\(^5\) energy storage,\(^6\) catalysis,\(^7\) and bio/fuel cells.\(^8\)–\(^9\)

The use of large surface area supports can potentially improve the performance of devices, e.g., the sensitivity of an affinity-based biosensor can be increased by increasing the number of immobilized receptor molecules. Sensitive biosensors have been developed for the detection of DNA,\(^4\) prostate specific antigen,\(^5\) and thrombin.\(^10\)

The development of electrochemical devices based on direct electron transfer (DET) between an immobilized enzyme and an electrode surface, has been hampered by the (a) limited number of redox enzymes exhibiting DET at the surface of an electrode (approximately 5% of known redox enzymes),\(^11\) (b) limited stability of enzyme electrodes, and (c) low, observed, current densities.\(^12\) Achieving DET requires selective modification of the electrode surface to enable optimal orientation of the enzyme and to minimize the distance between the electrochemically active center and the electrode surface.\(^13\) Correct orientation is crucial as the rate of electron transfer decreases exponentially with distance.\(^14\) The high surface-to-volume ratio and the ability to functionalize the surface of np-Au with alkanethiolate self-assembled monolayers (SAMs) can result in high loadings of optimally orientated enzymes. Such high loadings ensure that the maximum signal is achieved and factors such as substrate diffusion or enzyme kinetics limit the response. Immobilization of an enzyme within the protective sheltered surroundings of a nanopore, as opposed to on an exposed flat surface, may afford additional protection for the enzyme from external environmental conditions, potentially preventing desorption and stabilizing the protein against reversible unfolding.\(^15\) Any desorption of enzyme
that does occur is more easily compensated due to the abundance of enzyme initially adsorbed in comparison to that at a planar Au electrode.

Cytocrome c (cyt c) is a small (104 amino acid residues) redox protein capable of DET between its heme cofactor and a suitably modified electrode. The role of cyt c in the respiratory chains of mitochondria, coupled to its stability, wide availability and comprehensive physiochemical characterization ensures that it is widely used as a model for larger, more complex electron transfer (ET) systems. A stable electroactive monolayer of cyt c may be physically adsorbed by electrostatic interaction of lysine residues to a carboxy-terminated alkane thiolate SAM on Au. Alternatively, cyt c can be covalently attached using carbodiimide linkages, with molecular masses of 52–64 kDa, and belong to a group of “blue” multicopper oxidases that include laccases, ascorbate oxidase, and ceruloplasmin. BOD from the fungi Myrothecium verrucaria (MbBOD) and Trachyderma tsunoda (TtBOD) have been purified and used as cathode biocatalysts in biofuel cells. They catalyze the four-electron reduction of O2 to water without producing H2O2 and do so at neutral pH and relatively low overpotentials. Ikekda et al. initially described the use of BOD as a biocathode, reducing O2 to water in the presence of the mediator 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) at low overpotentials. High current densities for O2 reduction were achieved via a mediated electron transfer mechanism by incorporating MbBOD and TtBOD into osmium-based redox hydrogel polymers.

Here, we describe the fabrication and characterization of two np-Au electrodes of different morphology and surface roughness. np-Au electrodes were fabricated by sputtering a Au–Ag alloy on to a glass support with subsequent dealloying of Ag. In contrast to electrodes fabricated from 50% Ag:50% Au (white gold), the electrodes were mechanically robust, which greatly facilitated their ease of use. A series of electrochemical probes was used to characterize the electrochemically addressable surface area, Arec.

The importance of using a probe appropriate for the intended application is evident from the range of surface areas that were obtained. For applications such as biofuel cells, which entail immobilization of enzymes, cyt c was used as a model system to ascertain the surface area accessible to modification by a redox protein, Amacro. Significantly increased (11-fold) loadings were observed. Preliminary studies on the preparation of np-Au electrodes modified with osmium redox polymer hydrogels and MbBOD as a biocathode were performed.

**EXPERIMENTAL SECTION**

Fabrication of Nanoporous and Planar Au Electrodes. Metal targets for substrate deposition, Au (AJA International Inc., USA), Ag and Ti (Kurt J. Lesker Company Ltd., UK), were >99.99% pure. Magnetron sputtering was carried out at room temperature in an ultrahigh vacuum chamber (ORION-5-UHV custom sputtering system) onto plain precleaned glass microscope slides. Prior to metal deposition, the glass slides were cleaned in the vacuum chamber by Ar plasma. Sample substrates were tilted at ∼70° from the surface normal and rotated at 20–40 rpm to ensure uniform deposition. Sputter deposition rates were calibrated using a quartz crystal thickness monitor. The planar Au substrate was prepared by deposition of a ∼10 nm thick Ti adhesion layer followed by a ∼100 nm thick Au layer. A thinner Au substrate layer (35 nm) was used as a base for the deposition of a composite layer of AgxAu1−x with a uniform distribution of Ag and Au. The ∼35 nm thick Au layer improves adhesion and prevents delamination of the AgxAu1−x alloy layer during dealloying. In addition, any potential redox activity of the underlying Ti layer is suppressed. Each metal was sputtered at a constant sputtering rate to produce a ∼300 nm thick AgxAu1−x alloy layer with a uniform distribution of Ag throughout the alloy. AgxAu1−x substrates with a nonuniform distribution of Ag were prepared by simultaneously depositing Au at a constant sputtering rate while Ag was deposited at a gradually increasing sputtering rate. This procedure produced a ∼300 nm thick AgxAu1−x layer with a higher distribution of Ag near the surface of the alloy layer. Both “uniform” and “nonuniform” substrates were dealloyed in concentrated (70% w/v) nitric acid for 15 min at 38 °C. Upon removal from the nitric acid bath, the substrates were thoroughly rinsed with deionized water and dried in a stream of nitrogen.

The electrodes were electrically connected via a Ag wire attached to the Au substrate using indium and an epoxy glue. A circular electrode area (0.28 cm radius) was defined using an insulating paint (Gwent Electronic Materials Ltd., UK).

The structure and chemical composition of np-Au was characterized using a scanning electron microscope (SEM; Hitachi SU-70) equipped with an energy dispersive X-ray spectrometer (EDS, Oxford Instruments). In order to minimize charging effects, beam voltages were adjusted between 3 and 5 kV, and a corner of the sample was electrically connected to the sample holder with silver paint. The Au pore, ligament, and crack sizes were determined manually by identifying a minimum of 10 of each feature and making measurements across the shortest distance using ImageJ software.

Preparation of Nanoporous Gold Leaf Electrodes. 12-carat Au–Ag leaf (30 wt % Ag) was obtained from Wilhelm Wasner Blattgold, Germany. After dealloying in (70% w/v) nitric acid for 15 min at 30 °C, the free-standing np-Au films were washed several times with ultrapure water and floated on water. The floating np-Au films were attached to a polished glassy carbon (GC) electrode and allowed dry for 12 h at 4 °C to create a high surface area np-Au/GC electrode, as described previously.

Electrochemical Measurements and Reagents. All electrochemical studies were performed using either a CHI832 bipotentiostat or CHI630A potentiostat (CH Instruments, Austin, Texas, USA) in a standard three-electrode electrochemical cell with a 0.5 mm diameter Pt wire counter electrode (ALS Co. Ltd., Tokyo, Japan) and Ag/AgCl|3 M KCl reference electrode (I) Cambria Scientific Ltd., UK). Immediately prior to use, np-Au electrodes were electrochemically cleaned by cycling the potential between 0.0 and +1.5 V in 0.5 M sulfuric acid until a stable Au oxide cyclic voltammogram was obtained. Planar Au electrodes were cleaned by dipping in piranha solution (1:3 (v/v) mixture of 30% H2O2: 98% H2SO4) for 30 s followed by rinsing in ultrapure water in order to prevent electrochemical roughening of the surface. (Warning: piranha solution reacts strongly with organic compounds. Handle with extreme caution at all times. Do not store the solution in a closed container.) All reagents were of analytical grade, purchased from Sigma-Aldrich Ireland, Ltd., unless stated otherwise, and used as received. All solutions were prepared in ultrapure water (resistivity of 18.2 MΩ cm) from an Elgastat maxima-HPLC (Elga, UK) with the exception of thiocyanate solutions, which were prepared in absolute ethanol (Lennox Ltd., Ireland). Cyt c from horse heart (Sigma C7752) was used as received. MbBOD was purchased from Amano Enzymes, Inc. (Amano-3, 2.63 unit mg−1) and used as received.
Electrochemical Characterization. Surface-confined or diffusion-controlled probes were used to determine the electrochemically addressable surface areas \((A_{\text{real}})\) of each electrode design. With the exception of the Au oxide stripping technique (see below), \(R_T\) values were calculated by comparison of either surface coverage \((\Gamma, \text{ pmol cm}^{-2})\), double layer capacitance \((C_{\text{dl}}, \mu \text{F cm}^{-2})\), or current density \((I, \text{ mA cm}^{-2})\), at a planar Au electrode to that at a np-Au electrode. \(\Gamma, C_{\text{dl}}\) and \(I\) values (Table S1) were normalized to the geometric electrode surface area \((A_{\text{geo}}) 0.246 \text{ cm}^2\). \(\Gamma\) values for copper, 6-ferrocenyl)hexanethiol \((\text{FcHxSH})\) and \(\text{cyt c}\) were obtained by integration of the anodic current and converted to \% monolayer coverage using theoretical \((\text{FcHxSH})\) and \((\text{cyt c})\) surface coverage (see below), \(\Gamma\) values of 2000, 450, and 15 pmol cm\(^{-2}\) for complete monolayer coverage of \(\text{Cu}^{2+}\), \(\text{FcHxSH}\), and \(\text{cyt c}\) respectively, \(A_{\text{real}}\) values were determined by multiplying \(A_{\text{geo}}\) by \(R_T\).

Surface-Confined Electrode Reactions. (a) The charge required to strip a Au oxide layer in 0.5 M H\(_2\)SO\(_4\) was determined, and a conversion factor of 390 \(\mu\)C cm\(^{-2}\) was applied to obtain \(A_{\text{real}}\).\(^{30}\) (b) \(C_{\text{dl}}\) values were calculated using \(^{31}\)

\[
C_{\text{dl}} = \frac{I_{\text{total}}}{(2\nu A_{\text{geo}} \times 10^{-6})}
\]  

where \(I_{\text{total}}\) is the sum of the anodic \((i_a)\) and cathodic \((i_c)\) currents and \(\nu\) is the scan rate. \(i_a\) and \(i_c\) were obtained after dealloying \((A, C)\) a uniformly sputtered Au film and \((B, D)\) a nonuniformly sputtered Ag-Au film, in 70% (w/v) nitric acid at 38 °C for 15 min.

**RESULTS AND DISCUSSION**

SEM and EDS Characterization of np-Au Electrodes. Representative top-down and cross-sectional SEM images of both np-Au electrode designs are shown in Figure 1. The “uniform” and “nonuniform” methods of sputtering Au–Ag alloys resulted in np-Au films with clear morphological differences. The electrodes consisted of three-dimensional networks of quasi-periodic nanoporous voids (or pores) and interconnected gold ligaments. Significant volume contraction (20–30%) of the films during dealloying induces stresses that are manifested as cracks.\(^{39}\) np-Au films produced using the nonuniform sputtering method had relatively fine structures and an increased number of cracks between islands of ligaments. The pore, ligament, and crack sizes were 12–20 nm, 15–18 nm, and 55–65 nm, respectively. Uniformly sputtered np-Au films were coarser with pore, ligament, and crack sizes of 17–23 nm, 18–25 nm, and 40–55 nm,
respectively. Cross-sectional SEM images (Figure 1C, D) demonstrated that the wormlike structure of interconnected ligaments was regularly distributed throughout the entire volume of both films after dealloying. The pure Au adhesion layer remained intact after dealloying (Figure S1). Relative to uniform np-Au, the finer pore structures and increased crack densities in nonuniform
np-Au electrodes resulted in higher surface roughnesses, as outlined below. Successive batches of both np-Au electrode designs were prepared in a reproducible manner in terms of pore, ligament, and crack sizes, as confirmed by SEM and $A_{\text{real}}$ (calculated from the charge required to strip a gold oxide layer). \(^{30}\) EDS data (not shown) confirmed the removal of Ag with only 0.6 and 2.8 wt % Ag remaining on the uniform and nonuniform np-Au surfaces, respectively, post dealloying.

**Electrochemical Characterization.** Cyclic voltammetry of planar and np-Au electrodes in 0.5 M H$_2$SO$_4$ showed two broad oxidation peaks at 1.2 and 1.4 V, attributed to the formation of Au surface oxides, \(^{30}\) and a sharp reduction peak at 0.9 V due to subsequent removal of the oxides (Figure 2A). The absence of any additional peaks confirmed that Ag was removed in the dealloying process, in agreement with EDS data. Cyclic voltammograms in a 50 mM KH$_2$PO$_4$ and 29.1 mM NaOH, pH 7, buffer solution confirmed the formation of AuOH at a potential of $\sim$300 mV at each Au electrode design (Figure 2B). At potentials where faradaic processes were occurring, $-100$ mV for planar and $-200$ mV for both np-Au electrodes, large increases in $C_{dl}$ were observed at np-Au electrodes. The $R_f$ values obtained by both the Au oxide stripping and capacitance-based methods for nonuniform np-Au electrodes were in agreement with each other. However, larger than expected $R_f$ values were calculated at uniform np-Au electrodes and greater electrode-to-electrode variability was observed at each Au electrode design for the capacitance-based method (Table 1).

At both np-Au electrodes, the magnitudes of the observed current signals were enhanced significantly in the potential range of Cu UPD, in comparison to those at planar Au electrodes (Figure 2C). No significant current enhancements were observed in the potential range for bulk Cu deposition (Figure 2D). Bulk Cu deposition is controlled by the diffusion of aqueous Cu$^{2+}$ to the electrode surface, whereas Cu UPD is a surface-confined electrochemical process. Similar currents were observed for bulk Cu deposition at planar and np-Au electrodes, indicating that, on the time scale of the experiment, overlap of diffusion zones occurs between individual nanopores at the np-Au surface establishing a semi-infinite linear diffusion field. Thus, Cu$^{2+}$ in solution is reduced only at the surface and, perhaps, the upper pore regions of np-Au. The current densities of the diffusion-controlled and surface-confined processes are dependent upon $A_{\text{geo}}$ and $A_{\text{real}}$, respectively. Submonolayer coverage of Cu was observed at the planar Au electrode. The UPD response was unchanged on increasing the concentration of CuSO$_4$ from 1 to 2 mM, indicating that a concentration of 1 mM CuSO$_4$ was sufficient to achieve maximum coverage. However, the magnitude of the bulk deposition peak current doubled, as expected for a diffusion controlled process (Figure 2E). The $R_f$ value for a uniform np-Au electrode calculated from the Cu UPD surface coverage was in good agreement with the values obtained from the Au oxide stripping method. However, at nonuniform np-Au electrodes, the $R_f$ value from the Cu UPD was less than that measured by the Au oxide stripping technique (Table 1). Nonuniform Au electrodes have smaller pore and ligament sizes, as confirmed by SEM, than uniform np-Au, indicating that some of the nanopores may be inaccessible to Cu$^{2+}$.

Mixed monolayers of FchxSH/HO(CH$_2$)$_n$SH were coadsorbed on planar and np-Au electrodes in ethanol with a FchxSH solution mole fraction ($X_{\text{mol}}$) of 0.05. Cyclic voltammograms obtained in 1 M HClO$_4$ contained two peaks on both the anodic and cathodic sweeps for each electrode design (Figure 2F). Such multiple peaks are indicative of adsorption of FchxSH moieties in isolated (lower-potential) and clustered (higher-potential) states, respectively. \(^{28}\) The anodic and cathodic peak potentials decreased to less positive values at np-Au electrodes, indicative of stabilization of the ferrocenium form of the probe. This stabilization may arise from a more hydrophilic environment on the surface of np-Au. $C_{dl}$ was consistently larger at potentials positive of the formal redox potential, $E^{\text{red}}$, for ferrocene oxidation. This observation, in agreement with previous reports, \(^{24}\) may be due to increased permeability of the monolayer to electrolyte ions and/ or solvent on oxidation of ferrocene. $X_{\text{mol}}$ did not correlate directly with surface mole fraction ($X_{\text{surf}}$) as the adsorbate with the longest alkyl chain, FchxSH, is preferentially adsorbed. \(^{28}\) At a planar Au electrode, $X_{\text{surf}}$ for FchxSH was 0.36 (i.e., 36%)

<table>
<thead>
<tr>
<th>probe</th>
<th>roughness factor, $R_f$</th>
<th>% monolayer formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>uniform np-Au</td>
<td>nonuniform np-Au</td>
</tr>
<tr>
<td>UPD Cu$^{2+}$/0</td>
<td>15.50 ($\pm$0.02)</td>
<td>19.00 ($\pm$0.03)</td>
</tr>
<tr>
<td>FcHxSH</td>
<td>7.40 ($\pm$0.05)</td>
<td>9.3 ($\pm$2.8)</td>
</tr>
<tr>
<td>cyt c</td>
<td>9.3 ($\pm$1.2)</td>
<td>10.8 ($\pm$1.2)</td>
</tr>
<tr>
<td>Au Ox/Red</td>
<td>16.1 ($\pm$1.3)</td>
<td>28.1 ($\pm$1.5)</td>
</tr>
<tr>
<td>$C_8$</td>
<td>21.2 ($\pm$2.3)</td>
<td>28.8 ($\pm$3.2)</td>
</tr>
<tr>
<td>bulk Cu$^{2+}$/0</td>
<td>1.05 ($\pm$0.14)</td>
<td>1.35 ($\pm$0.15)</td>
</tr>
<tr>
<td>Fe(CN)$_6$$^{3-/4-}$</td>
<td>1.16 ($\pm$0.07)</td>
<td>1.25 ($\pm$0.08)</td>
</tr>
<tr>
<td>Ru(NH$_3$)$_6$$^{3+/2+}$</td>
<td>1.09 ($\pm$0.02)</td>
<td>1.02 ($\pm$0.01)</td>
</tr>
<tr>
<td>O$_2$</td>
<td>2.07 ($\pm$0.12)</td>
<td>2.09 ($\pm$0.18)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Averages and standard deviation data from a minimum of three electrodes tested. \(^b\) UPD and bulk Cu$^{2+}$/0 data presented for experiments carried out with 1 mM CuSO$_4$ in solution. \(^c\) Theoretical % monolayer formation of cyt c at a planar Au electrode.
monolayer coverage). Assuming that $X_{\text{surf}}$ remains constant at 0.36 for both np-Au electrodes, this would yield $R_f$ values of 7.4 ($\pm 0.05$) and 9.3 ($\pm 2.8$) for uniform and nonuniform np-Au electrodes, respectively. These values were considerably lower than those calculated using all other surface confined techniques, even for the immobilization of more bulky cyt c molecules, discussed below. Control experiments were carried out to confirm that all pore surfaces of np-Au could be coated with thiol molecules. A layer of 1-hexadecanethiol proved extremely effective in completely suppressing the redox responses of potassium ferricyanide (Figure 3C) and AuOH (data not shown) at planar and np-Au electrode surfaces, indicative of complete monolayer coverage. This observation eliminated the inability of thiol molecules to access and modify all areas of the np-Au nanostructure as a factor in accounting for the low FcHxSH surface coverages and, hence, the low $R_f$ values observed.

A possible explanation for the observed low surface coverages is that different electrode surface morphologies produce nonequivalent adsorption sites and thus different $X_{\text{surf}}$ values for FcHxSH.41 The major morphological difference between planar and np-Au electrodes is the presence of a higher density of defect-type sites (such as step-edges) in the latter.41 Defect sites are unlikely to be places of easily exchangeable molecules. Thus, any thiol adsorbed at a defect site will remain adsorbed and is less likely to exchange with free thiols in solution. Effectively, diluent molecules stabilized at defect sites no longer exchange with FcHxSH. The adsorption of diluent subsequently becomes more favored than at planar Au and $X_{\text{surf}}$ values for FcHxSH are reduced.

Cyclic voltammograms for the diffusion-controlled redox probes ruthenium(III) hexamine chloride (Figure 3A) and potassium ferricyanide (Figure 3B) show no significant increase in current density on substituting planar with np-Au electrodes. As observed for bulk Cu deposition, semi-infinite linear diffusion fields are established on the time-scale of the experiment limiting the observed current densities. Switching from planar to np-Au did, however, have a significant influence on the response observed for oxygen reduction (Figure 3D). A positive shift and splitting of the oxygen reduction peak was observed, from $-343$ mV at planar Au to $-210$ (peak 1)/$-346$ (peak 2) mV, and $-196$ (peak 1)/$-307$ (peak 2) mV, at uniform and nonuniform np-Au,

Figure 3. Electrochemical characterization of nonuniform nanoporous (dashed lines), uniform nanoporous (dotted lines), and planar (solid lines) gold electrodes using diffusion-confined probes: (A) Ru(NH$_3$)$_6$$^{3+/2+}$ and (B) Fe(CN)$_6$$^{3-/4-}$ in 0.1 M KCl. (C) Suppression of the Fe(CN)$_6$$^{3-/4-}$ redox response in 0.1 M KCl by the formation of a SAM monolayer on immersion in a 10 mM CH$_3$(CH$_2$)$_{15}$SH solution for 72 h. (D) O$_2$ reduction at a scan rate of 50 mV s$^{-1}$. Currents were normalized to the geometric surface areas of the electrodes. Unless stated otherwise, a scan rate of 100 mV s$^{-1}$ was used.
respectively. Also, a doubling of the current density was observed at the np-Au electrodes. As described previously,\textsuperscript{42} such observations are a result of oxygen reduction proceeding at planar and np-Au via different mechanisms due to the catalytic effect of defect sites present in np-Au. Oxygen reduction at np-Au electrodes proceeds via a four electron process that takes place in two steps. Oxygen is first reduced to hydrogen peroxide and then, due to the high reactivity of the defect sites in np-Au, undergoes reduction to water. At a planar Au electrode, oxygen reduction proceeds via a two electron reduction to hydrogen peroxide. Any subsequent reduction to water that may take place at planar Au occurs too slowly to be observed under the experimental conditions employed.\textsuperscript{42}

Direct comparisons between planar and np-Au systems for (a) adsorption of mixed monolayers of redox active thiols and (b) oxygen reduction are not valid due to the significant influence the electrode morphologies, in terms of defect site densities, have on the observed response. Comparisons of current densities at planar and np-Au systems for diffusion-controlled redox reactions, as a reliable method to determine the electrochemically addressable surface areas \((A_{\text{real, np-Au}})\), are not valid as the overlapping diffusion zones at np-Au limit the current response. Minimal increases in the electroanalytical signal for redox probes with fast kinetics can provide a better measure of the electroactive surface area. Cu UPD, for example, is a suitable method to determine \(A_{\text{real}}\), although it may give slightly lower values for very fine pore structures. The most commonly used, and most accurate, method to determine \(A_{\text{real}}\) is measuring the charge required to strip a Au oxide layer. This method is widely used to characterize Au based nanostructured electrodes.\textsuperscript{35,43,44,47} A less widely used technique, determining the \(C_{\text{dl}}\) at potentials where no faradaic processes are occurring is a suitable complementary method to estimate \(A_{\text{real}}\) for np-Au electrodes. This capacitance-based method should not, however, be used as a stand-alone technique to estimate \(A_{\text{real}}\) due to the relatively large electrode-to-electrode variations, compared to the Au oxide stripping technique, and possible overestimations of \(A_{\text{real}}\) observed.

**Voltammetric Characterization of Cyt c Immobilized on Planar and np-Au Electrodes.** The voltammetric responses of cyt c covalently immobilized at planar and np-Au electrodes modified with a HS(CH\textsubscript{2})\textsubscript{10}COOH/HS(CH\textsubscript{2})\textsubscript{6}OH mixed SAM are shown in Figure 4. In comparison to homogeneous SAMs modified with long chain homogeneous carboxylic acid terminated SAMs,\textsuperscript{35,43,44,47} plots of the anodic and cathodic peak currents versus scan rate were linear (Figure 5A), indicating that cyt c was adsorbed on the electrode surface. Voltammograms were nearly uniform (dotted line), and nonuniform (dashed line) np-Au electrodes in 4.4 mM K\textsubscript{2}HPO\textsubscript{4}–KH\textsubscript{2}PO\textsubscript{4} buffer, pH 7, at a scan rate of 100 mV s\textsuperscript{-1}. Current densities were normalized to the electrodes geometric surface area.

**Figure 4.** Cyclic voltammograms of 50 µM cyt c covalently immobilized on a 50/50 mol % HS(CH\textsubscript{2})\textsubscript{10}COOH/HS(CH\textsubscript{2})\textsubscript{6}OH mixed SAM at planar (solid line and inset), uniform (dotted line), and nonuniform (dashed line) np-Au electrodes in 4.4 mM K\textsubscript{2}HPO\textsubscript{4}–KH\textsubscript{2}PO\textsubscript{4} buffer, pH 7, at a scan rate of 100 mV s\textsuperscript{-1}. Current densities were normalized to the electrodes geometric surface area.

**Table 2. Voltammetric Characterization of Cyt c/HS-(CH\textsubscript{2})\textsubscript{10}COOH—HS(CH\textsubscript{2})\textsubscript{6}OH SAM-Modified np-Au Electrodes\textsuperscript{a}**

<table>
<thead>
<tr>
<th>electrode design</th>
<th>(E^{0}) (\text{mV}) \textsuperscript{b}</th>
<th>(\Delta E_p) (\text{mV}) \textsuperscript{c}</th>
<th>fwhm (\text{mV}) \textsuperscript{d}</th>
<th>(k_{et}) (\text{s}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>uniform np-Au</td>
<td>–12 (\pm 2)</td>
<td>18 (\pm 1)</td>
<td>115 (\pm 1)</td>
<td>0.53 (\pm 0.9)</td>
</tr>
<tr>
<td>nonuniform np-Au</td>
<td>–11 (\pm 1)</td>
<td>18 (\pm 1)</td>
<td>114 (\pm 1)</td>
<td>0.50 (\pm 0.9)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Averages and standard deviations represent data from four electrodes.

\textsuperscript{b} Data obtained from scan rates in the range 10–1000 mV s\textsuperscript{-1}.

\textsuperscript{c} \(\Delta E_p = (E_{p,u} - E_{p,a})\).

\textsuperscript{d} \(\Delta E_{p} = f_{np-Au} - f_{planar}\).

\textsuperscript{f} fwhm data obtained at a scan rate of 50 mV s\textsuperscript{-1}.

No changes in the voltammetric response with continuous potential cycling (30 scans) in aqueous buffer (not shown) were observed, demonstrating the stability of the adsorbed protein.

Electrode topography, low \(R_{t}\) planar versus high \(R_{t}\) np-Au surfaces, and heterogeneous mixed SAMs noticeably influence the voltammetry of adsorbed cyt c. Rough electrode surfaces modified with mixed SAMs to generate monolayers with higher defect densities provide the optimal faradic response.\textsuperscript{31,53} The irregularly textured surfaces described here provide such a response (Figure 4), which is in contrast to the poor response obtained at surfaces with minimal defects or smooth surfaces modified with long chain homogeneous carboxylic acid terminated SAMs.\textsuperscript{53} The response (inset Figure 4) of cyt c at planar Au electrode surfaces modified with a heterogeneous mixed SAM displayed more asymmetrical cyclic voltammograms (larger \(\Delta E_p\) of 70 mV), with broader, less defined, anodic peaks in comparison to identical immobilization conditions on np-Au electrodes.

The electron transfer coefficient \((\alpha)\) and heterogeneous rate constant \((k_{et})\) for cyt c at both np-Au electrodes were obtained using Laviron’s method\textsuperscript{38} (Table 2). An irreversible response (peak separation >200 mV/n, where \(n = 1\)) occurred for scan rates greater than 1.25 and 1.75 V s\textsuperscript{-1} for uniform and nonuniform np-Au electrodes, respectively. Values of \(\alpha\) were determined from the slopes of the linear regions of plots of peak
potential versus scan rate (Figure 5B, C), while $k_{\text{cat}}$ was determined for scan rates in the range 1.25–4 V s$^{-1}$ and 1.75–4 V s$^{-1}$, for uniform and nonuniform np-Au electrodes, respectively. A range of rate constants, with average values of $k_{\text{cat}}$ of 2.4 ± 0.9 s$^{-1}$ for uniform np-Au (range of 1.3–3.5 s$^{-1}$) and 3.3 ± 0.9 s$^{-1}$ for nonuniform np-Au (range of 2.1–4.3 s$^{-1}$) was obtained. This distribution of $k_{\text{cat}}$ values likely arises due to the variety of possible orientations cyt $c$ molecules adopt on the negatively charged mixed SAMs as discussed earlier. The rate constants were ca. 2 orders of magnitude less than those achieved at similarly modified planar gold electrodes. The lower rate constants may arise from changes in the composition of the SAM, which have been observed with FcFxSH. Such changes can significantly change the faradaic reponse of the protein.

Surface coverages of electrochemically active cyt $c$ were ~9 and ~11 times that possible at a planar Au surface (assuming full monolayer coverage per geometric surface area) for uniform and nonuniform np-Au electrodes, respectively (Tables S1 and 1). Considering that the sensitivity of affinity-based biosensors is limited by the binding capacity of immobilized receptor molecules, increasing the quantity of immobilized receptor molecules on substrates such as np-Au has the potential to significantly increase the sensitivity of such sensors.

$R_0$ values, particularly at nonuniform np-Au electrodes, were less than those obtained using other surface-confined techniques (Table 1). Whereas immobilization of cyt $c$ can not provide an accurate estimate of $A_{\text{real}}$, it does provide an indication of the accessible area of a particular nanostructure to a protein or large macromolecule, $A_{\text{macro}}$. Values of $A_{\text{macro}}$ were 2.288 (±0.295) and 2.657 (±0.295) cm$^2$, for uniform and nonuniform np-Au electrodes, respectively, compared to $A_{\text{real}}$ values of 3.961 (±0.164) and 6.931 (±0.238) cm$^2$ (from the Au oxide stripping method, Table 1). The more pronounced decrease in $A_{\text{macro}}$ for nonuniform np-Au electrodes reveals that a considerable portion of the finer nanostructure was inaccessible to the bulky cyt $c$ molecule (diameter of 6.6 Å). As discussed above, a control experiment with a “blocking” SAM, showed that the walls of the nanopores could be fully modified and that incomplete surface modification would not account for the reduced surface coverages of electrochemically active cyt $c$. In contrast to other surface-confined methods, cyt $c$ immobilization can be used to provide a more accurate probe of the loading capacities of large molecules on Au nanostructures.

**Comparative Study with np-Au Leaf Modified GC Electrodes.** The np-Au electrodes were mechanically robust and securely attached to the underlying glass support by a Ti/pure Au adhesion layer. This allowed ease of handling/manipulation and, as outlined, facile functionalization of the electrode surface with thiols and large biological molecules. An alternative method of fabricating np-Au electrodes involved using free-standing Au–Ag leaf as a precursor film of np-Au. Attempts were made to modify an np-Au/GC electrode with a mixed thiol SAM in an identical manner as described for cyt $c$ immobilization on sputtered np-Au electrodes. However, thiol modification of the Au was found to disrupt the weak attractive forces securing the np-Au film to the underlying GC electrode, detaching the np-Au film from the GC. Attempts to modify the np-Au film with thiol molecules prior to attachment on GC were not successful, either due to the film breaking in solution or failure of the thiol modified films to attach to GC. Improved attachment of the np-Au film to GC has been reported by dropping a suspension of Nafion on a dry np-Au/GC electrode. This, however, would preclude the

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**Figure 5.** (A) Plots of the anodic and cathodic peak current densities for immobilized cyt $c$ as a function of scan rate at uniform (hollow circles) and nonuniform (solid circles) np-Au electrodes; (B) dependence of the anodic and cathodic peak potentials for adsorbed cyt $c$ on the logarithm of the scan rate at uniform (hollow circles) and nonuniform (solid circles) np-Au electrodes; (C) linear regions of the plots in (B) used to calculate $\alpha$ (peak separation >200 mV/n, with $n = 1$).
Biocathode Development at Planar and np-Au Electrodes. The mediator Os(bpy)$_2$·PVI has previously been screened for use with biocathodes employing MbBOD. Fast electron transfer kinetics and appreciable current densities were achieved. A drop-cast coating consisting of 38.6 wt % MbBOD, 38.6 wt % Os(bpy)$_2$·PVI, and 22.8 wt % PEGDGE was utilized. This enzyme/redox polymer weight ratio of 1:1 was previously optimized for the mediator [Os(4,4'-dichloro-2,2'-bipyridine)$_2$Cl]$_{2/3}^{2+}$ complexed with polyacrylamide—poly(N-vinylimidazole), [(PAA—PVI)–Os(dcl-bpy)$_2$]$_{2/3}^{2+}$, in combination with MbBOD. The catalytic current will be limited by the enzyme-catalyzed rate of O$_2$ reduction if too little enzyme is present or by increased resistance in the film if too much nonconductive enzyme is present. At 22.8 wt % of cross-linker, the films were stable, showing no variation in response after 100 scans in the absence of O$_2$, while enabling appreciable current densities to be achieved in the presence of O$_2$.

Near identical symmetric responses were observed for the Os$_{2+/3+}$ complex at each electrode design in terms of $E_{m}^{o}$, $\Delta E_{p}$, half-peak widths, current density, and total charge transferred (Figure 6). Voltammograms differed only in the magnitudes of the double layer capacitances (C$_{dl}$) observed, with C$_{dl}$ increasing with R$_{q}$ as expected. $E_{m}^{o}$ values of 224 (±2) mV were in agreement with previous results. At 5 mV s$^{-1}$ $\Delta E_{p}$ values of 35 (±5) mV and half-peak widths of 115 (±5) mV were measured, indicating minor deviations from the theoretical values for an ideal Nernstian one-electron transfer reaction, as discussed for cyt c. These deviations may be attributed to repulsive interactions between redox centers or small variations in the environments of redox centers giving rise to a range of redox potentials. Scan rate (ν) studies of the polymer film in the absence of the biocatalyst at nonuniform np-Au electrodes (not shown) revealed thin-film behavior for ν $<$ 20 mV s$^{-1}$ ($i_0$ increasing linearly with ν), followed by diffusion-controlled behavior at ν $>$ 20 mV s$^{-1}$ ($i_0$ increasing linearly with $\nu^{1/2}$). Identical surface coverages of the immobilized osmium complex, calculated from the charge under the anodic redox peaks following comprehensive electrolysis at 5 mV s$^{-1}$, of 44.2 (±2) nmol cm$^{-2}$ were detected at planar and both np-Au electrodes. On the time-scale of the experiment, the diffusion layer thickness (δ) exceeded the film thickness (Φ), as indicated by the linear increase in $i_0$ for scan rates <20 mV s$^{-1}$, at planar and both np-Au electrodes. Increasing the surface roughness of the electrodes did not increase the quantity of addressable Os$^{2+/3+}$ centers in the hydrogel since, at slow scan rates, all of these highly mobile Os$^{2+/3+}$ centers were already fully oxidized and reduced during a potential cycle at a planar Au electrode.

At each electrode design, the onset of the O$_2$ reduction current began at a potential slightly positive of $E_{m}^{o}$, and a limiting peak current was reached at $E_{m}^{oc}$, which decayed gradually to a potential-independent plateau current at potentials $\sim$150 mV more negative than $E_{m}^{oc}$. The catalytic current response at both np-Au electrodes revealed a splitting of the reduction peak, in particular for uniform np-Au (Figure 6B). This may arise from the mediator/enzyme complex inhabiting different environments within the pores; such peak splitting was not observed for laccase and cellobiose dehydrogenase (data not shown). The rate of O$_2$ reduction catalyzed by MbBOD and mediated by Os(bpy)$_2$·PVI in the film was high when compared to the rate of O$_2$ diffusion in the electrolyte, resulting in depletion of O$_2$ at the electrode surface. Thus, concentration polarization, a consequence of not rotating the electrode, was manifested as a peak

Figure 6. Cyclic voltammograms in the absence (dashed line) and presence (solid line) of O$_2$ at (A) nonuniform nanoporous, (B) uniform nanoporous, and (C) planar gold electrodes modified with MbBOD (38.6 wt %), Os(bpy)$_2$·PVI (38.6 wt %), and PEGDGE (22.8 wt %) in 50 mM phosphate buffer saline, at 37°C, and pH 7.4. Scan rate of 5 mV s$^{-1}$.
current on the voltammogram. Near identical peak current densities of 476 (±74), 478 (±29), and 521 (±20) μA cm⁻² were obtained at planar, uniform nanoporous, and nonuniform nanoporous Au electrodes, respectively. As outlined for diffusion-controlled electrode reactions, overlapping diffusion zones between adjacent nanopores establish limiting semi-infinite linear diffusion fields on the time-scale of the experiment and the maximum current density was dependent on Ageo.

The observed current densities in Figure 6 are in agreement with the results obtained for similar biocathodes at GC electrodes which were subject to moderate rotation speeds of 100 rpm.²¹ Heller described a MoBOD cathode “wired” using a [(PAA—PVI)—Os(dcl-bpy)₂]⁺/²⁺/₂⁺ mediator, which was capable of operating with a maximum current density of 700 μA cm⁻² under stagnant physiological conditions at carbon fiber microelectrodes.⁵⁷ Using similarly modified carbon cloth electrodes under physiological conditions, maximum current densities of 5 mA cm⁻² were obtained at 1000 rpm.⁵⁵ These higher current densities arise from (a) the use of microelectrodes and high rotation rates, which increase the rate of O₂ transport to the electrode surface, and (b) the use of different osmium redox polymers, which significantly influence the catalytic current.²¹

Detailed studies examining a range of redox polymers and enzymes for use as anodes and cathodes in biofuel cells are underway. Improvements in stability expected within a sheltered nanoporous environment, currently under investigation for the redox polymers used in this study, may be further augmented by anchoring and tethering redox polymers and enzymes onto suitably pretreated surfaces. Improved stability by anchoring functionalized redox complexes and enzymes to suitably derivatized Au⁴⁸ and carbon⁵⁹ surfaces, using methods easily adaptable for use at np-Au, was demonstrated. The anchoring surface can be designed to contain amino, carboxyl, hydroxyl functional groups, and so forth, so that different preformed redox complexes, enzymes, or polymers can be tethered to the anchoring layer using conventional coupling reactions. The response of biosensors and biofuel cells can be improved under flowing conditions by increasing the rate of mass transport of substrate to the electrode surface. However, employing flowing conditions may increase the rate of desorption, and thus reduce the stability, of surface immobilized enzymes. Securely tethered enzymes within the protected conductive environment of np-Au can overcome this barrier and provide a stable, more sensitive response.

CONCLUSIONS

np-Au electrodes of differing morphology and surface roughness were fabricated and characterized for use in biosensors and biofuel cells. The suitability of a range of electrochemical probes was examined to determine (a) the electrochemically addressable surface area, Areal, and (b) the surface area accessible to modification by a biological macromolecule, Amacro. Measuring the charge required to strip a Au oxide layer and applying a conversion factor of 390 μC cm⁻² was the most accurate and reliable method to determine Areal. Values for Areal ranged from 0.251 (±0.013) cm² [the geometric electrode area, Ageo was 0.246 cm² giving a roughness factor, R₉ of 1.01 (±0.05)] at planar Au, to 3.961 (±0.164) cm² [R₉ of 16.1 (±1.3)] and 6.931 (±0.238) cm² [R₉ of 28.1 (±1.5)] for uniform and nonuniform np-Au electrodes, respectively. In addition, Cdl and UPD of Cu²⁺ were identified as suitable complementary methods to estimate Areal. The degree of steric hindrance a redox protein may experience at a nanostructured electrode was demonstrated using cyt c as a probe. R₉ values of 9.3 (±1.2) and 10.8 (±1.2), which equated to Amacro values of 2.288 (±0.295) and 2.657 (±0.295) cm², for uniform and nonuniform np-Au electrodes, respectively, were considerably less than Areal. The difference between Areal and Amacro was particularly large for nonuniform np-Au electrodes due to the confines of the smaller pores becoming increasingly inaccessible to cyt c. Surface coverages of electrochemically active cyt c were still, however, multiples of that possible at a planar Au surface (assuming full monolayer coverage per geometric surface area). A comparative study with np-Au film modified GC electrodes highlighted the importance of a robust electrode design as thiol modification of np-Au/GC electrodes, a crucial step for DET-based biological devices, caused the np-Au film to detach. The presence of a Ti/pure Au adhesion layer for np-Au electrodes produced via a sputtering method avoided such complications. Initial studies on the use of np-Au as a biocathode, using an osmium redox polymer and MoBOD, demonstrated that the diffusion layer thickness exceeded the film thickness, and diffusion zone overlap occurred between adjacent nanopores at v = 5 mV s⁻¹ yielding equivalent charge transfer and current densities at planar and np-Au electrodes. Splitting of the observed reduction peak indicated that the mediator/enzyme complex occupied different sites on the surface of the electrode. Future work will involve detailed studies of a range of redox polymers and enzymes for use as anodes and cathodes in biofuel cells. The combined advantages of a sheltered environment within a nanopore and novel enzyme/redox polymer immobilization strategies at np-Au will be applied to the development of bioloelectrochemical devices of optimal stability with improved responses under flowing conditions.

ASSOCIATED CONTENT

Supporting Information. (Table S1) Summary of the surface coverages (Γ, pmol cm⁻²), charges (Q, μC), double layer capacitances (Cdl, μF cm⁻²) and current densities (J, mA cm⁻²) determined using surface-confined and diffusion-controlled electrochemical techniques at each electrode design. (Figure S1) Cross-sectional image of a uniform np-Au electrode indicating the presence of the pure gold adhesion layer. This material is available free of charge via the Internet at http://pubs.acs.org.

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Direct electron transfer of bilirubin oxidase (Myrothecium verrucaria) at an unmodified nanoporous gold biocathode

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Abstract

Well defined mediatorless bioelectrocatalytic reduction of oxygen with high current densities of 0.8 mA cm\(^{-2}\) was obtained on nanoporous gold electrodes modified with Myrothecium verrucaria bilirubin oxidase. A stable faradaic response was observed when the enzyme modified electrode was coated with a specifically designed electrodeposition polymer layer. The response of the enzyme electrode was only slightly inhibited by the addition of F\(^{-}\).

1. Introduction

Bilirubin oxidase (BOD) and laccase have been used extensively in the cathodes of biofuel cells. Both enzymes are ‘blue copper’ proteins that reduce oxygen to water. Their redox potentials depend on the source of the enzyme. Values of 190 and 460 mV (vs Ag/AgCl, 3 M KCl at pH 7) were reported for the T2/T3 and T1 sites, respectively, for BOD from Myrothecium verrucaria (MvBOD) [1–3]. MvBOD is a monomeric enzyme with a molecular mass of 66 kDa and an isoelectric point of 4.2 [4]. In comparison to laccase, MvBOD possesses high catalytic activity at neutral pH while utilising relatively low overpotentials for the reduction of O\(_2\). The activities of laccase and BOD are inhibited by Cl\(^{-}\), with the effect on BOD being far less pronounced [5–8].

Direct electron transfer (DET) between immobilised blue multicopper oxidases, including BOD, on modified gold electrodes has been intensively studied [1–2, 9]. However, in comparison with carbon based surfaces, there are few examples of the use of metallic films in the preparation of biocathodes. DET on bare gold electrodes is difficult with unstable bioelectrocatalytic responses for the few systems that have been examined [9]. Modification of the gold electrode surface with thiol monolayers enables orientation of MvBOD on the electrode in a manner which promotes efficient DET. The current density depends on the nature of the terminal groups of the monolayers used to modify the gold electrodes. Carboxyl terminated monolayers enable higher electrocatalytic currents for the reduction of oxygen in comparison to –NH\(_2\), –OH and –CH\(_3\) terminated monolayers [10]. Covalent binding of MvBOD to a gold surface modified with 3-mercaptopropionic acid and stabilised with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) also showed a catalytic response [2]. Improvements in DET on bare gold electrodes can be achieved by changing the morphology of the electrode, with current densities of 0.5 mA/cm\(^2\) reported for MvBOD immobilised on 15 nm diameter gold nanoparticles at pH 7. Further increases in the current density were achieved when a highly porous carbon paper was used as a support for gold nanoparticles. Maximal current densities of ~4.9 mA/cm\(^2\) and power densities of 0.87 mW/cm\(^2\) at 300 mV were obtained for a biofuel cell composed of such a biocathode in combination with a fructose dehydrogenase bioanode [pH 6.0, stirred at 1000 rpm] [10].

Nanoporous gold is an attractive material due to its excellent stability over a wide pH range, high conductivity and biocompatibility [11–12]. Nanoporous gold is mechanically stable and easy to fabricate. It has a high surface-to-volume ratio with tunable pore sizes that allow increased loadings of immobilised proteins. In this study, DET-based bioelectrocatalytic reduction of oxygen by MvBOD immobilised on nanoporous gold electrodes was observed with high current densities of ca. 0.8 mA/cm\(^2\) under aerobic conditions. The inhibitory effect of F\(^{-}\) on MvBOD that is usually observed was substantially decreased for the immobilised enzyme.
2. Experimental section

2.1. Materials and methods

Sulphuric acid, citric acid and disodium phosphate were obtained from J. T. Baker. Sodium fluoride was purchased from Sigma-Aldrich. All chemicals were of analytical grade and used as received without further purification. Buffers were prepared with water (0.055 μS/cm) purified with Ultra Clean UV (SG). Aerobic and anaerobic conditions were established by bubbling oxygen and argon (Air Liquide), respectively through the solutions for 20 min.

Electrochemical measurements were performed with an AutoLab potentiostat (PGSTAT12, Eco Chemie) using a three-electrode cell with Pt wire, Ag/AgCl (3 M KCl) and nanoporous gold as the counter, reference and working electrodes, respectively. The supporting electrolyte consisted of 0.1 M citrate-phosphate buffer solution at pH 7.0. Current densities were estimated using the geometric surface area of the working electrode. All potentials are reported vs. Ag/AgCl (3 M KCl).

2.2. Enzyme

Partially purified preparations of MvBOD were kindly provided by Amano Enzyme Inc. (Nagoya, Japan). The enzyme was purified to homogeneity using a published procedure [13].

2.3. Synthesis of copolymer P017-epoxy

Polymerisation was carried out by reacting polyethylene glycol methacrylate (395 mg, 0.75 mmol), allyl methacrylate (631 mg, 5.0 mmol), and butyl acrylate (545 mg, 4.25 mmol) in methanol. Excess monomer was removed by precipitation from water. The solid copolymer was re-dissolved in MeOH to give the final polymer suspension (P017; 4.7% w/w). The synthesis of dimethyldioxirane (DMDO) and epoxidation of P017 was performed according to a published procedure [14]. The dry polymer obtained was re-dissolved in MeOH (1.0 mL). Water was added drop-wise, until the suspension turned milky, to give the final polymer suspension (P017-epoxy).

2.4. Modification of nanoporous gold electrodes

Nanoporous gold electrodes with uniform distribution of Ag were prepared as described previously [15]. The electrodes were cleaned by scanning (10 scans) from 0 to 1.5 V at 200 mV/s in 0.5 M H2SO4, followed by scanning at 50 mV/s between 0 and 0.8 V in 0.1 M citrate-phosphate buffer at pH 7.0. The electrodes were then placed in a vacuum chamber at 10⁻² mbar for 10 min. 20 μL of a MvBOD solution (40 μg/ml) 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 μL of P017-epoxy solution and allowed to dry in air at room temperature for 15 min. As a control experiment, an electrode was modified by drop-casting 20 μL of BOD solution (40 μg/mL) which was then kept at 4 °C for 2 h prior to use.

3. Results and discussion

Bioelectrocatalytic current densities of ca. 0.04 mA/cm² were obtained at MvBOD modified electrodes prepared by placing a drop of enzyme solution onto the surface of an electrode that had been first dried under vacuum (Fig. 1A). On changing the deposition conditions by first drying the electrodes in vacuum, followed by deposition of the enzyme solution in vacuum, significantly higher current densities of ca. 0.3 mA/cm² (Fig. 1B) were obtained. More importantly, these high bioelectrocatalytic current densities were observed in DET mode for MvBOD immobilised on unmodified, bare gold electrodes. The high catalytic responses observed arise from the high surface areas of the electrodes (roughness factor of 26 (inset Fig. 1B) [15]) combined with the high loadings of enzyme which were achieved by using vacuum to dry the electrode and then adsorb the enzyme. The use of heating to evaporate the solution in the pores...
was avoided in order to prevent further morphological changes in the electrode arising from thermal stresses [16].

On repeated potential scans the response of the electrode was observed to be unstable with a 50% decrease in the current (Fig. 1B). On modifying the electrode with P017-epoxy, current densities of 0.8 mA/cm² were observed which were stable on repeated potential scans (Fig. 1C). This indicates that BOD is relatively loosely bound and easily removed from the surface of the electrode. The role of P017-epoxy is to stabilise the adsorbed MvBOD by creating covalent bonds with nucleophilic groups such as amine, thiol and hydroxyl groups on the surface of the enzyme [17]. Effectively, a cap-like structure was formed on the electrode which prevents leakage of the enzyme (Fig. 2) and results in very stable currents (Fig. 1C).

In an oxygen saturated solution the onset potential for the biocatalytic reduction of oxygen at MvBOD-modified nanoporous gold electrodes was ca. 500 mV (Fig. 1), in good agreement with the redox potential of 460 mV for the T1 site [1–2, 18]. The theoretical limiting current density of ca. 0.6 mA/cm² (calculated from the Randles–Sevcik equation and utilising values of 2.6×10⁻⁵ cm²/s and 420 μM for the diffusion coefficient and concentration of oxygen, respectively [19] and a roughness factor of 2.1 for the reduction of dioxygen [14]), indicates that the observed current density of 0.8 mA/cm² is controlled by diffusion of O₂ from the bulk solution. At high currents, the response was quite noisy over the potential range of 0.35–0.1 V, which may have arisen from the highly porous and uneven nature of the electrode. Variations in the amount of oxygen (as well as the amount of adsorbed enzyme) may result in localised variations in the concentration of oxygen, disrupting the response observed. As expected both electrodes did not demonstrate a catalytic current in argon saturated electrolyte. A nanoporous gold electrode coated with P017-epoxy in the absence of enzyme did not show any oxygen reduction current (data not shown).

It is well established that F⁻ inhibits MvBOD activity by binding to the T2/T3 copper site, blocking the transfer of electrons from the T1 site to the T2/T3 cluster [20–21]. Surprisingly, the addition of F⁻ had little influence on the biocatalytic current of BOD immobilised at nanoporous gold electrodes under aerobic conditions. The maximal loss of activity of a biocathode stabilised with P017-epoxy was ca. 3.5%. Such a low inhibitory effect indicates that the normal deleterious effect of F⁻ on the response was not observed. The significantly reduced inhibitory effect may arise from exclusion of F⁻ from the pores by the polymer and from confinement of the enzyme in the pores of the electrode in a manner which precludes binding of F⁻ to the T2/T3 site.

The high catalytic current density of ca. 0.8 mA/cm² observed on MvBOD modified nanoporous electrodes is in contrast to that at a planar gold electrode where no catalytic response was observed in this and previous reports [1–2, 9]. It is feasible that enzymes within the 3D porous structure are in an environment where they are in intimate contact with the gold walls of the pores and able to undergo direct electron transfer in an efficient manner (Fig. 2). The use of these electrodes as supports for other redox enzymes in the development of biofuel cells is currently under investigation.

4. Conclusions

MvBOD immobilised within the pores of nanoporous gold electrodes can undergo efficient DET without surface modification of the electrode. Significantly higher stability was observed when the BOD-nanoporous gold electrode was coated with a P017-epoxy cap. High bioelectrocatalytic current densities of up to 0.8 mA/cm², with minimal inhibition of the response by F⁻, were obtained.

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