Review

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Recent advances in the electrochemistry and spectroelectrochemistry of membrane proteins

Abstract: Integral membrane proteins are encountered in fundamental natural processes, such as photosynthesis and respiration. The relation between the structure of the proteins and their function and dynamics are still not clear in most cases. Once fully understood, these processes could ultimately help researchers to develop alternative methods for producing energy, either from light or biomass. They could also lead to more efficient antibi-otics, which would selectively inhibit a specific membrane protein of pathogenic bacteria. Since the chemical reactions involved in both photosynthesis and respiration are redox reactions, electrochemical methods can play a considerable role in uncovering their mechanisms. The electrochemical characterization of membrane proteins is, however, quite challenging. An overview on the techniques used for the characterization of membrane proteins, including classical approaches such as voltammetry and spectroelectrochemistry, and recent developments, such as their combination with surface-enhanced techniques is given.

Keywords: bioelectrochemistry; bioenergetics; membrane proteins; spectroelectrochemistry.

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Introduction

Integral membrane proteins account for approximately one-third of the coding capacity of the genes in an average organism. They are critically important for multiple central functions in any living cell. It is estimated that membrane proteins provide 80–90% of the targets relevant for the chemical and pharmaceutical industries. However, due to their hydrophobic and amphiphilic nature, membrane proteins are extremely difficult to study. For instance, they account for <1% of the high-resolution protein structures thus far deposited in the databases. Their overproduction, purification and biochemical and biophysical characterization are far more challenging than for soluble proteins. The interaction of membrane proteins with their ‘solvent’, the membrane lipids, is not well understood. The function of membrane proteins is modulated by specific interactions with lipids, but also by specific interactions with cytosolic proteins, adding a further level of complexity. Some membrane proteins are bound only to the membrane surface, whereas others have one region buried within the membrane and domains on one or both sides of it. Domains within the membrane, particularly those that form channels and pores, transfer molecules and protons or other ions across the membrane. Protein domains on the extracellular membrane surface are generally involved in cell-cell signaling or interactions. Thus, the characterization of membrane proteins and their interaction with biological membranes is a highly challenging and emerging research field.

Dynamic electrochemical techniques have proven to be powerful tools by which to study the thermodynamic and kinetic properties of proteins that are electrochemically connected or ‘wired’ to the electrode surface. As described above, membrane proteins are more difficult to manipulate experimentally than soluble proteins, however, their electrochemical properties are of significant interest due to their rich redox catalytic properties. They can be studied after solubilization or after immobilization on the surface of electrodes. The studies in solution require the use of detergent to disperse the protein in the medium as well as various soluble redox molecules to mediate the electron transfer between the electrode and the cofactors. In such studies, electrochemistry is usually coupled with spectroscopy [ultraviolet (UV)-visible (VIS), infrared, electron paramagnetic response (EPR)] to determine the midpoint potentials and to monitor the changes in the cofactors or in the protein structure upon reduction.
and oxidation. For studies on the electrode surface, the main challenge is to adsorb proteins in their native state onto the electrode while efficiently exchanging electrons.

**Membrane protein film voltammetry**

Protein film voltammetry consists of the direct electrochemical characterization of thin films of redox proteins confined to the surface of electrodes (Armstrong, 1990; Armstrong et al., 1997; Butt and Armstrong, 2008). Most often, immobilized proteins are investigated by cyclic voltammetry, square wave voltammetry or differential pulse voltammetry, which are three dynamic methods based on the measurement of current (or differences of current) upon application of a time-dependent potential and thus providing so-called current-potential curves (Bard and Faulkner, 2001). Alternatively, chronoamperometry, which consists of the measurement of current vs. time at a constant potential after application of a perturbation, can be used. The main idea here is that the electrode is playing the role of the redox partner of the enzyme, allowing a perfect control of the redox states of the cofactors and consequently of the catalytic activity by the application of the potential. This approach offers a number of advantages such as:

- Only a small amount of the sample is needed: given the size of redox proteins, $10^{-12}$–$10^{-11}$ mol/cm$^2$ is usually sufficient to obtain a monolayer on the surface.
- Various conditions of temperature, pH, concentration of substrates or inhibitors can be screened with only one modified electrode.
- Complications originating from protein diffusion are suppressed.
- Thermodynamic and kinetic information on the redox proteins can be simultaneously obtained from the voltammograms.
- Potential scan rates can be finely tuned from 1 mV/s up to 1000 V/s, which allows us to study both slow and fast chemical reactions coupled to electron transfer.

In general, the current-potential curves of immobilized enzymes contain three principal contributions: the inherent catalytic properties of the protein, the kinetics of interfacial electron transfer, and the transport properties of the substrate and products. The limitation relating to the latter contribution can be avoided by using rotating electrodes, which allows a constant flux of substrate to move towards the enzyme. The second contribution critically depends on the nature of the support, as well as on the orientation of the protein on the support. For membrane proteins in particular whose surfaces are comprised of both highly hydrophobic and hydrophilic or charged domains, efficient immobilization is one of the most difficult aspects of the study. If the most accessible cofactor of the enzyme can be brought within a 14 Å distance from the electrode, a direct electron transfer (DET) between the immobilized protein and the electrode can be achieved through this cofactor (Page et al., 1993). This is not always possible with large membrane proteins (Burgess and Hawkridge, 2002), because their cofactors are usually buried inside the protein shell. In this case, some redox mediators or smaller proteins can be added to the electrolyte or co-immobilized to improve communication with the electrode (mediated electron transfer). These small redox mediators are typically natural redox molecules [such as ubiquinone, nicotinamide adenine dinucleotide (NADH), etc.] or synthetic organic compounds with a fully reversible electrochemistry in the potential range of interest (Szentrimay et al., 1977).

Once the support and the transport of substrates is optimized, valuable information about the catalytic mechanisms can be gained from the voltammograms. In simple cases of DET, enzymes exhibit their catalytic activity in cyclic voltammetry by a sigmoidal wave at a potential corresponding to the reduction or oxidation of their cofactors in the absence of substrate but with a significant increase of current, traducing multiple catalytic turnovers. Substrate concentration can be varied to determine the substrate’s affinity for the enzyme, usually measured by the Michaelis-Menten constant, $K_m$. In comparison, catalysis in mediated electron transfer processes occurs close to the redox potential of the mediator, and thus requires larger overpotentials.

**Methods of immobilization**

Membrane proteins or their subcomplexes lacking the membrane-anchored subunits have been immobilized on carbon, metal or semiconductor electrodes. Various types of binding of these proteins to the electrode surface have been considered.

**Films on carbon electrodes**

Some soluble subcomplexes of quinoliumurate reductase (QFR) from *Escherichia coli*, related succinate quinone oxidoreductase (SQR) (Sucheta et al., 1992; Hudson et al., 2005), and mitochondrial NADH quinol oxidoreductase...
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(Zu et al., 2003) have been shown to form quite stable films on bare graphite electrodes.

Films on metal electrodes

The electrochemical behavior of membrane proteins can be accessed through immobilization on metal electrodes. The large enzymes are prone to denaturation when adsorbed on bare metal surface, however, which requires modification of the surface (Burgess and Hawkridge, 2002). The most frequently studied supports are gold and silver, which can be easily modified through self-assembly of thiol derivatives (Bain et al., 1989). Bifunctional thiols are usually used, with various head groups that can direct the enzyme into the best orientation on the electrode, thus minimizing the distance between the active redox site and the electrode (Prime and Whitesides, 1991; Willner and Katz, 2000). In addition to alkanethiols, which mimic the hydrophobic environment of the membrane, thiols bearing amino, hydroxyl, carboxylic acid and aldehyde end-groups have been considered for the immobilization of membrane proteins, such as cytochrome oxidases and photosystems. Thin films of proteins are usually formed on the electrode surface over the course of several hours. A rapid and versatile method has been reported recently in the case of photosystem I (PSI) (Faulkner et al., 2008). It consists in applying vacuum (30 mTorr) after deposition of the protein on the electrode surface and leads to dense monolayers of the protein within 1 h.

Mixed protein/lipid films

Lipid membranes on electrode surfaces can provide a native-like environment for full membrane proteins and improve their electron transfer properties. Different strategies have been reported to modify electrodes with enzymes embedded in lipid membranes, see Figure 1 (Murgida and Hildebrandt, 2005; Jeuken, 2009; Naumann et al., 2011).

The first immobilization of bovine cytochrome c oxidase (cco) within a phosphatidylcholine lipid bilayer freely suspended on a semiconductor indium tin oxide electrode was reported in the early 1990s (Salamon et al., 1993). The reconstitution of the enzyme in the phospholipid membrane was performed by detergent dilution of concentrated solutions of the enzyme. At almost the same time, the same protein was incorporated into a hybrid lipid bilayer assembled on a gold electrode (Cullison et al., 1994). In this study, the gold surface was first modified with a submonolayer of octadecyl mercaptan, and a deoxycholate dialysis procedure was used to add the second layer and incorporate the oxidase. This dialysis is commonly used to reconstitute the oxidase into vesicles (Hinkle et al., 1972) with its cytochrome c binding site preferentially oriented outward. The stability of the membrane was later improved by adding a layer of electrodeposited silver onto the gold surface (Burgess et al., 1998). Sulfur binds more strongly at silver surfaces than gold, and the thiol hydrocarbon tail is believed to adopt a nearly normal orientation on such surfaces.

In the previously described assemblies, the lipid bilayers are held very close to the electrode surface, which might not provide sufficient space and flexibility for incorporating membrane proteins with large extramembranous domains. In addition, it seems important for their activity to maintain an ionic reservoir between the lipid bilayer and the electrode. For these reasons, the tethered bilayer lipid membrane (tBLM) methodology was developed. The tether is usually a bifunctional thiol consisting of a lipophilic end-group and hydrophilic spacer (Jenkins et al., 1999; Naumann et al., 1999; Schiller et al., 2003). After modification of the metallic surface with the tether, the lipid membrane incorporating the enzyme can be built by fusion of preformed phosphatidylcholine liposomes. Several terminal oxidases have been immobilized with
this methodology: bovine heart cco (Naumann et al., 1999) and cytochrome bo, from E. coli (Jeuken et al., 2006, Weiss et al., 2010).

Alternatively, the enzyme can be immobilized on the electrode prior to reconstitution of the lipid bilayer, which allows a better control of its orientation on the surface. This assembly was thus termed the protein-tethered lipid bilayer (ptBLM) and was developed mainly for cco (Ataka et al., 2004; Giess et al., 2004). The enzyme was anchored to a gold electrode by taking advantage of the histidine-tag (His-tag) genetically engineered on its surface to render its isolation easier. This tag can complete the coordination of a nickel – or copper – nitritriacetic acid complex covalently attached to a metal surface. For further reconstitution of the lipid bilayer the detergent needs to be removed, which can be done by in-situ dialysis, or by adding biobeads.

Finally, multiple bilayer films, such as those formed by insoluble surfactants upon drying on electrodes (Rusling, 1998), have also been considered as immobilization matrices. Films of phosphatidylcholine or dimyrstoylphosphatidylcholine have allowed the immobilization of photosynthetic reaction centers (Kong et al., 1998; Munge et al., 2003; Alcantara et al., 2006).

**Mixed protein/nanomaterial films**

For future applications of membrane proteins in solid-state devices, it remains crucial to increase the amount of immobilized proteins and improve their coupling with the electrode. Nanomaterials are now considered very promising electrode supports, thanks to their unique electrical properties, their large specific surface and excellent biocompatibility. New hybrid materials can be designed that combine the unique electro-optical properties of the nanomaterials with the exceptional catalytic activities of the proteins (Katz and Willner, 2004; Baron et al., 2007; Yeh et al., 2012).

Among the nanomaterials known, gold and silver nanoparticles (NPs) are probably the easiest to prepare with various sizes and surface modifiers (Daniel and Astruc, 2003; Grzelczak et al., 2008). Their interaction with proteins has been studied in detail (Mahmoudi et al., 2011). A major breakthrough in the electrochemistry of proteins was achieved with the report of glucose oxidase ‘nanowiring’ onto an electrode covered with a monolayer of gold nanoparticles (Xiao et al., 2003), which clearly demonstrated that the gold NPs, thanks to their small size, can mediate the long-range electron-transfer between the electrode and the enzyme cofactors. Gold NPs have thus started to be examined as supports for membrane proteins in particular photosystems for sensing applications (Maly et al., 2005; Miyachi et al., 2010). In a preliminary study, our group has shown that gold NPs constitute an efficient support for the immobilization of several respiratory membrane proteins fragments from the thermophilic bacterium Thermus thermophilus (Meyer et al., 2011). The immobilization of full terminal oxidases on gold NPs is now possible (Melin et al., 2013).

Another approach deals with carbon nanotubes (Iijima, 1991), which belong to the family of new carbon allotropes, and consist of one or several graphene layers rolled up into concentric cylinders. Their attractiveness as a support for redox proteins comes from their unique physical and electronic properties, including their electrical conductivity (Tans et al., 1997) and electrocatalytic activity (Pumera, 2009). Functionalization of nanotubes and PSI by covalent, hydrogen, or electrostatic bonds and the photoelectrochemical properties of such systems have been reported (Simone et al., 2009; Kaniber et al., 2010). A synthetic complex incorporating lipids, nanotubes and photosynthetic reaction centers has been shown to assemble and disassemble reversibly upon the removal or addition of surfactant (Ham et al., 2010).

**Film characterization**

The characterization of immobilized proteins is usually made by a combination of analytical methods. Atomic force microscopy and scanning electron microscopy give insight into the topography of the films. It can be determined whether or not the film totally covers the substrate, and whether or not a monolayer of proteins is present on the surface. On gold or silver surfaces, the optical thickness of the films can be obtained by surface plasmon resonance. This technique is based on the measurement of reflectivity differences at prism-metal interfaces upon binding of the biomolecules (Salamon et al., 1996, Abdulhalim et al., 2008). The amount of protein on the surface can be estimated by quartz-crystal microbalance, a technique that consists of measuring the change in frequency of a piezoelectric crystal oscillator upon the formation of a film on its surface (Marx, 2003). These latter two techniques can also serve to measure the affinity of a given surface for the protein of interest.

The electrical properties of the membrane protein-electrode interface, i.e., its resistance and capacitance, can be studied using electrochemical impedance spectroscopy (EIS) (Bard and Faulkner, 2001, Lisdat and Schäfer, 2008). In the context of membrane protein film electrochemistry,
EIS serves to control the quality of the supported or tethered lipid bilayers before and after incorporation of the protein. Perfect planar lipid bilayers on electrode surfaces should exhibit capacitance values of about 0.5 μF/cm² and high electric resistance (Lang et al., 1994). Increased capacitance values indicate a reduced thickness of the bilayer, which can be due to some disorder in the lipid chains or some tilting of these chains relative to the normal of the bilayer surface. Incorporation of a protein also increases the capacitance values, as a result of higher dielectric constant and disorder in the bilayer. The lipid bilayers tethered to gold surfaces with a cholesteryl-type tether exhibited almost ideal capacitance values (0.7–0.9 μF/cm²; Jenkins et al., 1999). Interestingly, further incorporation of cytochrome bo inside the bilayer did not significantly change these values, which suggests that the protein does not create too many defects in the structure of the bilayer (Jeuken et al., 2006). In contrast, peptide-tethered lipid bilayers with or without embedded cco exhibited high capacitance values (10–20 μF/cm²) since they contained more defects (Naumann et al., 1999). This could be due to the relative dielectric constant of the peptide linker that limits the flexibility of the whole structure (Naumann et al., 2011). Protein-tethered BLMs on gold surfaces have higher amounts of protein, with only few lipids between them. They are expected to have relatively high capacitance values. In line with this expectation, values of 6–7 μF/cm² have been found for protein-tethered bilayer lipid membranes (BLMs) with cco (Giess et al., 2004). The presence of vesicles on the surface instead of a planar bilayer can also be detected by EIS (Jeuken et al., 2005).

**Influence of the electrode interface on electrocatalytic efficiency**

Importantly, the electrochemical behavior of membrane protein-based electrodes dramatically depends on their environment on the surface. The following representative examples of immobilized membrane proteins highlight this influence.

**Quinol fumarate reductase and succinate quinone oxidoreductase**

QFR is a complex membrane protein that couples the reduction of fumarate to the oxidation of ubiquinol; whereas SQR catalyzes the reverse reaction. These structurally highly-related proteins (Ohnishi et al., 2000) were among the first to be immobilized on surfaces and the corresponding studies have helped to establish protein film voltammetry as a very efficient analytical technique for multicentered enzymes (Butt and Armstrong, 2008). The redox chemistry of fumarate/succinate occurs at the flavin adenine dinucleotide active site, which is well buried inside the soluble domain of the protein. A chain of iron-sulfur clusters (one 2Fe-2S, one 4Fe-4S and one 3Fe-4S) facilitates connection with the surface.

Subcomplexes of both proteins immobilized on carbon electrodes exhibited very good DET rates and interesting catalytic activity upon the application of a potential. The voltammogram of SQR (Figure 2) in the presence of a 1:1 mixture of succinate and fumarate shows that the enzyme catalyzes both fumarate reduction and succinate oxidation at pH lower than 7.7 and in the potential range corresponding to the fumarate/succinate redox potential (Sucheta et al., 1992; Hirst et al., 1996). Comparison of the currents suggests better activity for fumarate reduction. At high negative overpotentials, however, this activity drops significantly, although the electron transfers no longer limiting any longer. The catalytic curve is thus similar to the current-potential characteristic of a ‘tunnel-diode’. This behavior is still not completely understood for this specific protein. It is suggested that reduction of the flavin could slow down the release of succinate, possibly after a conformational change. In related QFR, a similar behavior is observed below pH 7, and seems to be due...
to hyper-reduction of the 3Fe-4S cluster at high negative potentials, which blocks the electron transfer to the active site (Hudson et al., 2005). This is the closest cluster to the protein surface and thus the probable site of entry of the electrons.

The full QFR complex from E. coli also directly exchanges electrons with alkanethiols-modified gold electrodes (Kinneir and Monboquettte, 1993). A mixture of octadecyl and dodecyl mercaptans seemed to be the most suitable support for this enzyme. Indeed, catalytic waves were reported in the presence of fumarate, close to the redox potential of this molecule. The catalytic activity was reversibly altered upon addition of the inhibitor oxaloacetate. Direct electron transfer was also demonstrated for full SQR from Bacillus subtilis immobilized on 6-mercapto-1-hexanol modified gold electrodes, but this time catalysis occurred at larger overpotentials, which suggested that the electron transfer rate between the enzyme and the electrode in this assembly was rather slow (Christenson et al., 2008). SQR was also studied in tethered vesicles on a gold surface, with its soluble domain pointing into the solution (Jeuken et al., 2005). The active site is thus too far from the electrode for DET, but catalytic activity was reported in the presence of quinones as electron mediators. Succinate oxidation was possible in the presence of ubiquinone in the vesicles.

**Terminal oxidases**

Cco has been immobilized on almost all electrode surfaces, and can thus be considered a model protein to in the study of the influence of the immobilization surface on electron transfer characteristics. Achieving satisfying electron transfer rates for the catalytic reduction of oxygen with this particular protein, however, has turned out to be quite challenging.

In the absence of bilayer lipid membranes, bovine cco covalently immobilized on 3-mercapto propionic acid-modified gold electrodes (Li et al., 1996) seemed to exhibit a DET with the cyt-a₃ cofactor, but not on 3-mercaptopropanol-coated gold electrodes (Haas et al., 2001). Cco reconstituted either within a supported lipid bilayer on an ITO electrode (Salamon et al., 1993) or within a hybrid bilayer assembled on a gold electrode (Gullison et al., 1994) also exhibited a DET. The values of the redox potentials suggest a communication with the Cu₄₃ and cyt-a₃ cofactors respectively. It may be suggested that the orientation of the enzymes differs in these mixed protein-lipid films. No reaction with oxygen, however, was reported for any of these modified electrodes. In a phospholipid-tethered BLM on a gold electrode, it was also not possible to observe this reaction in absence of soluble mediators.

In contrast to these experiments, cco from Rhodobacter sphaeroides bearing a His-tag close to the CuA cofactor (subunit II) and immobilized within a ptBLM assembly on a gold electrode exhibited a complex catalytic curve with two peaks (Friedrich et al., 2008). The first one at -0.2 V vs. standard hydrogen electrode (SHE) is believed to correspond to the electrocatalytic reduction of oxygen itself, while the second one at -0.42 V vs. SHE was attributed to the reduction of the protons pumped towards the electrode surface during the multiple catalytic turnovers. Such proton transport also results in a decrease in electrical resistance, which can be measured by EIS (Naumann et al., 1999, Giess et al., 2004). However, catalysis occurred at much lower potentials than expected from the values of the cofactors’ midpoint potentials, which could be due to conformational changes in the protein upon exposure to oxygen (Schach et al., 2010). In comparison, cco with a His-tag on subunit I did not show any direct electrocatalytic behavior in similar ptBLM assemblies, which again emphasizes the importance of protein orientation. We have recently shown that oxidases immobilized on 15 nm gold NPs modified with a mixture of hexane thiol and 6-mercapto-1-hexanol also exhibit a clear electrocatalytic activity in presence of oxygen, see Figure 3 (Melin et al., 2013). Larger NPs mediate the electron transfer less efficiently.

In an alternative approach, cytochrome c, the natural redox partner of the protein can be used as a soluble redox mediator. Such mediated electron transfer electrocatalytic processes were reported for cco physisorbed on 3-mercapto-1-propanol modified gold electrodes (Haas et al., 2001) or for cco with a His-tag on subunit I in ptBLM assemblies (Friedrich et al., 2008). In the case of cbb₃ oxidase, an artificial redox mediator was used:

![Cyclic voltammetry of cytochrome bo₃ immobilized on 15 nm gold nanoparticles in the presence of O₂](image-url)
N,N,N',N'-tetramethyl-p-phenylenediamine (Todorovic et al., 2008).

**Photosystems**

The motivation for the immobilization of photosystems on electrodes is based on interest in the development of an application for future solid-state devices capable of converting light energy into electricity. In the case of PSI, it was established that the orientation of the protein can be controlled by the functional groups present on the electrode surface (Lee et al., 1997). Detergent-stabilized PSI does not bind on hydrophobic alcanethiol self-assembled monolayers (SAMs) (Ko et al., 2004). On hydroxyl-terminated monolayers, 70% of the immobilized proteins are oriented with the primary electron donor (P700 cofactor) close to the electrode. Clear reversible peaks for P700 were observed in the cyclic voltammetry of PSI from spinach leaves adsorbed on 6-mercapto-1-hexanol modified gold electrodes in the absence of light (Ciobanu et al., 2007). The signals of the terminal electron acceptors (the iron-sulfur clusters F_A and F_B) further away from the electrode surface were detected by using the more sensitive square wave voltammetry technique only. An increased current was observed by chronocamperometry in the presence of light and the electron scavenger methyl viologen, which suggests that PSI is still photoactive when adsorbed on these modified gold electrodes. Covalent immobilization taking advantage of the numerous surface lysine residues close to the P700 center (Faulkner et al., 2008) was also successful. In multiple lipid films on pyrolytic graphite electrode (PGE), in contrast, the electrochemical communication with the iron-sulfur clusters F_A and F_B was established more efficiently (Munge et al., 2003). In the presence of ferredoxin, which is the native redox partner of PSI, an electrocatalytic effect was observed. Finally, binding through a polyhistidine tag genetically engineered on a subunit of the protein was also reported for both PSI (Das et al., 2004; Krassen et al., 2009) and PSII (Badura et al., 2006). In these later studies, due to the large distance between the electrode and the protein, soluble electron carriers were required to mediate the electron transfers.

For PSII immobilized within multiple dimyristoylphosphatidylcholine films on a carbon electrode, an interesting pH and scan-rate dependency was reported for the signal of the manganese cluster (Alcantara et al., 2006). In basic conditions, or at fast scan rates, reversible peaks were observed. At neutral pH and low scan rate, the oxidation peak was smaller than the reduction peak. In acidic conditions, only the reduction peak was visible. It was suggested that reduction of the cluster is followed by protonation, while re-oxidation requires prior de-protonation. This is an example of ‘proton-gated’ electron transfer.

**Spectroelectrochemistry**

Spectroelectrochemistry couples electrochemistry and spectroscopic approaches, and thus enables us to follow a specific molecular response as a consequence of a chosen electrode potential. Importantly a redox-dependent marker needs to be available in the spectral range being studied. Coupling to any spectroscopic technique is in principle possible. The electrodes are adapted to the type of experimental cell required for the chosen spectral range, including cuvette type, window material or solvent. Specific parameters such as temperature or detector positioning need to be considered. The different spectral ranges and the respective spectroscopic techniques then provide access to a complete picture of the changes in the molecules being studied. As discussed before, the surface of the metal electrodes needs to be modified to avoid denaturation of the protein during the experiment and redox mediators that are able to penetrate the protein structure are needed to facilitate electron communication between the electrode and the redox site. Here we will introduce studies performed by coupling electrochemistry to UV/Vis, EPR, and vibrational spectroscopies.

**UV/Vis spectroscopy**

The UV/Vis spectra of a protein are often specific for the redox state of a cofactor, e.g., hemes, FeS clusters or flavins. This feature is extremely useful in combination with electrochemistry for identifying compounds that undergo heterogeneous redox conversion. Many soluble redox proteins, such as cytochrome c (Kreishman George et al., 1980; Taniguchi et al., 1980; Rubinson and Mark, 1982; Kwee, 1986; Moss et al., 1990), blue copper proteins (Sailasuta et al., 1979), soluble spinach ferredoxin (Landrum et al., 1977, Crawley and Hawkridge, 1981), myoglobin (Heinemann et al., 1979), and hemoglobin (Song and Dong, 1988) have been studied with UV/Vis spectroelectrochemistry. The technique was then adapted to large membrane proteins, including several proteins from the respiratory chain. One of the first examples was the study of the bc complex from *Rhodobacter capsulatus* (Baymann et al., 1999). Cytochrome bc complexes are transmembrane proteins working as proton pumps in the photosynthesis of green plants, algae,
heliobacteria, and cyanobacteria, and are involved in respiration by eukaryotes and prokaryotes. Several hemes participate in the catalytic mechanism. In order to distinguish the individual contributions, UV/VIS spectroelectrochemical studies have been performed. Spectroelectrochemical results are then usually evaluated by fitting the spectroelectrochemical titration data to the Nernst equation (see black line in Figure 4) (Dutton, 1978).

Figure 5 shows the different spectra of the cytochromes from the $bc_1$ complex as calculated from redox titrations. The data reveal the different spectral properties of each heme during redox, ligation and the spin state. The data were similarly described by other groups for $bc_1$ complexes from other organisms (Ritter et al., 2003; Iwaki et al., 2005). The use of the UV/VIS range for the study of the redox properties of membrane proteins is now well established; recent examples include the study of the PSII and different enzymes from respiration, such as complex II (Christenson et al., 2008; Kato et al., 2009; Nakamura et al., 2011).

Electron paramagnetic resonance

EPR, or electron spin resonance, is an attractive technique for the identification and study of paramagnetic systems such as radicals, radical ions, and certain transition metals. The signal in an EPR experiment relies on the spin possessed by an electron. Essentially there are two different approaches. Either the electrodes are directly introduced into the EPR tube or the redox titration is performed in a separate electrochemical cell and then transferred to the respective tube. The advantage of the first approach is evident: the redox state can be probed much more precisely and there is no perturbation due to transfer into the EPR tube. However, since the experiments are often performed at liquid nitrogen temperature or lower, the second approach allows the full redox titration series to be frozen.

EPR is the method of choice for investigating complex redox reactions that proceed via radical intermediates or the formation of EPR-visible cofactors, and metal-containing redox-active sites of proteins and enzymes. Thus, EPR spectroelectrochemistry is a very powerful tool in the study of the formation of free radicals during the catalytic turnover of redox enzymes (Whittaker, 2003), and in monitoring the redox states of iron-sulfur clusters, and some other metal-containing sites. The technique is often the only possibility for cofactors that are silent in many other spectroscopic measurements. All iron-sulfur centers in the respiratory chain and several quinone binding sites have been successfully studied by EPR. This includes, for example, the quinone radical stabilized during electron transfer in the quinol oxidase from $E. coli$ (Hellwig et al., 2002). In NADH:ubiquinone reductase, all iron-sulfur centers identified by this technique were later confirmed by crystallography (Ohnishi, 1998).

Electrochemically-induced vibrational difference spectroscopy

Experimental approach

The combination of electrochemistry with vibrational spectroscopies [either Raman or Fourier transform infrared spectroscopy (FTIR)] is very successful. The possibility of following the reaction of a protein by a trigger such as light or the redox potential became important for the analysis of reaction mechanisms in enzymes. The so-called infrared difference spectroscopy was essentially pioneered by Rothchild and colleagues (Rothschild et al., 1981; Engelhard et al., 1985; Dollinger et al., 1986). The
technique was reviewed several times (Gerwert, 1999; Vogel and Siebert, 2000, Zscherp and Barth, 2001). Here we will focus on the combination of the technique with electrochemistry.

The success of this approach is based on the possibility of monitoring the vibrational absorption bands of a single -COOH group, or any other residue, in a protein that contains in the order of $10^3$ residues. The difficulties are obviously substantial. In practice, this amounts to being able to monitor the absorption of about $10^5$ optical density (OD) in a background of about 1 OD, which requires very low random noise and drift (one part in $10^6$). Data reported are thus typically obtained by cycling the reaction of interest and averaging a large number of scans and cycles. Several approaches have been exploited to obtain difference FTIR spectra. The goal in all cases is to keep the sample at a constant concentration and path length, while perturbing the state of the sample in a way that is informative.

The analysis is based on the presence of specific vibrational marker modes of reactant, intermediate or product states. The outcome or kinetics of the reaction induced can then be interpreted by inspection of the transitions of these marker modes. Site-specific information is obtained for vibrational marker modes that involve nuclear motions of specific molecular side groups. When vibrational normal modes are involved in nuclear motions of extended parts of the molecules, no direct structural insight is usually obtained. Isotopic substitution reveals the involvement of certain nuclei in the vibrational motions. When comparing the experimentally-observed vibrational mode pattern with predictions from quantum chemical calculations a full correspondence is possible, the three-dimensional structure is derivable. Current quantum chemical calculation routines, such as density functional theory, allow for the estimation of the electronic ground state structure of medium-sized molecules. For excited electronic states, including intermediate and product states, reliable results can be obtained with the \textit{ab initio} complete active space self-consistent field routine, albeit for mid-size molecules not much larger than 20 atoms. New developments in numerical procedures, such as time-dependent density functional theory, may prove beneficial in the estimation of larger molecular structures in excited electronic states.

The discrimination of individual contributions in the spectrum and their unequivocal assignment is thus very difficult when using the induced reaction technique. A number of strategies have been developed over the years to overcome this, including the variation of external parameters such as pH, pressure, temperature, specific and non-specific isotope labeling or, in the case of proteins, site-directed mutagenesis on crucial amino acids.

**Type of cells**

For \textit{in situ} infrared spectroscopic investigations of electrochemical reactions, a number of different techniques were developed. The first thin-layer cells were reported in the 1970s (Heineman et al., 1972; Norris et al., 1976).

It should be noted that implementation for infrared spectroscopy is considerably more complicated when it comes to measurements in aqueous solution, as is usually the case for membrane proteins. A cell was developed that became successful for use in redox-induced FTIR difference spectroscopic studies of biological molecules in aqueous solution in transmission mode, see Figure 6 (Moss et al., 1990). Fourier-transform infrared spectra are obtained on the basis of thin-layer electrochemistry of the protein at a modified gold grid electrode. Here, the modification or functionalization of the electrode surface is often done with the help of thiols that covalently bind to the electrode and thus protect the protein from irreversible adsorption onto the gold surface. This spectroelectrochemical thin layer cell allows fast, accurate and reproducible control of the redox situation of the protein. The resulting reduced minus-oxidized infrared difference spectra show the changes in the frequencies and intensities of molecular vibrations that arise from the redox-linked conformational change and coupled protonation changes. The concept of the transmission cell is not significantly changed for the mid-infrared (MIR) spectral range. In the far infrared (below 600 cm$^{-1}$),

![Figure 6](image-url)
where metal-ligand vibrations can be observed, a cell with silicon or diamond windows was created on the basis of the same cell geometry (El Khoury and Hellwig, 2011; Marboutin et al., 2011). This technique is complementary to metal-ligand studies performed by coupled electrochemical approaches and Raman spectroscopies in this spectral range.

Another possibility when working with samples that require aqueous solutions is to use a set-up based on the reflection of infrared light. Attenuated total reflection FTIR spectroscopy devices have been successfully combined with electrochemistry (Marshall et al., 2006). The measurements are performed in reflection mode; therefore the working electrodes may be of any metal or of glassy carbon. Unfortunately, a large sample volume of 20 μl is needed. A technical solution that enables a significantly lower sample volume to be used was developed based on surface-enhanced approaches (see below), where the sample is directly adsorbed onto the electrode surface (Figure 7). A profound knowledge of the molecular structure and dynamics of immobilized proteins and enzymes that can be obtained by these methods is a prerequisite for the rational design of bioelectronic devices of technological importance. In addition, surface-enhanced vibrational spectroscopies create new possibilities by which complex biomimetic systems can be studied, thereby providing novel insight into fundamental biological processes.

**Surface-enhanced resonance Raman and FTIR difference spectroscopy**

Proteins adsorbed on metal surfaces can also be studied using vibrational spectroscopy, which attracted significant attention after the pioneer work carried out by Fleischman et al. with pyridine molecules on silver surfaces (Fleischmann et al., 1974; McQuillan et al., 1975). It was demonstrated that on such metal surfaces, the Raman scattering is enhanced by at least six orders of magnitude (Albrecht and Creighton, 1977; Jeanmaire and Van Duyne, 1977). Interestingly, the infrared signals of adsorbed molecules are also enhanced, albeit to a lesser extent (Hartstein et al., 1980; Osawa, 2001; Ataka and Heberle, 2006, 2007). The enhancement is believed to originate mainly from a coupling between the light electric field and the surface plasmons of the metal substrate (electromagnetic mechanism; Moskovits, 1985; Campion and Kambhampati, 1998). The adsorption process can also contribute (chemical mechanism). The metal substrate should present some heterogeneity at the nanometer scale. Considerable efforts have thus been devoted to finding efficient supports allowing both a good enhancement of vibrational signals and adsorption of proteins (Banholzer et al., 2008; Lal et al., 2008; Grosserueschkamp et al., 2009; Feng et al., 2008, 2010). In the context of protein-film characterization, this technique offers several advantages. Due to the enhancement, the sensitivity of the method is very high and even low enzyme coverage can be detected. It can easily be coupled to electrochemistry, by using the metal substrate as the working electrode.

Surface-enhanced resonance Raman spectroscopy allows us to exclusively probe the cofactors of the proteins and the direct environment. Water, buffers, and the polypeptide backbone are almost invisible, provided the choice of the laser excitation wavelength is correct. In particular, in hemoproteins several marker bands are known to be very sensitive to the spin, oxidation and ligand state of the heme, and are thus often used to confirm the integrity of the adsorbed proteins. Three porphyrin modes are particularly easy to identify in the spectra upon excitation in the Soret band region, the so-called ν4 band in the 1350–1370 cm⁻¹ region, the ν3 band at about 1500 cm⁻¹, and the ν2 band in the 1550–1600 cm⁻¹ region. A first indication of correct wiring of the protein to the electrode surface can be obtained through the application of a
potential. Spectra taken at sufficiently negative and positive potentials should be similar to spectra obtained in presence of a chemical reductant (dithionite) and oxidant (ferrocyanide), respectively. Such analyses were reported for cco in ptBLM assemblies (Friedrich et al., 2004; Hrabakova et al., 2006; Todorovic et al., 2008) and confirmed that the structure of these proteins is largely preserved after immobilization.

Surface-enhanced FTIR spectroscopy, in contrast, can give additional information on the polypeptide backbone, as well as on amino acid side chains. The attachment of the protein to the electrode surface can be monitored through the amide I band in the 1700 to -1600 cm\(^{-1}\) range and amide II band in the 1600–1500 cm\(^{-1}\) range (Ataka et al., 2004; Badura et al., 2006; Krassen et al., 2009). These bands correspond to the C=O stretching and N-H in-plane bending vibrations, respectively, of the polypeptide chain (Barth, 2007). Within these lines, it was confirmed that the transmembrane helices of the cbb oxidase adopt a near-normal orientation relative to the gold surface in protein-tethered BLM assemblies (Todorovic et al., 2008). Importantly the techniques allow the protein to be reconstituted in phospholipid membranes and thus allow us to study the effect of the membrane on the properties of the proteins. In addition to the interaction of the lipids with the protein’s surface, the creation of the membrane potential may influence the catalytic activity and its regulation (Jiang et al., 2008; Kozuch et al., 2012; Schkolnik et al., 2012).

Examples of electrochemically-induced FTIR difference spectra and approaches for the assignment of signals

Redox-induced FTIR difference spectroscopy was successfully applied for the characterization of the redox-sensitive vibrational modes of several cofactors, such as hemes, flavins and quinones. Together with the redox-dependent transitions, signals from the reorganization of the direct environment upon electron transfer become available. These reorganizations may include protonation state changes or conformational movements. The positive and negative signals in the difference spectra correspond to changes that occur upon oxidation or reduction, respectively. Generally the data include all changes that happen upon the induced reaction. The structural changes are involved in the so-called amide I band that is typically observed between 1700 and 1600 cm\(^{-1}\) and includes the \(\nu(C=O)\) vibration of the polypeptide backbone in a hydrogen bonding-dependent manner. Furthermore, the amide II signal seen from 1580 to 1520 cm\(^{-1}\) may provide information on structural rearrangements. Specific contributions for the rearrangement of individual amino acids may also be observed in the spectra. Cytochrome c and other small cytochromes are well characterized by the electrochemically-induced FTIR difference spectroscopic approach (Moss et al., 1990; Berthomieu et al., 1992; Calvert et al., 1997). The approach was then developed for all major membrane proteins starting with photosynthesis and also studies on enzymes involved in respiration and metabolic pathways.

Indeed, reaction-induced FTIR spectroscopy was first developed for the field of photosynthesis. FTIR spectroscopic and electrochemical studies on the bacterial reaction center have been widely presented. Studies on quinones as well as on the signals specific for the chlorophyll centers have been performed, helping with the identification of the mechanism of electron transfer to the quinones and the role of residues in direct proximity, for example (Nabedryk et al., 1990; Moss et al., 1991; Leonhard and Maentele, 1993; Iwaki et al., 2002; Nabedryk and Breton, 2008). Most infrared spectroscopic approaches published on PSI and II are based on light-induced approaches (Vogel and Siebert, 2000; Breton, 2001; Chu et al., 2001; Kim et al., 2001). The redox active tyrosine in PSI, however, was the focus of several spectroelectrochemical studies (Kim and Barry, 1998; Berthomieu and Hienerwadel, 2005).

Cco was studied in great detail using this technique. Figure 8 shows the oxidized-minus-reduced FTIR difference spectrum of cco from Paracoccus denitrificans (Hellwig et al., 1998). The positive and negative signals correspond to changes that occur upon oxidation or reduction, respectively. The vibrational modes observed include redox-dependent changes of the hemes themselves, of the environment of the electron acceptors/donors, and of any

![Figure 8](https://example.com/figure8.png)
coupled protonation or conformational change. In the amide I range (1680–1620 cm\(^{-1}\)), the differential signals indicate changes in absorption arising from alterations of the C=O groups from the polypeptide backbone. These difference signals can be related to subtle perturbations in the structure after the redox process. In addition, contributions from the formyl group of hemes \(a\) and \(a_1\), the heme propionates and from individual amino acid side chains (Asn, Gln and Arg) have been described in this spectral region. In the spectral region from 1560 to 1520 cm\(^{-1}\) (the amide II range), essentially contributions from aromatic amino acid side chains and heme C=C groups are observed, as well as antisymmetric COO modes caused by protonation/deprotonation or perturbation of COO groups (Asp, Glu and heme propionate modes). At 1748 cm\(^{-1}\), a positive signal, correlating with the oxidation of the enzyme, can be seen. In the spectral region above 1710 cm\(^{-1}\), contributions from the \(\nu(C=O)\) vibrational mode of protonated Asp and Glu side chains are expected exclusively (Vogel and Siebert, 2000; Barth, 2007).

The identification of the contribution of one individual amino acid clearly demonstrates the sensitivity of the reaction-induced FTIR difference spectroscopic technique. Several studies have identified contributions from individual amino acids within redox-active enzymes. The possible functions of individual amino acid side chains as proton acceptors/donors, and thus their participation in the proton pathway, are often investigated together with site-directed mutagenesis that involves biochemical perturbation of the individual amino acids. The molecular processes concomitant with the redox reactions of a wild-type and mutant enzyme can be analyzed by combining protein electrochemistry and FTIR difference spectroscopy. Using these techniques, the protonation state of glutamic acid 278 in the cco from \(P.\) denitrificans was assigned. This residue is the key proton transporter in the structure after the redox process. In addition, comparisons between the as isolated form of the protein and the oxidase, in which all COOH propionate functions were \(^{13}\)C-labeled, the vibrational modes of heme propionates within a heme protein were distinguished for the first time.

Further studies on membrane proteins from the respiratory chain have been carried out, for example analysis of the bc, complex (Baymann et al., 1999; Ritter et al., 2003), and applied to all proteins from the respiratory chain independent from the size of the protein. The most recent approach concerns the NADH:ubiquinone reductase or respiratory complex I, which has about 1 MioDa in mitochondria. Electrochemically-induced FTIR difference spectroscopy was used to provide evidence of the coupling of electron transfer and the conformational reorganization. Evidence was found for the role of the cluster \(N\), and specific residues participating in proton translocation were identified (Hellwig et al., 2000; Flemming et al., 2003; Flemming et al., 2005; Marshall et al., 2006).

**Summary and outlook**

Integral membrane proteins are critically important for multiple central functions in any living cell. Their ability to catalyze chemical reactions with a high degree of specificity and efficiency makes them an interesting target for use in biosensors. Significant extensions of knowledge have been made recent years about the expression and purification of these membrane proteins, providing the possibility of having sufficient material available for analytical applications. Electrochemistry on soluble proteins is a well-established field, with a very diverse and rich history of applications in the field of fundamental research and applied methods.

Here we reviewed the recent developments in electrochemistry for membrane proteins. On the basis of the high degree of importance of these approaches for fundamental research but also for the field of analytical biochemistry and biomedicine, we believe that the techniques presented here will be further developed in the near future and become important routine applications.

Received December 3, 2012; accepted January 8, 2013; previously published online January 28, 2013
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