Electrochemical Hybridization Assay for DNA and Asymmetric PCR Products Detection by Labeling with Osmium Tetroxide

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Xiaoqi Ni

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Gutachter:

Prof. Dr. Gerd-Uwe Flechsig
Institut für Chemie, Universität Rostock (bis August 2013)
Dept. of Chemistry, University at Albany, SUNY

Prof. Dr. Peter Langer
Institut für Chemie, Universität Rostock

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Abstract

Electrochemical Hybridization Assay for DNA and Asymmetric PCR Products Detection by Labeling with Osmium Tetroxide

Xiaoqi Ni

Institut für Chemie der Universität Rostock

Dr. -Lorenz-Weg 1

18051 Rostock

Deutschland

This dissertation describes the hybridization assay on gold electrodes for electrochemical detection of DNA and polymerase chain reaction (PCR) products by means of the electrochemically active compound osmium tetroxide bipyridine. Single base mismatches are introduced to the capture probe section, or the reporter strand section or both, can be discriminated for their different thermal behavior. For the first time, our approach of protective strand has been applied to produce osmium tetroxide-labeled reporter strands. There was no strand displacement involved in the hybridization between target and capture probe at the gold electrode surface. Rather, the strand displacement of the protective strands by the target took place in the homogeneous hybridization solution. We further optimized protective-strand design, surface-probe density, and hybridization temperature so that a sensitive, fast electrochemical DNA sequence detection assay, which does not require high hybridization temperatures, could be attained. Furthermore, we also investigated how to obtain osmium tetroxide complex-labeled single stranded DNA targets from asymmetric PCR after labeling forward primer with osmium tetroxide compound for electrochemical detection. The forward primer, hybridized with protective strands and labeled with osmium tetroxide bipyridine, was used in a 10-fold excess used for the asymmetric PCR. The two-mismatch protective strand yielded 10 times larger differential-pulse voltammetric (DPV) response than fully complementary protective strand. Coupling this with different thermal behavior is promising for PCR and DNA detection assay.

Keywords: Sandwich hybridization; osmium tetroxide bipyridine; gold electrode; single base mismatch; hybridization temperature; asymmetric PCR

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List of Abbreviations

°C	Degree centigrade
µA	Microampere
µL	Micro liter
µg/L	Microgram per Liter
µM	Micromol per liter
A	Ampere
AC	Alternating Current
AdSV	Adsorptive stripping voltammetric
AdTS	Adsorptive transfer stripping
AE	Auxiliary Electrode
Au	Gold
AuNPs	Gold nanoparticles
bp	Base pair
CR	Current range
CV	Cyclic Voltammetry
DBT	Dynabeads oligo(dT)25
dc	Direct current
DIG	Digoxigenin
DME	Dropping mercury electrode
DNA	Deoxyribonucleic Acid
dNTPs	deoxyribonucleoside triphosphates
DPV	Differential Pulse Voltammetry
dsDNA	double stranded DNA
E	Potential

EDTA	Ethylenediaminetetraacetic acid
EE	End potential
Ep	Peak potential
F	Faraday constant
GPES	General Purpose Electrochemical System
h	hour
HMDE	Hanging Mercury Drop Electrode
HRP	Horseradish peroxidase
I	Current
i_0	Current at the beginning of the scanning
ip	Peak current
M	Mol per liter
M-1	Per mol
mA	Miliampere
MB	Methylene blue
MBs	Magnetic beads
MCH	Mercaptohexanol
mM	Milimol per liter
MM	Mismatches
MON810	Type of genetically modified maize
MWCO	Molecular weight cut-off
MΩ	Mega Ohms
nA	Nanomol per liter
NHE	Normal Hydrogen Electrode
pA	Picoampere
PCR	Polymerase Chain Reaction

RE	Reference Electrode
RNA	Ribonucleic Acid
RP	Reporter probe
RT-PCR	Reverse transcription
SAM	Self Assembled Monolayer
SE	Start potential
SG	Specific gravity
SNPs	Single nucleotide polymorphisms
ss-DNA	Single Stranded DNA
SWV	Square Wave Voltammetry
T	Temperature
TAE	Buffer solution containing a mixture of Tris base, acetic acid and EDTA
tRNA	Transfer ribonucleic acid
TEA	Triethanolamine
WE	Working Electrode

1. Introduction and Objective

Deoxyribonucleic Acid (DNA) as carrier of genetic information is an interesting biomolecule and the nucleic acid hybridization has become an important approach in analysis and detection of specific DNA sequences, and the sequence-specific detection of DNA was reported at 1993 by S. R. Mikkelsen [1]. After that, lots of new DNA-testing technologies have emerged. Among them, the DNA biosensor, which converts the Watson-Crick base pair recognition event into a readable analytical signal, has been developed dramatically. A basic DNA biosensor is always designed by the immobilization of a single stranded (ss) oligonucleotide (probe) on a transducer surface to recognize its complementary (target) DNA sequence via hybridization. The analytical signal converted by the transducer can be obtained after the DNA duplex formed on the electrode surface [2,3,4]. The electrochemical DNA hybridization assay is a novel biosensor that is mainly based on the differences in the electrochemical behavior of the labels with or without double-stranded DNA (dsDNA) or single-stranded DNA (ssDNA). It combines nucleic-acid layers with electrochemical transducers to produce the biosensors [5]. Electrochemical DNA biosensor has been investigated immensely around the world, various reviews of it have been reported [6,7,8].

Sandwich-type assays are widely used in immuno-assays with antibodies or aptamers but also work with nucleic acids. Sandwich-type electrochemical DNA hybridization assays have attained considerable attention, because they allow the use of both simple or sophisticated covalent labels while avoiding the labeling of target strands [9,10,11]. One region of the target single-stranded oligonucleotide is usually hybridized with a capture-probe immobilized on an electrode surface or onto magnetic beads. The other region of the target is hybridized with a chemically labeled oligonucleotide called a reporter probe, signaling strand, or detection probe. Extreme sensitivity has been achieved by employing enzymes as biocatalytical reporter labels producing large amounts of electrochemically active products. Single base mismatches can be detected while they are located either in the capture probe or in the reporter section of the target. Dequaire and Heller reported about an enzyme-labeled sandwich assay in a redox polymer layer (containing Os(II/III) complexes as redox mediator) on screen-printed electrodes. This assay was successfully tested also with one or two mismatches in the capture probe section of the target strand. Hybridization was carried out for 15 min at thermally stringent 53 °C [9]. Before that, Heller et al. had introduced conductive redox polymer layers

for the immobilization of capture oligonucleotides. Containing osmium complexes, these layers provided an immobilized redox-mediator for soybean peroxidase. Different hybridization temperature was adjusted using thermostated cells, which allowed for discrimination of single and four base mismatches. In this first paper, the enzyme was attached to the target DNA [12]. Mascini et al. used electrochemical DNA sandwich assays with enzyme-labeled reporter strands to detect bacteria [13]. The eSensor™ represented an early example of hybridization sandwich assays with reporter strands covalently modified with simple redox labels, in this case ferrocene [14]. Remarkably, the thiol-linker was placed in the middle of the partial DNA duplex of target, reporter strand and capture probe. As a result, the captured target in its duplex with the Fc-labeled reporter strand layed flat on top of the probe SAM. This sophisticated mixed probe SAM on the eSensor™ gold electrodes also contained organic "electric wires" [15] to foster the electron transfer between gold surface and ferrocene labels. It was possible to detect single base mismatches due to effects on thermal stability of duplexes coupled with dual labeling using two different ferrocene derivatives [16].

Osmium tetroxide 2,2'-bipyridine (OsO_4(bipy)), introduced in the 1980s for labeling of DNA [17], is a redox reagent that reacts with thymine bases in single-stranded DNA in a click-reaction, forming a labeled nucleic acid which is then electrochemically detectable. The labeling can be performed in-house in any lab, and does not require purchase of commercially modified DNA. Thymine bases labeled with OsO_4(bipy) prevent formation of a base-pair at that nucleotide; However, the reagent does not readily react with thymines in intact double-stranded regions, and thus selective labeling of specific thymines can be achieved using protective strands. These protective oligos are hybridized to a given strand before labeling, and preserve the recognition site either at the target strands for later hybridization with capture-probes on gold electrodes [18] or at reporter probes for hybridization at magnetic beads followed by adsorptive transfer stripping-voltammetry (AdTSV) on carbon electrodes [19].

Until now, detection assays using this method involved labeling of the DNA analyte before hybridization and detection at gold electrodes, which involved an extended period of time before a sequence in a solution sample could be analyzed. Here, we describe a protocol that avoids modification of the DNA analyte, and instead uses osmium tetroxide-labeled reporter strands, which hybridize specifically to the target analyte sequence alongside capture probes immobilized on gold electrodes. Furthermore, this method would used to examine the

asymmetric PCR products and discriminate single base mismatches in the capture probe section, or the reporter strand section, or both due to their different thermal behavior.

Besides, we have investigated the electrochemical detection of PCR products by means of labeling DNA with [OsO_4(bipy)] and protective strand. If the primers can be appropriately designed before PCR, the target can also be avoided for more treatment but for more sensitive detection after PCR amplification. Therefore, lots of investigations on single/double labeled primers for electrochemical detection of PCR amplified DNA have been published [20,21]. A. Escosura-Muniz and coworkers have reported a novel design of isothermal amplification using for the first time primers labeled with both AuNPs and MBs for obtaining double-labeled amplified products has been successfully developed for a DNA sequence characteristic of L. infantum kinetoplast tested in dogs. The double label allows the rapid magnetic purification/ preconcentration of the product followed by direct AuNP electrocatalytic detection, avoiding DNA hybridization procedures [22]. M. I. Pividori research group has developed an assay performed by double-labeling the amplicon during the PCR with a -DIG and -SH set of labeled primers. The thiolated end allows the immobilization of the amplicon on the nano-AuGEC electrode, while digoxigenin allows the electrochemical detection with the antiDIG-HRP reporter in the femtomole range [23]. M. Koets has also utilized end-labeled primers to introduce tags during nucleic acid amplification; the specific amplicon is double-tagged with a biotin and a distinguishing tag such as fluorescein or digoxigenin [24].

This tagging PCR strategy opens new routes not only as an easy strategy for labeling during PCR process, but also combines the advantages of special labeling purposes with rapid electrochemical detection of nucleic acids so that can make the assay more flexible and sensitive. Herein, we initially investigated the electrochemical detection of PCR products by means of [OsO_4(bipy)]-labeled forward primers. The asymmetric thermocycler PCR with labeled primers was followed by hybridization of the obtained labeled PCR products with the thiol-linked capture probes at the surface of gold electrode. After hybridization, the [OsO_4(bipy)]-labeled PCR products yielded the observed SWV peaks as analytical signals. Since the protective strand is needed for labeling of primer with osmium tetroxide compound to protect the sequence, which is necessary for annealing during PCR process, it would make sense to separate the primer and it's protective strand after labeling to prevent the inhibition in the PCR. However, the initial investigations in the separation of primer and its protective

strand are not satisfactory. This approach should be pursued further, since our colleague proposed a promising way to remove the protective strands with magnetic beads and get relative high peak signal, and also there are more available labeling ways in publication to overcome this issue. It would be a novel way for developing the real-time electrochemical detection of DNA if it can be treated properly.

2.Theoretical Foundations

After Watson and Crick present the double helix structure of DNA in 1953 [25,26], the nucleic acid hybridization research has become an important approach in life science, gene analysis, molecular diagnosis detection, pharmaceutical, environmental and forensic applications. Several different techniques and strategies are involved in those areas, including acoustic, optical, quartz crystal microbalance, surface plasmon resonance spectroscopy and electrochemistry [27,28,29]. Among those techniques, electrochemical assay possess an important position, especially after the concept of 'the electrochemical DNA hybridization biosensor' was proposed by Millan and Mikkelsen for the first time [1], this area has received considerable attention from scientists around the world, and there are also large number of reviews of electrochemical nucleic acid sensors have been published [30,31,32,33]. With experimental convenience, simplicity, low cost, fast detection, high sensitivity and good selectivity, electrochemical nucleic acid hybridization assay coupled with other modern micro-fabrication technologies, makes it fabulous applications in biological studies, drug discovery, gene sequencing and clinical diseases diagnosis [34, 35]. Traditionally, electrochemical nucleic acid assay powerfully convert the hybridization event into a readable analytical signal by means of electrochemical methods, such as square wave voltammetry (SWV), differential pulse voltammetry (DPV), cyclic voltammetry (CV), amperometry, impedance spectroscopy and chrono potentionmetry [36,37]. Moreover, coupling with PCR amplification makes electrochemical hybridization an excellent advantage in DNA detection and other genetic identifications [38].

2.1. DNA and Electrochemical DNA detection

As we all know, DNA is a molecule that carries most of the genetic instructions used in the development, functioning and reproduction of all known living organisms and many viruses. In 1953, Watson and Crick proposed the structure of DNA that it consists of two biopolymer strands coiled around each other to form a double helix in opposite directions. Each strand has a backbone made up of (deoxyribose) sugar molecules linked together by phosphate groups. The 3' C of a sugar molecule is connected through a phosphate group to the 5' C of the next sugar. This linkage is also called 3'-5' phosphodiester linkage. This structure is schematically illustrated in Fig. 1.

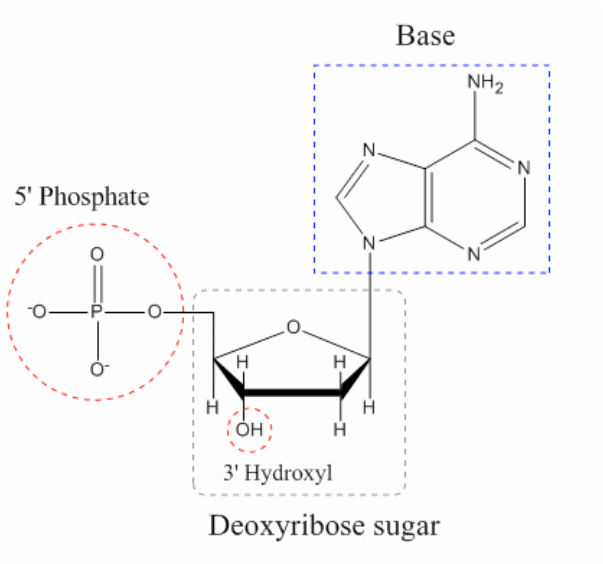


Figure 1: Schematic structure of the nucleotides taken from http://mcat-review.org/molecular-biology-dna.php

The pyrimidine bases are thymine and cytosine, purine bases are adenine and guanine, which in the DNA bases adenine and thymine via two hydrogen bonds and cytosine and guanine are connected complementary three hydrogen bonds [39], as shown in Fig. 2.

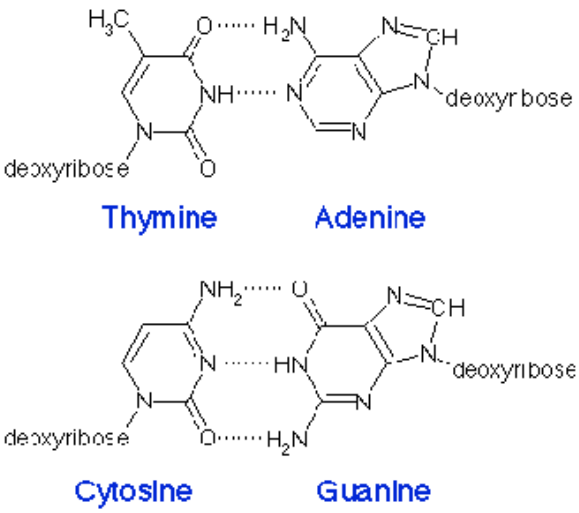


Figure 2: Structure of DNA bases taken from http://ciano. altervista. org/ index. php? section= bionotes/ node5.html.

Because of the complementary nature of base pairing, DNA can transmit genetic information through replication during cell division, the sequence of the DNA is preserved and thus the genetic information is transmitted to the next generation. Furthermore, it forms the basis of

various detection methods of nucleic acids in molecular biology that a probe hybridizes with a complementary target strand and then this hybridization can be detected in a certain form.

DNA hybridization has been considered a fundamental province in molecular biology for the detection and analysis of specific DNA sequences, especially after the discovery of the electrochemical activity of nucleic acids [40], electrochemical DNA detection assays have been explored deeply and attracted increasing attention. Traditionally, Electrochemical DNA assay based on DNA hybridization converse the base-pair hybridization event into a readable and measurable electrical signal [1], therefore, this kind of electrochemical DNA hybridization has also been called electrochemical DNA biosensor and plays a more and more important role in DNA analysis.

Generally, an electrochemical DNA sensor comprises three parts (Fig.3): electrode as recognition layer to immobilize probe and transduce signal; Probe sequence that is immobilized on the electrode surface to hybridize with its complementary strand forming a double helix structure; The analyte (or target) which hybridize with probe, bring electrochemical active indicator to produce useful electrochemical signal.

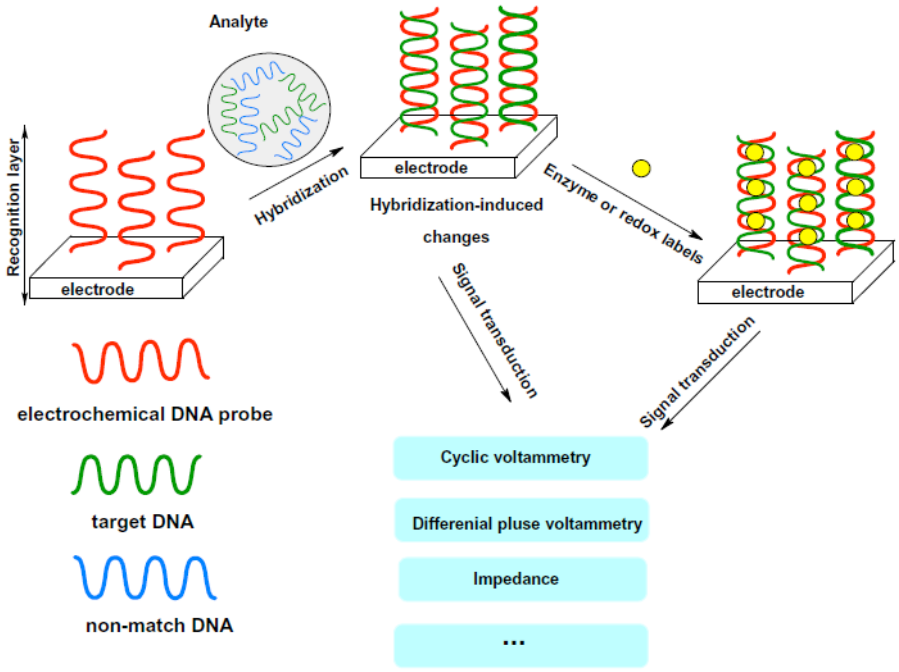


Figure 3: General design of DNA biosensor [32].

Regarding to the electrode, various materials have been employed, such as glassy carbon [41], carbon paste [42], mercury [43], gold [44], silver [45], and platinum [46]. As the signal transducer,

the electrode must be good for probe immobilization and recognize the electrochemical indicator after hybridization events. Due to the proprietary character of gold, such as inert, stable in the ambient conditions, and easy to form S-Au covalent bonds for monolayer molecular assembly, therefore, gold electrode is the most widely and frequently used as transducer lay in the electrochemical DNA biosensors [47]. To date, in order to improve the sensitivity and selectivity of electrochemical DNA biosensors and strengthen the signal, much effort has been done to improve the status of the electrodes, such as modification of nanoparticles [48], graphene [49], polythionine [50] and quantum dot [51].

In terms of probe DNA immobilization, a variety of strategies have been developed for immobilizing single-stranded DNA probes, such as covalent coupling [52], electrostatic binding [53], biotin-streptavidin interaction [54], physical absorption [55], and self-assembly [56]. Among them, the self-assembly method based on S-Au covalent bonds formed by thiol group at the end of DNA probe and gold on the gold electrode surface has been demonstrated a stable immobilization approach and obtained considerable interest in fabricating the self-assembled monolayer (SAM) in electrochemical DNA biosensors [57]. After S-Au bonds formed, the single probe DNA was lying on the gold electrode surface resulting in a low hybridization efficiency [58], Consequently, mercaptohexanol (MCH) has been explored to overcome this shortcoming due to the advantages of dislodging the non-specifically adsorbed DNA from the gold surface and remaining the probe strand standing. Besides, there are still other conditions can affect the probe DNA immobilization and hybridization efficiency, such as the length of probe strand, the density of probe on the surface of electrode, many excellent papers have reported and discussed about that [59,60,8].

After DNA hybridization, a measurable current signal is usually detected due to redox indicator, electroactive label or the hybridization-induced changes of electrochemical behavior of DNA strands. According to the difference of signal generation, electrochemical detection of DNA hybridization has mainly divided into two types, direct methods and indirect methods. The direct methods are always based on the electrochemical characteristic of DNA strands. For example, guanine and adenine are the most electroactive bases of DNA [61,62]. The indirect methods of DNA hybridization detection are always use mediators to enable the reversible exchange of electrons in the electrode surface, it is based on two main ways of electrochemical signals: electroactive intercalator and electroactive molecule label. Plenty of intercalators have been explored in DNA detection of electrochemical hybridization,

among them, the most common intercalators are methylene blue (MB) [63], Fe $[(CN)]_6^{3-/4-}$ [64], $Ru(bpy)_3^{3+/2+}$ [65], ferrocene [66], $Co(phen)_3^{3+}$ [67], and osmium (Os) complex [18]. Redox-active molecule labels are very easily and efficiently labeled to some groups or bases in DNA strands to form a strong affinity and hybrid, so it has been widely employed in electrochemical DNA hybridization assays. In this step, the hybridization format which immobilized probe hybridizes directly with labeled target strand to generate a detectable signal is the most commonly applied. As an alternative, the sandwich-type hybridization assays are also explored to identify the target. The target strand is hybridized both with the immobilized probe and also with another appropriately labeled strand (reporter strand). In this sandwich-type assay, the target strand does not need any labeling; therefore, it can improve the specificity of the assay. In addition, this dual hybrid would make numerous DNAzyme adjacent to the electrode surface, thus providing amplification for the target DNA detection [68].

2.2 Electrochemical DNA Single Base Mismatches Detection

DNA biosensors are increasingly applied in various areas, including sensitive trace detection of oligonucleotides, biomolecules and heavy metals, genomic sequencing, mutation detection, and hybridization reactions [69,70]. Since mutations between bases are able to cause a series of human genetic diseases, a highly successful DNA biosensor should have high ability to discriminate single base pair mismatch after hybridization event. Compared to fully matched hybridization, the mismatched duplex is not so stable, it is possible to differentiate between both from the differences of redox active intercalators behavior [71], rate of charge transport [72] or kinetic in the strand exchange reactions [73]. A variety of methods have been widely explored for mismatch detection, such as surface plasmon resonance spectroscopy [74], Raman spectroscopy [75], UV-vis spectroscopy [76], mass spectroscopy [77], fluorescence techniques [78], electrochemical methods [79], and so on. Among them, electrochemical method for DNA hybridization and mutation analyze is an attractive alternative and has been widely developed [80,81]. For example, H. Joda and coworkers have reported a simple approach to enhance solid-phase hybridization-based single base mismatch discrimination at high ionic strength based on the deliberate insertion of a natural DNA base mismatch in the surface-tethered probe [82]. Fig. 4 shows the schematic of hybridization of different probes with single base mismatched target.

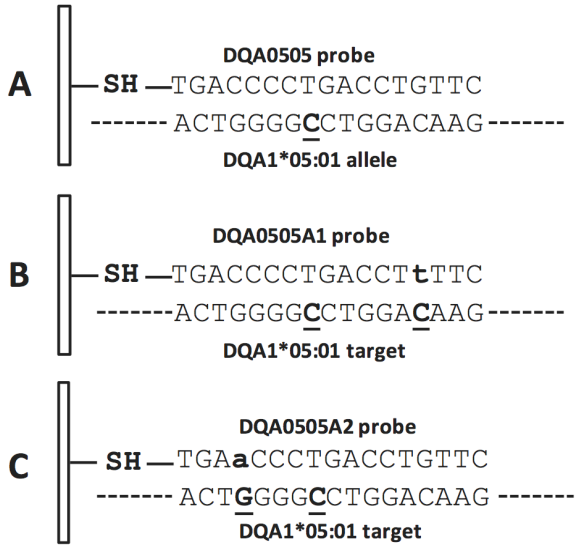


*Figure 4: Schematic highlighting hybridization of different probes with single base mismatched DQA1*05:01 allele: (A) DQA0505 probe; (B) DQA0505A1 probe; (C) DQA0505A2 probe. Base in lowercase letter indicates deliberated mismatch, and base in bold and underlined letter indicates mismatch* [82].

Besides, single nucleotide polymorphisms (SNPs) which refers to single base pair mutations are very common variations in the genetic heterogeneity [83,84], in recent years, detection of SNPs received considerable attention due to its importance for the early diagnosis and treatment of gene disease [85,86].

2.3 The application of reporter (signaling) strands in electrochemical assay

As we all know, most of electrochemical DNA biosensors comprise three procedures: the ssDNA capture probe which immobilized onto a solid electrode surface to form a recognition layer for selectively detecting a specific sequence of DNA; the hybridization between target and capture probe strands; a measurable electrochemical signal is detected to prove the presence of the hybrid at the recognition layer has occurred. Therefore, remaining electroactive for detecting DNA hybridization in the electrode surface is one of the most important components in the development of a DNA biosensor. There are various strategies to obtain the readable electrochemical signals after the hybridization that we have elaborated in part 2.1. In short, electrochemical detection of DNA hybridization has mainly divided into two types, direct methods and indirect methods, based on the difference of signal generation. The direct and label-free electrochemical bio-assays have been developed in structure-sensitive DNA analysis and the detection of DNA damage due to its intrinsic electroactivity

and surface activity [87,88]. The changes of the electrochemical response come from the nucleobase accessibility [89,90] or the intrinsic nucleobase electroactivity, such as oxidation of the guanine or adenine moieties [91,92]. However, these methods have some limitations, for example, the limitation of electrode material, insufficient sensitivity, the irreversibility of the nucleobase oxidation or reduction, and the increase of charge transfer resistance. Compared with the shortcomings of these approaches, the indirect electrochemical bio-assays with electroactive labels and indicators exhibit its advantages: first of all, it improves selectivity and sensitivity of the analysis. Secondly, many kinds of labels and indicators have maturely explored in various ways in different kinds of electrodes according to special requirements. Furthermore, due to the peculiar potentials of labels and indicators, they can discriminate explicitly their responses from the DNA intrinsic responses. Besides, the capture DNA probe immobilized in electrode surface does not need to be destroyed during detection.

Electroactively labeled reporter strands represent one of the important strategies for the detection of specific interactions in the development of electrochemical DNA biosensors [93,94]. M. Fojta group has showed that covalent reporter probe labeling with the osmium tetroxide complexes can be used for “multicolor” electrochemical DNA coding and parallel hybridization analysis of multiple target DNAs [19]. As the Fig. 5 shows, target DNA (tDNA) is captured at magnetic Dynabeads oligo (dT)25 (DBT) via DNA duplex formation between T25 chains covalently attached to the DBT surface and A20 stretches present in the target ODNs. After magnetic separation and washing of the beads, a reporter probe (RP) (Os,L) is hybridized with the tDNA. The DBT-tDNA-RP (Os,L) “sandwich” is formed only in the case of nucleotide sequence complementarity between tDNA and the RP. After another washing step, the hybrids are denatured by heat, the DBT removed, and the released RP (Os,L) determined using AdTS, SWV. Besides, they also demonstrated that utilization of this technology is not restricted to homopurine- homopyrimidine sequences, but it can be applied for labeling of any (mixed-sequence) reporter probe with the oligo (T) tail when appropriate modification procedure is used. Furthermore, they successfully applied an analogous technique to analyze PCR- amplified natural genomic DNA sequences.

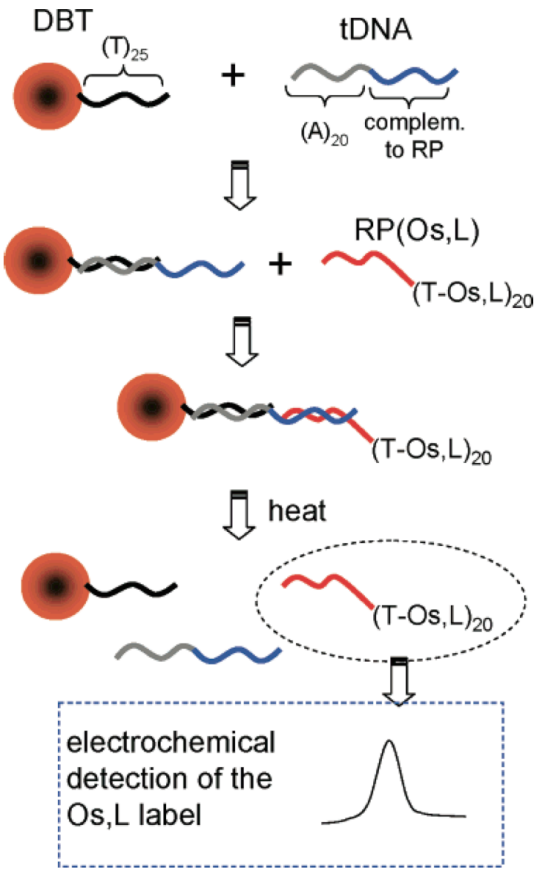


Figure 5: Scheme of the electrochemical double-surface (DS) DNA hybridization assay [19].

Introducing reporter strands into electrochemical DNA detection assays exhibits some obvious advantages for DNA hybridization. The electroactive moieties are only covalently modified with the reporter strands, therefore the modification of the target strands is avoided during the sample preparation. Since the labeling procedure independently take place before hybridization with target strands, and meanwhile, the reporter strands can only hybridize with part sequence of target strands, so the reporter strands do not bring any significant, unexpected effects in capture probe strand and its interaction with the target DNA. Furthermore, the reporter strands can be designed to have similar characteristic of nucleic acid thermodynamics as the target strands so that the mixture which reporter strands are added into sample solution is allowed to reach binding equilibrium at the appropriate temperature and time. Due to all the advantages mentioned above and also their facile procedure for detection, outstanding sensitivity, and invariant response for the target structures and sequences. The reporter strands seem to possess great potential in the future for electrochemical analysis of DNA hybridization.

2.4 PCR and Electrochemical PCR Products Detection

Polymerase chain reaction (PCR) is a technique that exponentially amplifies the single copy of a DNA strand by repetitive cycles to a detectable amount of products within a short term. Since its development in the early 90s [95], the PCR has become a golden technique for gene expression analysis, forensic analyses, diseases diagnostics, food safety, environmental science, medical science and molecular science [96].

For the PCR, two short single-stranded oligonucleotides (primers) which must bind to a specific region on either side of the target DNA sequence and initiates replication of the target DNA at that point is required. Primers specify the DNA sequence to be amplified. Moreover, the target sequence must be present as a template. Furthermore, deoxyribonucleoside triphosphates (dNTPs) and a thermo-stable polymerase that makes a new strand of DNA through the sequential addition of nucleotides and the appropriate buffer system are required [97,98].

The PCR is generally carried out according to the following scheme (shown graphically in Fig. 6):

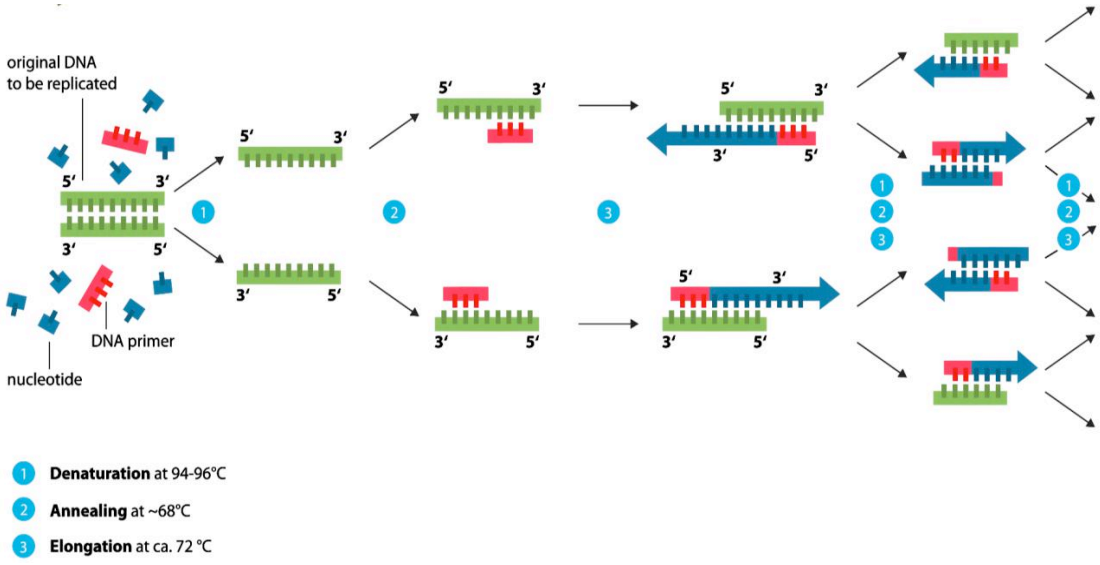


Figure 6: Graphic representation of the PCR, taken from https://en.Wikipedia.org/wiki/Polymerase_chain_reaction.

1. Denaturation of the DNA: The DNA is heated to 95°C. This breaks the weak hydrogen bonds that hold DNA strands together in a helix, allowing the strands to separate creating single stranded DNA.

2. Annealing of primers: The primers anneal to the complementary portions in the template DNA. This step is usually carried out at 50-60 °C. It is important to ensure that the annealing temperature is below the melting temperature of the primers.

3. Extension: The synthesis of DNA occurs through incorporation of dNTPs by the polymerase in accordance with the complementary template. The temperature of this step is determined by the optimum temperature of the polymerase used and is around 72-75 °C.

These steps are repeated for 20-35 times. The amplification is exponential for each cycle, the DNA strands made in the previous cycles can serve as template for next cycle.

Since the impressive amplification of PCR in various DNA-related applications, the combination of PCR amplification with electrochemical detection provides a sensitive and specific method for an integrated DNA biosensor [99]. Various research groups have reported electrochemistry based DNA and PCR detection assays [100,101], R. Y. Lai and coworkers have explored an electrochemical DNA based sensor that use methylene blue (MB) as a reporter molecule conjugated to the 3' end of an amino- and thiol-modified stem loop oligonucleotide through succinimide ester coupling for the electrochemical sequence-specific detection of unpurified amplification products of the gyrB gene of Salmonella typhimurium [100]. Fig. 7 shows the sensor was fabricated by self-assembly of the MB-labeled DNA probe on a gold electrode surface. In the absence of target, the stem-loop structure holds the MB tag close to the electrode surface, thus enabling efficient electron transfer. Upon hybridization with the target PCR amplicon, a large change in the reduction peak of MB was observed.

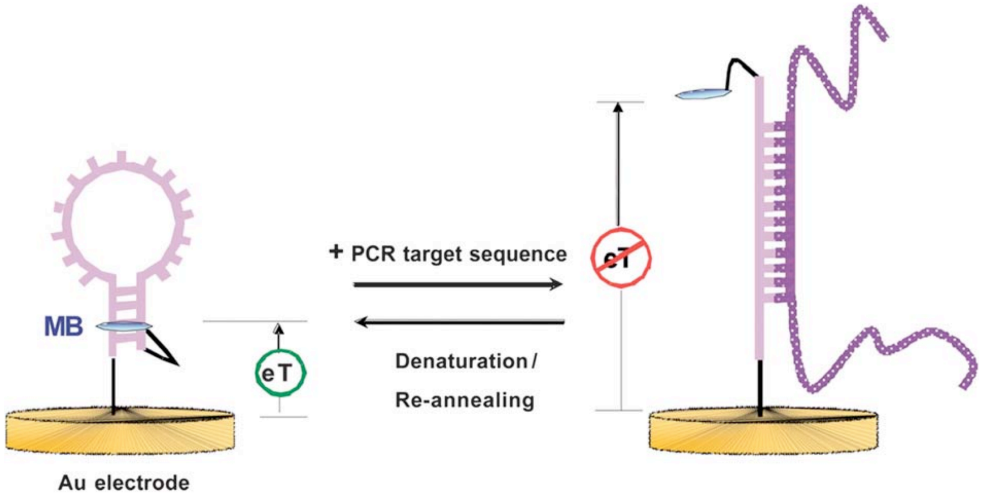


Figure 7: Electrochemical DNA-based PCR sensor fabricated by self-assembly of a MB-labeled DNA probe on a gold electrode surface [100].

Besides, Hsing's group firstly reported to electrochemically monitor DNA amplification during PCR [102]. During those approaches, external electroactive labeling [103,104,105,106], intrinsic electroactive moiety [107] and chemical modification of single stranded amplicons [108] are most often utilized ways to get the readable electrochemical signals of PCR product in the detection principles. In order to strength the effect of amplification and enhance the sensibility, some nanoparticles are also introduced to DNA/PCR based detection system [109,110]. Besides, due to excellent advantages of electrochemical techniques-based assay, such as simplicity, experimental convenience, fast detection, good selectivity and sensitivity, and also the special temperature related PCR procedures, lots of research groups put those two techniques together to explore convective PCR [111], microfluidic electrochemical PCR detection devices [112] and lab-in-chip [113,114]. DNA amplification by the PCR plays a profound role in electrochemical DNA detection assays.

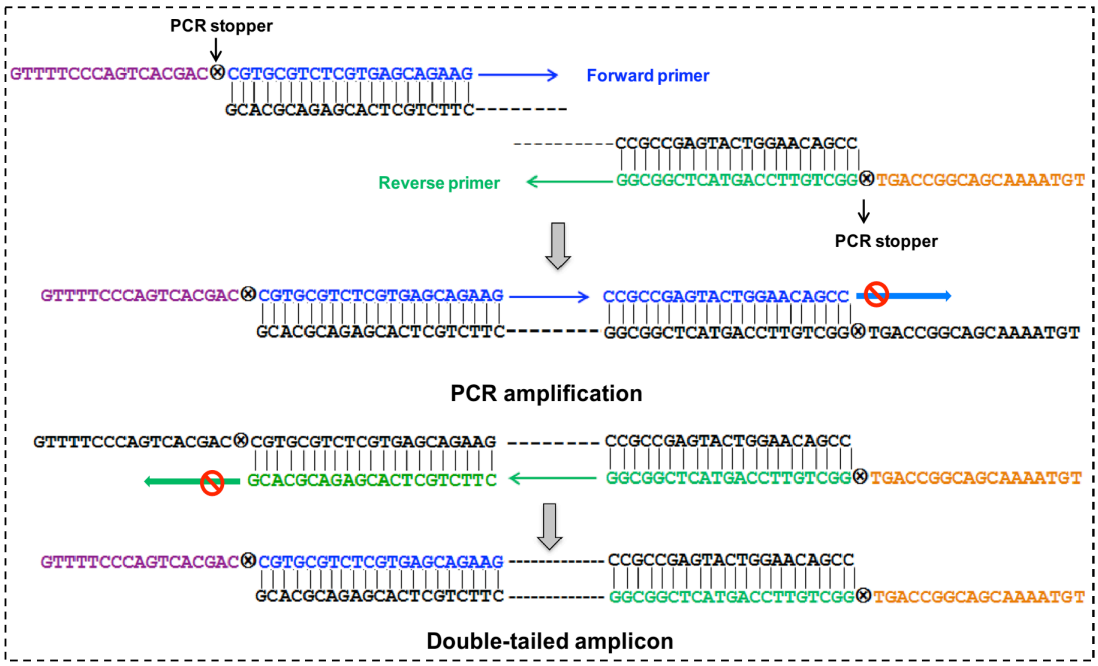
2.5 Modified-Primers for PCR and electrochemical analysis

With the development of PCR in biochemical and electrochemical analysis, improvement and exploitation of PCR process and products to get the target DNA have gained more and more attention recently. However, the use of labeled nucleotides often yields high background signals due to non-specific nucleotide incorporation in fragmented endogenous mispriming [115]. As one of most important factors in the PCR constituents, primers (forward primer and reverse primer) have also been explored a lot. Combining primer labeling with

electrochemical technique could explore a profound development in real-time detection and chips. In order to avoid the post-modification, or make the final detection procedure simple, rapid and sensitive, or fulfill some special requirement, some research group have developed various kinds of ways to label the primers. E. Tartour group reported first in 1999 to use biotinylated primers for direct in situ reverse transcription (RT-PCR), consequently found that this type of label provided much better results than digoxigenin or fluorescein conjugated oligonucleotides and showed that in situ RT-PCR using biotinylated oligonucleotides was the most specific direct method for the detection inside cells, of mRNA encoding for various human cytokines [116]. Besides one primer labeling, double labeling is common and useful in electrochemical analysis. C. K. O'Sullivan and coworkers have explored polyoxometalates (POM)-forward primer and biotin-reverse primer, have demonstrated, for the first time, the synthesis and characterization of POM-DNA conjugates, their electrochemical detection after hybridization to surface-tethered probes, the application of these POM-labeled oligo-nucleotides in PCR, and the subsequent direct electrochemical detection of POM-labeled PCR products [117]. Merkoci and coworkers have designed a novel assay of isothermal amplification using for the first time primers labeled with both gold nanoparticles (AuNPs) and magnetic beads (MBs) for obtaining double-labeled amplified products has been successfully developed for a DNA sequence characteristic of L. infantum kinetoplast tested in dogs [118]. For the first time, Pividori group has designed a double tagging PCR strategy for the detection of Salmonella sp which was performed by double-labeling the amplicon during the PCR with a -DIG and -SH set of labeled primers. The thiolated end allows the immobilization of the amplicon on the gold nanocomposite electrode (nano-AuGEC), while digoxigenin allows the electrochemical detection with the anti-Digoxigenin-horseradish peroxidase (antiDIG-HRP) reporter in the femtomole range. This double tagging PCR strategy opens new routes not only for immobilization purposes but also as an easy strategy for labeling with gold or quantum dots during PCR [119].

Besides labeling primers with compound, nanoparticles and magnetic beads, O'Sullivan group have exploited to tag primers with single stranded DNA [120]. This novel sequence specific primer design produces a PCR product flanked by two single stranded DNA tails. These tails are used for hybridization with probes immobilized onto the surface of electrode array and with enzyme labeled reporter probe, allowing for rapid and multiplex detection of several targets. Fig. 8 demonstrates clearly about the procedure of PCR and electrochemical

detection. Two specific sequence were tagged in the end of forward and reverse primers, after PCR cycling, two tail-double DNA strand was gained and used to hybridize directly with a surface immobilized probe and an enzyme labeled reporter probe, eventually get the readable electrochemical signal.



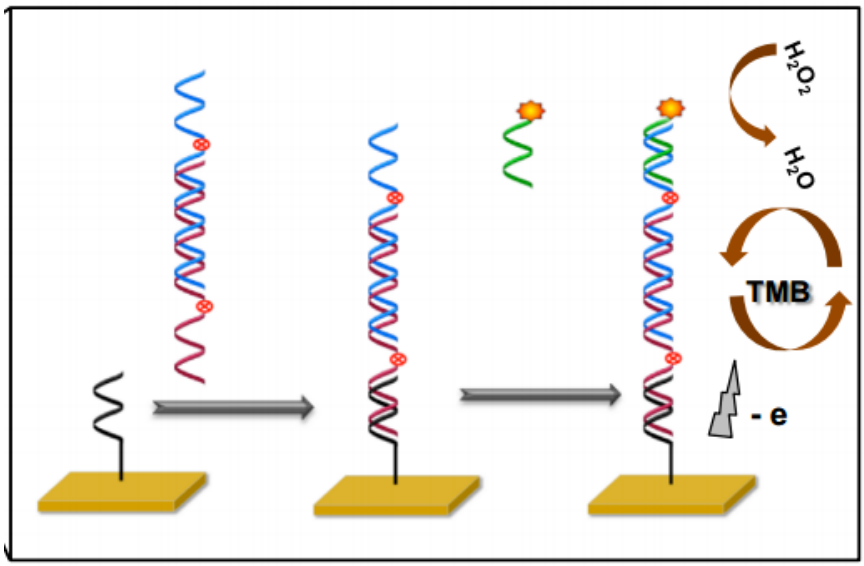


Figure 8: (Above) Schematic of double tailed PCR amplification using the proposed primer design. (Below) Shows schematic of electrode array and electrochemical detection of the double tailed PCR product [120].

2.6 Gel electrophoresis

DNA electrophoresis is one of the most important tools in modern molecular biology and biotechnology. It's defined as the migration of DNA molecules in a supporting medium under the influence of an electric field. Several of studies found that the mobilities of DNA molecules in agarose gels mainly dependent on the molecular mass and electric field applied to the gel [121, 122]. The most common used gel is agarose and polyacrylamide. In this study, we used agarose gels for the determination of DNA and PCR products. Agarose is an alternating copolymer of 1, 3-linked β-d-galactose and 1, 4-linked 3, 6-anhydro-α-l-galactose [123]. It can separate DNA molecules ranging in size from ≈100 bp to ≈20 kilo base pairs (kbp) in unidirectional electric fields.The gels are usually characterized by the percentage (w/v) of agarose in the matrix (%A). The pore size is determined primarily by the amount of dissolved agarose (for example, 1% [w / v] agarose ≈150 nm). In agarose gel can separate DNA fragments with a size of 25 kbp to 80 bp without special protocols [124]. In this work the commercial dye GelRed™ was used, which was usually added before boiling the agarose.

2.7. Electrochemical methods

2.7.1. Cyclic voltammetry (CV)

Cyclic voltammetry is the very common used technique for obtaining qualitative information about electrochemical reactions. It offers a rapid location of redox potentials of the electroactive species by measuring the current at the working electrode during the potential scans. The potential is applied between the working electrode and the reference electrode while the current is measured between the working electrode and the counter electrode. The result of this potential curve is recorded as current (i) vs. applied potential (E). Referring to statements about the characteristic of the analyte oxidation and reduction potentials, the CV can be used to obtain information about the stability of reaction products, the presence of intermediates in redox reactions, and the reversibility of a reaction. Furthermore, the CV information can show the kinetics of heterogeneous electron transfer processes, and adsorption on the electrode [125,126,127]. The potential cycle can be repeated, and the scan rate can be varied. Fig. 9 shows a typical cyclic voltammogram result that obtained with the Fe^{2+}/ Fe^{3+} redox system in an aqueous electrolyte.

In this work, the cyclic voltammetry was used to clean the electrode to the quality control of the electrodes.

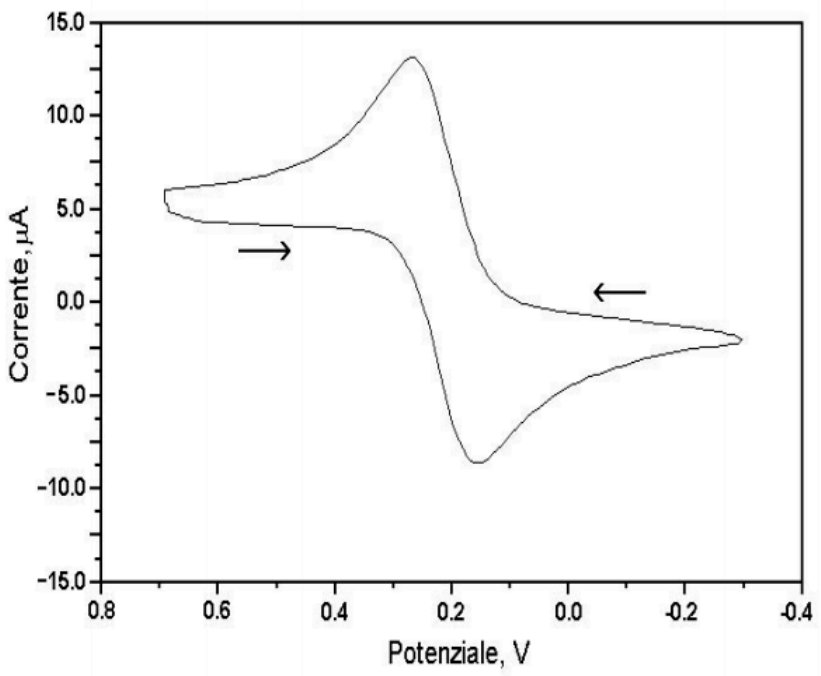


Figure 9: Cyclic voltammogram of the Fe^{2+} / Fe^{3+} redox system in aqueous electrolytes [128].

2.7.2 Square-wave voltammetry (SWV)

The Square-wave voltammetry (SWV) is a form of linear potential sweep voltammetry. The starting potential is a median of extreme potentials of the square-wave signal. To each tread of the staircase signal a single square-wave cycle is superimposed, so the waveform can be considered as a train of pulses towards higher and lower potentials added to the potential that changes in a stepwise manner. The current is measured at the end of the positive and negative pulse, as shown in Fig.10. It is the current difference between the two measurements is calculated and plotted against the voltage in the diagram, thereby resulting in a peak-shaped current-voltage curve, which represents predominantly the Faraday current [129]. The peak height is directly proportional to the concentration of the electroactive species. This method is relatively sensitive and the measurement can be carried out very quickly that can furthermore offer the possibility to use it for measurement in flow systems or for kinetic studies [130].

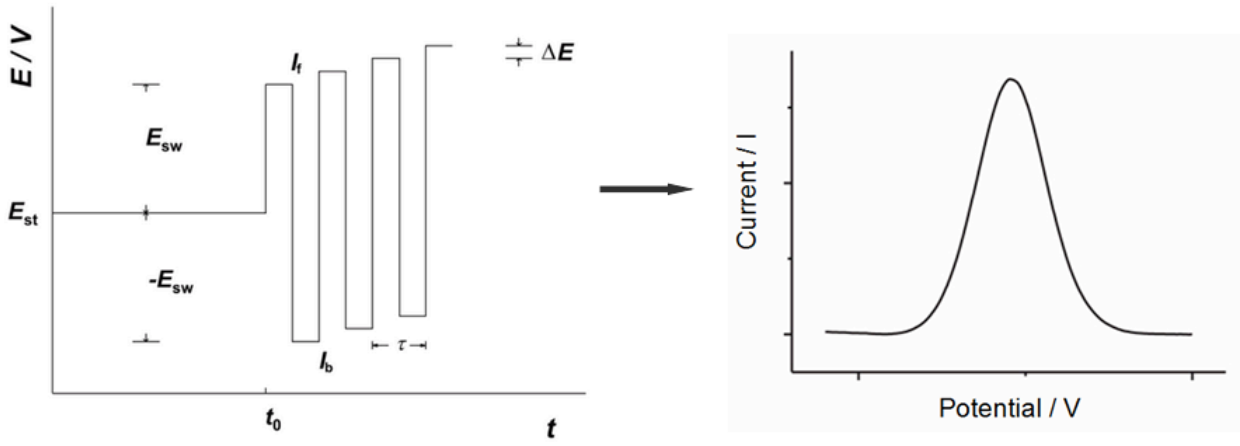


Figure 10: Scheme of the voltage-time function (left [131]) and current-voltage curve of the SWV (right). Est: starting potential, Esw: pulse height, ΔE: potential increment, τ: staircase period, t_0: delay time and If and Ib denote the points where the forward and backward currents are sampled, respectively.

2.7.3. Differential Pulse Voltammetry (DPV)

As we all know, voltammetry is an analytical technique based on the measure of the current flowing through an electrode dipped in a solution containing electroactive compounds, while a potential scanning is imposed upon it. The working electrode in the measurement system, could be made with several materials, if a drop of mercury rhythmically dropping from a capillary forms the electrode, the analytical technique is called Polarography [132].

Polarography has been developed in 1922 by Jaroslav Heyrovsky, it is a technique in which the electric potential (or voltage) is varied in a regular manner between two sets of electrodes (indicator and reference) while the current is monitored [133]. The shape of a polarogram depends on the method of analysis selected, the type of indicator electrode used, and the potential ramp that is applied.

The dropping mercury electrode (DME) was used in direct current (dc) polarography as working electrode, which consisting of small reproducible drops of mercury flowing from the orifice of a capillary tube connected to a mercury reservoir. The electrode surface is being constantly renewed in a cyclic fashion, the current increases from a small value as the drop begins to form to a maximum value as the drop falls [133,134].

Differential Pulse Voltammetry/Polarography (DPV/P) can be considered as a derivative of linear sweep voltammetry or staircase voltammetry, with a series of regular voltage pulses

superimposed on the potential linear sweep or stairsteps. The current is sampled twice in each Pulse Period (once before the pulse, and at the end of the pulse), the difference between these two current values is immediately measured and plotted as a function of potential. The consequence of double sampling is also that the differential pulse voltammograms are peak-shaped [135,136]. The plot of DPV can be seen in the Fig. 11.

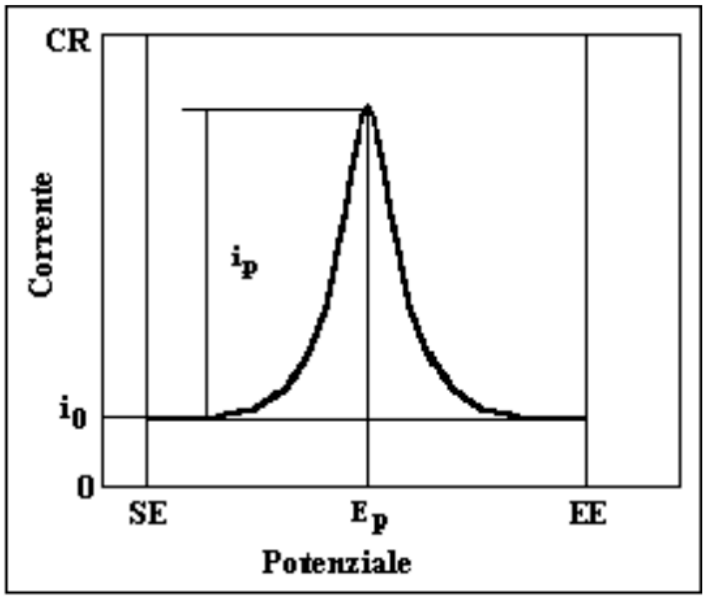


Figure 11: Plot of DPV voltammogram SE = Start potential; EE= End potential; i0=Currentat the beginning of the scanning; ip = Peak current; Ep= Peak potential; CR = Current range [137].

2.7.4 Electrochemical Impedance Spectroscopy (EIS)

Electrochemical Impedance Spectroscopy (EIS) is a traditional electrochemical method and now widely established as one of the most powerful and reliable methods for investigating the mechanisms of electrochemical reactions, measuring the dielectric and transport properties of materials, exploring the properties of porous electrodes and investigating passive surfaces. This technique has grown tremendously and used in a wide range of applications [138]. Important areas of application are corrosion, electrode kinetics, batteries, semiconductors, fuel cells, membranes, surface treatment, and membranes, interfaces [139,140,141].

EIS measurement is similar to other electrochemical methods in either a three-electrode mode or a two-electrode mode, depending on the purpose of the measurement. It is conducted using instruments such as potentiostats that have EIS functions to determine charge transfer

resistance, double layer capacitance and ohmic resistance with an impedance spectra in a wide frequency range such as from 100 kHz to 1 mHz.

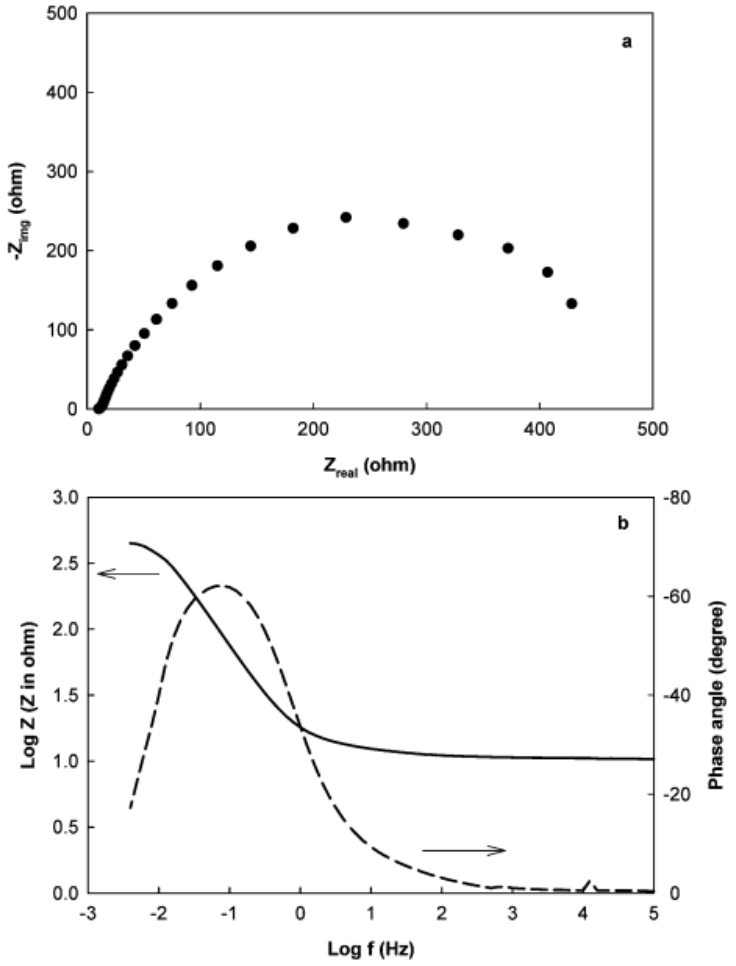


Figure 12: The impedance of the anode in a microbial fuel cell using acetate solution as electrolyte: (a) the complex plan plot; (b) Bode plot [142].

We herein refer to a well known textbook 'Impedance Spectroscopy Theory, Experiment and Applications' edited by Barsoukov and Macdonald for its fundamental theory [143]. The results of the EIS for electrochemical cells are most often represented in Nyquist plots (also called the complex plane plot) and Bode plots as shown in Fig. 12 [144]. A Nyquist plot for the anode of a microbial fuel cell is showed in Fig. 12(a). It depicts the impedance with a real part (plotted on the X-axis) and an imaginary part (plotted on the Y-axis that is negative) as a semicircle. Each point on the complex plane plot represents the impedance at a certain frequency. Nyquist plots have the advantage that activation-controlled processes with distinct time-constants show up as unique impedance arcs and the shape of the curve provides insight into possible mechanism or governing phenomena. However, a shortcoming of the Nyquist

plot is that it does not show the values of the frequency of the applied ac signal and the phase angle. The Bode plot, on the other hand, refers to representation of the impedance magnitude (or the real or imaginary components of the impedance) and phase angle as a function of frequency. Fig. 12b is the Bode plot for the data in Fig. 12a. Because both the impedance and the frequency often span orders of magnitude, they are frequently plotted on a logarithmic scale. The axes of both impedance modulus |Z| and frequency f are logarithmic. Since both data formats have their advantages, it is usually best to present both Nyquist and Bode plots.

2.8 The application of [OsO_4(bipy)] compound in electrochemical detection assay

The DNA structure based electrochemical device always requires electroactive indicator to recognize DNA sequences in a hybridization process or the surface of electrodes. Various electrochemical indicators have been investigated [1,145]. Among them, osmium tetroxide complexes containing an atom with oxidation number VIII have been first explored as selective electroactive marker in DNA assays in the early 80s by Palecek group [17,146,147]. It has been reported that osmium tetroxide, pyridine (([OsO_4(py)]), 2,2-bypiridine ([OsO_4(bipy)]) and phenantroline ([OsO_4(phen)]) can bind covalently to pyrimidine bases through addition to the 5, 6 double bond of the pyrimidine ring in single stranded (ss) or distorted double stranded DNA (dsDNA) regions to form stable adducts. Subsequently, an electrochemical response as a hybridization signal can be gained after the bindings of the indicator with ssDNA and dsDNA [148,149].

Compared with other osmium tetroxide complexes, [OsO_4(bipy)] is much more stable and has more advantages. Firstly, pyridine requires higher concentration to react with osmium tetroxide and stabilize the formed osmate complexes [147]. However, 2, 2-bypiridine can be utilized as the same concentrations as the osmium tetroxide to form the resulting compound, which can be applied as a DNA marker in both vitro and vivo [150]. Secondly, [OsO_4(bipy)] only reacted with ssDNA while [OsO_4(phen)] can not only combine to ssDNA, but also to dsDNA [151]. This kind characteristic of [OsO_4(bipy)] shows considerable selectivity for ssDNA. Furthermore, [OsO_4(bipy)] is sensitive to DNA structure, the procedure of labeling can be easily achieved in the laboratory. In addition, due to the electrochemical reversibility and stability in both oxidized and reduced states, [OsO_4(bipy)] could also be developed as redox mediators in biocatalytic processes [152,153]. Besides, when [OsO_4(bipy)] complex with

nitrogen ligands reacts with the pyrimidine ring, thymine residue exhibits about 10-fold more reactive than cytosine, in the meantime, purine bases do not combine to [OsO_4(bipy)] complex [154,155], therefore, the reactivity of [OsO_4(bipy)] complexes with ssDNA strands offers an attractive proposal of a single-nucleotide resolution in DNA modification [151,154,156].

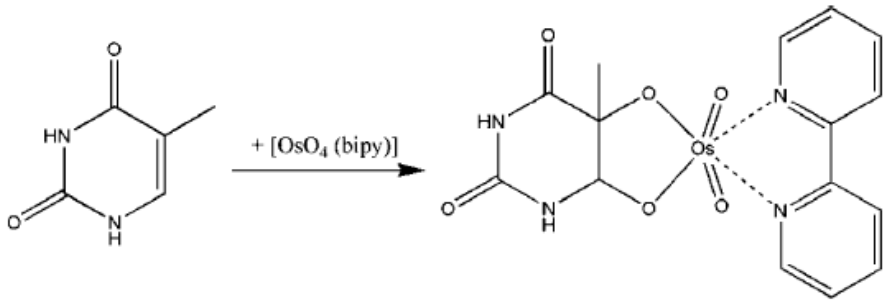


Figure 13: Oxidative labeling of thymine with [OsO_4(bipy)] [18].

At last, the resulting stable adducts can be measured electrochemically by means of several different methods and electrodes, such as mercury [148, 154,157], carbon [155,19, 158], amalgam [159], and gold electrodes [160,18]. On the basis of those advantages, [OsO_4(bipy)] compound as an electroactive indicator has been widely investigated not only in electrochemical DNA assays, DNA biosensors, and the study of DNA structure, it has also been applied in labeling of peptides [161] and proteins [162].

3. Experimental section

3.1 Materials

2, 2'-Bipyridine, Osmium tetroxide as a 2% aqueous solution was obtained from Sigma-Aldrich (St. Louis, MO, USA). The Tris-buffer (containing 10 mmol/L tris-(hydroxymethyl)-aminomethane (Sigma-Aldrich) and 0.5 mol/L sodium sulfate (Sigma-Aldrich) was adjusted to pH 7.5 using sulfuric acid. Highly purified water (>18 MΩ cm. TOC<2 ppb) prepared using a system of SG Water (Barsbuttel, Germany) was used in all experiments in chapter 4.1 to 4.3. Barnstead Mega-pure water (Thermo Scientific) was used in all experiments in chapter 4.2 and 4.4. All chemicals were of analytical grade. Buffer solutions, beakers and pipette tips were autoclaved twice prior to use.

The probe, target, signal, and protective DNA oligonucleotides were delivered by Friz Biochem GmbH (Munich, Germany), 3 kinds mismatch target DNA for each two sequence were obtained from IDT (Coralville, Iowa, USA). Table 1 lists the sequences of all DNA-strands together with their length and designations used in this work. The probe strands contained 15 thymine or adenine bases as a space to allow high hybridization efficiency on the electrode surface. The protective oligonucleotides were designed to have mismatches (highlighted in Table 1) to facilitate their later displacement by the immobilized probe or target strand. One of the forward primers was designed to have 5 more thymines that could be labeled with [OsO_4(bipy)] compound.

Table 1. DNA oligonucleotides, the mismatches (MM) in the target strands are highlighted.

Designation	Length	Sequence
		Capture probes
P-caceto (chapter4.1 4.2,4.3)	42	5'-AAGTCCTTGAGCTGATTTTACTGTATTttttttttttttt[Dithio]3-3'
P-ivrp (chapter4.1 4.2)	44	5'-CACGTGAGAATTTCCGTCTACTCGAGCCTAAAAAaaaaaaaaaa[Dithio]$_3$-3'
P (chapter4.4)	41	5'-AGATACCAAGCGGCCATGGACAACAAAAAAAaaaaaaaaaa[Dithio]$_3$-3'

Reporter strand and its protective strand		
Reporter （chapter 4.1, 4.2,4.4）	27	5'-ttttTACGCTTGTCAAAGTGGTAGTA-3’
Protector1 (chapter4.1 4.2,4.4)	22	5'-AAGAACCACTTTGACAAGGGAA-3'
Protector2 (chapter4.4)	22	5’-AACAACCACAAAGACAAGGGTA-3’
Protector-1	21	5’-TCCCAAAACAATTCCTGTAAG-3’
Protector-2	21	5’-TCC<u>A</u>AAAACAATTCC<u>C</u>GTAAG-3’
Target strands		
T-caceto-1 (chapter4.3)	136	5’- CTTACAGGAATTGTTTTGGGACTTGCAAGGGCTTTTGGAGAGGCATTAGCAGTTCAAATGGTA ATAGGTAATACAGTAAAATCAGCTCAAGGACTTTTTTCTCCTACTACCACTTTGACAAGCGTA CTTACAATGG-3'
T-caceto (chapter4.1,4.2)	57	5’-AATACAGTAAAATCAGCTCAAGGACTTTTTTCTCCTACTACCACTTTGACAAGCGTA-3'
T-ivrp (chapter4.1,4.2)	64	5’- TTTTTAGGCTCGAGTAGACGGAAATTCTCACGTGTTTTCTCCTACTACCACTTTGACAAGCGT A-3’
1MM-P-caceto (chapter4.2)	57	5’-AATACAGTAAAATCAGCTCAAGGA<u>**G**</u>TTTTTTCTCCTACTACCACTTTGACAAGCGTA-3'
1MM-R-caceto (chapter4.2)	57	5’-AATACAGTAAAATCAGCTCAAGGACTTTTTTCTCCTACTACCACTTTGACAAGC<u>**C**</u>TA-3'
2MM-caceto (chapter4.2)	57	5’-AATACAGTAAAATCAGCTCAAGGA<u>**G**</u>TTTTTTCTCCTACTACCACTTTGACAAGC<u>**C**</u>TA-3'
1MM-P-ivrp (chapter4.2)	64	5’- TTTTTAGGCTCGAGTAGACGGAAATTCTCAC<u>**C**</u>TGTTTTCTCCTACTACCACTTTGACAAGCGT A-3’
1MM-R-ivrp (chapter4.2)	64	5’- TTTTTAGGCTCGAGTAGACGGAAATTCTCACGTGTTTTCTCCTACTACCACTTTGACAAGC<u>**C**</u>T A-3’
2MM-ivrp (chapter4.2)	64	5’- TTTTTAGGCTCGAGTAGACGGAAATTCTCAC<u>**C**</u>TGTTTTCTCCTACTACCACTTTGACAAGC<u>**C**</u>T

		A-3’
T (chapter4.4)	61	5’-TTTTTTTGTTGTCCATGGCCGCTTGGTATCTTTTTCTCCTACTACCACTTTGACAAGCGTA-3’
		DNA Strands for PCR (besides target)
F1-Primer (chapter4.3)	27	5’-ttttCTTACAGGAATTGTTTTGGGA-3’
R1-Primer (chapter4.3)	21	5’- CCATTGTAAGTACGCTTGTCA-3’
F-Primer (chapter4.1 caceto)	23	5’-AATACAgTAAAATCAgCTCAAgg-3’
R-Primer (chapter4.1 caceto)	23	5’-AATACAgTAAAATCAgCTCAAgg-3’
F-Primer (chapter4.1 ivrp)	24	5’-AGG CTC GAG TAG ACG GAA ATT CTC-3’
R-Primer (chapter4.1 ivrp)	24	5’- TAC GCT TGT CAA AGT GGT AGT AGG -3’

For these sequences, lower case ‘t’ in reporter and forward primer denotes the thymine bases labeled with [OsO_4(bipy)], and lower case ‘a’ in capture probe denotes adenine bases used as a spacer.

3.2 Preparation of the probe SAM-modifies gold electrodes

The gold disk electrode (Metrohm, Switzerland) was polished using corundum 0.3 and 0.05 µm (Buehler, Germany), and thoroughly rinsed with water. After this, the electrode was electrochemically treated using cyclic voltammetry with 25 voltammetric cycles between -0.2 and +1.65 V at 100 mV/s in 0.5 mol/L sulfuric acid and again rinsed with water. For immobilization, a droplet of 5 µL probe solution (30.3 µmol/L) was placed onto the electrode surface in order to form the probe SAM (self-assembled monolayer) via immobilization of the [dithio-serinol]$_3$-linker onto gold. After overnight at room temperature in a water-saturated atmosphere, the electrode was rinsed and immersed in 1 mmol/L aqueous mercapto-hexanol (MCH) solution for 1 h. EIS measurements were performed after rinsing with water before and after application of MCH, and after the final dehybridization of a hybridization series.

SWV and DPV measurements were performed as background response after application of MCH. Concentrations of probe solution and timing of drop applications vary and are discussed explicitly in the subsequent section.

3.3 Preparation of the Reporter

The [OsO_4(bipy)]-labeled signaling oligonucleotide by means of protective strands was named after reporter in this work. For the labeling process, 25 µL 100 µM signal oligo solution and 25 µL 100 µM protective strand solution were mixed and left for 2 h at room temperature to allow hybridization. And then 12.5 µL 10 mM [OsO_4(bipy)] solution (10 mM osmium tetroxide and 10 mM 2, 2'-bipyridine in This-buffer solution) was added and left at room temperature for 2 h for labeling. After this period, the excess of the osmium complex was removed by dialysis against pure Tris-buffer by means of Slide-A-Lyzer MINI Dialysis Units, 3500 MWCO (Rockford, IL, USA) at room temperature for overnight. After dialysis, the purified reporter solution (osmium-labeled signal oligo) was filled up to 500 µL with Tris-buffer and used for the hybridization experiments.

3.4 Hybridization and dehybridization procedure

For the hybridization step, the probe-SAM-modified gold disk electrodes were immersed in a beaker containing the stirred target and reporter duplex strands solution at given concentrations, times and temperatures in a water bath. A magnetic stirring was utilized at 1000 rpm during hybridization. After hybridization, the electrodes were rinsed with Tris-buffer to remove nonspecific interactions and then used for square wave voltammetry (SWV) and differential pulse voltammetry (DPV) measurements. The single-stranded probe SAM was then regenerated by a dehybridization step rinsing with a stream of water followed by immersion of the modified electrode surface in 70 °C water for 60s. A SWV measurement was then performed to confirm absence of hybridization signal. The dehybridization procedure was repeated until absence of signal was detected if required.

3.5 Electrochemical Measurements

Electrochemical measurements were performed using a µAutolab controlled by a PC with GPES 4.9 software (Ecochemie, B.V., Utrecht, Netherlands) and Autolab PGSTAT 128N with NOVA 2.1 software (Metrohm-Autolab B.V., Utrecht, Netherlands) and FRA32 module

for impedance measurements, using reference electrodes of Ag/AgCl (3M NaCl) for CHI and BASi and Ag/AgCl (3M KCl) for Metrohm and either glassy carbon (Metrohm) or platinum (CHI and BASi) counter electrodes and an electrode stand inside a Faraday cage. For square-wave voltammetry (SWV) measurements, 20 mL 10 mM Tris-buffer solution (10 mM Tris and 0.5 M Na_2SO_4 were adjusted to pH 7.5 using sulfuric acid) was used as electrolyte. For electrochemical impedance spectroscopy (EIS) measurements, $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$, 5 mM in each component, in 100 mM KCl solution was used. For this solution, potassium hexacyanoferrate(II) trihydrate (Sigma-Aldrich) and hexacyanoferrate(III) solids (Chem-Impex, Wood Dale, IL) were dissolved in aqueous potassium chloride (Sigma-Aldrich). A three-electrode cell was used with a gold disk electrode, an Ag/AgCl (3 mol/L KCl) reference electrode and a carbon counter electrode. The square wave voltammograms (SWV) was conducted at 200 HZ frequency and an amplitude of 40 mV within a scan range from -0.55 V to 0.0 V (potential step 1.95 mV) in the Tris-buffer (pH 7.5) at the room temperature. The peak-shaped measuring signals were smoothed with a level 4 Savitzky-Golay filter and baseline-corrected (moving average, peak width 0.004) using the GPES 4.9 and NOVA 2.1 software.

3.6 PCR conditions and agarose gelelectrophoresis

A synthetic 136-base and 57-base-DNA strand (sequence from the genome of Clostridium acetobutylicum [1855609-1855744]) was used as the template. The asymmetric PCR was performed with 1µL 100 ng of template DNA (136bp) /1 µL 5 µM (57 bp), 1 µL 10 mM dNTPs, 5 µL 10 µM of the primer C.aceto Forward, 0.5 µL 10 µM C.aceto Reverse, 0.5 µL 5 U Sawady-Taq-DNA-Polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany) and 5 µL 10x buffer in 50 µL reaction volume according to the manufacturer's instructions. The PCR was performed with the following parameters: 1 × 94 °C, 2 min; 30 × (94 °C, 30 s, 57 °C, 45 s, 72 °C, 30 s); 1 × 72 °C, 7 min.

The agarose gel electrophoresis was performed with a 2% [w/v] agarose gel in 1 × TAE-buffer (40 mM tris-(hydroxymethyl)-aminomethane, 20 mM glacial acetic acid, 1 mM EDTA) for 45 min at 80 V. The gels were stained with Gelred (Cambridge BioScience, UK) for 30 min and visualized on a Transilluminator UST-20M-8PC (Biostep, Jahnsdorf, Germany) under a Darkhood DH-40/50 (Biostep, Jahnsdorf, Germany) and photographed with an Olympus C-7070 digital camera with an Ethidiumbromide-filter (540-640 nm). All

DNA-concentrations were evaluated visually in the agarose gel (2%[w/v]) using the DNA-ladder (Fermentas GmbH, St. Leon-Roth, Germany) as a comparison.

3.6.1 Modification of fragment oligonucleotide

The modification was as the similar as the preparation of the reporter. For labeling the PCR product with non-labeled forward primer, 200 μL of PCR product solution (containing the single target strand fragment) were used without further purification. The fragment at its maximum concentration and the protective strand (10 μmol/L in the hybridization solution) were mixed and left for two hours at room temperature to allow hybridization. A solution containing 10 mmol/L osmium tetroxide and 10 mmol/L 2, 2'-bipyridine (forming [OsO_4(bipy)]) was then added and left at room temperature for 2 hours. After this period the excess of the osmium complex was removed by dialysis against pure Tris-buffer by means of Slide-ALyzer MINI Dialysis Units, 3500 MWCO (Rockford, IL, USA) at 4 °C for overnight. After the dialysis, the purified solution (containing the osmium-labeled target) was used for the hybridization experiments.

For labeling forward primer, it was the same procedure as reporter labeling. 60 μL 10 mM OsO_4(bipy) compound solution was added after the reaction of 50 μL 100 μM forward primer with 5 more thymine and 50 μL 100 μM protective strands (protector-1/ protector-2) solution for 2h at room temperature. Another 2h later, the labeling solution moved to a centrifuge tube with a filter to centrifuge for three times. After that, the purified labeled forward primer was used for asymmetric PCR, which product solution was used for following step without any further labeling.

3.7 Electrochemical detection at HMDE

The adsorptive stripping voltammetric (AdSV) experiments were carried out by adsorptive accumulation of the [OsO_4(bipy)]-forward primer or [OsO_4(bipy)]-PCR product at the given potential and for the given time at room temperature (22 °C), followed by a DPV stripping step at a hanging mercury drop electrode (HMDE) controlled by a 797 VA Computrace polarograph from Metrohm (Switzerland). The differential pulse voltammetry (DPV) experiments were performed in a electrochemical cell comprised of 20 mL acetate buffer (0.1 M, pH 4.8), HMDE, also an Ag/AgCl (3 M KCl) reference and a platinum counter electrode (Metrohm).

5 μL 20.83 μM forward primer with protector-1 and 5 μL 20.41 μM forward primer with protector-2 solutions were added separately into 20 mL acetate buffer (pH 4.64) each time. 200 μL solution of PCR product with each kinds of [OsO_4(bipy)] modified forward primer was diluted to 1000 μL, then10 μL diluted PCR product solution was taken to add separately into 20 mL acetate buffer for measurement. Besides, 10 μL solutions from 200 μL undiluted PCR product solution was also performed to add into 8 mL acetate buffer. Prior to the measurements, purified nitrogen or argon had been passed through the solution for a period of 300 s. Deposition time 60 s, deposition potential -0.9 V, pulse amplitude 50 mV, pulse time 40 ms, potential step 10 mV, sweep rate 25 mV/s.

4. Results and Discussion

4.1 Electrochemical sandwich hybridization assay for DNA detection using tetroxide-labeled reporter strands

4.1.1 Feasibility and control experiment

Fig. 14 outlines the design of the sandwich hybridization assay that we used to study the DNA detection by means of [OsO_4(bipy)]-labeled reporter. The recognition sites of capture probe and reporter strand in the target were separated by an 8-base gap to prevent electron transfer from the five terminal osmium tetroxide-labeled thymines through the double strands. As depicted in Fig. 14, the capture DNA probe with thiol group at 3' end was immobilized firstly on the electrode surface in order to form the capture probe SAM. After immersed in aqueous mercaptohexanol solution for 1 h, the probe-SAM-modified gold disk electrode was dipped for hybridization into a beaker containing the target and reporter solution. Then the target DNA was hybridized with the capture probe and reporter strands. After hybridized with target and reporter, the surface-confined reporter strands yield the observed response peaks. Electrochemical response of osmium tetroxide compound was monitored using SWV to detect the target DNA.

In this case, two recognition sites at the target strand are needed, one site for the capture probe, and the other one for the reporter strand. Hence, two hybridization events have to take place in order to observe any electrochemical response. We compared the electrochemical result, which two hybridization events are set in a same beaker, with the result that the hybridization was separately operated one step after another, found out that the results were no difference. Thus, we chose to let these two hybridizations take place synchronously to simplify experimental procedure and save experimental time. It performed by taking the probe-SAM-modified electrode into target and reporter mix solutions for hybridization.

Two different kinds of target oligonucleotide strands were used to check the feasibility of this sandwich hybridization assay: target caceto, target ivrp and its relative probe and reporter. Fig. 15 illustrates the detection of two kinds target in gold electrode surface. As expected, no signals were observed on gold electrodes with SAMs of mercaptohexanol and capture probes.

Small signal peaks showed up probably due to some low unspecific adsorption in the surface of gold electrode.

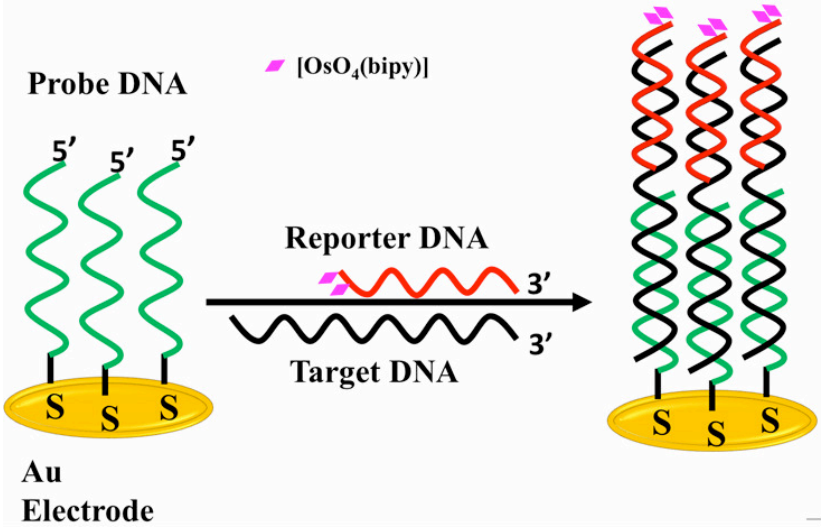


Figure 14: Scheme of the sandwich hybridization assay at the surface of a gold electrode.

The maximal voltammetric response was obtained after hybridization with [OsO_4(bipy)]-modified reporter and target strands. The peak at ca. -0.28 V could be addressed to osmium (IV/VI) in the complex with bipyridine and thymine glycol, which means this sandwich hybridization assay was well designed for target DNA strands detection by means of labeled reporter strand. This way, any modification of the target is not necessary, permits fast, cheap and easy sequence-specific DNA detection. Moreover, the fully non-complementary capture probes were used to do the control experiment. It can be seen from Fig. 16 that the fully non-complementary capture probes gave the smallest signals for both targets, which revealed the very low unspecific adsorption tendency of this assay. The error bars represent the standard deviation of measurements obtained in 3 independent series, each with a freshly prepared probe SAM.

G. Hartwich group has reported a high-sensitive DNA hybridization assay based on a reporter system, which gives electrochemical response after dsDNA formation without any target modification [163]. In this assay, proflavine was chose as a kind of parent molecule which was further modified with biotin moieties due to its special property of intercalation into dsDNA after hybridization in the gold electrode surface. The biotin moieties introduced at proflavine act as anchor groups to bind streptavidin/alkaline phosphatase (AP) conjugate.

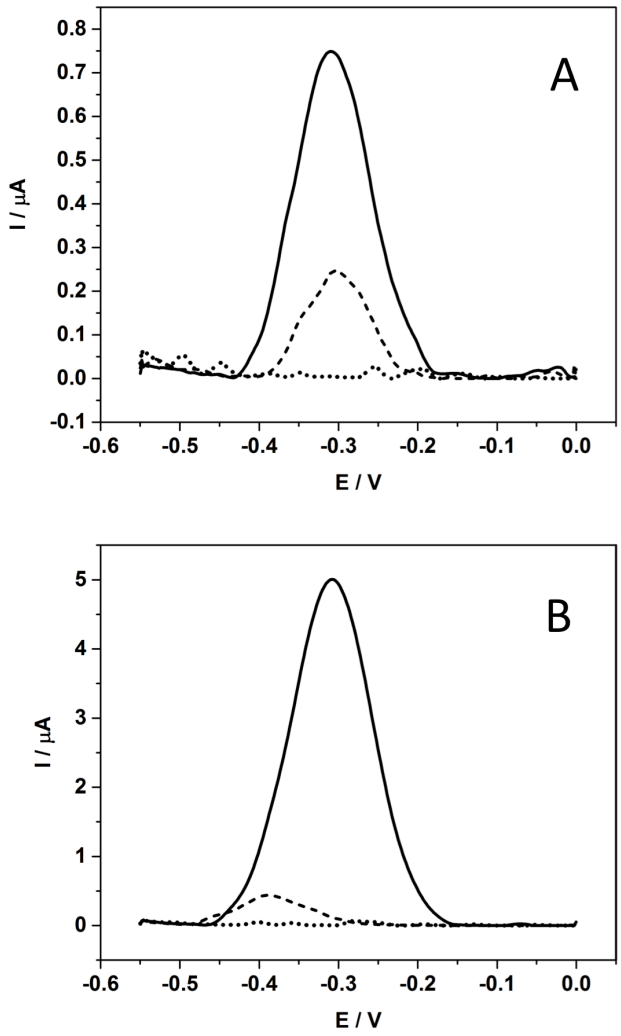


Figure 15: SWV signals obtained at gold disk electrodes modified with A) immobilized caceto capture probes and B) ivrp capture probes, representing the background with capture probes and MCH (dotted line), blank values obtained with SAM of immobilized capture probes and MCH after hybridization with reporter strands (dashed line), and signals obtained by 75 nM fully matching target strands hybridization with immobilized capture probes and reporter strands (solid line).

As we can see from the Fig. 17, hybridization was detected by oxidation of p-AP formed in the enzyme-catalyzed hydrolysis of p-APP (p-aminophenylphosphate).

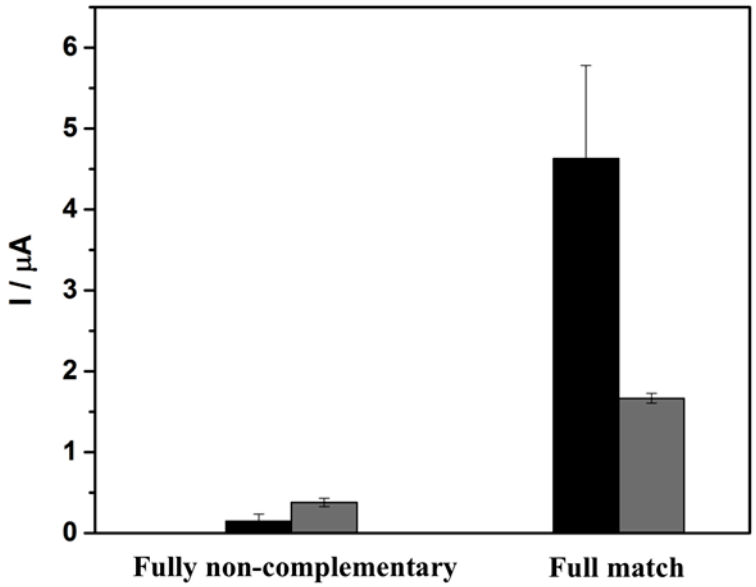


Figure 16: Hybridization signals of targets ivrp (black) and caceto (gray) with non-complementary and full complementary capture probes. The concentration of each kind of targets is 75 nM.

This method has many advantages. Owing to biotinylated proflavine, unspecific adsorption was avoided at the monolayer, and also prevents binding to ssDNA as proflavine exclusively binds to dsDNA. Since the labeling was operated after the hybridization with target strands, the target strands did not need any further modification.

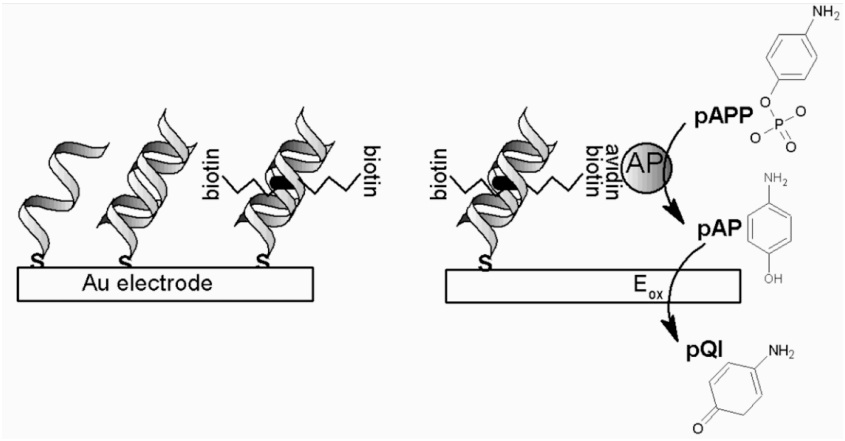


Figure 17: Proposed assay strategy for post-labeling of dsDNA using biotinylated proflavine and further binding of a streptavidin/alkaline phosphatase conjugate [163].

Furthermore, streptavidin could be conjugated with enzyme system, therefore, signal output is enhanced as expected. However, from the other hand, this approach seems more complicated.

Except for the synthesis of the compound of biotinylated proflavine for one week, biotin needs to incubate with avidin/AP conjugate to form specific biotin-avidin interaction. Additionally, the polyethylene glycol spacer was needed to improve solubility during incubation of biotin-avidin interaction. Moreover, the interface had to be designed to prevent as much as possible unspecific binding of the avidin/AP conjugate. All those propose make this design complex and more extra processes. Nevertheless, compared with their approach, our DNA hybridization assay looks more compact, simpler and efficient. With introduction of reporter strands, the target strands do not need more modification as well. Meanwhile, the labeling process can be easily performed and only for two hours at room temperature, the compound could be saved in dark for long time under -20 °C, the labeled DNA still can be used and have activity stored in 4 °C up to one month. The hybridizations of two parts can be performed at the same time and do not need extra incubation. Besides, this sandwich hybridization can also be well used to distinguish the mismatches. This DNA hybridization assay, thus, we think it is advantageous and potential if is explored in biosensor chips and amplification studies.

4.1.2 Effect of hybridization time

Fig. 18 exhibits the effect of hybridization time upon the square-wave voltammetric response of the targets hybridized with [OsO_4(bipy)]-modified reporter strands and the immobilized capture probes from 10 to 60 min at 50 °C. With increasing hybridization times, growing electrochemical signals were obtained. An increase can be obviously observed from 30 min hybridization for both two kinds of targets system. The hybridization process reached at a highest state for 45 min hybridization. After 45 min, a signal decrease was observed, presumably due to hybridization saturation between targets, reporters and catching probes. The trend in each series in this figure indicated that 45 min is indeed the optimal hybridization time.

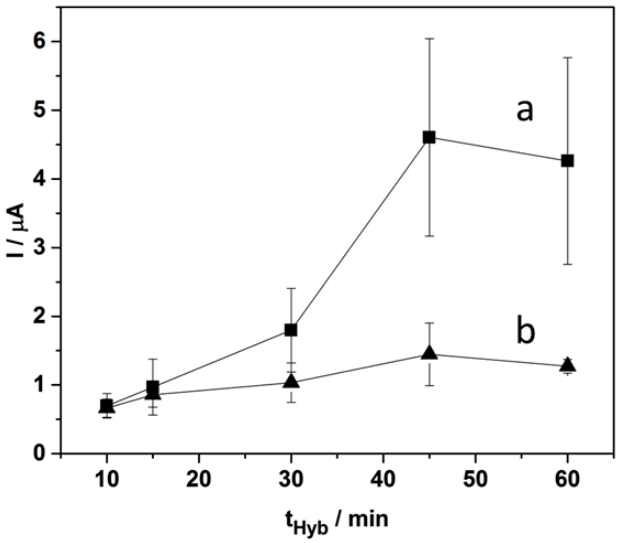


Figure 18: Effect of hybridization time upon the normalized SWV response of immobilized c.aceto probe (triangle) and ivrp probe (square) with their 75 nM target from 10 to 60 min at 50 °C.

4.1.3 Effect of hybridization temperature

We further investigated the effect of hybridization temperature. Fig. 19 shows how temperature affected the hybridization process.

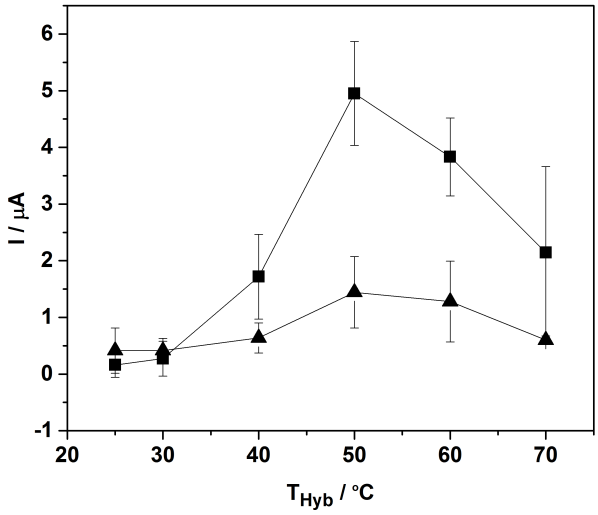


Figure 19: Effect of c.aceto (triangle) and ivrp strands (square) hybridization temperature upon the normalized SWV response at from 25 to 70 °C with 75 nM targets for 45 min.

It illustrates that elevated hybridization temperature had a positive effect up to 50 °C. At higher temperature, the signal height decreased rapidly, especially at temperatures higher around 70 °C, the disruption of the probe layer resulting at least in a loss of hybridization capability, as we observed earlier [164]. Furthermore, compared with the behavior of caceto strands, ivrp strand had more sensitive and higher signal response for hybridization temperature.

4.1.4 Effect of target concentration

Fig. 20 depicts the effect of target concentration upon the voltammetric response of the [OsO_4(bipy)]-modified reporter and catch probe strand. The signal heights increased with the target DNA concentration from 25 nM and reached a highest state at 150 nM and 200 nM for caceto and ivrp strands respectively are displayed in Fig. 19. However, no matter for caceto or ivrp strands, 75 nM was in the rising stage and most steady target concentration during hybridization process, hence, 75 nM target concentration was indeed the optimal concentration for exploring DNA / PCR products hybridization assay.

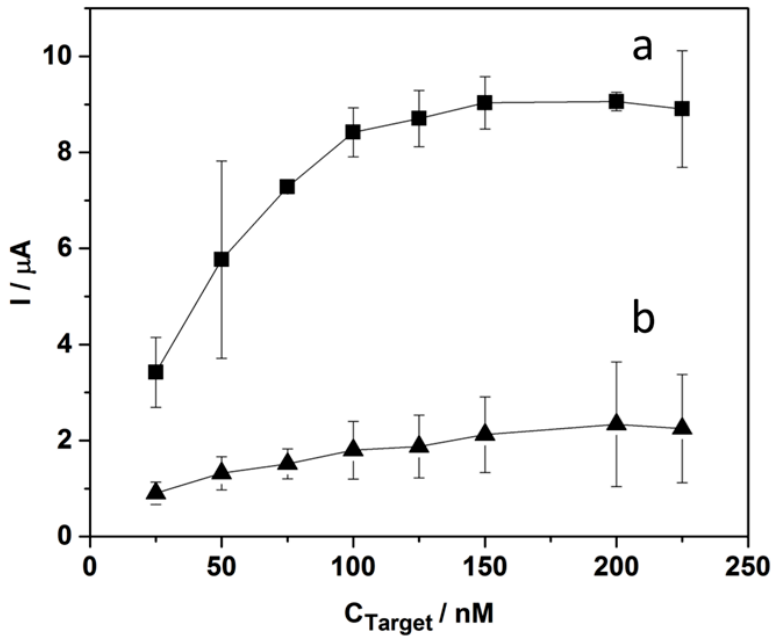


Figure 20: Effect of c.aceto (triangle) and ivrp target (square) concentration upon the normalized SWV signals hybridization with their probe strands from 25 nM to 225 nM for 45 min at 50 °C.

4.1.5 Detection of PCR product

Fig. 21 depicts square-wave voltammograms representing the detection of the asymmetric PCR products (c.aceto target strand was used as template) together with only SAM on the electrode surface. As expected, a large reversible peak at 0.29 V (vs. Ag/AgCl, 3 mol/L KCl) was found after hybridization with asymmetric PCR products and reporter strands for 45 min at 50 °C, and the capture probe layer (SAM) did not show any voltammetric response. The peak was caused by the reversible redox couple osmium (IV/VI). This voltammetric signal of the osmium tetroxide bipyridine-labeled reporter hybridized with PCR product indicates a good hybridization efficiency with the immobilized capture probe at 50 °C.

The sandwich electrochemical detection of DNA-sequences amplified by PCR (polymerase chain reaction) has been reported in a couple of publications in the last few years. In most cases the template is amplified by symmetric or asymmetric PCR, and avoided further modification, the main response signal comes from the signaling or reporter strands. C. L. Manzanares-Palenzuela and coworkers have reported a sandwich assay for quantitative analysis of genetically modified soybean with the event GTS-40-3-2, also known as Roundup

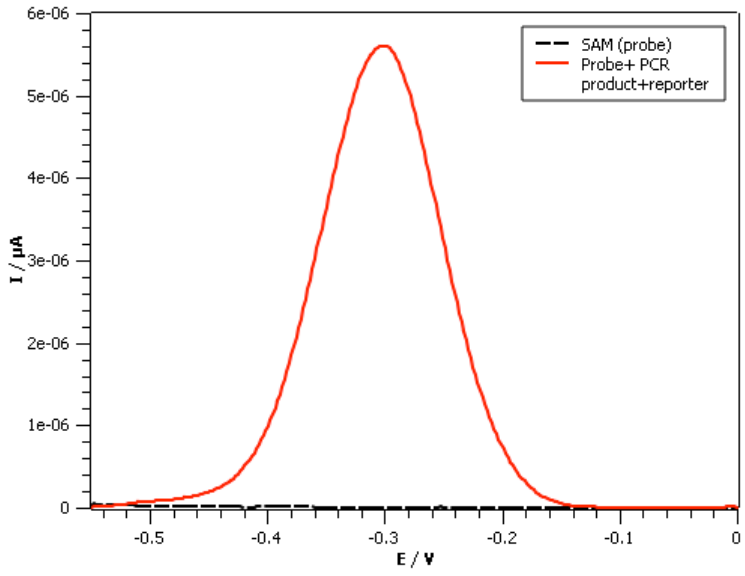


Figure 21: Square-wave voltammetric signals at gold disk electrodesin Tris-buffer with immobilized probe strands. Black dashed line: only SAM of immobilized capture probes and MCH. Red line: 5-times diluted asymmetric PCR products hybridized with immobilized capture probe and reporter strands.

Ready (RR) soybean, using magneto assay with electrochemical detection, coupled to DNA amplification by end-point polymerase chain reaction (PCR) [165]. R. Miranda-Castro research group has developed an electrochemical hybridization genosensor that combined with a previous non-ended-point PCR-amplification step to determine the DNA of L. pnemophila in environmental samples [166]. Y. Lei has described a sandwich sensing mode of electrochemical method for the sequence-specific detection of double-stranded PCR products of PML/ RARα fusion gene in acute promyelocytic leukemia (APL) [167]. However, to our knowledge, these methods suffer from some of the problems, for example, a denaturation step is necessary to separate the double strand (produced during the PCR) into two single strands, time-consuming cleaning steps are often needed, the synthesis of some kind of DNA sequence-labeling seems to be time-consuming and complicated. Besides, cost is another concern to be considered.

Compared with those problems of the sandwich electrochemical PCR detection mentioned above, our assay is more promising as it is more simple and low cost. The most important advantage is that it has a high signal-to-baseline ratio, therefore this proposed sandwich-like assay seems to can offer a high sensitive method for detection of PCR-amplified DNA hybridization, and analysis of real samples.

4.2 Detection of DNA single base mismatches on gold electrodes by means of sandwich hybridization assay and osmium tetroxide-labeled reporter strands

4.2.1 Determination of target DNA single base mismatches

Fig. 22 outlines the design of the sandwich hybridization assay that we used to study the influence of single base mismatches located in the target either in the recognition site of the capture probe or the reporter strand, or both. The recognition sites of capture probe and reporter strand were separated by an 8-base gap to prevent electron transfer from the five terminal osmium tetroxide-labeled thymines through the double strands.

Like described in part 4.1.1 Fig. 14, the target strands hybridized with capture probe and reporter strands at the same time and then the electrochemical response of osmium tetroxide compound was followed. Single base mismatches located in three positions in the target

strands, hence, the mismatch target was respectively named after 1-MM-Probe, 1-MM-Reporter and 2-MM if its position in the target DNA where hybridize with capture probe, reporter strand, or both.

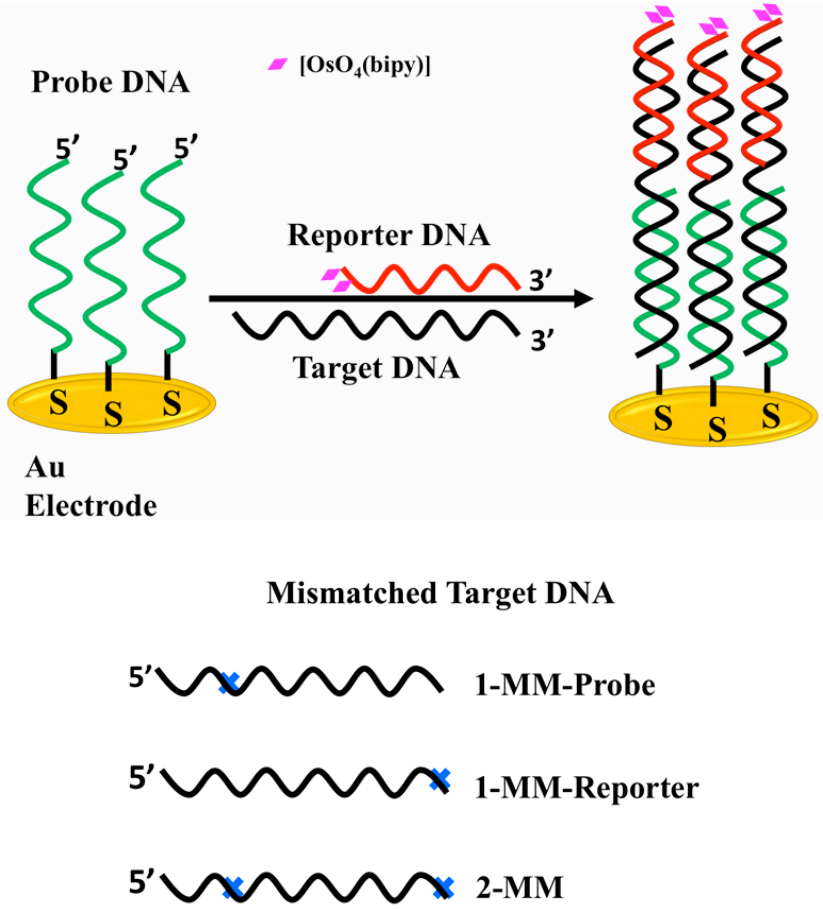


Figure 22: Scheme of the sandwich hybridization assay for DNA single base mismatches detection at the surface of a gold electrode. Single base mismatches located in three positions in the target strands (1-MM-Probe, 1-MM-Reporter and 2-MM.) were marked with blue X.

Fig. 23 illustrates the detection of mismatches that are located either in the capture probe or in the reporter strand section of the target. Although we used one and the same reporter sequence for both targets, the capture probe sequences were different. Surprisingly, the single base mismatch in the capture probe induced a very different hybridization behavior of the target. Whereas sequence ivrp behaved as expected, the caceto sequence yielded the greater response with the probe mismatch, and a much smaller signal with the full match. Also the reporter mismatch signal was smaller than in case of the fully matching caceto target. Only with two mismatches in both the probe and the reporter section, the hybridization signal was significantly lower than for the full match. In contrast, the target sequence ivrp yielded the

highest response without any mismatches. The reporter mismatch signal was considerably lowered and at a similar level as for the caceto sequence. This could be expected since the reporter sequences including the studied reporter mismatch of both caceto and ivrp were identical.

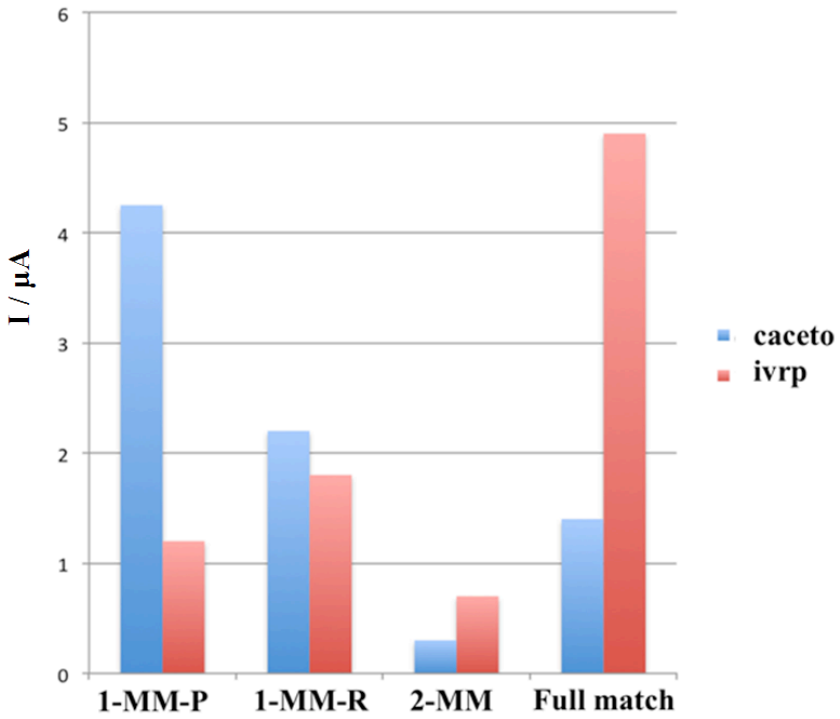


Figure 23: The detection of mismatches that are located either in the capture probe or in the reporter strand section of the two kinds target (caceto blue, ivrp red). 75 nM target strands (mismatch and fully matched) was used for hybridization.

On the other hand, the probe mismatch delivered a dramatically decreased response in comparison to both fully matching ivrp target and probe-mismatched caceto target. Having both mismatches in the ivrp target further lowered the hybridization response. The fully non-complementary capture probes gave the smallest signals for both targets, which revealed the very low unspecific adsorption tendency of this assay.

4.2.2 Effect of hybridization temperature

The findings in Fig. 24 reveal for caceto sequence that the probe mismatch would yield the highest response at all temperatures between 30 and 70 °C. Also the reporter mismatch in the caceto target gave larger response after hybridization between 40 and 70 °C compared with the full match. Both mismatches together, however, resulted in a dramatic decline in SWV

response. Due to the 8-base gap between capture probe and reporter strand recognition sites at the target, the both double stranded parts have to be considered as separate strands.

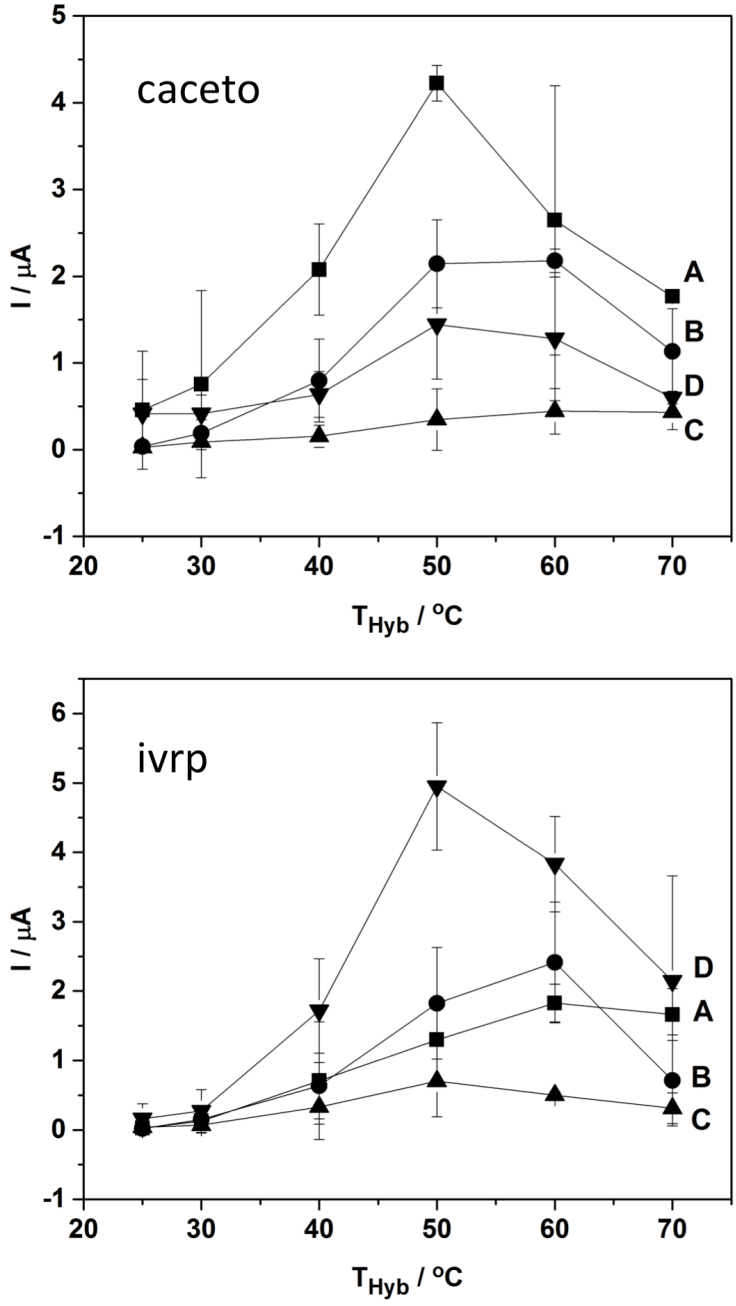


Figure 24: Effect of hybridization temperature upon the normalized SWV response of immobilized c.aceto and ivrp probe hybridized with their reporter and 75 nM (A) 1MM-Probe (near 5' end), (B) 1MM-Reporter (near 3' end), (C) 2MM, and (D) full match, respectively.

Latest reports reveal that the G-G mismatch (caceto) leads to much greater distortion than the C-C mismatch (ivrp) [168]. One consequence of this could be G-G-mismatch-induced DNA

bending that would decrease the distance of the distal osmium labels from to the electrode surface leading to increased voltammetric response. Thermodynamically, however, the effect of C-C mismatch is larger, i.e. the melting temperature of a short 13 bp duplex was lowered by 16 K, compared with 8.5 K for a G-G mismatch [169]. Interestingly, the temperature effect (Fig. 23) is much larger for the ivrp full match than for the caceto fully matching target, with 25-fold and 3-fold increase, respectively, when going from 25 to 50 °C hybridization temperatures. The optimal hybridization temperature was 50 °C for both fully matching targets.

4.3 Electrochemical detection of PCR products by means of [OsO_4(bipy)]-labeled primers

A schematic representation of the detection procedure is illustrated in Fig. 25. The whole process includes three steps: PCR, hybridization at electrode surface, and voltammetric detection. The asymmetric thermocycler PCR with labeled primers was followed by hybridization of the obtained labeled PCR products with the thiol-linked capture probes at the surface of gold electrode. After hybridization, the [OsO_4(bipy)]-labeled PCR products yielded the observed SWV peaks as analytical signals.

The classic symmetric PCR provides an optimal yield of the double stranded amplicon, raising the concentration of one primer, the (now asymmetric) PCR yields ssDNA besides dsDNA. Here, we performed the asymmetric PCR with a 10-fold excess of the forward-primer to get ssDNA for the following hybridization step. To avoid the need for post-amplification sample treatment, here, the forward primer was designed to have 5 more thymines (T5) at 5' end, and then labeled with [OsO_4(bipy)] by means of protective strand which our group had developed earlier [1]. After the asymmetric PCR, the PCR products amplified using the labeled forward primer were tagged with [OsO_4(bipy)] tails at each end of the amplicon can be used for direct hybridization with a gold electrode surface-confined probe, allowing for rapid and direct electrochemical detection.

In order to make sure the protective stand was replaced after DNA extension, a two mismatches-containing protector (protector-2) was also designed to protect the forward primer, besides the complementary protector (protector-1).

After PCR process, the PCR product was collected and used to hybridize with the capture probe immobilized in the surface of gold electrode. Unexpectedly, no SWV signal was obtained according to the scheme. Since the asymmetric PCR is usually prone to limited efficiency, we made an asymmetric PCR but labeled the PCR product with [OsO_4(bipy)], rather than the forward primer, and then used it for hybridization assay to check if electrochemical response of the asymmetric PCR with 10 fold excess of the forward primer could be gained.

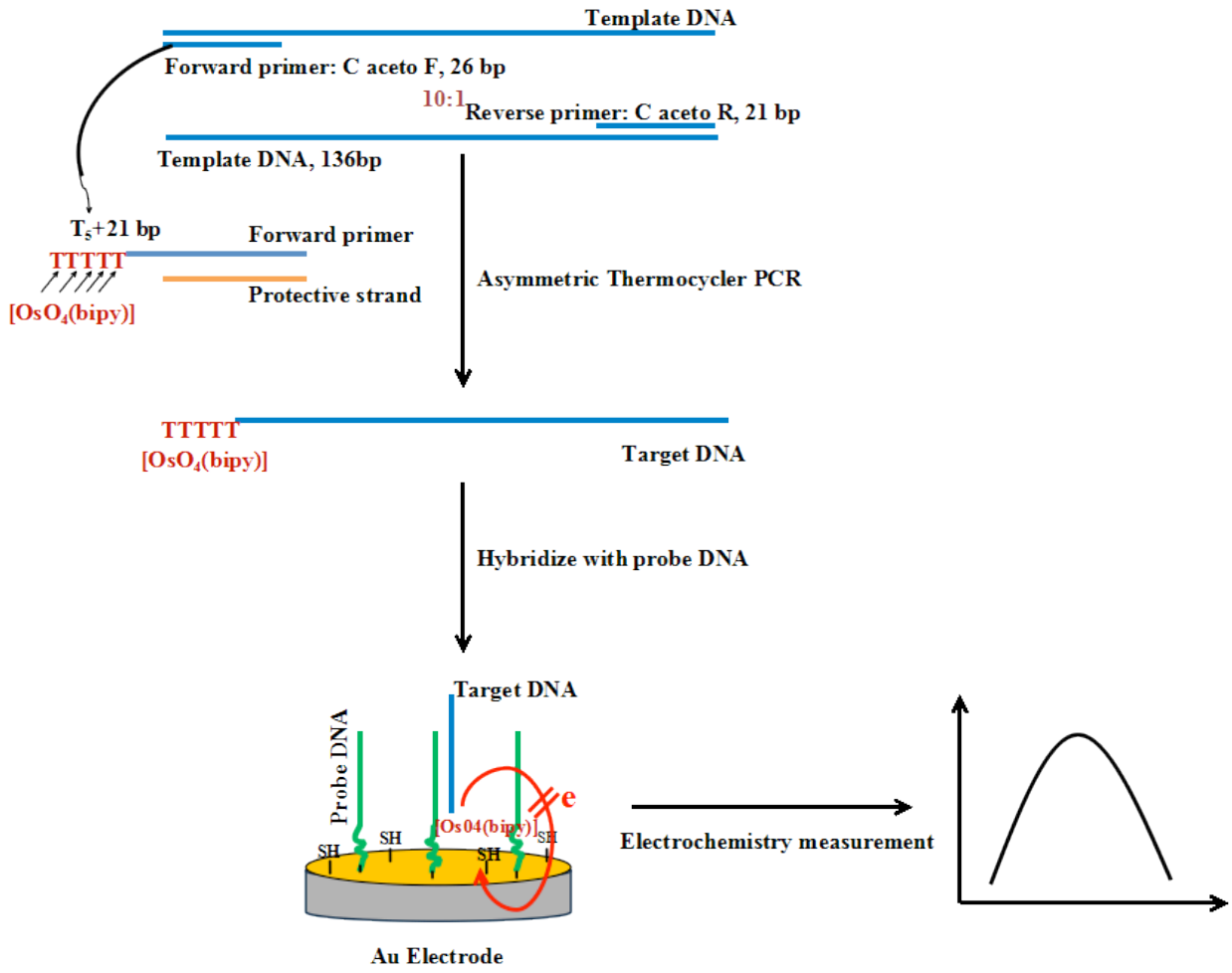


Figure 25: Scheme of the electrochemical detection of PCR products with [OsO_4(bipy)]-labeled forward primer at the surface of a gold electrode.

Fig. 26 shows SWVs representing the detection of the single-stranded PCR product of [OsO_4(bipy)]-labeled forward primers and PCR product of non-labeled forward primer hybridization with immobilized capture probe strands. Here, the protector-2 (two mismatches protector) was utilized to protect the forward primer. For the asymmetric PCR with non-

labeled forward primer, the PCR product was labeled in a separate step after PCR procedure. The large voltammetric signal of the osmium tetroxide bipyridine-labeled PCR product (curve b in Fig. 26) not only indicates a good hybridization efficiency with the immobilized capture probe in the electrode surface, but also implies something wrong in asymmetric PCR after the forward primer was labeled (curve a in Fig. 26).

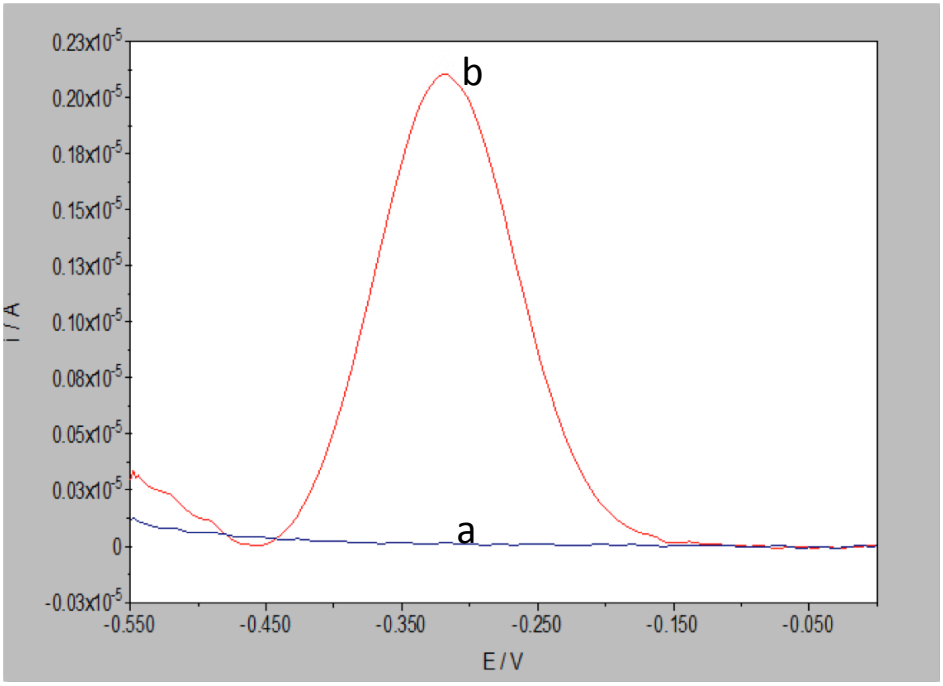


Figure 26: SWV signals of comparing PCR product hybridization at gold disk electrodes. Curve a: PCR products with protector-2 labeled forward primer; Curve b: PCR products with non-labeled primer and then were labeled with [OsO_4(bipy)]. The asymmetric *PCR product was diluted 1:5 with Tris-buffer for hybridization.*

In order to make sure the forward primer was successfully labeled and still intact and included in the PCR products after the PCR process, the techniques of DPV on the hanging mercury drop electrode and gel electrophoresis were used. Fig. 27 shows the DPV responses of [OsO_4(bipy)]-labeled forward primer with two kinds of protective strands, protector-1 (a) and protector-2 (b). Each kind of forward primers was added into cell for four times. The peak height of signal for first addition was 1.5 µA (with protector-1) and 7.28 µA (with protector-2). This result illustrates well that both of the forward primers were indeed labeled with [OsO_4(bipy)].

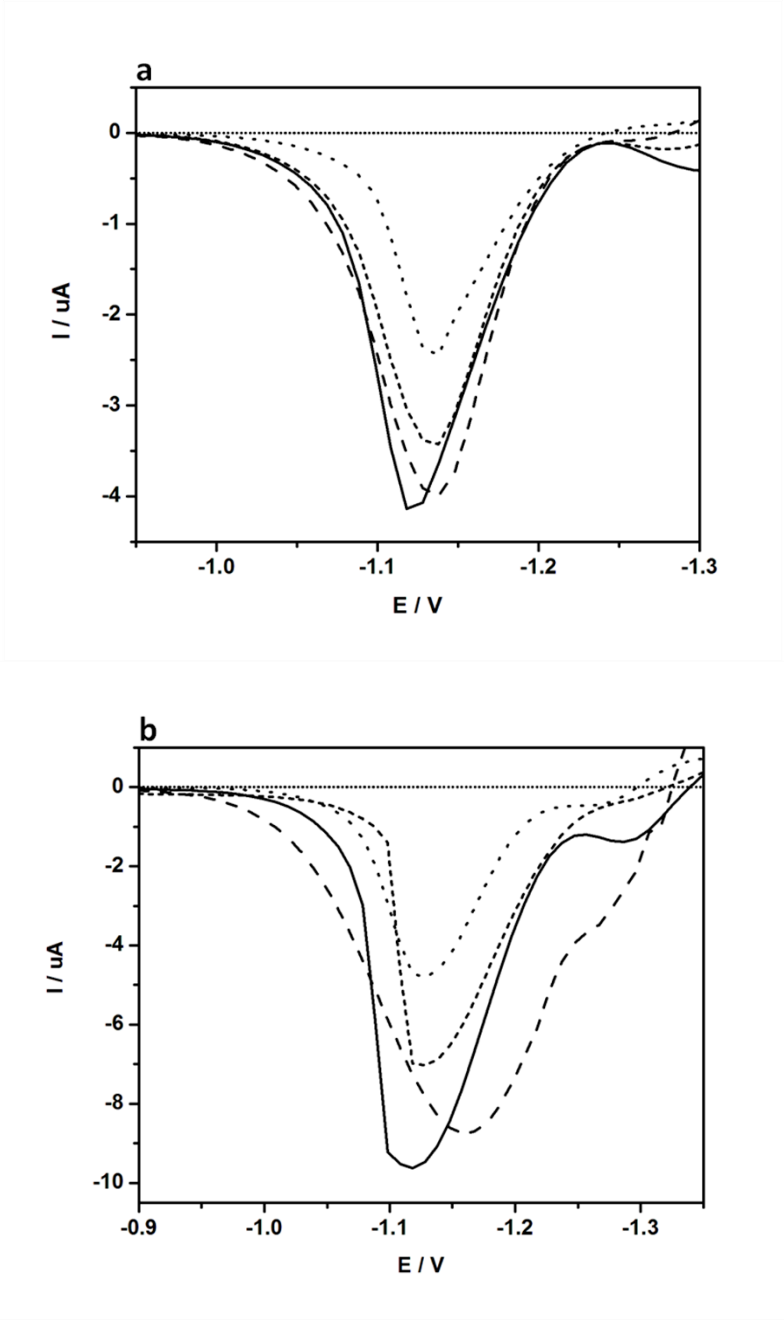


Figure 27: DPV stripping signals of [OsO_4(bipy)] modified forward primer, a and b is treated with complementary and two mismatch complementary strand, respectively. From short dot line to solid line is blank, first, second, third and fourth addition of forward primer solutions, respectively. The concentration of forward primer with protector-1(a) in cell is 5.21 nM, 10.42 nM, 15.63 nM and 20.84 nM after each addition. The concentration of forward primer with protector-2 (b) in cell is 5.1 nM, 10.2 nM, 15.3 nM and 20.4 nM, respectively.

Meanwhile, Fig. 28 displays the electrophoresis analysis of asymmetric PCR products with labeled (with two kinds protective strands) and non-labeled forward primers at agarose gel. It shows that the PCR product obtained with a non-labeled forward primer (lane 2) and labeled

primers with protector-2 (lane 3) are visible on the gel, surprisingly, PCR product containing labeled forward primer with protector-1 (lane 4) shows a much weaker trace. The polymerase was inhibited by the perfectly matching protector-1.

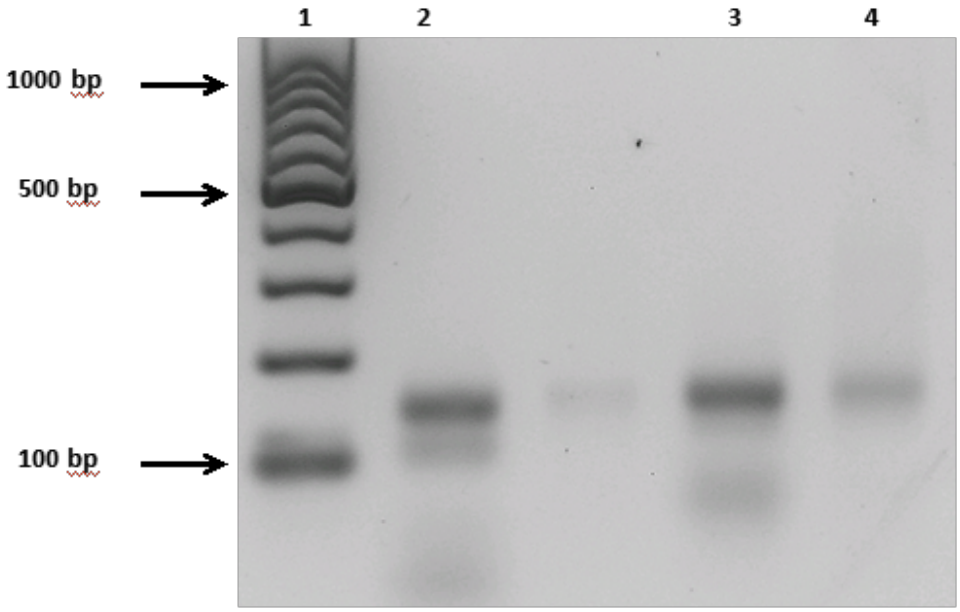


Figure 28: Gel electrophoretic results of PCR product. 2% [w/v] agarose-gel stained with Gelred; lane 1: Gene Ruler™ 100 bp DNA Ladder; Lane2: Asymmetrical PCR-product with unlabeled forward primer. Lane 3+4: Asymmetrical PCR-product with [OsO_4(bipy)] labeled forward primer protected by two mismatch (protector 2) and complementary strands (protector1), respectively.

These findings were supported by adsorptive stripping voltammetry at the HMDE to check the availability of [OsO_4(bipy)] labels in the asymmetric PCR products. In comparison to the response of short [OsO_4(bipy)]-forward primers, considerably smaller peak signals were obtained for the much longer PCR products as shown in Fig. 29. The AdSV response of the two kinds of PCR products was 0.544 nA and 5.77 nA, respectively. It seems that, after the PCR procedure, the signal of [OsO_4(bipy)] has a dramatical loss, probably this is the reason why we can not get obvious SWV signals in the surface of gold electrode. However, the peak of PCR with protector-2 is much higher than protector-1 (around 10 times larger), presumably the protector would not replaced during the extension process, after all, if the protector contains a few mismatches and can be replaced effortlessly. Since osmium tetroxide complexes still have electroactive ability after heating for DNA dehybridization temperature [140], therefore, we also explored to heat the [OsO_4(bipy)]-labeled forward primers with protector in a beaker at 65 °C for 3min to remove the protector, and then put the modified

primers for asymmetric PCR, unfortunately, the SWV and DPV results were as similar as untreated with heating.

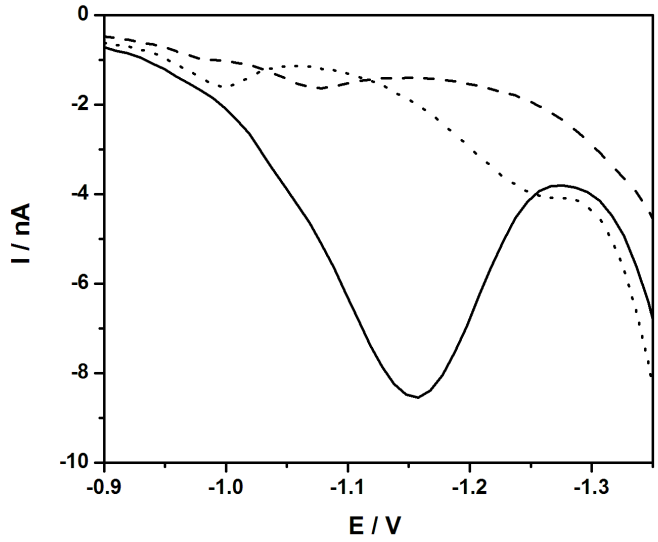


Figure 29: DPV stripping signals of PCR product with [OsO_4(bipy)] modified forward primer protected by complementary strand and two mismatch complementary strand, respectively. The dash line is the PCR product with protector-1, solid line is protector-2, and dot line is blank.

Except for heating the modified forward primer to solve the issue of protective strands, we also tried the following possibilities (the protector-2 was used in the following procedure):

1. Perform symmetric PCR with labeled forward primer, and then heat the PCR product at 95 °C for 10 min to denature the dsDNA to ssDNA; The results were negative;

2. Label forward primers without protective strands to check whether the PCR would happen, it turned out impossible;

3. Try different concentration ratio of forward and reverse primers, the gel electrophoresis in the Fig. 30 could tell that 10:1 is the best ration between forward and reverse primers, and furthermore, it also demonstrated that protective strand is necessary in the labeling procedure.

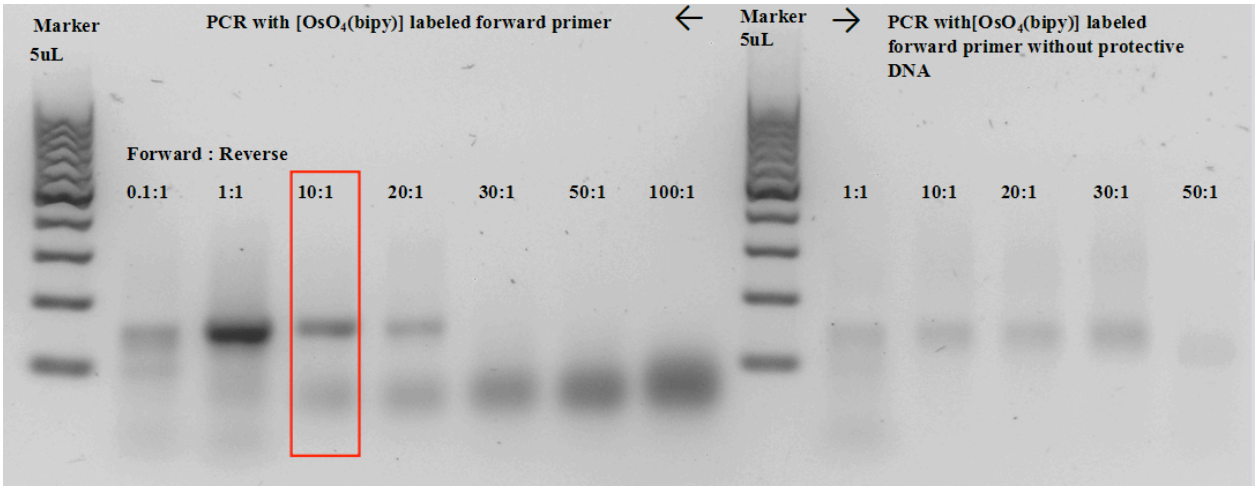


Figure 30: Electrophoresis results of different cycler PCR products in thermo cycler.

4. Due to the response of [OsO_4(bipy)] in the forward primer sharply declined after PCR process, we wondered whether the temperature during denaturation would have any influence on the activity of [OsO_4(bipy)], hence, we heated the modified forward primer solution at 94 °C for 2 min. It can be seen in Fig. 31 that after heating, the DPV peak signal declined by almost 50% (5.1 nM forward primer (with protector-2) in acetate buffer), nevertheless, the signal is still approximately 1000 times higher than for the PCR products. Therefore, the high temperature during the PCR process is probably not the main reason why the response of [OsO_4(bipy)] dropped rapidly.

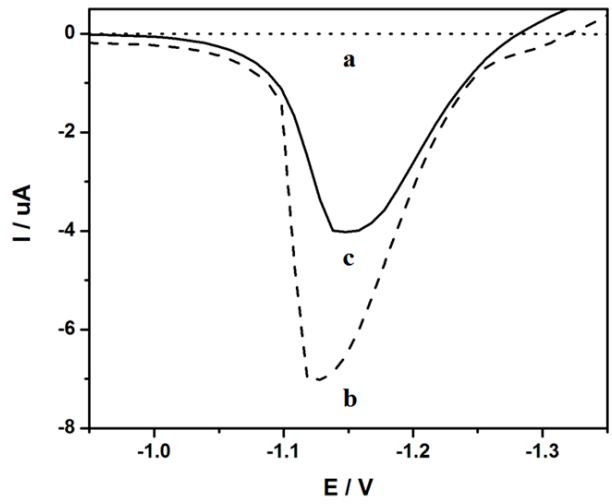


Figure 31: DPV stripping signals of [OsO_4(bipy)]-labeled forward primer with protector-2 before and after heating. a: blank, b: before heating, c: after heating.

4.4 Strand-displacement and hybridization efficiency considerations for the Tethered Surface of the hybridization Sandwich Assay

The work in this chapter was cooperatively carried out with our colleagues in the research group of Prof. G. U. Flechsig (State University of New York, USA). H. Jodab, A. Sedovab and K. Biala have designed the oligonucleotides used in the assay that as the same as the scheme in chapter 4.1.1 (Fig. 14), and optimized the experimental conditions, including surface probe density, hybridization temperature and time, so that a sensitive, fast electrochemical DNA sequence detection assay can be obtained. This cooperation result has been published.

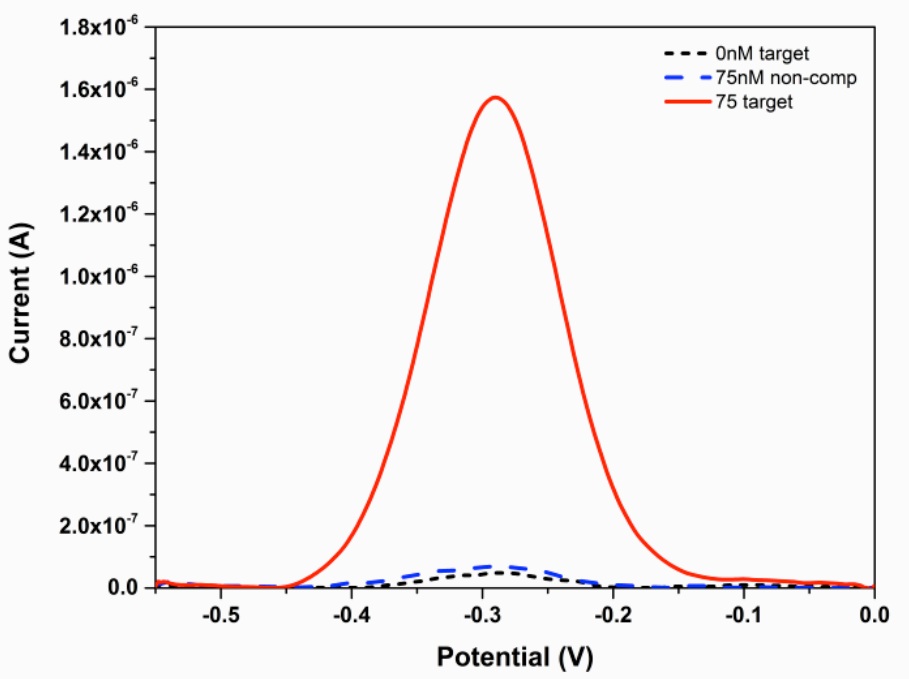


Figure 32: SWV response of reporter assay for complementary target (red solid line), compared with blank solution (black dotted line) or with a solution containing non-complementary DNA (blue dashed line).

In order to further investigate the sandwich assay, which schemed in chapter 4.1 and 4.2 (Fig 14 and 22), and also to make it fully applicative for the future real-time detection of DNA strands and PCR product in a more simple and smaller device, our colleagues redesigned some of the oligonucleotides and optimized the experimental conditions on the base of the works in chapters 4.1 and 4.2. Fig. 32 shows the response signal of this hybridization assay for the complementary target-strand as opposed to a non-complementary strand and as

compared to the baseline signal due to nonspecific interactions with the labeled reporter. Signal-to-baseline ratio is approximately 32:1 by means of 75 nM concentrations of both target and reporter. Achievement of maximal signal height, minimal nonspecific signal, reduced time requirements for hybridization, consistent reproducibility of response and sensitivity at concentrations of target below 10 nM required optimization of protective-strand design, probe density, and backfill application.

4.4.1. Effect of surface probe density and backfill

The high and low-density probe SAMs were performed to explore the effect of surface probe density and back fill of this hybridization assay. The high-density probe SAM was performed by immobilization of 1 μM thiolated probe/high-salt buffer solution for 18 hours, and then a 1 hour application of 1 mM MCH, while the low density probe SAM was created by immobilization of 200 nM probe solution in the low-salt buffer for 30 minutes, and then with a 90 minutes MCH application. For the high density probe SAM, after the first hybridization, a low SWV response signal was obtained. Besides, it is interesting that, after a series of the first hybridization and dehybridization, the final hybridization response were found to make a large jump, and then to stabilize at a maximal height after about 3 consecutive hybridizations. However, the results show that, using the lower density probe SAM could eliminate this unresponsive first hybridization result, but showed a slight decrease in signals of subsequent hybridizations. Meanwhile, the EIS measurements results demonstrated that there was a large Rct of the high density SAM, and then the Rct decreased after MCH application, and decreased further after a series of hybridizations. But for low density SAM, the EIS showed a low Rct. After MCH application, the Rct increased somewhat. Fig. 33 shows the effect of multiple hybridizations on peak signal height for the high and low density versions as a function of number of consecutive hybridization reactions with the same DNA probe SAM, along with Nyquist plots from EIS.

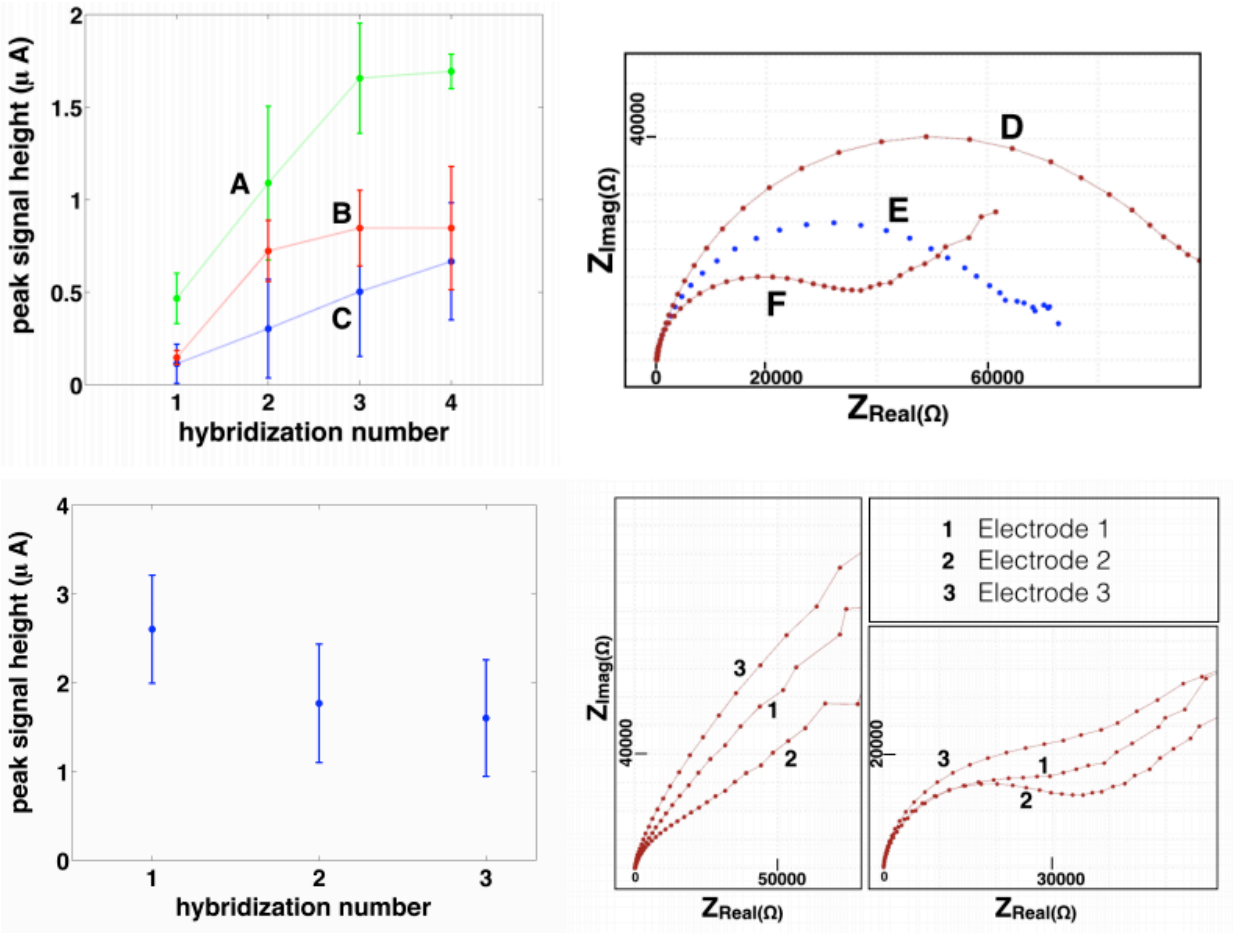


Figure 33: Effects of probe density. Top: High-density probe. Left-peak signal response (SWV) after multiple hybridizations: 30 minute (A), 15 minute (B), and 5 minute (C) hybridization times, all performed at 50 °C, results are mean and error bars for three independent experiment series each with a new probe SAM; right-Nyquist plots of EIS measurement after DNA immobilization (D), after further MCH application (E), and after a series of repeated hybridizations (F). Bottom: Low-density probe, left-peak signal response (SWV) for 3 consecutive hybridizations of 15 minutes each, all performed at 50 °C, results are mean and error bars for three independent experiment series each with a new probe SAM; right-Nyquist plots of EIS measurement for three low-density SAMs before and after MCH application. All hybridizations used 75 nM each of target and reporter.

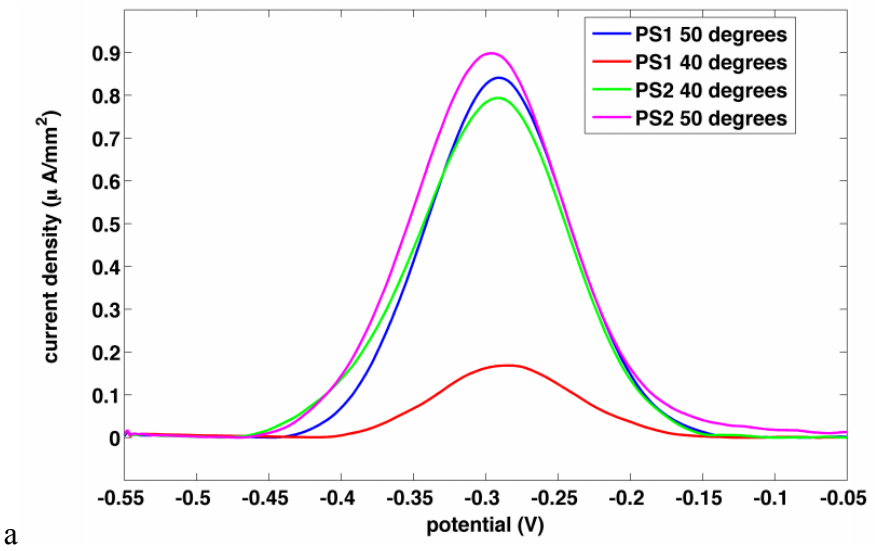
The lower initial signal response and the increased signal in the following repeated hybridizations for the high density SAM is potentially due to reduced hybridization efficiency for the large signaling-duplex at high surface densities, and a potentially annealed or adsorbed probe conformation as a result of the multiple adenine bases used as a spacer. These effects are reduced by hybridizations possibly due to a combination of SAM conformational changes, such as upright positioning of the probe strands and reduction of adsorption of the spacers, and of removal of some of the immobilized DNA strands from the surface. It has been found

that DNA has an extremely strong adsorption affinity for gold, which can even outcompete the thiol binding; adenine has been found to have the highest affinity [170]. Both our reporter strand and its protector have a high number of adenines (6 and 12 As, respectively), and may displace some thiolated probe molecules. The use of an adenine spacer has been suggested as an alternative to an alkane-thiol backfill due to this affinity [171], but it is also possible that our poly-A spacer is creating a less permeable, annealed layer, and prevents upright positioning and hybridization of the probe recognition sequence initially. Repeated exposure to a heated solution and electrochemical potential may also affect the SAM. Further optimization of both probe and backfill density, together with spacer design will be pursued in future efforts.

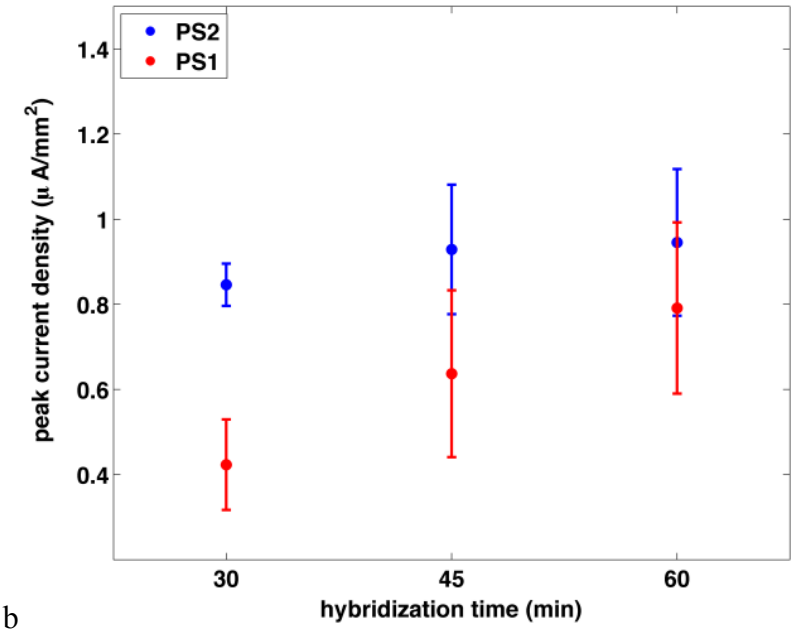
4.4.2. Design of protective strand design and effects of reduced melting temperature of immobilized probe duplexes

Protective-strand design is an important part in this hybridization assay, as the protective strand must be replaced by target strand to form another more stable duplex. Furthermore, the high temperature used in this hybridization would also affect the displacement between protective strand and target strand. Therefore, the protector/reporter duplex must be melted before the probe/target duplex. While solution-based calculations have been shown repeatedly to offer accurate predictions for solution melting temperatures (Tm), it has been found that hybridization efficiency, conformational ensemble composition, and melting temperature can all be altered in numerous ways at the tethered surface [172,173]. Previous studies have found that DNA duplex melting temperatures at tethered surfaces can be significantly reduced as compared with solution temperatures [174]. Estimates for the adjustment to be made to the solution-based melting temperature have been proposed to be as much as 20 °C [175]. Two protective strand were designed in this work: one with a calculated melting temperature about 20 °C lower than the solution-calculation for our probe/target duplex, which is named after PS1, and the other one, called PS2, with a calculated melting temperature about 15 °C lower than the reduced probe/target Tm.

Fig. 34 demonstrates the SWV signal response of this hybridization assay with those two kinds of protective strands. The value of the solution Tm was calculated by the DINMelt Web Server (http://unafold.rna.albany.edu/?q=DINAMelt) is about 82°C for the probe/target duplex, 73°C for target/reporter duplex, 56°C for PS1 and reporter, and 46°C for PS2 and reporter, respectively.



a

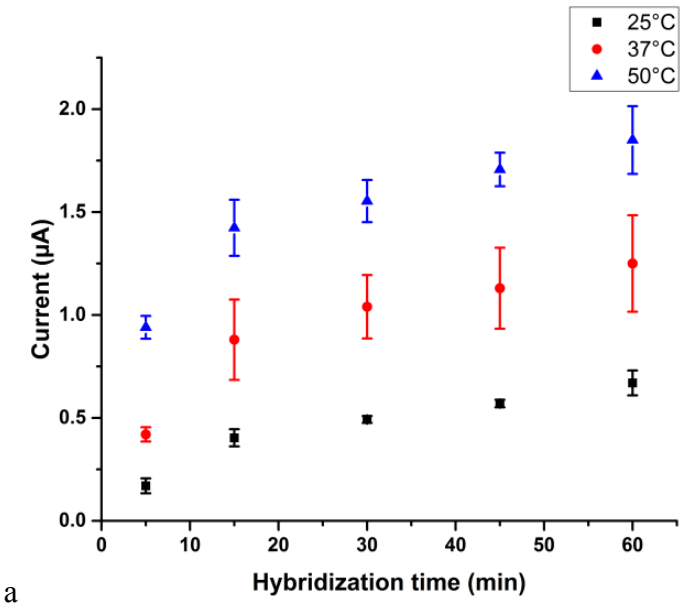


b

Figure 34: Effects of protective-strand melting temperature. a: SWV signal response using protective-strand 1 (PS1) and protective-strand 2 (PS2) at 40 and 50°C. b: Peak SWV signal for varying hybridization times using PS1 or PS2. Hybridization was performed at 50°C. All hybridizations used 75 nM each of target and reporter.

As can be seen in Fig. 34a, the SWV response signal was low for hybridization at 40 °C by means of PS1, while with the PS2, the height of hybridization signal at 40 °C was no big difference with the 50 °C signal for PS1. The maximum signal was achieved at 50 °C with PS2. Under 60 or 70°C, the PS1/report duplex would be melted, and lead to a more full hybridization. However, the signals begin to drop at 60 °C and decreased by nearly half of the

maximum at 70 °C, this potentially due to the melting of target/reporter/probe at the tethered surface.



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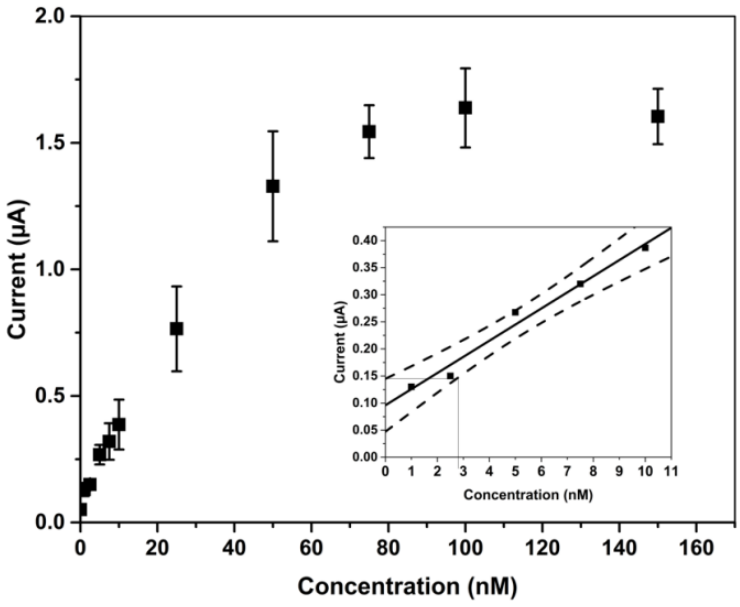

b

Figure 35: a: Dual effects of temperature and hybridization time on peak SWV signal height. Target and reporter solutions were each 75 nM. b: concentration calibration for the sandwich assay. Peak signal (SWV) measured after 15 minute hybridization at 50°C.

Furthermore, using PS2 could accelerate the strand displacement and hybridization kinetics. Fig. 34b shows that a near-maximal signal could be attained fora 30 minutes hybridization. As can be seen Fig. 35a, using protective strand 2, a substantial signal response can be achieved

after a 15 minute hybridization, even at room temperature, the signal response still can be expected within 15 minutes. At 50 °C, a large signal response is attainable in only 5 minutes.

4.4.3. Sensitivity of optimized hybridization assay

Using protective strand 2 and an optimizedsurface SAM, which is a stable high density SAM after several repeated hybridizations, we tested the sensitivity of this sandwich hybridization assay. Fig. 35b shows SWV signal height response to different target concentrations, together with a limit of detection (LOD) calculation using the prediction-band method. 75 nM report strand was used for target strands. Our estimated lower LOD was determined to be approximately 2.8 nM.

5. Summary and Outlook

This study demonstrates that the electrochemical detection of sequence-specific DNA with reporter strand which covalently modified with [OsO_4(bipy)] is possible. In this assay, the labeling of the reporter strands has been performed using protective strands to preserve the recognition site of these single strands for hybridization with the target strands. The target strands hybridized with both immobilized capture probe and reporter strand with electroactive labels to yield electrochemical response. We have found that, with optimization of protective-strand design, surface-probe density, and hybridization temperature, we are able to attain a sensitive, fast electrochemical DNA sequence detection assay that does not require high hybridization temperatures. In fact, a clearly detectable signal can be achieved at room temperature.

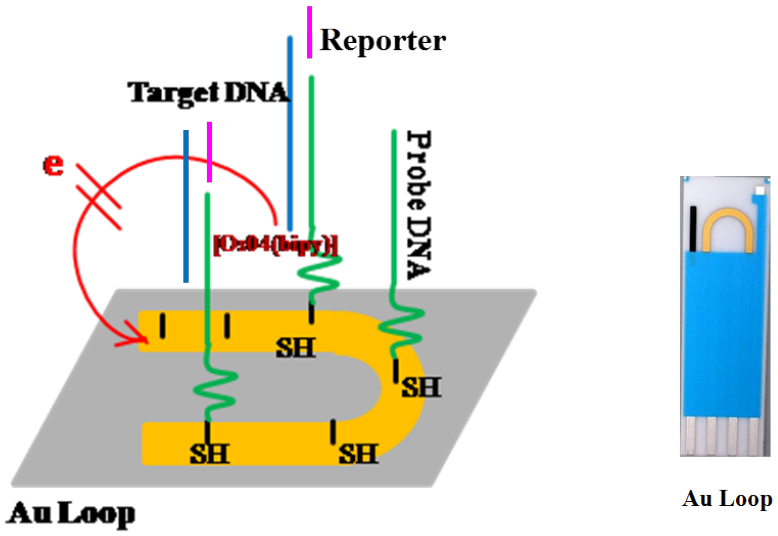

Figure 36: Schematic illustration of electrochemical detection of DNA hybridization using [OsO_4(bipy)] labeled reporter DNA on gold loop electrode.

Furthermore, using a mildly heated hybridization solution, a high signal is achievable in five minutes. This assay does not involve chemical labeling of the DNA analyte, it paves the way for a rapid, field-level genetic screen that can ultimately be ported to a miniaturized device that would not require special training to utilize. Furthermore, this sandwich-type assays can also discriminate single base mismatches in the capture probe section, or the reporter strand section, or both due to their different thermal behavior.

Due to the introduction of reporter strands, the modification of the target strands was avoided. We think that this approach of sequence-specific DNA detection is very useful under the some complicated design requirement or in elaborate real-time and label-free hybridization assay. We initially investigated to perform this sandwich-type assay on the gold loop (scheme in Fig. 36) by means of heating control via a Thermalab (Gensoric) and the software (PSTrace) controlled by a computer. Herein, 0.5 µM target strand was used to hybridize with 0.3 µM capture probe and 0.5 µM report strands in 2000 µL hybridization solution for 1 hour at 30 °C (Stirring at 1500 rpm during hybridization step).

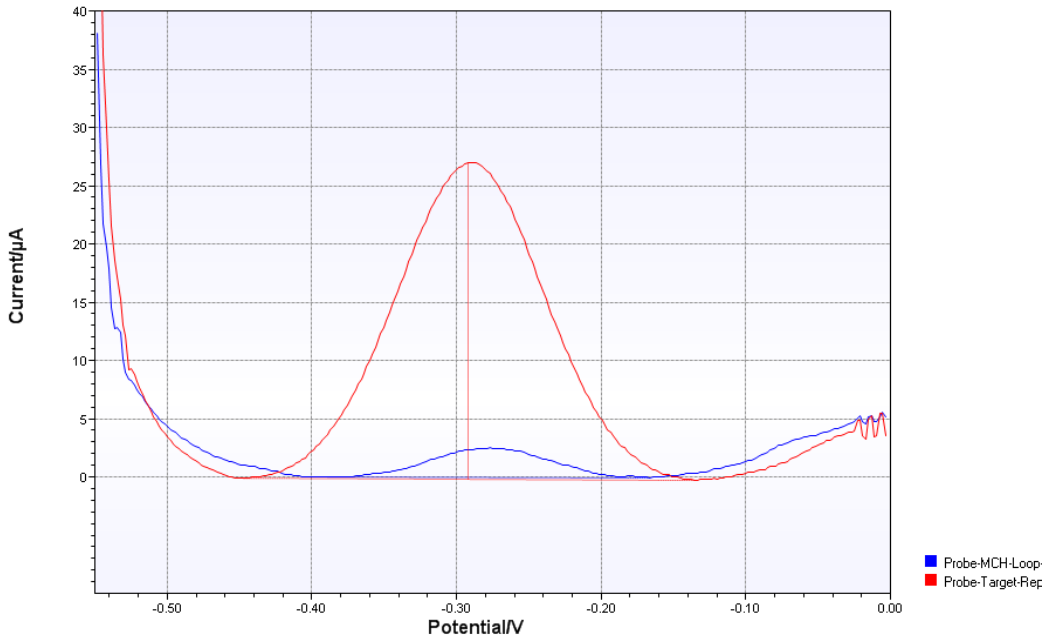

Figure 37: SWV signals of the immobilized probe (blue), and caceto probe hybridized with 0.5 µM cacetotarget and reporter (red) for 1h hybridization under 30 °C controlled by Thermal Lab.

Fig. 37 displays the SWV signal at gold loop in Tris-buffer with immobilized probe strands. 0.5 µM target was used in the hybridization with capture probe and reporter strands. The red curve, which obtained after hybridization, shows 10.8 times higher signal response compared with non-hybridization (blue curve). The result was better than expectation, however, it would need further investigation. The combination of DNA hybridization detection and heating electrode is advantageous, as it could realize elimination of target modification, temperature control and real-time detection at the same time, which makes it promising for further development in miniaturization biosensors or micro-fluidic chip and its spread in real-sample

practice. Moreover, combining PCR or convective PCR with electrochemical hybridization detection and chip would be proposed to a new kind of compact genetic testing instruments. In addition, the electrochemical approach based on this kind of sandwich structure can be further extended to other DNA-based systems to detect and quantify wider ranges of analytes.

Furthermore, we have investigated the electrochemical detection of PCR products by means of [OsO4(bipy)]-labeled forward primers. The result of DPV response at HDME shows the forward primer was well labeled with [OsO4(bipy)]. Surprisingly, no satisfactory result could be found from SWV and DPV response after asymmetric PCR procedure. Since the protective strand is needed for labeling of primer with osmium tetroxide compound to protect the sequence, which is necessary for annealing during PCR process, it would make sense to separate the primer and its protective strand after labeling to prevent the inhibition in the PCR. However, the initial investigations in the separation of primer and its protective strand are not satisfactory. The amplification of target without separation from its two mismatches protective strand primers could be seen successfully in gel electrophoresis, unexpectedly, this kind target from PCR product could not be detected electrochemically, as we described earlier [176,177].

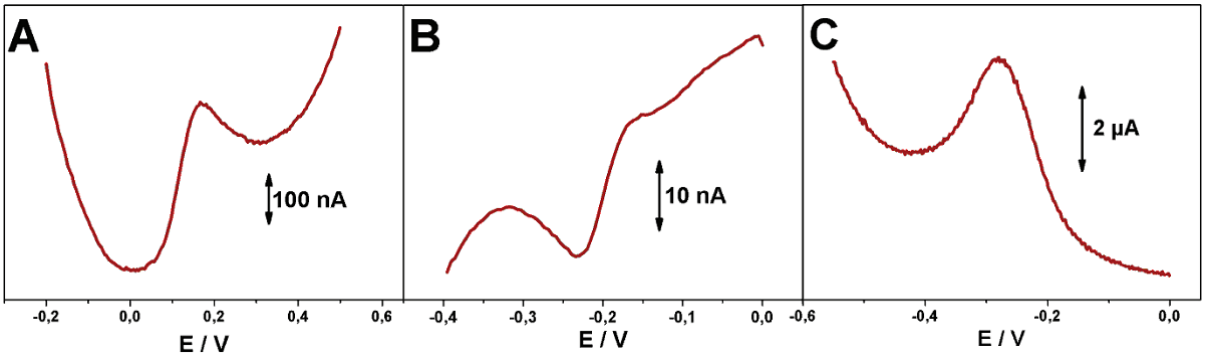

Figure38: Voltammetric signals of PCR products obtained with labeled primers. The asymmetric PCR was performed with A) one ferrocene-labeled primer, B) one methylene-blue-labeled primer and C) one [OsO_4(bipy)] labeled primer. The signals were recorded using A) alternating current voltammetry, B) differential pulse voltammetry and C) square wave voltammetry [178].

Nevertheless, this approach should be pursued further, since our colleague proposed a promising way to remove the protective strands with magnetic beads and get relative high peak signal, which can be seen, in Fig. 38C. It demonstrated the successful target

amplification and labeling during the polymerase chain reaction, which would be potentially applied on in-situ electrochemical PCR product detection [178].

The magnetic beads (MBs) have been widely developed and applied in separation and detection methodologies recently owing to its low toxicity, high biocompatibility, high surface area and chemical and physical stability [179]. Because of these properties, MBs are also used as an attractive platform for the design of electrochemical biosensors. The application of MBs in the development of electrochemical DNA hybridization sensors has been reviewed [180]. Moreover, MBs has been explored as a platform for immobilized DNA probes before the PCR amplification to simplify the concentration and purification process of the target DNA [181].

Besides these extensive applications of MBs in DNA biosensors, combination of MBs with labeled-primers PCR process and appropriate electrochemistry has been an attractive way to get highly sensitive detection of DNA hybridization. Pividori research group has designed a genomagnetic assay for the electrochemical detection of food pathogens based on in situ DNA amplification with magnetic primer [21]. Besides used for sensitive detection of PCR products, this strategy can also detect changes at SNP level. Furthermore, they have presented another genomagnetic assay combining immunomagnetic separation (IMS), double-tagging PCR, and electrochemical magneto genosensing of the double-tagged amplicon for *Salmonella* in milk [182]. Merkoci group has explored the isothermal amplification by means of primers labeled with both AuNPs and MBs for the first time [183]. This double-labeled amplified product has been successfully applied for a DNA sequence characteristic of *L. infantum* kinetoplast tested in dogs, chosen as the model. Hasenknopf and coworkers have investigated direct electrochemical detection of ssDNA, which magnetic-bead-mediated generated from PCR amplification with POM-forward primer and biotin-reverse primer [22].

Fig. 39 demonstrates the double-labeled PCR amplifications were separated by streptavidin-coated MBs into single strands and used for electrochemical detection after hybridization to surface-immobilized DNA strands. Owing to the fast development of all these new strategies above in electrochemical DNA biosensors combining with MBs and labeled-primers PCR, the work of electrochemical detection of PCR products by means of $[OsO_4(bipy)]$-labeled primers (part 4.3, scheme Fig. 25) would take several possibilities into consideration to overcome the present tough situation and realize the real time detection of PCR product detection.

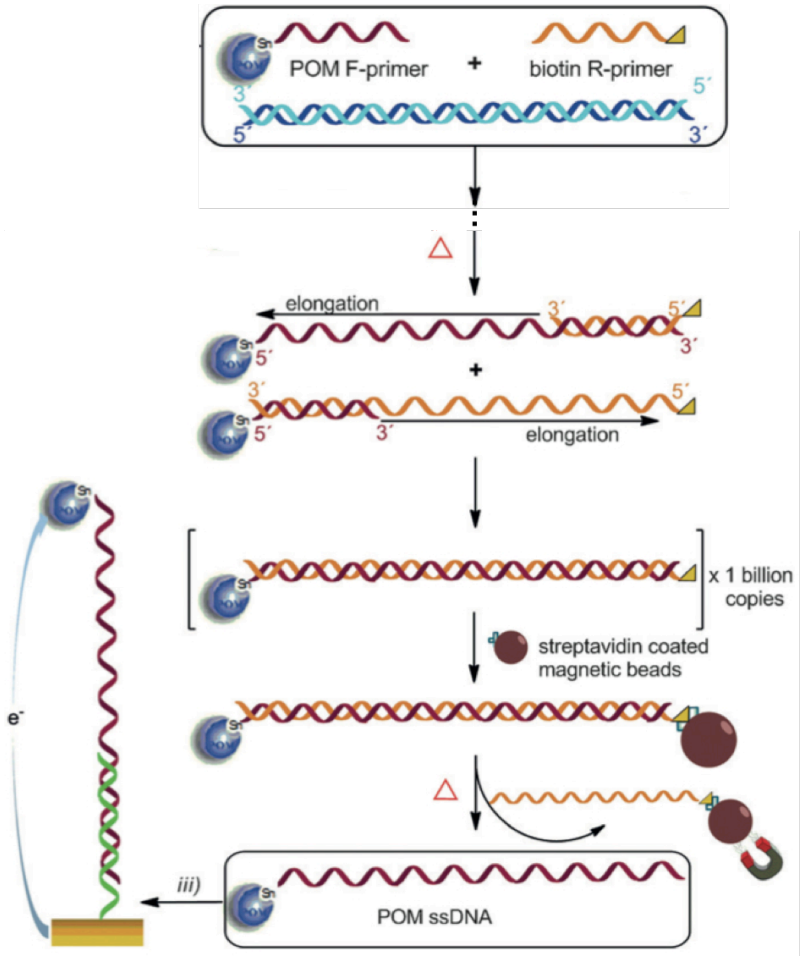

Figure 39: Scheme of PCR amplification with POM-forward primer and biotin-reverse primer, followed by streptavidin-coated magnetic-bead-mediated generation of single-stranded DNA (ssDNA) and hybridization of ssDNA to a surface-tethered probe and electrochemical detection [22].

On the one hand, MBs could be used to remove the protective strands, which protect the forward primer from labeling, to eliminate the negative effect of protective strands on PCR amplification procedure. Since classic symmetric PCR provides an optimal yield of the double stranded amplicon, asymmetric PCR is usually prone to limited efficiency, thus on the other hand, MBs would be explored to split the double-strands PCR products into single-strands after symmetric PCR to make sure the high yield of target amplicons and improve the sensitivity of the assay. It should not be a problem to utilize the MBs more than once during one assay. M. Fojta and coworkers have utilized the magnetic separation for three times to obtain osmium-labeled reporter for electrochemical DNA detection [158]. Furthermore, besides labeling of forward primer with osmium tetroxide compound, reverse primer could also be labeled with a $HS(CH_2)_6$ moiety. After PCR amplication, the thiolated end allows the direct

immobilization of the PCR product on the gold electrodes, such as gold loop/wire/chips, while osmium tetroxide compound allows the electrochemical detection. This double tagging PCR strategy opens good ways not only for simplify the whole experimental process, but also as a promising strategy for miniaturized systems for real time PCR with electrochemical detection. Pividori research group has demonstrated for the first time that a double tagging PCR strategy with a thiolated primer for the detection of *Salmonella sp*. The rapid electrochemical verification of the amplicon coming from the pathogenic genome of Salmonella performed by PCR with a set of two labeled primers was demonstrated to be an easy way for the thiolation of the PCR product [109].

6. References

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