Fabricating third-generation lactose biosensors using cellobiose dehydrogenase on gold and glassy carbon electrodes

Daoyuan Yang
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Under the guidance of Dr. Roland Ludwig, BOKU, Vienna

1. Introduction

Biosensor

A biosensor is the device made of a biological element in intimate contact with a transducer, which is in turn connected to an electronic indicator. Biosensors can be classified by the method of transduction, and electrochemical biosensors are a subset using electrodes as the transducer. The most common biological elements related to the electrochemical biosensors include enzymes, antibodies, microorganism, plant and animal tissues, etc. (Corcuera, 2015).

Due to the lack of the industrial instrumentation for analysis, accurate analyses of biological materials are expensive and often need to be performed in the external laboratories. Even though the standard and conventional analyses methods are reliable to use, sometimes they are not capable of doing on-line implementation because of the long previous purification process time (Corcuera, 2003). Therefore, even though the biosensor technology was originally designed for medical diagnostics, its research was mostly driven by the lack of specific, low-cost, rapid, sensitive, and easy methods to detect biomolecules. Besides clinic usage, the biosensors also have great potential application value in other areas like agriculture, food, environment, military, veterinary services, etc. (Bahadır and Sezgintürk, 2015). In food and agricultural areas, biosensors can be a rapid analysis method for food composition, spoilage, pathogen contamination, pesticide, fertilizer residues, packaging, etc. (Biji et al., 2015; Corcuera, 2015; Narsaiah et al., 2012). It is desirable that biosensors can be as reliable as the conventional instruments for the reason of its specificity (Barthelmebs et al., 2010).

Biosensors were first introduced in 1960s, and since then it became a popular research topic till today. As for the commercial biosensors, the first one was a glucose biosensor by Yellow Springs Instruments back in 1973. By 2015, it was estimated that the global biosensor market reached approximately 12 billion dollars (Bahadır and Sezgintürk, 2015). Despite the many potential application areas mentioned before, the majority biosensor market is still restricted to the medical care for people with diabetes, obesity, cancer, etc. Biosensors for diabetes patients detecting glucose and for people doing pregnancy tests are the most commonly commercialized ones (Bahadır and Sezgintürk, 2015). Therefore, even though there are a huge number of publications each year, the efficiency of transforming the technology to the real products on markets is very low.

One reason is the high price of the biosensor system. Some expensive systems are used in research facilities, while the market usually requires a much lower cost. Some biosensor systems require thousands of dollars and each sensor transducer assembly can cost up to 80 dollars (Goode et al., 2014). Another important reason is the low stability. The limited biological components lifetime made it very difficult to be applied to the on-line analyses. Frequent maintenance will increase the expense, so an unstable biosensor also increases the cost. Due to the two reasons, the most successful and commonly used commercial biosensors today are the low-cost disposal biosensors to measure blood glucose for diabetes patients (Halalipour et al., 2016). In food industry, similar to other areas, only a few commercial devices are available and they are primarily to determine the glucose and lactic acid (Barthelmebs et al., 2010).

Three generations of glucose biosensors

Ever since the first enzyme-based glucose oxidase biosensor and an oxygen electrode were introduced, researches have been done to improve the system of monitoring glycemic levels. To summarize, there are in total three different stages, corresponding to three different generations of biosensors.

First generation glucose biosensors measured the glucose concentration by monitoring the oxygen consumption by a Clark-type oxygen or hydrogen peroxide liberation with a Pt electrode polarized at +0.6 V vs. Ag|AgCl. Oxygen was the electron acceptor for that generation biosensors. The turning point from first to second generation of biosensors was the introduction of low molecular weight artificial redox mediators. These mediators were able to make the enzyme reaction at a much lower overvoltage by facilitating the electron transfer between the electrode and the enzyme active site. Therefore, the electron acceptor for the second generation biosensors became the electron mediators (redox dyes or artificial electron acceptors). This new electron acceptor was monitored colorimetrically or electrochemically; and apart from oxygen, it avoided the interference from other redox species. In the later research, the redox enzyme and the mediator were bound together through flexible polymeric backbones. In this way, the substrates and products can diffuse freely in and out. Thanks to this, the third-generation biosensors had direct electron transfer (DET), avoiding the toxic artificial electron mediators and lowering the errors when there were other interferences to the oxygen in the samples (Ferri et al., 2011; Stoica et al., 2006).

DET is believed to occur at considerable rates only over distances shorter than 20 Å between prosthetic groups and electrode surfaces and is reported for only about 50 redox enzymes out of 3500. For the application of redox enzymes in biosensors and biofuel cells, an efficient DET is certainly a great feature to achieve leading to a simple electrode setup and avoiding expensive or harmful mediators (Ortiz et al., 2012).

The rate of DET between redox enzymes and an electrode is dependent on many features: 1. The location of the redox enzyme center; 2. The orientation of the enzyme on the surface of the electrode; 3. The denaturation degree of the enzyme when contacting with the electrode surface; 4. Whether the enzyme is glycosylated or not. If so, the rate of the electron transfer will show an exponential decrease due to the extra insulating shell forms by sugar and also the increased distance the electrons need to pass (Stoica et al., 2005). Based on Marcus theory, the features are: "i) the distance between the redox site and the electrode surface; ii) the reorganization energy, which reflects the structural rigidity of the redox site in its oxidized and reduced forms; iii) the thermodynamic driving force of the ET, which is related to a proper synchronization between the redox potential of the protein and the polarization of the electrode surface" (Kovacs et al., 2012).

Lactose biosensors

Food products and their raw materials require extensive control in order to keep consumers safe, by protecting them against the sickness or death coming from food pathogens or harmful components.

Milk products are major constituents of our daily diet, and lactose is the main carbohydrate present in milk at a concentration of 4-5%. Lactose is a disaccharide formed from one galactose and one glucose molecule bonded through a β-1→4 glyosidic linkage. Determining lactose is a routine monitoring step in mild and dairy industry for quality control and also for safety. Lactose is a basic evaluating indicator for milk quality and also a detecting indicator for abnormal milk (coming from cows with mastitis). Determining the lactose concentration is also important for controlling the fermenting process for those fermented dairy products (Gürsoy et al., 2014). Another important impact of lactose to human is that a large population is lactose intolerant and suffering digestive disorders because of the deficiency of enzyme β-galactosidase. The principle is as followed. Lactose should be hydrolyzed into monosaccharides by β-galactosidase, and the monosaccharides will be bound to the mucosal membrane of the small intestine and then absorbed

by it. However, those people who is lactose intolerant have low or absent intestinal lactase activity and cannot metabolize the lactose into galactose and glucose. Therefore, the non-absorbed lactose will pass into the colon, causing water influx, diarrhea, and be fermented by intestinal microorganisms to cause cramping and flatulence (Gürsoy et al., 2014). So measuring lactose is essential to both for the consumers and food producers in dairy industry.

There are many methods being used to determine the lactose content, including titrimetry, gavimetric analysis, polarimetry, spectrophotometry, chromatography (gas, liquid and high-pressure liquid chromatography), and infrared spectroscopy (Stoica et al., 2006). But most of the methods are expensive, time consuming or labor intensive. The gravimetric method is a conventional method and quite tedious while infrared and polarimetric methods are not specific to lactose. Although HPLC methods are accurate, these are expensive and require sophisticated instruments and training workers. It is required by the need of the industry to find something can be used routinely possible. The enzymatic methods are the most preferred ones since they are highly specific and relatively cheap. The cost is reduced since the enzyme can be recovered and used several times before disposing (Jasti et al., 2014). Therefore, among the different methods, biosensor is cheap, fast, reliable, and can be operated by unskilled personnel, which makes it having great potential to be an alternative to determine lactose routinely in industry.

Amperometric biosensors determining the lactose concentration were built by various researchers using different principles. Some were summarized as followed: (i) a cascade of three enzymes using β-galactosidase (GAL), glucose oxidase (GOx) and horseradish peroxidase (HRP) and based on electrochemical monitoring of 5-aminosali- cylic acid used as mediator for HRP; (ii) a bi-enzyme lactose biosensor consisting of co-immobilized GAL and GOx and based on measuring the production of H_2O_2 using a Pt electrode; (iii) a glucose noninterference lactose sensor using co-immobilized GAL and galactose oxidase (GalOD) with or without HRP, and with ferrocene as efficient mediator for HRP; or (iv) using oligosaccharide dehydrogenase (Stoica et al., 2006).

Cellobiose dehydrogenase

As mentioned above, the third-generation biosensor approach uses enzymes that are capable of establishing direct electron transfer (DET) between their cofactor and the electrode surface. This transfer doesn't need any mediating substances. This kind of biosensor is more applicable with nanostructured electrode surface designs (Wang, 2005). A limited number of enzymes are capable of directly transferring electrons without any electrode modifications. This is due to the fact that for the majority of enzymes the active site is deeply buried within the protein structure in combination with the lack of a built-in electron transfer pathway connecting the active site with the surface of the protein (Matsumura et al., 2012). But with the enzymes having the feature, they allow the application of low polarization potentials close to the midpoint potential of the enzyme's cofactor (Felice et al., 2013).

Therefore, multi-domain enzymes are drawing more attention than before due to its feature that may support DET. These enzymes include flavocytochromes, quinohemoproteins, and multicopper oxidases, etc. The multi-domain often means they have both a catalytic domain and a redox domain (electron transfer pathway). This is of great interest since the redox domain can act as a built-in mediator like a bridge connecting the active site of the enzyme, which is the catalytic domain in this case, and the electrode (Matsumura et al., 2012).

Cellobiose dehydrogenase (CDH) is among these enzymes and is a hot research topic for decades. CDH is a monomeric oxidoreductase consisting two domains, flavodehydrogenase

domain (DH_{CDH}) and cytochrome domain (CYT_{CDH}). Molecular masses are usually within 90 to 100 kDa and the mass percentage of its glycosylation is around 10%. They are secreted by wood degrading fungi phyla Ascomycota and Basidiomycota. These fungi can be white or brown, can be phytopathogenic or composting (Tasca et al., 2011). The CDH enzyme family is a heterogenous group of proteins with protein sequences (749 to 816 amino acids) and sequence identities as low as 35% (Tasca et al., 2011). Depending on the origin the biochemical properties, CDH can vary in size (usually between 80 and 100 kDA), isoelectric point (usually below pH 5) substrate spectrum and pH optimum (Cipri et al., 2016). CDH also has an important function in wood degradation, due to the reasons that CDH is widely existed in fungi and it constitutes a considerable fraction of the lignocellulolytic enzymes secreted by these fungi (0.5–12%). Actually transcriptomic and proteomic analyses were applied to study the composition of lignocellulolytic enzyme cocktails and the up- and downregulation of single constituents recently. The results implied that CDH genes are up-regulated during growth under cellulolytic conditions from samples ascomycete fungus Neurospora crassa and on the white-rot basidiomycete model organism Phanerochaete chrysosporium (Ludwig et al., 2013).

CDH can be classified into three different groups or branches. Class I CDHs are formed by Basidiomycete CDH sequences (from the Atheliales, Corticiales and Polyporales). Class II is mainly from one sequence of ascomycete origin (Sordariales, Xylariales and Hypocreales). But class II has two sub-classes: class IIA and class IIB CDHs (Ludwig et al., 2013). As for class III, it is also suggested to be from the sequenced genomens, but it is still unknown the exact secretion source for it (Schulz et al., 2016). But it may be from the members of Eurotiales, Helotiales and Pleosporales since encoding sequences of CDH from a separate phylogenetic branch were found in these sequenced genomes (Ludwig et al., 2013). Concerning of the molecular architecture of these CDHs classes, there are some minor difference between class I and class II. There is a carbohydrate binding site on the DH_{CDH} in class I; while it is a small C-terminal family 1 carbohydrate binding module (CBM1) in class IIA CDHs which is lacked in class IIB CDHs (Ludwig et al., 2013). Comparing class I and II in a different point of view, class I has a shorter sequence but is with a highly conserved linker region; while class II has a less conserved linker region. As for the catalytic properties, class I CDHs (basidiomycete to be more specific) is able to discriminate glucose very well and the internal electron transfer is only efficient at $pH < 5.5$ with optima between 3.5 to 4.0. On the other hand, class II CDHs has little discrimination to glucose, broader substrate spectrum, and its internal electron transfer pH can be neutral or alkaline. Therefore, this class II CDHs feature can further be explained as the adaptation behavior of fungi to the natural habitat and a polymer degradation mechanism (Tasca et al., 2011).

CDH plays an important part in the degradation process of cellulose from wood. Its natural substrate is cellobiose, which is a decomposition product from cellulose. CDH oxidizes cellobiose and reduces lytic polysaccharide monooxygenases (LPMOs), which in its reduced form supports the decomposition of cellulose (Cipri et al., 2016). But even though all CDHs prefer cellodextrines and cellobiose as substrates, they also convert lactose and some class II CDHs show activity for glucose as well (Cipri et al., 2016). Therefore, CDHs are good for making biosensors and bioelectronics tongues to detect lactose and glucose.

CDH is in the extrinsic group of the redox enzymes, and this can be concluded from the following statements cited from Guo and Hill: “With the former the catalytic reaction between an enzyme and its substrates takes place within a highly localized assembly of redox-active sites. There need be no electron-transfer pathways from these sites to the surface of the enzyme, where, it is presumed, it would interact with an electrode. For such intrinsic redox enzymes, electrode

reactions may require i) that the sites of the catalytic reaction be close to the protein surface, ii) that the enzyme can deform without loss of activity, iii) that the electrode surface projects into the enzyme, or iv) that electron transfer pathways be introduced by modification of the enzyme. With extrinsic redox enzymes, there is usually another protein involved in transporting electrons and therefore an electron-transfer pathway exists within the enzyme connecting the active sites to an area on the surface where the ancillary protein binds. If this area could be disposed toward an electrode, it would be possible for the enzyme electrochemistry to be obtained. (Guo et al., 1991)"

As mentioned above, CDH is a monomeric protein having two domains, the larger flavodehydrogenase domain (catalytically active) belonging to the glucose-methanol-choline (GMC) oxidoreductase superfamily and the smaller cytochrome domain containing haem b as a cofactor. These two domains have different structures and inherent properties. They are connected together by a "bridge", which is a polypeptide linker region (about 20 amino acids). The flavodehydrogenase domain is linked to the N-terminal of the cytochrome domain. This linker between both domains can be cleaved by proteases present in the culture supernatant (and in vitro by papain), with a still catalytically active DH_{CDH}. Thanks to the feature of multiple domains, CDH is capable of having efficient direct electron transfer between the active site and an electrode surface. And during the direct electron transfer, the haem b is the electron transfer protein between flavodehydrogenase domain and a terminal electron acceptor (Ludwig et al., 2013). The reoxidation of CDH can occur at the reduced flavodehydrogenase domain by transferring the reduction equicvalents to a two-electron acceptor or, the electrons can be sequentially shuttled from the reduced flavodehydrogenase domain to the haem b cofactor, then followed by the reduction of two one-electron acceptors (Ludwig et al., 2013). The overall reaction can be briefly summarized as below: "The reaction catalyzed by CDHs starts at the DH_{CDH} domain, which oxidizes cellobiose, cello-oligosaccharides, and some other carbohydrates (e.g., lactose) to their corresponding δ-lactones. The two electrons stored in the reduced FAD can be transferred onto small one- or two-electron acceptors, e.g., ferricyanide or quinones. Alternatively, the electrons can be transferred sequentially from the reduced FAD in the DHCDH to the heme b in the CYT_{CDH} by an intramolecular electron-transfer step and further on to a terminal electron acceptor like cytochrome c. (Ortiz et al., 2012)"

The flavodehydrogenase domain is peanut shaped and has the average molecular mass around 60 kDa without glycosylation, contributing to about 10% of the total mass. The isoelectric point of the flavodehydrogenase domain is often around 5, very low but varies for different enzymes. The non-covalently bound FAD cofactor in flavodehydrogenase domain is the actual component catalyzing the oxidation of carbohydrates (Ludwig et al., 2013).

The cytochrome domain is formed by two ellipsoidal antiparallel β-sheets and its molecular mass is about 22 kDa without glycosylation. The isoelectric point of the cytochrome domain is also very low around 3. The haem cofactor in cytochrome domain is coordinated by Met 65 and His 163. Haem b in cytochrome domain results in a low redox potential (100-160 mV vs. NHE at pH 7.0) (Ludwig et al., 2013).

DET is the most important feature for CDH when it is used in biosensor detection. But it is also important to know that CDH is also applicable for MET. When using the MET, electrons can be transferred to the electrode from either the flavodehydrogenase domain or the heme domain. For DET, it is necessary to have the heme domain, while as for MET, it is working either with the intact enzyme or just with the separated flavodehydrogenase domain (Tasca et al., 2008).

The main application of CDH is for detections, as biosensor, electronic tongues, etc. It has been used to fabricate biosensors to detect lactose and glucose, catecholamines, and also pollutants

such as phenolic compounds. The newly discovered CDH from the ascomycete Corynascus thermophilus (CtCDH) showed great potential in making the third-generation mediator-free glucose biosensors. This CtCDH can oxidase glucose at neutral pH and exhibits most efficient inter-domain electron transfer. CtCDH was researched to be absorbed on the surface of bare carbon electrodes and also the oxidatively shortened single-wall carbon nanotubes modified carbon electrodes. The analytical response by using the oxidatively shortened single-wall carbon nanotubes modified carbon electrodes was one-third higher than the one used with bare carbon electrode (Zafar et al., 2012).

Besides the detection, biofuel cell anodes involving of CDH also showed very promising application when using lactose and glucose as fuels. The advantage of CDH used in biosensors and in electronic tongues is, there is high catalytic current but also low electric potential, which is preferred to minimize the oxidation of any interfering compounds and to increase the cell voltage for the biofuel cells. Apart from these, CDH is also a promising catalyst for the precious chemical production like aldonic acids (Schulz et al., 2016).

Electrode requirements for DET

For the purpose of having a DET, the electrodes also need to be treated besides the usage of proper multi-domain enzymes. In particular, the high electron transfer rates require the correct orientation of the active sites and vicinity to the electrode surface (Tasca et al., 2011). If CDH is immobilized in a good position, an electrode can also be a terminal electron acceptor. DET between cytochrome domain and the electrodes has a slower rate than the internal electron transfer, so it is the rate-limiting step of CDH in solution (Ortiz et al., 2012).

In order to optimize the DET currents from CDH to electrodes, there are two directions mainly being researched. One direction is to use biochemical method to modify enzymes or the reaction cascade to increase the current of CDH-based electrodes. The other is to find new electrode materials, nanostructures, or to modify the electrode surfaces to increase the effective surface area for CDH binding; or to adjust to the proper orientation of the enzyme on the surface (Ludwig et al., 2013).

Novel nanostructures are being developed to increase the current density of bioelectrodes, based on the fact that a higher loading of the redox enzyme and a higher probability of correct orientation for DET can be obtained. The current nanostructure electrodes being researched include single-walled carbon nanotubes, gold nanoparticles, carbon nanotube-modified screen-printed electrodes, etc. (Ludwig et al., 2013).

Among all of the nanomaterials, metal nanoparticles (MNPs) is very important to modify electrodes, mainly due to their high surface area-to-volume ratios and high surface energy. This feature facilitates the immobilization of several kinds of proteins, and hence producing an electron conducting pathway between the prosthetic groups of enzymes and the surface of the electrodes. Using MNPs also makes the enzyme based electrode more stable. This can be interpreted as less enzyme is leached from the electrode due to the strong adsorption of proteins onto the uncoated nanometer-sized colloidal MNPs. This strong adsorption helps protein to retain its biological activity. MNPs is often deposited onto the electrode surface by drop-casting, covalent linkage, or electrodeposition directly from the metal precursor (Bollella et al., 2017).

As mentioned above, electrodes modified with nanomaterials have efficient DET reactions of redox enzymes due to the large surface area for protein attaching. And this is because the large surface area-to-volume ratios and the intrinsic biocompatibility. It was reported that the conventional electrodes (glassy carbon, graphite, screen-printed electrodes) modified with carbon

nanomaterials did have more efficient DET reactions because of the efficient CDH adsorption on carbon-based nanomaterials (Matsumura et al., 2012).

But for Au electrode, it has to be modified to be bounded with the CDHs. In other words, CDHs cannot be linked or bound to an unmodified Au electrode surface. Therefore, the modified gold nanoparticles (AuNP) is often used. But the shortcoming is the modified gold nanoparticles have very low current density (Matsumura et al., 2012).

Besides using the nanomaterials, the electrodes are also often modified with different chemicals, such as thiol. The most commonly used electrode modifiers for DET are self-assembled monolayers (SAM) of functionalized thiols on Au electrode. These SAM can facilitate the ordered immobilization of proteins. But now the trend is more likely using functionalized aryl diazonium salts to replace the SAM, since diazonium is more stable and has higher possibility to be used on both metal and carbon surfaces. Therefore, the diazonium can be applied to both Au and glassy carbon electrodes (Tasca et al., 2011).

Lactose biosensor using DET

Lactose biosensors based on cellobiose dehydrogenase are third-generation biosenosrs as CDH is capable of doing DET which is a result of its two domain structures. Lactose is oxidized at the FAD containing dehydrogenase domain (DH_{CDH}) and electrons are sequentially transferred to the heme b containing cytochrome domain (CYT_{CDH}). CYT_{CDH} then works as a built-in mediator and delivers the electrons to suitable electrodes like Au or glassy carbon electrodes (Tasca et al., 2013).

Research need

For DET, the initial problem of lacking communication between redox enzymes and the supportive electrode still remains. So DET is difficult to produce a high current density, which is needed for both sensitivity and power output needs of biosensors and biofuel cells. Some commonly used method to improve the situation includes: (i) co-immobilization of redox enzymes with a mediated electron-transfer method. The mediator can help as an electron-transferring bridge between the electrode and the enzyme. (ii) increase the electrode surface area using nanostructure designed to increase the enzyme contact area with the electrode. This may also increase the enzyme percentage oriented for DET. (iii) modifying the electrode surface to have a better enzyme orientation or attachment. (iv) protein engineering to the enzyme to improve the catalytic properties and orientation on the electrode. This may also minimize the distance between the redox-active cofactor of the enzyme and the electrode surface (Ortiz et al., 2012).

Though all four directions are being researched, it is of great interest for us to know how exactly the orientation of CDH placed on the electrodes can influence the response or DET rate from the enzyme to the electrodes. Therefore, we hypothesized that the orientation of CDH placed on the electrodes can change the DET rate from the enzyme to the electrode. And by achieving this, CDH was marked and only activated at different points by a postdoc in the lab, Su Ma. This ensured the CDH can only be bound to the electrode at certain orientation.

2. Objective

To prove out hypothesis, the objective of this study is to fabricate biosensors by binding labeled CDH to Au or glassy carbon electrodes. Comparing the responses got from different biosensors to find out the best orientation of CDH should be attached on the electrodes to have the best DET rate.

3. Experimental tests

3.1. Test the procedure by entrapping enzyme on the electrode with a membrane

Aim

i). Get familiar with the experiment protocol;

ii). Confirm the enzyme is still active and find any improvement to the protocol.

Materials and methods

Electrodes cleaning and polishing

Gold electrodes (diameter 1 mm) were prepared (cleaned and polished) and used for this test. They were dipped into a freshly prepared piranha solution (H_2SO_4:H_2O_2 = 3:1) for 10 min first. Then they were rinsed thoroughly and carefully with HQ-water. Cyclic voltammetry tests were conducted for all electrodes chemically cleaned in 0.1 NaOH with scan rate 100 mV/s in the voltage range of -0.205 to 1.205 V. This was another cleaning step in an electrochemical way. Then all electrodes were rinsed thoroughly and carefully with HQ-water.

After cleaning, the gold electrodes were polished with abrasive papers and alumina. For newly made electrode or electrodes haven't been used for a long time, the polishing should go through three polishing pads; while for the electrodes regularly used, the polishing just needs to go through the fine abrasive pads. First of all, electrodes were polished on a MQ pure water wetted abrasive paper (p1200/2500) until planar surfaces were observed. Then they were rinsed thoroughly and carefully with HQ-water. Secondly, the electrodes were polished on microcloth diamond pads (Buehler, Lake Bluff, IL, USA) in a diamond polishing suspension (1 μm, Buehler) for 5 to 10 min until planar surfaces were observed. Then they were rinsed thoroughly and carefully with methanol first, then with HQ-water. Finally, the electrodes were polished on microcloth alumina pad (Buehler, Lake Bluff, IL, USA) in a Masterprep polishing suspension (0.05 μm, Buehler) for 5 to 10 min until very find and planar surfaces were observed. Then the electrodes were rinsed thoroughly and carefully with HQ-water. After polishing, all electrodes were ultra-sonicated in HQ-water for 10 min to get rid of any alumina or other contaminant.

The electrodes were electrochemically cleaned again after the polishing and ultrasonic treatment. Cyclic voltammetry was conducted in 0.5 M H_2SO_4 for 20 cycles with a scan rate of 200 mV/s in the range of -0.205 to 1.745 V. Then they were thoroughly and carefully rinsed with HQ-water. If not to be used immediately, the electrodes should be stored in 0.5 M H_2SO_4. If the electrodes were used for making biosensors immediately, then they should be left in HQ-water.

Electrode modification

Firstly, diazonium salt was reduced electrochemically on the gold electrode. 8 mg diazonium salt and 165 mg tetrabutylammonium tetrafluoroborate (TBATFB) were dissolved in 6.5 ml acetonitrile to make the solution. The cyclic voltammetry was conducted for 10 cycles with a scan rate of 50 mV/s in the voltage range of 0 to -0.8 V. The solution needed to be made fresh every time and the color was light yellow. Then the electrodes were rinsed with ethanol and HQ-water. Then they were dipped into 2 M HCl and stirred for 45 min to get rid of the Boc-protecting group. The electrodes were cleaned again by rinsing with ethanol and left dry.

Secondly, the spacer was coupled to the electrodes with diazonium salt on them. 10% 3C spacer solution was prepared fresh every time. 7.6 mg Boc-β-alanine was mixed with 36.72 μl butyric acid, 180 μl N-(3-Dimethylaminopropyl)-N-ethyl-carbodimide hydrochloride (EDC) and 69 mg N-Hydroxysuccinimide (NHS) in 10 ml DMF. The electrodes were dipped into the solution and covered with petrifilm to prevent evaporation for 16 h. The electrodes were then cleaned with ethanol and HQ-water. Then the electrodes were cleaned again using HCl by dipping them into

the 2 M HCl solution and stirring for 45 min to get rid of the Boc-protecting group. The electrodes were then cleaned with HQ-water and ethanol and left dry.

Finally, the maleimide was coupled onto the electrodes. 8.45 mg maleimide was dissolved into the mixture of 60 mM NHS and 0.1 M EDC in DMF. The solution had to be made fresh every time and the NHS and EDC mixture should be made first. The electrodes were dipped into the solution and covered with petrifilm to prevent evaporation for 16 h. The electrodes were then cleaned with ethanol and HQ-water. All the electrodes in the above steps can be left in HQ-water temperately.

Enzyme immobilization

CDH enzyme Mu7 was diluted 1:1 with 50 mM Bis-Tris/ 30 mM $CaCl_2$ pH 7.4 buffer. In this case, the enzymes were not truly immobilized onto the electrode by were trapped by the membrane. A small amount of the diluted enzyme was transferred by a pipette to the center of a holder on the electrode. Then the enzyme was covered by a membrane and locked position with a rubber ring.

Tests

5 ml 50 mM Bis-Tris/ 30 mM $CaCl_2$ pH 7.4 buffer was transferred to the reacting vial. Ar was flushed into the solution for 15 min for mixing and conditioning before each cyclic voltammetry (CV). The CV was conducted for 2 cycles with the scan rate of 5 mV/s in the voltage range of 0 to -0.2 V. Buffer alone and buffer with 10 mM lactose were tested for 4 different electrodes.

Results and discussion

Differences between solutions with substrate (lactose) and without substrate (buffer only) can be distinguished from the CV plots easily. This indicated that the electrode modification protocol was applicable. But it didn't give any information about how well the enzyme can be immobilized onto the modified electrodes.

3.2. Fabricate biosensors using the same protocol

Aim

i). To confirm the immobilization step of the protocol worked;

ii). To check whether it was the spacer that made the response too low to be detected.

Materials and method

Electrodes cleaning and polishing

Gold electrodes were still used for this test. The method for cleaning and polishing was the same as described in session 3.1.

Electrode modification

Electrochemical diazonium salt reduction onto the electrodes were the same as described in session 3.1. The maleimide coupling was also the same as described in session 3.1.

As for the spacer, a new spacer thioglycerol was also used to compare the results with the spacer Boc-β-alanine. The detailed methods were described in session 3.1.

Enzyme immobilization

Enzyme was thawed fast under the warm water and diluted with pH 7 phosphate buffer (1:5). The diluted enzyme was then transferred onto the modified electrode surface. The whole surface area had to be covered with the enzyme solution. The electrodes were then covered with a small centrifuge tube to prevent any evaporation. The enzyme contact time was controlled between 30 min to 1 h. The electrodes were rinsed with the Bis-Tris buffer before CV tests.

Tests

Both DET and MET were tested. MET was conducted by CV for 4 cycles with scan rate 2 mV/s in the voltage range of 0.1 to 0.45 V, while DET was conducted by CV for 2 cycles with scan rate 1 mV/s in the voltage range of -0.3 to 0.15 V. 6 ml buffer was transferred into the vial instead of 5 ml. For MET, the tested solutions were 50 mM Bis-Tris/ 30 mM $CaCl_2$ buffer (pH 7.4) + 100 µl potassium ferricyanide, 50 mM Bis-Tris/ 30 mM $CaCl_2$ buffer (pH 5.5) + 100 µl potassium ferricyanide, 50 mM Bis-Tris/ 30 mM $CaCl_2$ buffer (pH 7.4) + 100 µl potassium ferricyanide + 10 mM lactose, 50 mM Bis-Tris/ 30 mM $CaCl_2$ buffer (pH 5.5) + 100 µl potassium ferricyanide + 10 mM lactose. For DET, the tested solutions were 50 mM Bis-Tris/ 30 mM $CaCl_2$ buffer (pH 7.4), 50 mM Bis-Tris/ 30 mM $CaCl_2$ buffer (pH 5.5), 50 mM Bis-Tris/ 30 mM $CaCl_2$ buffer (pH 7.4) + 10 mM lactose, and 50 mM Bis-Tris/ 30 mM $CaCl_2$ buffer (pH 5.5) + 10 mM lactose. The enzymes used in this test included Mu3 and Mu5.

Results and discussion

This tests included 7 treatments: no spacer, with spacer thioglycerol, with spacer Boc-β-alanine, enzyme Mu3, enzyme Mu5, buffer pH 5.5, buffer pH 7.4. And all electrodes were tested both DET and MET. The reason having this design is that too much spacer, or even the existence of the spacer can compete with the enzyme for the limited electrode surface areas. Whether having the spacer or not, the type of spacer, and also the concentration of the spacer solutions may all contribute to the failure of enzyme immobilization. Two enzyme mutations were also tested in case the enzyme couldn't attach due to the specific orientation requirement for the enzyme. As for pH, the optimal pH for lactose is around 6. Therefore, trying buffer with pH 5.5 was expected to have a better result than pH 7.4. Finishing all these combinations took around 1.5 months.

However, unfortunately, all experiments gave us bad results, showing no difference with and without the substrate added. A high concentrated lactose was also used to make sure there were enough substrate to give a strong signal when the enzyme was immobilized. But nothing worked, indicating no matter having the spacer or not, no matter the pH is 7.4 or 5.5, no matter what enzyme mutation used, the enzyme just couldn't be immobilized onto the surface of the gold electrode. Therefore, we moved to the glassy carbon electrode to give another shot.

3.3. Test with glassy carbon electrode using the same protocol

Aim

i). To check if the previous failures are due to the electrode material.

Materials and methods

Electrodes cleaning and polishing

For all glassy carbon electrodes, the electrodes were only cleaned physically which was polishing. No chemical cleaning was operated and the polishing protocol was the same as described in session 3.1. All polishing pads and polishing solutions were changed to the newly purchased ones.

Electrode modification

The electrode modification was mostly the same as described in session 3.1. The electrodes were stored in acetonitrile before and after the diazonium reduction. The spacer was Boc-β-alanine in this test.

Enzyme immobilization

Enzyme Mu3 was used and diluted with pH 7 phosphate buffer 5:1. And the time the enzyme stayed on the surface of each electrode was controlled to be at least 1 h.

Tests

Both DET and MET were tested with CV. The CV settings were the same as described in session 3.2. Argon was purged into the reacting vial for at least 20 min for DET test, while for at least 5 min for MET. The buffer was changed to the 50 mM acetic acid buffer pH 5.

Results and discussion

The results were the same as the gold electrode: no difference between the buffer with and without lactose, no matter it was MET or DET. It implied that the enzyme couldn't be immobilized to the glassy carbon electrodes with the old protocol, either. Therefore, it indicated that this problem was not a simple electrode material problem. Another protocol had to be developed in order to have better results.

3.4. Test with glassy carbon electrodes with new protocol

Aim

i). To test whether the newly developed protocol would work;

ii). To test whether the more concentrated enzyme can make any difference.

Materials and methods

Electrodes cleaning and polishing

The electrodes were only physically polished as described in session 3.1.

Electrode modification

A solution containing 2 mM N-Boc-1,6-hexanediamine, 18 mM N-(2-aminoethyl)-acetamide and 0.1 M TBATFB in acetonitrile was prepared and purged with Ar for 20 min in the electrochemical reacting vial. The covalent immobilization of the amines onto the glassy carbon electrodes was performed by chronoamperometry holding the electrode potential at 2 V vs. SCE for 180 s. The electrode was then washed with acetonitrile and the Boc-protecting group was removed in 4 M HCl in dioxane under gently stirring for 45 min. For the coupling of 6C-spacer, a solution containing 10 mM N-Boc-6-aminohexanoic acid, 60 mM NHS, and 0.1 M EDC in DMF was prepared and stirred for 15 min. Electrodes were immersed for 16 h in the solution, and then washed with acetonitrile and water and then dried. The Boc-protecting group was removed from the 6C-spacer in 4 M HCl in dioxane under gently stirring for 45 min. For the coupling of maleimide, a solution containing 25 mM N-maleoyl-β-alanine, 60 mM NHS, and 0.1 M EDC in DMF was prepared and stirred for 15 min. Electrodes were immersed in the solution for 16 h, then being washed with acetonitrile and water and then dried.

Enzyme immobilization

Enzyme was centrifuge filtered to be made more concentrated. The enzyme was recovered from -30^{o}C fridge and then thawed with 25^{o}C water and sit in ice. The liquid enzyme was transferred into the filtration tube and centrifuged at 4^{o}C (15 rcf) to concentrate it. Enzyme Mu7 was used for this test. 5 μl enzyme was mixed with 1 μl pH 7 phosphate buffer right before each test. The diluted enzyme was transferred onto the electrode surface for 1 h before running the CV test.

Tests

Both DET and MET were tested. MET was conducted by CV for 4 cycles with scan rate 2 mV/s in the voltage range of 0.1 to 0.45 V, while DET was conducted by CV for 2 cycles with scan rate 1 mV/s in the voltage range of -0.3 to 0.15 V. 6 ml Acetic acid buffer (pH 5) was used this time.

Results and discussion

The new protocol failed to give a good result. There was still no enzyme immobilized on the electrode to give any signals. Therefore, it was clear by now that the experiment failure was

not related to polishing (used new pads and solutions, polishing long time, CVs were scanned for every electrode right after polishing), buffer pH (pH 7.4, pH 5.5, and pH 5 were all tried), enzyme concentration (the enzyme was so concentrated by then). And probably it was also not a problem of the electrode materials. Therefore, it was worth checking step by step to see from which step it started to go wrong.

3.5. Step by step checking

Aim

i). Use substitute to test every step to find out the exact place going wrong;

ii). Compare the two protocols for the first electrode modification step in order to avoid doing the chronoamperometry.

Materials and methods

i). During the last test, it was not very confident when performing the chronoamperometry, especially when it didn't give any feedback like the cyclic voltammetry. But the good thing is there is one way to test whether the amino group was successfully attached to the electrode. The CV could give a typical plot when amino-modified electrodes react with the anthraquinone-2-carboxylic acid solution.

Therefore, 10 electrodes were cleaned and polished for this test. 5 electrodes were used the diazonium as described in session 3.1; the other 5 electrodes were used the chronoamperometry methods described in session 3.4. A solution containing 25 mM anthraquinone-2-carboxylic acid, 60 mM NHS, and 0.1 M EDC in DMF was prepared and stirred for 15 min. The electrodes were dipped into the solution with gently stirring for 16 h. After washing the electrodes with acetonitrile and water, the electrodes were tested using CV in 0.1 M phosphate buffer (pH 7) for 5 cycles with the scan rate 100 mV/s in the voltage range from -0.25 to -0.65 V.

ii). Since the spacer did the similar modification to the electrodes, whether the spacer was successfully attached on the electrode can also be checked partially using the method above. The shortcoming is the CV plot will show a typical plot even when the spacer is not attached but with a good diazonium layer. However, the spacer check step is not of vital importance since the enzyme can still be immobilized on the electrode without the spacer theoretically.

As for testing the maleimide-modified surface, the thiols can be a good substitute for enzyme to attach to the surface. The reaction between the nitrothiophenol and the maleimide is spontaneous and very fast. The electrodes to be tested are only needed to put into the nitrothiophenol solution for 30-60 min to finish the reaction. The CV plot in buffer or in acidic solutions can be the reference of a good maleimide-modified surface.

10 electrodes (GC1 to 10) were divided into two groups. GC1 to GC5 were modified the first step (diazonium) using the protocol described in session 3.1. GC6 to GC10 were modified the first step using the protocol described in session 3.4. All 10 electrodes were immersed into the spacer solutions for 16 h. GC 1, 2, 3, 6, 8, 9 were washed with 4 M HCl to remove the Boc-protecting group. GC 1, 2, 8, 9 were immersed into the maleimide solution overnight. GC1 and GC8 were continued doing the enzyme immobilization; while GC2 and GC9 were tested with nitrothiophenol. GC 3, 4, 5, 6, 10, 11 were tested with anthraquinone-2-carboxylic acid solution. These tests were repeated twice but the nitrothiophenol tests were not finished the second time.

Results and discussion

All electrodes showed the typical CV plot after reacting with anthraquinone-2-carboxylic acid overnight. Therefore, it implied that both protocols did well in the first step to modify the

electrode with amino. However, after adding the anthraquinone-2-carboxylic acid solution, the electrodes all showed the typical plot (shown in appendix) no matter they were washed by HCl or not. This indicated that either the reaction itself could remove the Boc-protecting group or the Boc-protecting group was off during the normal procedure before the HCl washing. All electrodes with the spacers also showed the plot with anthraquinone-2-carboxylic acid solution. This implied that at least the first step modification was successful, but not sure about the spacer.

DET and MET tests for GC1 and GC8 still didn't have the lactose peak. The nitrothiophenol tests failed the first time and were not finished the second time. Therefore, it is not certain that the maleimide was linked successfully or not. And now there are two steps that may resulted in the failure of the enzyme immobilization: the maleimide linkage and the enzyme immobilization.

4. Conclusions

The experiment results failed to prove our hypothesis. Actually, we encountered big difficulty even trying to immobilize the enzyme onto the electrode. Both protocols we used and both gold and glassy carbon electrodes we used had already been employed by other labs. Some of those labs are still using them and are having good results. I picked some results (mainly plots directly from the software) attached below in the appendix.

After trying to "debug" the protocol and refining the operations, I am certain that our operation was exact the same as the protocols derived from the published papers and also directly from other labs in Europe. The possible failure reasons are now narrowed down to the last step of electrode modification and the electrode immobilization. However, there must be some difference or details I was missing. The known differences include: i). Though we all use disk electrodes, the ones in the Austrian lab has a much smaller surface area than the other labs. This difference itself can make the response much smaller and make the immobilization process much more difficult; ii). The CDH enzyme we used was not the pure enzyme. For the purpose of testing our hypothesis, we modified the enzyme itself to control its immobilization direction to the electrode. This modification to the enzyme CDH may result in the enzyme incapable or more difficult to attach to the cross-linker.

Therefore, in my opinion, there are some experiments worth trying if the project needs to continue: i). Run the last test again and finish testing the maleimide modification. By doing this, we can either narrow down the problem to the enzyme immobilization step, or to the maleimide linking step; ii). Try either of the two protocols with either gold or glassy carbon electrodes, but with the native CDH enzyme instead of the modified ones. If the native enzyme works, then the whole experiment I did in Austria meant nothing. If the native enzyme doesn't work, then the focus can go back to the test i) to figure out how to solve the problem.

5. Acknowledgement

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6. Appendix

Here are some figures from this research.

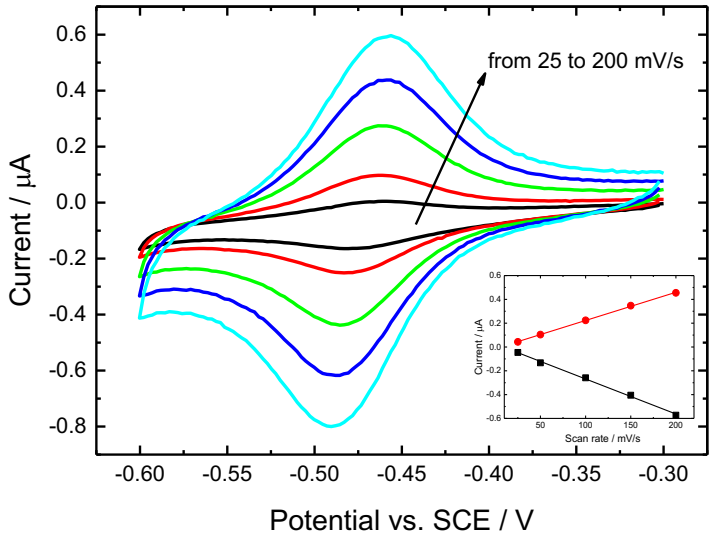

Figure 1. Typical cyclic voltammograms at AQ-modified gold electrode in 0.1 M phosphate buffer (pH 7). The electrode potential was swept from -0.3 to -0.6 V vs. SCE (5 cycles) at different scan rates: 25, 50, 100, 150 and 200 mV/s. The inset shows a plot of the peak currents as a function of scan rate. This plot was used as a reference to test the amino-modification using anthraquinone-2-carboxylic acid solution.

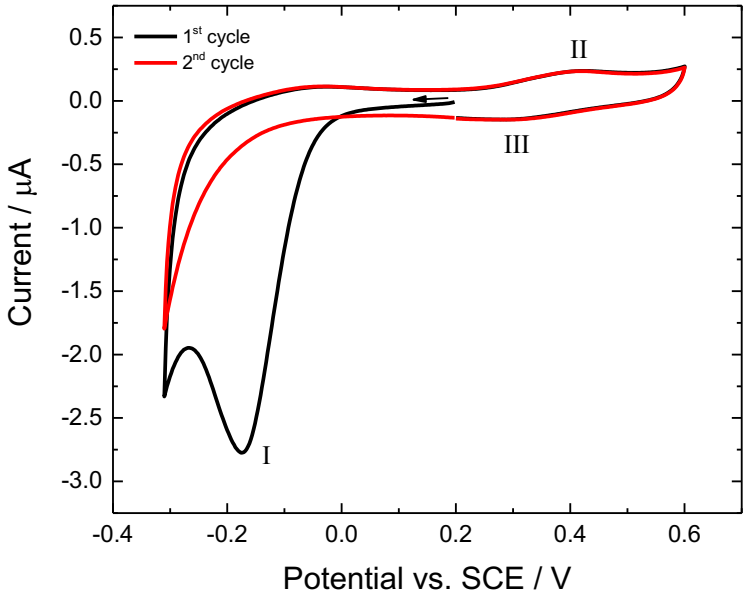

Figure 2. Typical cyclic voltammograms at NTP-modified gold electrode in 0.5 M sulfuric acid. The electrode potential was swept from 0.2 to -0.3 V, and then to 0.6 vs. SCE at 50 mV/s. This plot was used as a reference to test the maleimide modification using nitrothiophenol.

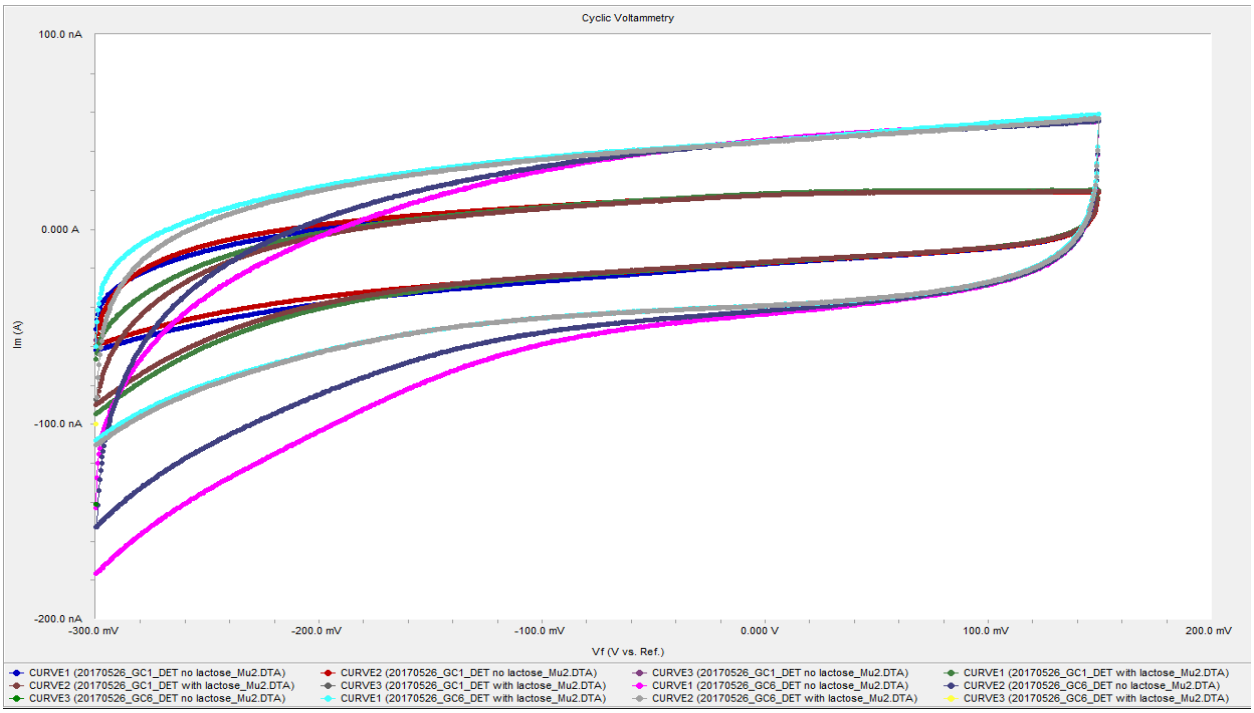

Figure 3. Examples of the DET results that couldn't tell the difference between buffer with lactose and without lactose. These data were from the last set of experiment described in session 3.5.

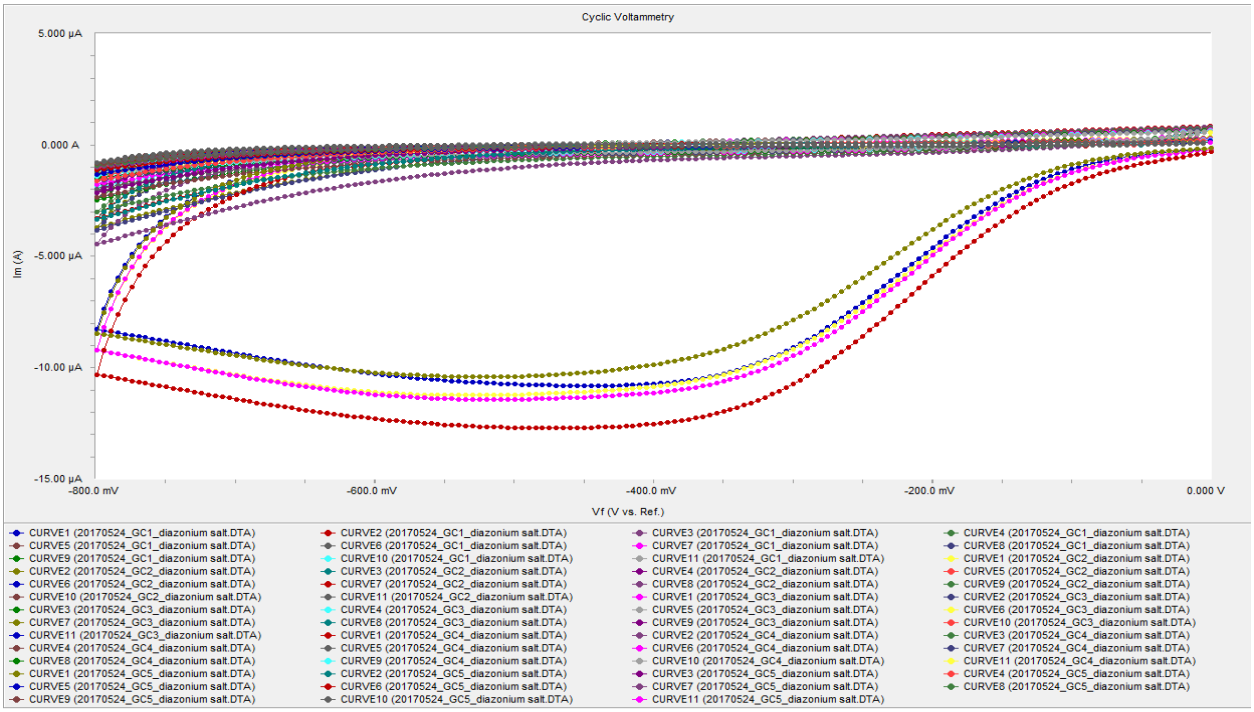

Figure 4. Example of the diazonium salt reduction on the electrode. These data were also from the last experiment described in session 3.5, but they clearly showed the reaction process.

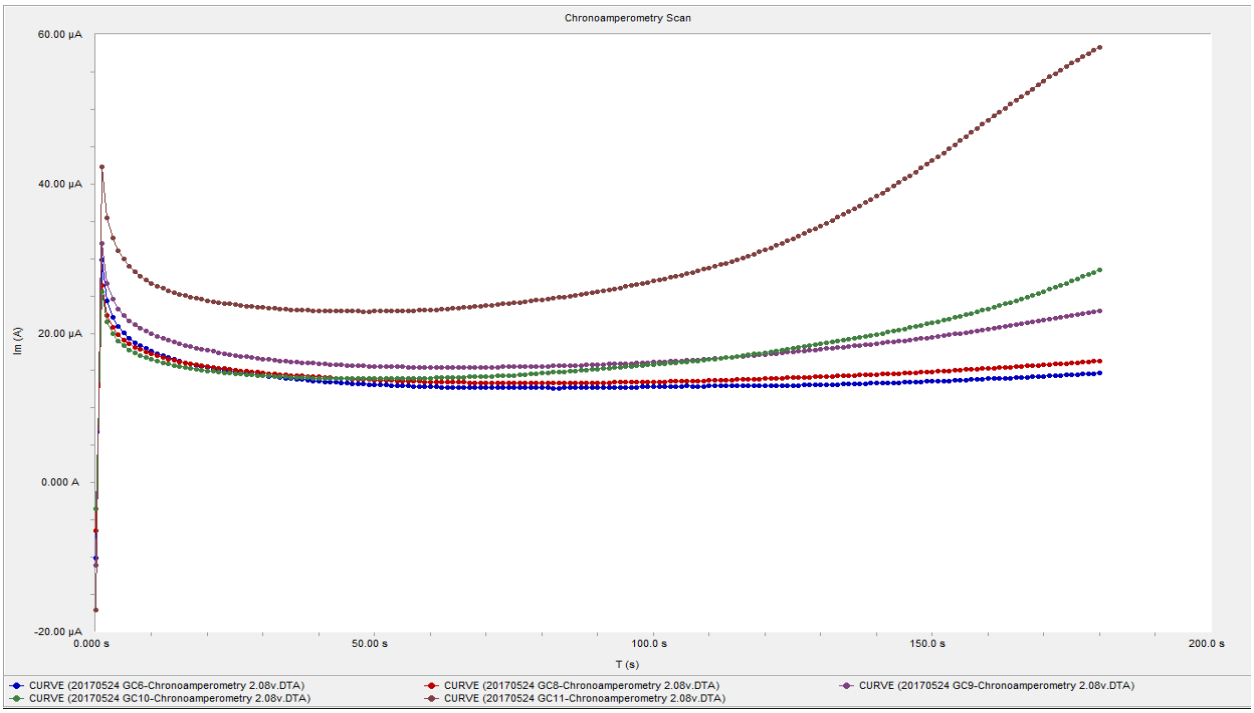

Figure 5. Example of the chronoamperometry. Compared with this, figure 4 gave a feedback with more direct information of how the process went

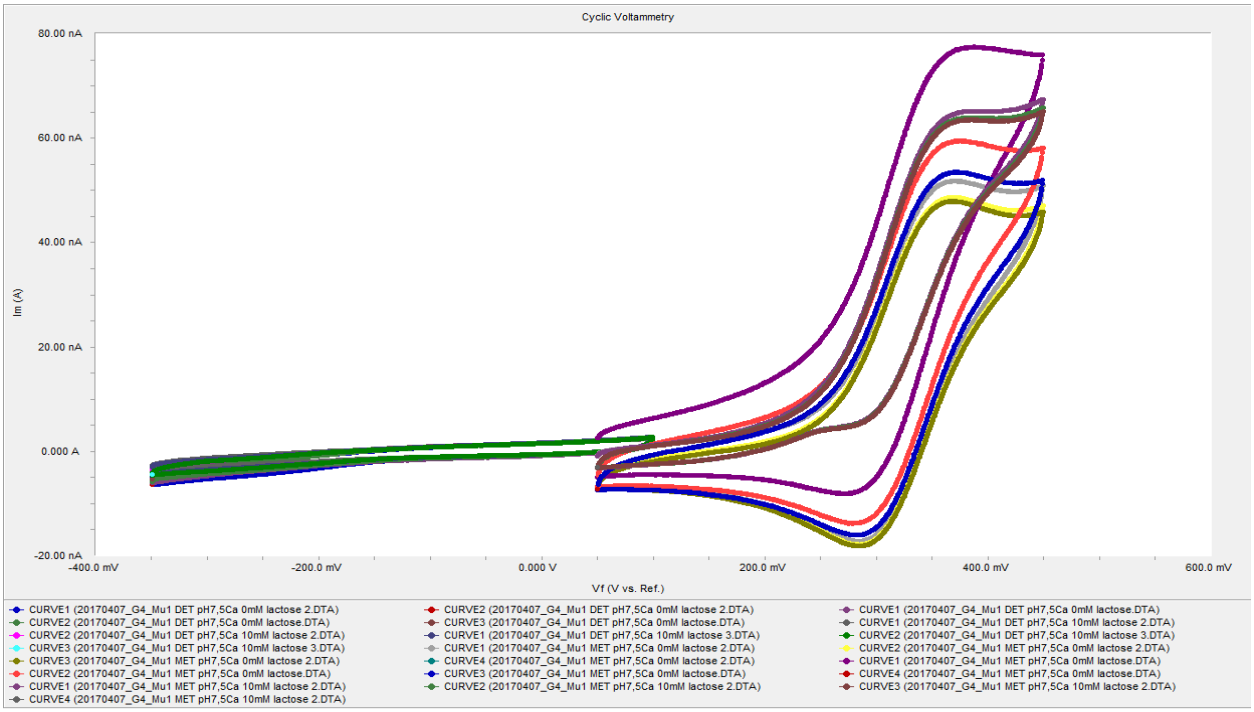

Figure 6. Example of the results putting DET (left) and MET (right) together.

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