Towards Electrochemical PCR-Chips - Contributions to Couple Enzymatic Amplification and Electrochemical Detection of DNA

Dissertation
zur
Erlangung des akademischen Grades
doctor rerum naturalium (Dr. rer. nat.)
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität Rostock

vorgelegt von

Heiko Duwensee
geb. 4.2.1980 in Hamburg

Rostock, April 2009

urn:nbn:de:gbv:28-diss2009-0121-6

So eine Arbeit wird eigentlich nie fertig,
man muß sie für fertig erklären,
wenn man nach Zeit und Umständen
das Möglichste getan hat.

(J. W. Goethe, Italienische Reise, 16. März 1787)

Die vorliegende Arbeit wurde im Zeitraum von September 2005 bis April 2009 im Institut für Chemie der Universität Rostock angefertigt.

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Datum des Rigorosums: 16. Juni 2009
Datum der Verteidigung: 23. Juni 2009

Acknowledgments

Firstly I wish to thank my advisor, PD Dr. Gerd-Uwe Flechsig for the excellent mentoring/supervision throughout the entire PhD thesis. I really appreciated his intellectual support and continual encouragement throughout my studies. Scientific discussions with him were always a pleasure. I count myself lucky for the freedom in research and the opportunities for development he offered me.

I thank Prof. Dr. Joseph Wang for giving me the opportunity to join his group and for his excellent mentoring throughout my two research stays at the Biodesign Institute. Terranie Vázquez helped me performing the measurements concerning the detection of dopamine in presence of gelatine. In this connection I want to thank the patent attorneys Maikowski and Ninnemann for helping us with the patent application.

In addition let me thank Prof. Dr. Ursula van Rienen, Aleksandar Markovic and Nico Mahnke who helped me to get in touch with mathematical simulations of heat allocation.

Furthermore I would like to thank Prof. Dr. Jan Gimsa. The concerted invention of the inductive bridge helped us to realize the heatable multi-electrode array as generally measurements at electrically heated electrodes. Marco Stubbe helped us to understand and interpret the processes in convective pcr by performing computational simulations.

Additionally I want to thank Dr. Stefan Fiedler, Michael Zwanzig and Ralf Schmidt from the Fraunhofer IZM who introduced galvanical surface modifications to our group and gave me the possibility for a short research stay at their institute.

I want to thank Prof. Dr. Inge Broer and her group for the collaboration around genetically modified plants and for supplying us with relevant sequences and samples.

Special thanks go to the Friz Biochem GmbH, especially Dr. Norbert Persike, Dr. Thomas Kratzmüller and Dr. Gerhard Hartwich for the collaborative development of the multi-electrode array and the heating multiplexer.
I want to thank cooperation partner Rational GmbH and especially Dr. Heyo Mennenga for the development and improvement of a new heat current generator within the framework of the Excellence Funding Program.

Furthermore I would like to thank the Forschungsverbund Mecklenburg-Vorpommern (FMV) and the Patent- und Verwertungsagentur Mecklenburg-Vorpommern (PVA-MV). Especially Lars Krüger (PVA-MV) and Frank Büttner (FMV) helped us to see the possibilities and helped to develop strategies for a commercialization of our research results.

In addition I want to thank Sabine Haack, Margit Bartenwerfer and Petra Sattler. It was a pleasure to work together with them in the practical courses we offered for the undergraduates.

I owe huge thanks to our electrical engineers Harald Grabow and Hilal Diab. Especially Harald Grabow, who helped me enormously with fixing broken instruments, developing new circuitries or even whole instruments and discussing several electrical phenomena with me.

Furthermore, I want to thank Prof. Peter Gründler who allowed me to perform my diploma thesis in his group and so sparked my interest in bioanalytical electrochemistry. I enjoyed the (informal) meetings in the last years and the scientific discussions.

A special thank goes to Richard Blakeborough for the proof-reading of this work.

For financial support I want to thank the DAAD, DFG, the University of Rostock and the federal state Mecklenburg-Vorpommern (Excellence Funding Program).

I want to thank all (former) members of Gerd-Uwe Flechsig´s research group and my friends and colleagues: Hilal Diab, Sven Ehlert, Martin Jacobsen, Simone Krüger,

Maren Mix, Jörg Peter, Thomas Reske, Hanna Sopha, Annette-Enrica Surkus, Falko Wachholz and Anne Walter. I want to express my gratitude especially to Simone Krüger and Maren Mix who performed lots of experiments together with me. I´m grateful to Martin Jacobsen for performing his diploma thesis about signaling oligos and so had to do lots of experiments on this topic even after having finished his diploma thesis.

Special thanks go to Dr. Annette-Enrica Surkus. Not only for the numerous scientific discussions but also I want to thank her for the proof-reading of this work.

Finally, I would like to thank my friends, my family and my wife who unremittingly supported me during my years of study. They made this work possible.

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List of Abbreviations

$\Delta_R S$	entropy of reactions
$\Delta_R G$	Gibbs energy of reactions
ΔE	potential difference
β	temperature coefficient
ΔT	temperature difference
°C	degree Celsius
µA	microampere
µg/L	microgram per liter
µm	micrometer
µM	micromol per liter
A	ampere
AC	alternating current
AE	auxiliary electrode
Au	gold
DNA	deoxyribonucleic acid
DPV	Differential Pulse Voltammetry
e^-	electron
E	potential
F	Faraday constant
I	current
iR drop	voltage drop
K_a	complex formation constant
kHz	kilohertz
LTCC	low temperature cofired ceramic
M	mol per liter
M^{-1}	per mol
mA	miliampere
MHz	megahertz
mm	milimeter

mM	milimol per liter
MON 810	type of genetically modified maize
nA	nanoampere
nM	nanomol per liter
P	pressure
pA	picoampere
PCR	polymerase chain reaction
ppt	parts per trillion
RE	reference electrode
RF	radio frequency
RNA	ribonucleic acid
SAM	self assembled monolayer
ss-DNA	single stranded DNA
T	temperature
THF	tetrahydrofuran
TPA	Temperature Pulse Amperometrie
TPV	Temperature Pulse Voltammetry
WE	working electrode
z	number of transferred electrons

1. Aims and Objectives

DNA (deoxyribonucleic acid) as carrier of the genetic instructions to construct proteins and RNA (ribonucleic acid) is one of the most important biomolecules. Classical methods for the detection of DNA in life sciences are blotting and gel electrophoresis. Whereas in the beginning of research radioactive labeling was used modern methods mostly base upon optical detection schemes. These methods suffer from the high costs of the equipment. Beside the size of the instruments is huge and so miniaturization is difficult. Electrochemical detection seems to be a good alternative as electrochemical sensors are generally considered to be cheap, good to miniaturize and no further signal transduction is needed.

The focus of this dissertation will be the electrochemical detection of DNA. Therefore, different strategies will be evaluated for their applicability for the detection of polymerase chain reaction (PCR) products. A strategy for the simultaneous labeling of several different sequences at the same time in one beaker as also a detection procedure at which no labeling of the target sequence is necessary, will be developed. In order to achieve selective and sensitive sensors, several aspects must be considered. One main effort will be the further development and adaption of the electrically heated electrodes and the development of the equipment needed to generate the heating current. Therefore the known procedures and instruments should be simplified as far as possible. Suitable sensors should be developed. Another field of activity should be the improvement of the sensitivity. Surface modifications of the metal sensors are one way to improve the sensivity. Another possibility for the sequence specific detection of trace amounts of DNA is the amplification of the DNA. The combination of an amplification step with the electrochemical detection seems to be very promising to detect sequence specific low concentrations of DNA.

2. Electrochemistry at Heated Electrodes

2.1. Introduction I

Classical electrochemical measurements are performed by controlling one of the following parameters: current, voltage, concentration or time. Several involved processes like diffusion and electron transfer are temperature dependent. During the last two decades an increasing number of papers dealing with the temperature influence onto the electrochemical signals have been published. Ducret *et al.* [Ducret 1966] described some experiments with directly heated platinum electrodes. Ten years later Harima *et al*. [Harima 1976] reported on heatable gold film electrodes which were used for electrochemical measurements. In the following years different methods for electrochemical measurements at elevated temperatures were described in literature. These methods were reviewed by Wildgoose *et al*. [Wildgoose 2004] and Gründler *et al*. [Gründler 2006, 2008].
The main advantages of measurements at heated electrodes are:

- Acceleration of kinetically sluggish reactions
- Enhanced mass transport caused by the micro stirring effect (due to the temperature gradient)
- Bulk solution can be kept at room temperature and so also temperature sensitive substances can be detected
- Fast temperature changes
- Thermal discrimination / enhanced selectivity

Applying these technological innovations, a lower limit of detection could be obtained with heated electrodes. In the chronopotentiometric determination of trace mercury the stripping signal of a solution containing 10 µg/L was 7-fold higher when performing the measurement at a 60 °C gold wire electrode compared to the measurement performed at room temperature. The limit of detection could be lowered to 80 ppt [Wang 1999c]. Using a heatable bismuthfilm electrode, the

cadmium and lead signal where 10-fold higher, for zinc 16-fold higher comparing the signals obtained at room temperature and at a heated (58 °C) bismuth film electrode. The authors could prove that the increase is not only due to the convection. Using a rotating disc the signals could only increased 5-fold [Flechsig 2002]. For the electrochemical DNA detection an even more dramatic effect was observed. Increases up to 20-fold for single stranded DNA and up to 34-fold for double stranded DNA were described comparing measurements at room temperature at a heatable carbon paste electrode (43 °C) [Wang 2004]. Hybridization signals at a capture probe modified gold wire electrode were enhanced 128-fold by heating the wire electrode to 42 °C during the hybridization step compared to the signal obtained for the hybridization at 3 °C [Flechsig 2005b].

2.2. Techniques for electrochemical Measurements at Elevated Temperatures

In addition to the possibility of heating the whole measuring solution which leads to isothermal conditions it is feasible to create non-isothermal conditions, which can be reached by several methods to be classified into two main groups:

a) Heating a layer near to the electrode
b) Heating the electrode itself

Several possibilities to heat a layer near to the electrode are mentioned in the literature. Compton *et al*. suggested using microwaves to heat the immediate vicinity of the electrode [Compton 1998, Marken 2000, Tsai 2001, Förster 2006]. Ultrasound is known to enhance the mass transport, prevent electrode fouling and accelerate electrode reactions [Brett 1997a, Brett 1997b, Matysik 1997]. Another alternative to heat the layer near to the electrode is the use of high frequency AC as Baranski *et al*. proposed [Baranski 2002, Boika 2008]. Here, the electrode and its connection act as an antenna to absorb the high frequency waves. In these designs the electrode itself stays at the solution temperature. Contrary to this the electrode changes its temperature in several other designs when the electrode is heated itself. Inductive coupling with radiofrequency (e.g. 8 MHz) has been shown by Qui *et al*. [Qui 2000]. The most common way is the Joule heating by radio frequency. This is an old idea, first published 1966 by Ducret *et al*. [Ducret 1966]. The main disadvantages were the huge disturbances caused by the iR drop and the so caused additional polarization of the electrode. Later higher frequencies (radio frequency) and electrical filtering were used and so the noise level could be lowered [Gabrielli 1983, Gabrielli 1993, Gründler 1993]. One major improvement was presented by Gründler *et al*. 1996 [Zerihun 1996b, Gründler 1998a]. By using a symmetrical electrode with a third contact (directly in the middle, between the other two contacts) the noise level could be reduced much better than in any other previuos experiment. The reason for this good result is a remaining iR drop at each side of the center contact as described

above. The quantity is exactly the same but the polarity is contrary, so the distortion compensates itself. This principle is shown in Figure 1.

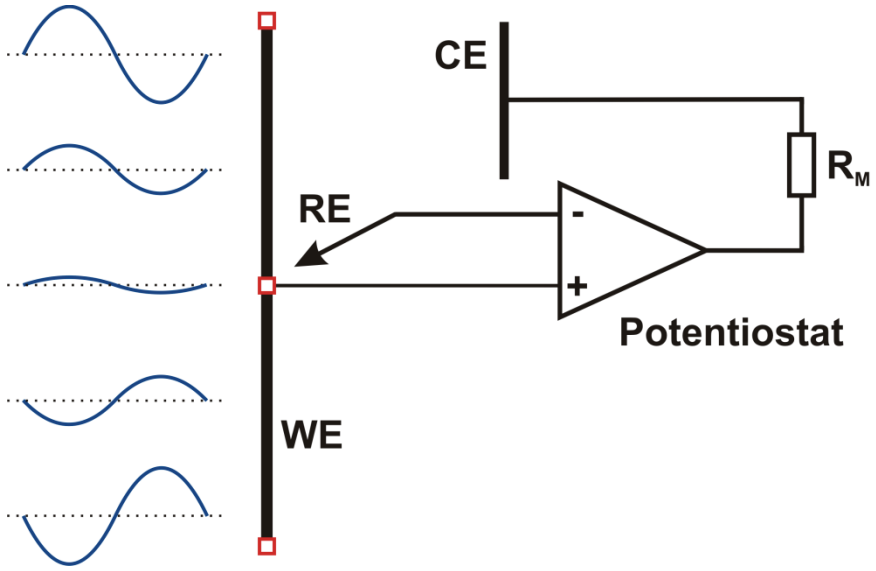

Figure 1: Principle of noise-reduction with symmetrical electrodes. The amount of iR-drop is equal in amount at each side but contrary in polarity. This allows compensation and so the noise-reduction at electrically heated electrodes (taken from [Kirbs 1999]).

Frequencies between 50 kHz to 100 kHz which are typical for the Joule heating are applied [Gründler 2006]. The needed equipment is not very complicated and consists of two main components: a radio frequency generator and an amplifier. The galvanical separation between the AC source and the measuring cell is assured and so the measuring cell is floating versus ground by using a RF transformer [Gründler 2006]. A scheme is shown in Figure 2. Symmetrical electrodes for electrochemical measurements have been described for many different applications (see chapter 2.4) and have been made of many different materials e.g. gold [Gründler 1998b, Peter 2007], platinum [Zerihun 1996b], carbon [Sur 2005], graphite [Wu 2007] and carbon paste [Wang 2000]. Due to the difficulties in the fabrication of the symmetrical electrodes there has been some research to simplify the needed technical equipment necessary for electrically heated electrodes. One approach is an electrode design developed in Gründler's group and applied by Lau *et al*. [Lau 2004]. Here the heating circuit is galvanically separated from the working electrode. This can be realized by

using the Low Temperature Cofired Ceramic (LTCC) manufacturing technique. This type of electrodes can be used with alternating or even with direct current without any electrical filtering.

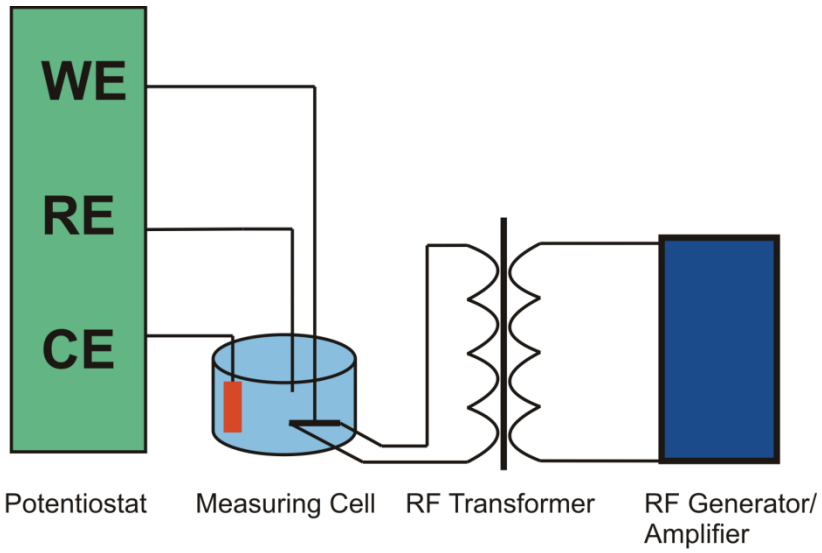

Figure 2: Experimental setup for radio frequency heating of symmetrical electrodes (in the style of [Gründler 2006]).

In the “Hot-Wire-Electrochemistry” which was introduced by Gründler and co-workers in 1993 two different techniques were developed. According to the first one the electrode (in most cases a 25 µm platinum or gold wire) is heated continuously for a long period of time (some seconds or even minutes) at the same temperature [Beckmann 1999, Beckmann 2000, Flechsig 2001]. Following the second method several short heat pulses (10-100 ms) are applied. This method is known as Temperature Pulse Voltammetry (TPV) [Gründler 1996, Gründler 1999, Gründler 2001a, Gründler 2001b] in line with the Differential Pulse Voltammetry (DPV). Contrary to the DPV where voltage pulses are applied to minimize the capacitive current and so lower the limit of detection in the TPV, heat pulses are used to amplify the analytical signal and to enhance systems with sluggish kinetics at room temperature (to detect analytes which are not accessible at room temperature) [Gründler 1999]. A further advantage is the possibility of reaching temperatures above the boiling point of the solvent. Some reports show electrode temperatures of 250 °C in water without working at high pressure in an autoclave [Gründler 2001a,

Gründler 2001b]. Recently a new method called Temperature Pulse Amperometrie (TPA) has been presented by Wachholz *et al*. [Wachholz 2007]. In this report several short heat pulses were applied during an amperometric measurement in order to distinguish the amount of picric acid at a gold electrode.

2.3. Temperature Calibration

The knowledge of the exact electrode surface temperature is most important. In the appropriate technical literature two methods are reported to be used for electrically heated electrodes. The easiest way is the measurement of the temperature dependant ohmic resistance [Voß 1995]. The more accurate method is the measurement of the potential difference between two electrodes (one electrode is kept at a constant temperature, the other one is heated) in a solution of a reversible redox couple with known temperature coefficient. The temperature dependency of the free energy of reactions is shown in equation 1:

$$\left(\frac{\partial \Delta_R G}{\partial T}\right)_P = -\Delta_R S$$

Equation 1

The coherence between the free energy of reactions and the electrode potential is presented in equation 2:

$$\Delta_R G = -zFE$$

Equation 2

Assuming that the pressure is constant equation 3 results:

$$\beta = \left(\frac{\partial E}{\partial T}\right) = \frac{\Delta_R S}{zF}$$

Equation 3

For a single electron transfer and assuming that $\Delta_R S$ is not temperature dependent the integration yields equation 4:

$$\Delta T = \frac{\Delta E}{\beta}$$

Equation 4

The term ΔT represents the temperature difference between the two electrodes. Taking a constant temperature of the solution and a constant temperature of the second electrode for granted, equation 5 describes the temperature of the heated electrode:

$$T_{Elektrode} = T_{Lösung} + \frac{\Delta E}{\beta}$$

Equation 5

Following the potential difference by zero current potentiometry the surface temperature can be calculated with the equation 5. Nevertheless, both methods (the resistance and the potential method) only provide information about the average surface temperature. It is possible, that there are some areas at the electrode surface which are warmer or colder than the average temperature. So these methods do not give any information about the homogeneity of the surface temperature.
Further methods to estimate the surface temperature are the thermography and digital simulations [Schneider 2000, Beckmann 2000].

2.4. Applications of Heated Electrodes

Heated electrodes were used for numerous applications. The elevated mass transport caused by accelerated diffusion and convection (micro stirring effect) often yields to lower limits of detection (higher current signals and same level of background noise). Stripping of heavy metals is the best investigated implementation. The detection of cadmium [Jasinski 2001], lead [Flechsig 2001], mercury [Wang 1999c] and arsenic [Gründler 1998b] were improved by using heated gold electrodes. Platinum electrodes were used for the detection of lead [Zerihun 1996a]. Even heatable bismuth film electrodes [Flechsig 2002] and heatable mercury film electrodes [Jasinski 1999, Jasinski 2001] were reported for the determination of trace levels of heavy metals. Trying to discriminate the effect of an elevated mass transport due to the micro stirring effect of the temperature influence to the diffusion coefficient, the rotating disc electrode was used as an example for the best known classical hydrodynamic electrode [Flechsig 2001]. In addition to the micro stirring the authors were able to show an extra temperature effect. Heated electrodes can lower the limit of detection for several heavy metals from one to two magnitudes [Flechsig 2001] compared to the measurements performed at room temperature.

Measurements of ferrocene/ferrocenium in non-aqueous solutions (THF) were described by Beckmann *et al*. [Beckmann 1999]. The detection of dissolved oxygen [Zerihun 1996b] and several organic compounds (methanol, formic acid, glucose and formaldehyde) [Zerihun 1998] at heatable platinum microelectrodes is mentioned in the technical literature. Heatable enzyme-modified platinum electrodes were successfully applied for the determination of glucose in the presence of ascorbic acid [Lau 2004]. The discrimination of maltose next to glucose could be performed by the immobilization of two enzymes on the surface of a heatable platinum electrode [Duwensee 2005].

In the last fifteen years several groups reported on the detection of biological important compounds at heated electrodes. The electrochemical behaviour of cytochrome c [Voß 1999] and nicotinamide adenine dinucleotide [Lau 2005] at a heated platinum electrode were described. The main focus of research is DNA

detection by means of heatable electrodes. Whereas first reports described the unspecific adsorption of DNA oligonucleotides at paste electrodes recent works show the improvement of sequence specific DNA detection at heated electrodes. Several strategies are presented. Some use magnetic beads for the separation of the target DNA sequence from the probe DNA sequence. A strategy with protective strands without magnetic beads has been published shortly [Flechsig 2007a]. The authors used osmiumtetroxidbipyridine [OsO_4(bipy)] to label the unprotected area of the single strand. This technique is described more in detail in chapter 3.3.

3. Electrochemical DNA-Detection

3.1. Introduction II

The number of publications dealing with DNA biosensors and DNA microarrays have grown enormously during the last fifteen years [Sassolas 2008]. Whereas in 1994 only ten publications with this topic were released the number increased to 300 publications in the year 2000 and almost 2000 publications in 2006. The reason for this development can be found in the variety of important applications of DNA sensors. DNA-based diagnostic tests for the following applications were described: gene analysis, DNA diagnostics, (fast) detection of biological warfare agents, food industry (detection of pathogens), forensic applications and more. Due to the low limit of detection on the molecular level reliable diagnostics can be carried out without any amplification of the target DNA even before any symptoms of an indisposition occur. The electrochemical detection became more and more popular among other things owing to their low cost, simple design, capability of real-time and label-free detection and their potential of miniaturization [Lucarelli 2008].

Preliminary experiments for the electrochemical detection of DNA started in the 1950s. The electrochemical activity of nucleobases was proven by Palecek *et al*. [Palecek 1958, Palecek 1960]. The characterization of nucleic acids being used for analytics was described by Joseph Wang and coworkers [Wang 1996a, Wang 1997, Wang 1999a, Wang 1999b] and Emil Palecek and coworkers [Palecek 1988, Palecek1990, Palecek 1994] in the 1990s. The early research could only show whether DNA was present or not. Therefore, the huge tendency of mercury, graphite and carbon paste electrodes to adsorb unspecific DNA was exploited. To overcome the disadvantage of unspecific detection, strategies with modified magnetic beads were presented [Wang 2001a, Wang 2004]. Biotinylated capture probes were coupled with streptavidine modified magnetic beads. After the hybridization with complementary target sequences the magnetic beads with the hybridized target were separated from the bulk solution, dissolved in another solution, denaturated and then electrochemically detected by using the oxidation signal of guanine. A highly

sensitive and selective detection method for DNA sequences could be achieved by this combination of an unspecific adsorption at graphite electrodes with the magnetic separation in advance. There are some publications describing the direct detection of hybridization processes at carbon paste, glassy carbon and graphite electrodes [Millan 1993, Millan 1994, Wang 1996b, Cai 1997]. Sometimes even covalent coupling of DNA to the electrode surface has been described. Modifications on the electrode surface have been reported to achieve a good adsorption. The aim was to create a positively charged electrode surface which forms a stable complex with the negatively charged phosphate groups of the DNA. Chitosan on glassy carbon electrodes [Cai 2002, Xu 2001] or a pre-oxidation step of the glassy carbon [Escosura-Muniz 2007] are two possibilities to create a positively charged surface. But the main disadvantages of graphite and carbon paste electrodes are still evident: unspecific adsorption of nucleic acids and adsorption of redox-active substances (added for the detection). This leads to false positive signals and a high background level. Suffering from these aspects graphite and carbon paste electrodes with adsorbed probes are rarely used anymore for specific DNA detection.

3.2. Immobilization Techniques

In the mid nineties the tendency towards sequence specific DNA detection started. The most important step for a sensitive and specific DNA sensor is the immobilization of the capture probe. One above mentioned immobilization is the adsorption at the electrode surface. This is possible because of the multiple sites of binding between the negatively charged sugar-phosphate backbone and the positively charged electrode surface. The adsorption of the capture probe at the electrode surface suffers from low hybridization efficiency. Trying to overcome this poor hybridization efficiency two other procedures are described in the appropriate technical literature: covalent coupling and avidin/streptavidin-biotin interactions.

Avidin-biotin coupling takes advantage of the high affinity between biotin and avidin ($K_a=10^{15}$ M^{-1}) and its high specificity [Sassolas 2008]. Avidin (as streptavidin) has four equivalent binding sides for biotin. Beside the simple adsorption of avidin to the electrode, described for silica [Fang 1999], carbon paste electrodes (neutravidin) [Metfies 2005] and screen printed carbon electrodes (streptavidin) [Hernandez-Santoz 2004, Marrazza 1999], the entrapment of streptavidin in a polymer deposited at the electrode surface is reported [Gajovic-Eichelmann 2003]. The deposition of a polymer consisting of monomers coupled with biotin was presented by Dupont-Filliard *et al*. [Dupont-Filliard 2001]. In a second step the avidin binds to the fixed biotin.

Coupling the capture probe via a covalent bond directly to the electrode surface has been described in different variations. Herne and Tarlov did pioneer work using the chemisorption between thiol modified DNA probes and metal [Herne 1997, Peterlinz 1997]. Due to the strong affinity between thiol groups and noble metals, self-assembled monolayers form just after giving a solution of mercaptoalkane on a noble metal. The most commonly used metal is gold. Adding a thiol modified DNA capture probe on a gold surface and adding e.g. mercaptohexanol in a second step leads to a well ordered and well defined surface with immobilized DNA probes in an upright position (Figure 3). These systems were described to have an excellent hybridization efficiency and even base pair mismatches can be detected [Kelly 1999]. Due to its

simplicity this method is widely used [Flechsig 2005a, Wong 2005, Pavlov 2004, Lucarelli 2005, Liu 2005].

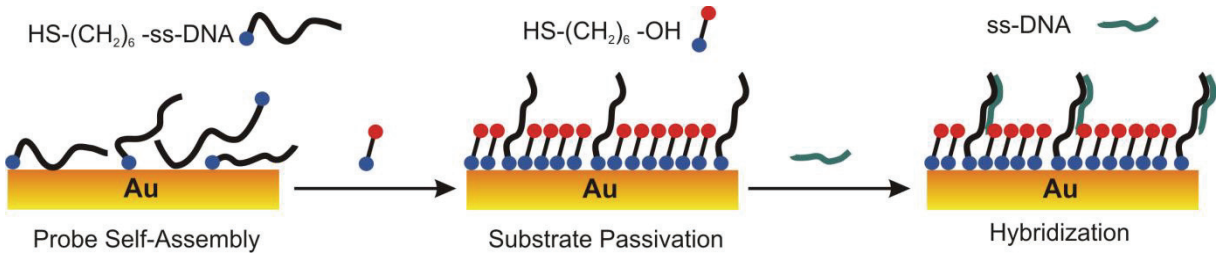

Figure 3: Probe immobilization by means of chemisorption between gold and thiol modified capture probe. In the second step unspecific adsorption is minimized by mercaptohexanol treatment.

Covalent coupling to the electrode surface can also be realized by using carbodiimide as coupling reagent. In a first step carbodiimide binds to an activated surface e.g. glassy carbon [Millan 1992] or plastic composite [Schülein 2002] and in a second step the DNA capture probe is coupled to the electrode surface. This is schematically shown in figure 4.

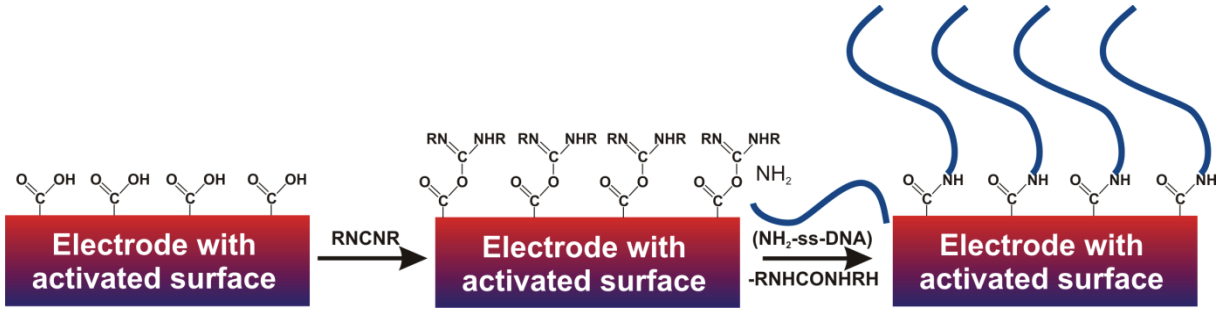

Figure 4: Probe immobilization by means of carbodiimide coupling between an amin modified capture probe and the activated electrode surface.

Activating the electrode surface by immobilizing reactive groups at the electrode surface has often been achieved by using polymers [Lee 2001, de Lumley-Woodyear 1996]. Another commonly used method is the formation of hydroxyl, carboxyl or amino terminated self assembled monolayers on gold electrodes [Zhao 1999].

A promising method seems to be the electroaddressable immobilization of aniline derivates. Here, the diazotation reaction of the aniline derivate is performed in a first

step that leads to the formation of an aryl diazonium which then can be electrochemically reduced to an aryl radical (shown in figure 5 at electrodes 1 and 4). This forms the bond between the (metallic) electrode surface and the aryl group. The DNA probe can be attached to the aryl group via carbodiimide coupling as described above and as shown in figure 5.

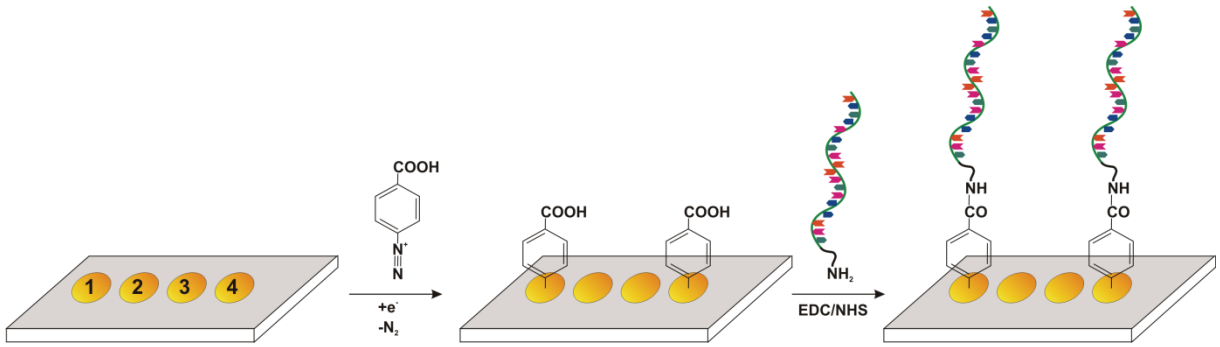

Figure 5: Electroaddressable probe immobilization. In the first step the electrochemical reduction of the aryl diazonium takes only place at polarized electrodes (electrode 1 + 4). In the second step the coupling between the amine modified probe and the carboxcylic group of the immobilized aromatic compound is performed (in the style of [Harper 2007]).

This type of immobilization has been reported for a variety of electrode materials like gold [Laforgue 2005], copper, nickel, cobalt, zinc, platinum [Bernard 2003], carbon [Saby1997], iron [Boukerma 2003] and silicon [Henry de Villeneuve 1997]. In general this immobilization method is considered to be less prone to oxidation and reduction [Harper 2007] and more robust against temperature [Liu 2007].

3.3. Principles of Detection

To detect the hybridization event between the immobilized DNA capture probe and the target sequence several different strategies are reported. These can be divided into four main groups:

(1) Without addition of redoxactive substances to the solution

Beside the direct guanine or adenine oxidation signal [Kara 2002, Ozkan 2002] impedance spectroscopy was successfully applied [Cougnon 2007] for the hybridization detection without adding any redox active substance or modifying the target. Redox active groups immobilized to the electrode surface changing their conformation and so their electrochemical properties during the hybridization were described by Jenkins *et al*. [Jenkins 2006]. Here ferrocene labeled hairpin probes were immobilized on a gold electrode. During the hybridization with a complementary target the ferrocene label removes itself from the surface and the redox current in cyclic voltammetry diminishes. The principle is shown in figure 6.

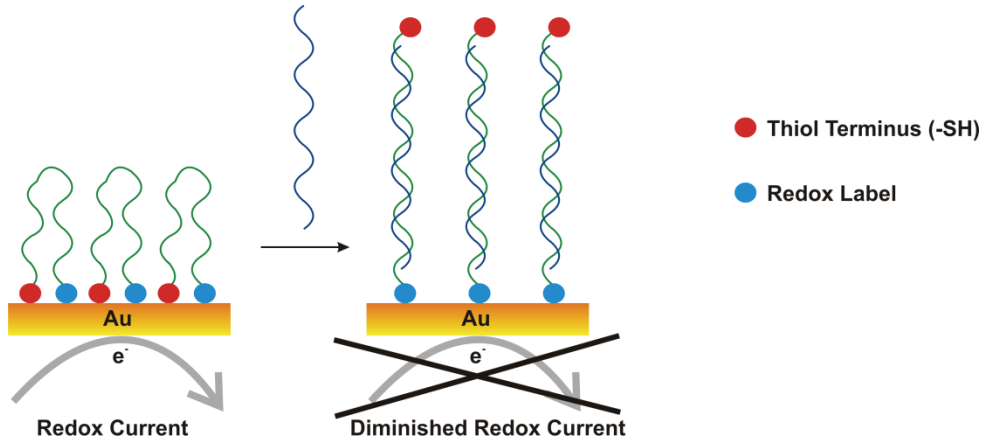

Figure 6: DNA-detection by means of hairpin probes. Due to the hybridization with the added single strand DNA the distance between redox label and electrode surface grows. This leads to a diminished redox current after the hybridization.

(2) Adding a redoxactive substance to the solution

The addition of redoxactive substances (intercalators) which bind or accumulate in the minor or major groove of double stranded DNA was examined in numerous

publications and is the most common electrochemical technique to detect DNA hybridization. Daunomycin [Hashimoto 1994], Hoechst 33258 [Kobayashi 2004] and ethidium bromide [Liu 1996] are only a few of the reported intercalators allowing to discriminate between single and double stranded DNA and so can be used for hybridization detection. The principle of intercalation is shown in figure 7. Some intercalators cause an increase of the electrochemical signal after the hybridization; others show a decrease of the signal.

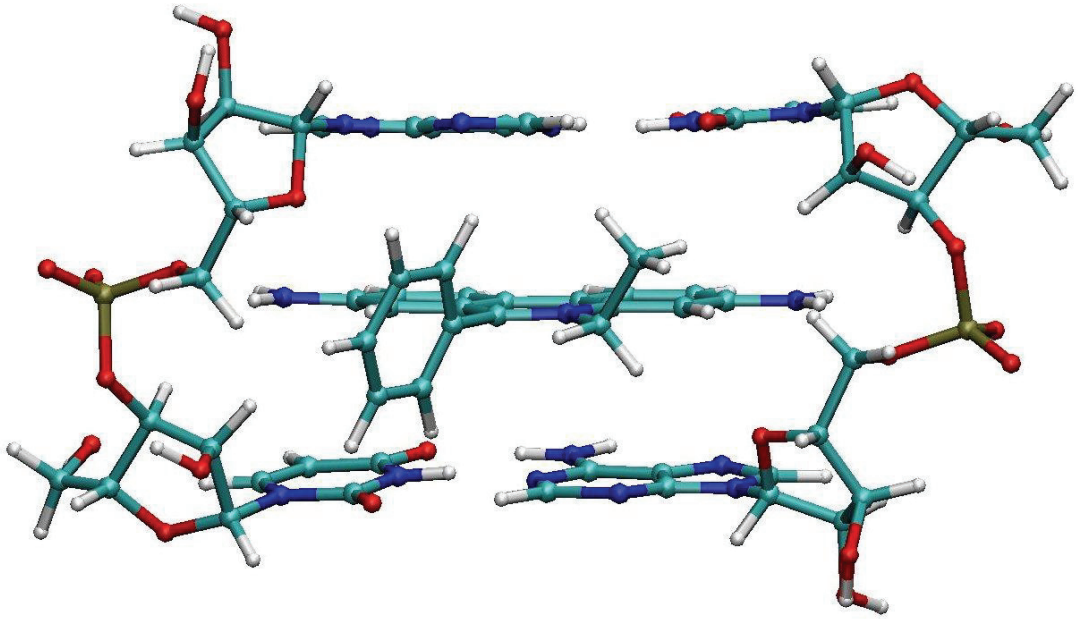

Figure 7: Intercalation of ethidium bromide in double stranded DNA.
http://en.wikipedia.org/wiki/File:DNA_intercalation2.jpg

(3) Addition of labeled DNA sequences (reporter/signaling oligo)

Redox labeled single stranded DNA can be used for the sequence specific detection as well. One strategy is the displacement of labeled single stranded DNA. Here a labeled signaling oligo is hybridized to the immobilized capture probe. As soon as the target sequence is present the labeled signaling oligo is displaced by the target sequence. The displacement takes place due to the more stable complex formed by target DNA and capture probe [Liepold 2008]. The reasons for this are either mismatches between the capture probe and the labeled single strand or varieties in length between capture probe and the labeled single strand. Another strategy is the use of so-called reporter probes. Reporter probes are labeled single stranded DNA

sequences which only lead to an electrochemical signal when hybridization between capture probe and target DNA is successful [Fojta 2004, Nakayama 2002]. Commonly used labels are ferrocene and osmium derivatives. Fojta *et al.* reported the use of protective strands to label reporter oligos [Fojta 2007]. They used magnetic beads to remove the protective strands. Both strategies (signaling oligos and reporter oligos) are shown in figure 8.

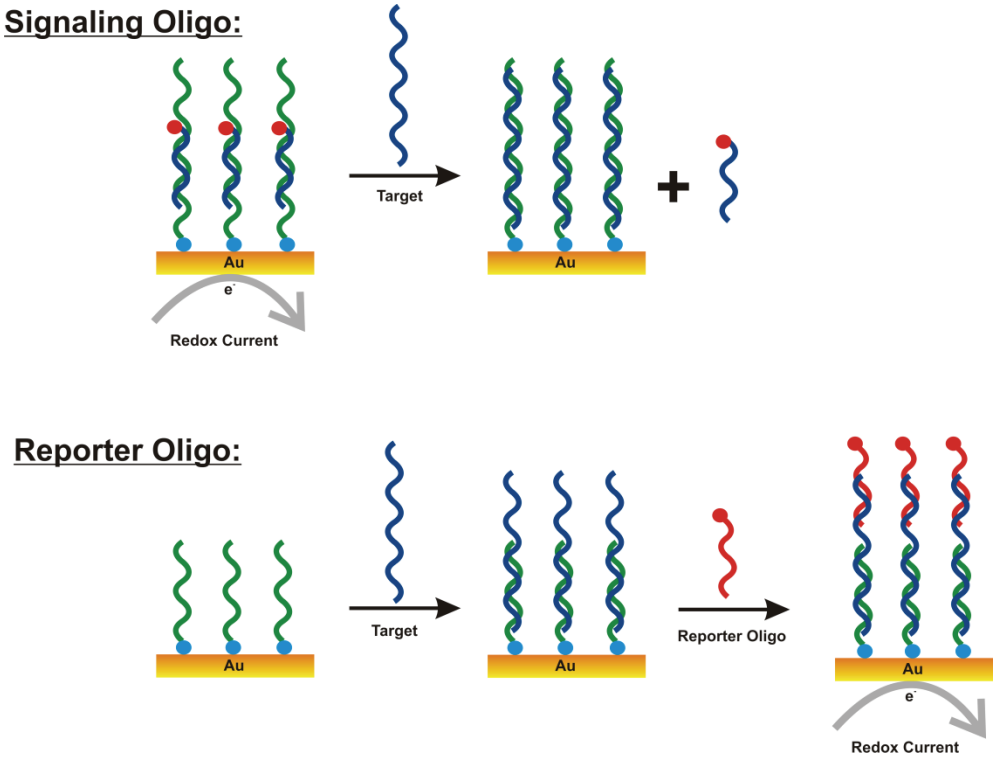

Figure 8: DNA-detection by means of signaling oligos and reporter oligos. Whereas the analytical current decreases with the hybridization when using labeled signaling oligos, the current increases with the hybridization when using a labeled reporter oligo. Here the successful hybridization between immobilized capture probe and target sequence is the precondition.

(4) Modifying the target sequence

Modifying the target DNA with a redox active compound is another possibility for an electrochemical detection of hybridization. The polymerase chain reaction (PCR) is a powerful tool to insert redox active groups into DNA sequences. This can be achieved by using a labeled primer or modified nucleobases. Ferrocene derivates of nucleoside triphosphates have been investigated by Fojta's group [Brázdilová 2007].

Fojta's group also reported on aminophenyl- and nitrophenyl-labeled nucleoside triphosphates and their applications to the electrochemical detection of DNA sequences [Cahová 2008]. Wjatschesslaw *et al*. reported about ferrocene derivates of uracil which were inserted into DNA strands during the polymerase chain reaction instead of thymine bases [Wjatschesslaw 2002]. Another approach is the direct labeling of the target DNA. Palecek reported on a labeling procedure with osmium compounds [Palecek 1992, Palecek 2001]. Osmium (VIII) compounds (e.g. osmiumtetroxidebipyridine [OsO_4(bipy)]) oxidize the double bounds of pyrimidine bases especially thymine. Kinetically investigations showing differences in reaction kinetics of the four nucleosides were performed by Flechsig and coworkers [Reske 2009]. The product of the labeling is the diester of osmium (VI) acid which shows several reversible electrochemical signals in the negative potential range. The reaction of thymine, which is the fastest reacting nucleobase, with [OsO_4(bipy)] is presented in figure 9.

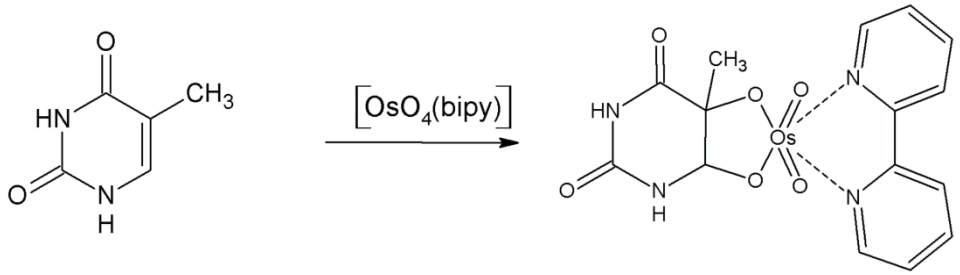

Figure 9: Reaction between thymine and osmiumtetroxidbipyridine. This reaction can be used to label single stranded DNA.

The catalytic peak can be used to detect labeled DNA at mercury or amalgam electrodes. This signal is not accessible at gold electrodes but there are several articles reporting a signal at -0.35 V. This signal can be used for the detection of labeled single stranded DNA. To ensure the ability of hybridizing to a double strand, the area complementary to the capture probe must be protected by means of protective strands [Flechsig 2007a]. This protection is necessary because osmium-labeled single strands lose their skill of hybridization. The method of protective strands has been reported for several applications including the detection of polymerase chain reaction products [Peter 2007, Reske 2007] respectively labeled

RNA strands [Sopha 2008]. Fojta *et al.* used magnetic beads to remove the protective strands after the labeling process [Fojta 2007]. The principle of labeling single strands with [OsO_4(bipy)] without removing the protective strand is presented in figure 10.

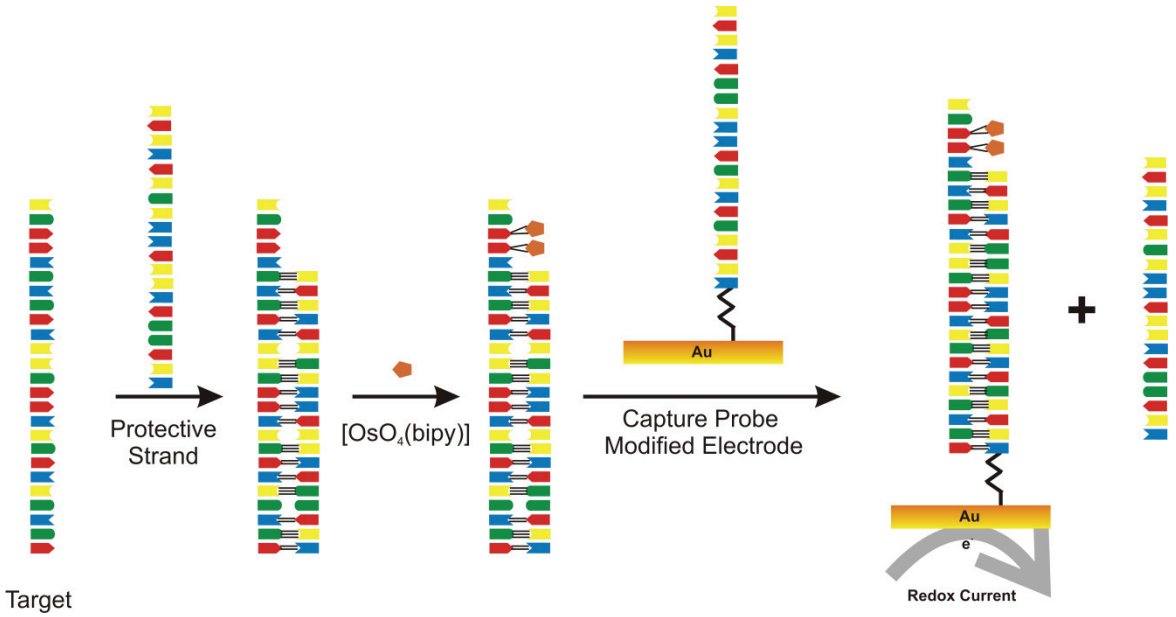

Figure 10: Single strand labeling procedure with [OsO4(bipy)] and protective strands. In the first step the protective strand, which is partly complementary, is added to the single stranded target sequence. After a hybridization time [OsO4(bipy)] is added. [OsO4(bipy)] reacts primarily with the unprotected thymine nuclebases.The labeled target can be detected using a fully complementary capture probe which is immobilized on a gold electrode.

[OsO_4(bipy)] has reported to be a redox label which delivers relatively high electrochemical signals and therefore can be used to detect low target DNA concentrations. The reason for this high voltammetric signals can be found in two facts: firstly the reversible two electron reduction/oxidation between osmium (VI) and osmium (IV); secondly every unprotected thymine base can be labeled and so several redox labels can be attached to the target DNA sequence.

3.4. Ultrasensitive DNA-Detection

In addition to the capability of a sequence specific detection a further requirement is a detection limit as low as possible. Only this allows e.g. the detection of diseases before any symptoms appear. The detection of low DNA concentrations can either be achieved by modifying the sensing system to make it as sensitive as possible or by the amplification of the target sequence.

(A) Improving the sensing system

Enzymes can be used to generate multiple redox active molecules after a successful hybridization event. Mainly used enzymes are alkaline phosphatase [Patolsky 2003, Horakova-Brazdilova 2008] and horseradish peroxidase [Zhang 2002]. A detection scheme for a DNA sensor based on enzymatic detection is shown in figure 11.

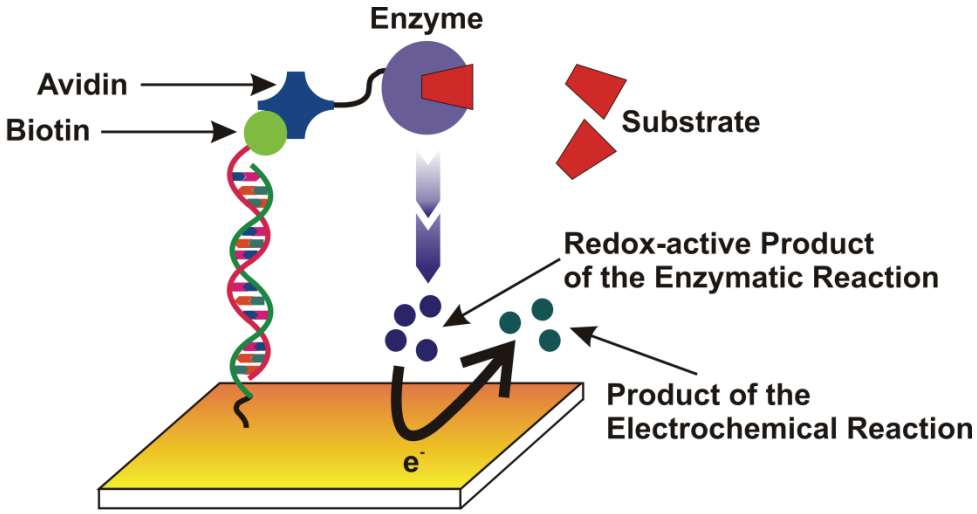

Figure 11: Enzyme amplified DNA-detection. After the hybridization between the immobilized capture probe and the biotin labeled target DNA, the avidin modified enzyme is added. The added redox-inactive substrate is transformed by the enzyme to a redox-active product which can be detected at the electrode surface.

The combination of two enzymes for a simultaneous detection of two different target sequences was also described [Wang 2002]. Another possibility for developing ultrasensitive DNA sensors is the use of nanoparticles. So the detection of one biorecognition event can be used as an origin of the accumulation of several detectable atoms. One possibility is the use of gold nanoparticle modified targets or

reporter probes. After the hybridization event silver can be deposited on the gold nanoparticles. In the following step, the silver is either detected directly or dissolved and subsequently electrochemically detected [Wang 2001b]. The principle is shown in figure 12.

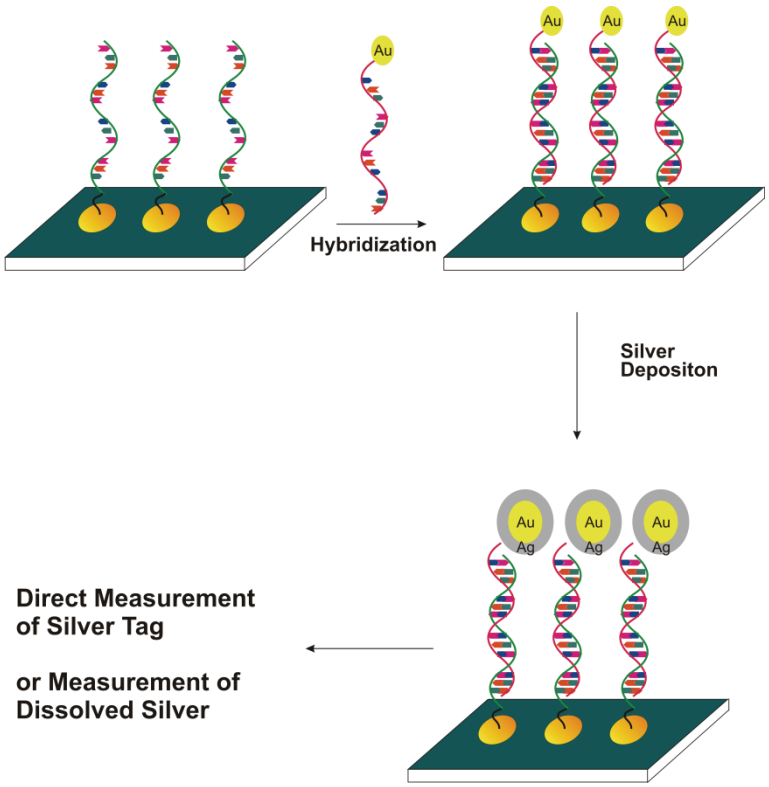

Figure 12: DNA-detection by means of nanoparticles. After the hybridization between the immobilized capture probe and the nanoparticle modified target, silver is deposited at the nanoparticles by applying a potential at the electrode. The deposited silver can either be detected directly or dissolved first and detected in a second step.

The combination of different metal nanoparticles allows the simultaneous detection of a few DNA targets. Wang *et al*. and Hansen *et al*. used cadmium sulfide, zinc sulfide and lead sulfide nanoparticles for this purpose [Wang 2003, Hansen 2006].

(B) Amplification of the target sequence

Another way to improve the sensivity of a system is the amplification of the target sequence. An additional amplification step previous to the electrochemical detection has been reported by many groups [Marrazza 2000, Ye 2003, Li 2004, Lai 2006]. The combination of target amplification and following electrochemical detection of the

amplified target is reported by Hsing *et al*. [Lee 2003, Yeung 2008]. They used a microfluidic chip with separated amplification and detection chambers. Supplementary to the amplification of the searched target sequence (and so enhancing the sensivity) the polymerase chain reaction enhances the specifity of the detection. This is due to the fact that by choosing suitable primers only the target sequence is amplified. All other DNA strands which are present in the original sample are not amplified and so can not disturb the detection. Nevertheless, the amplification requires additional handling procedures with the sample which are not necessary when the electrochemical detection of the target can be performed directly with the original sample.

The combination of both strategies (improving the sensing system and amplification of the target sequence) allows to lower the detection limit.
An efficient overview of different electrochemical DNA sensors and their sensitivity is given in a review article written by Sassolas *et al*. [Sassolas 2008]. Sequence specific DNA detection down to a femtomolar level and below is possible.

3.5. DNA-Detection at Elevated Temperatures

Beside ionic strength of the solution, the base sequence and the length of the single strands, temperature is the most important parameter influencing hybridization processes. The adaption of the temperature to the respective application is standard in molecular biological laboratories. One example is blotting. During the hybridization step, elevated temperatures are applied to achieve higher hybridization efficiency. The second advantage is a higher selectivity. Each mismatch between the single strands lowers the melting point of DNA. The temperature where half of the DNA is single stranded and half of the DNA is double stranded is defined as the melting point. Several groups published formulas to calculate the melting point of a given DNA sequence [Wallace 1979, Howley 1979, Breslauer 1986]. Another example in the field of molecular biology is the polymerase chain reaction. Depending on the base sequence, the annealing temperature has to be adjusted.

Though temperature is not used very often in electrochemical experiments concerning DNA detection, there are still some reports. Experiments with adsorbed DNA at heatable carbon paste electrodes and intercalators such as daunomycin and cobalt (III) phenanthrolin were reported by Flechsig [Flechsig 2006]. In order to overcome the disadvantages of adsorbed capture probes and the disadvantages of intercalators (tendency of unspecific interaction with single and double strands; just increase/decrease of the signal due to the hybridization of 20-50%; degree of intercalation temperature dependent) further experiments were performed at a heatable gold wire electrode with covalent redox markers like ferrocene [Flechsig 2005b]. The authors could show a 140-fold enhancement of the obtained signal by changing the hybridization temperature from 3 °C to 48 °C. Further experiments at a rotating disc electrode were performed [Flechsig 2007b] to investigate whether the signal enhancement is due to the enhanced mass transport or to a faster hybridization at elevated temperatures. The authors proved a significant temperature influence to the hybridization process additionally to the enhanced mass transport. The combination of osmium labeling by means of protective strands and the hybridization detection was also presented. Polymerase chain reaction products could be detected by using an enzymatically treatment to decomposite one single

strand of the double stranded polymerase chain reaction product followed by the osmium labeling procedure with protective strands [Reske 2007]. UV-spectroscopic measurement is often used to detect single pair mismatches. The absorption at 260 nm is recorded in dependency on temperature changes and so melting curves are recorded. Electrochemically recorded melting curves can be received by using [OsO_4(bipy)] labeled target sequences which are allowed to hybridize to a capture probe (immobilized at a heatable electrode) over night. When the temperature is raised slowly, simultaneously electrochemical measurements are performed. The melting curves are obtained by plotting the electrochemical signals versus the temperature. Single base mismatches in the target can be detected by a shift of the melting point to lower temperatures compared to the fully complementary target [Surkus 2009].

4. Results

4.1 Novel Heatgenerator [Publication I]

For electrochemical measurements with directly heated electrodes, symmetrical electrodes were necessary, as described in chapter 2, to overcome problems with the iR-voltage drop along the length of the electrode which causes polarization and therefore strong interferences with the measuring device (potentiostat). Usually the heating current is the mA to A range, whereas the electrochemical current/signal is in the nA or µA range. Symmetrical electrodes can minimize these distortions but beside the difficulties in manufacturing, a residual noise can be observed. We tried to improve the situation by inventing a new circuitry which we called “inductive bridge”. Instead of using symmetrical electrodes (with three contacts) we used regular electrodes with two contacts (e.g. a metal wire). In order to separate the heating current from the measuring current, we used two inductors which were symmetrically contacted to the electrode. In the middle between the inductors the measuring signal can be recorded by a potentiostat. The effect to electrochemical measurements is shown in figure 13.

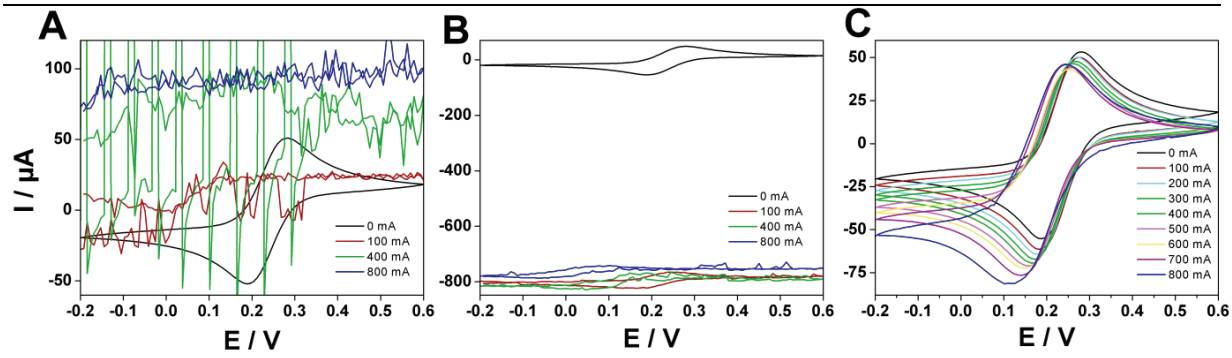

Figure 13: Heated glassy carbon electrode: Cyclic voltammograms in a solution containing 0.1 M KCl and 5 mM $K_3[Fe(CN)_6]$ vs. Ag/AgCl (3 M KCl). The voltammograms are recorded using a A) asymmetrically connected electrode, B) symmetrically connetected electrode and C) asymmetrically connected electrode but with inductive bridge. (Publication I, figure 7)

The inductors filter the high frequencies of the heating current as they have a high inductive resistance but allow the measuring signal to pass through as the ohmic

resistance is very low. Integrating this circuitry into our newly developed and compact heating generator allowed us to simplify the measuring setup and the work with heated electrodes.

We tested our equipment at the well known redoxsystem potassium ferricyanide/ferrocyanide. Measurements showed the improvement at a gold wire electrode as an example for electrodes with low ohmic resistance. Even electrodes with high ohmic resistances as glassy carbon electrodes could be successfully used with our new equipment as figure 13 demonstrates. The improvement to symmetrical electrodes is also obvious.

The invention of the inductive bridge allows using a more simple electrode design. Instead of three contacts, which before were required for symmetrical electrodes, in the new electrode designs only two contacts are necessary per electrode. This simplifies especially the design of multi-electrode arrays as typically used in DNA analysis and allows one to have more electrodes with the same number of electrically contacts on an array. One example, is the heatable 16-electrode array presented in chapter 5. This design is only possible when using the inductive bridge.

4.2 Biofouling [Publication II & III]

After the successful tests with the new heating device at model systems, we decided to investigate a more difficult problem. In environmental research with electrochemical sensors, electrode deactivation occurs frequently. The reason can primarily be found in either matrix components which adsorb at the electrode surface or in products of the electrochemical reaction that stick to the electrode surface and cause suppression and distortion of the analytical signal. Our idea was to use heated electrodes to minimize these effects. The temperature gradient at the electrode surface causes convection and this hinders electrode blocking. We chose the dopamine detection in presence of gelatine as a model. First experiments did not show the expected improvement with heated electrodes. The combination of electrochemical and thermal pretreatment could enhance the situation enormously. The signal drops to 40 % from the first to the seventh measurement without pretreatment, with thermal pretreatment or with electrochemical pretreatment. In contrast, the signal stays almost constant for at least 12 repetitive measurements with a combined thermal and electrochemical pretreatment. We suppose the negative potential in combination with the caused hydrogen evolution untightens or lifts the adsorbed molecules and they can be transported away because of the convection caused by the temperature gradient. The signal stability after different pretreatment methods is presented in figure 14.

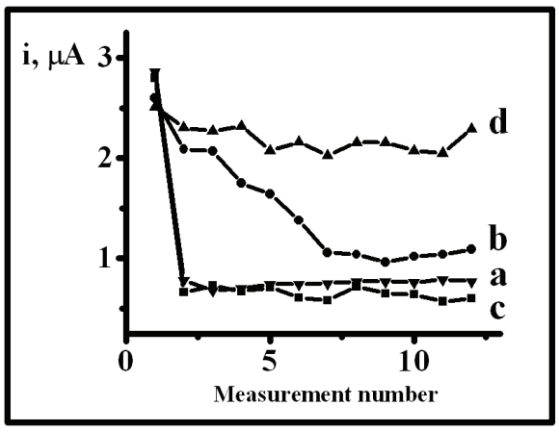

Figure 14: Electrochemical response to dopamine depending on the pretreatment. The square wave voltammetric signals were recorded with a) no treatment, b) electrochemical pretreatment, c) thermal pretreatment and d) combined thermal and electrochemical pretreatment. (Publication II, figure 3)

The careful and conscientious cleaning of the electrode surface with mild conditions is of enourmous significance for biosensors and DNA sensors. The biolayers immobilized on the electrode surface are usually prone to high temperatures and/or reduction/oxidation. The combination of low temperatures and low potentials will be very useful e.g. for the regeneration of DNA sensors (to control the dehybridization process) and to minimize unspecific adsorption/hybridization.

4.3 Electrode-Design Optimization [Publication IV]

Using heatable electrodes for DNA detection poses a further challenge. In addition to the immobilization method for the capture probe, a homogenous temperature allocation at the electrode surface is necessary. Otherwise a precise control of e.g. hybridization processes and well-founded results are hardly attainable. The disadvantage of conventional electrode designs like metal wires, which are soldered to a printed circuit board, is a source of heat loss at the solder point. Here we estimated a non-constant electrode temperature along the wire. Due to the fact that the methods for temperature calibration (see chapter 2.3) only deliver information about the average surface temperature, we decided to apply mathematical simulations of the heat allocation. As expected, first results showed a hot area in the middle of the wire and two colder areas near to the solder contacts. This behavior was reported earlier by Schneider *et al.* [Schneider 2000]. Their results for the temperature allocation along the electrode are presented and compared with our results in figure 15.

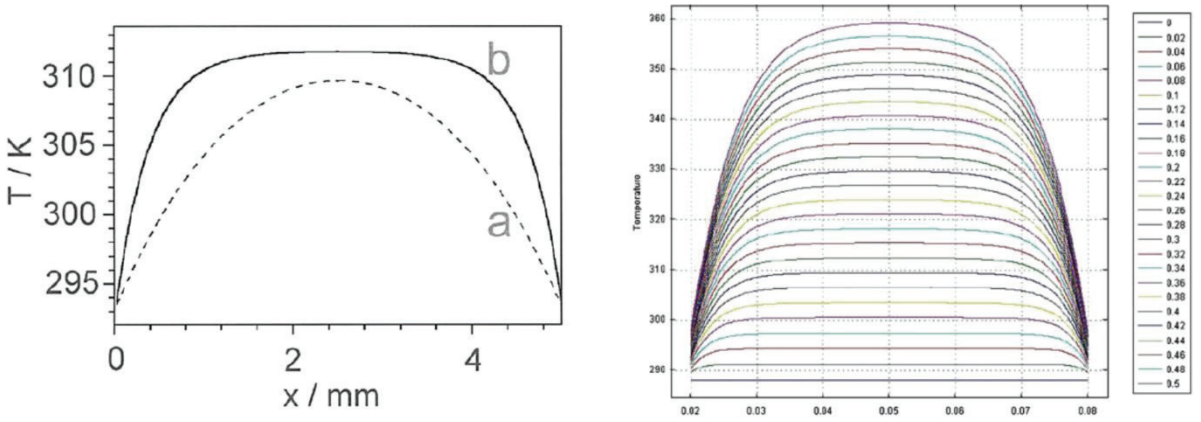

Figure 15: Surface temperature of a heated electrode. On the left the surface temperature of a heated 25 µm gold wire in air (a) and in water (b) after 0.1 s is shown (taken from [Schneider 2000]. On the right side our results for different times are displayed (in water). (Publication IV)

In further simulations we tried to minimize this gradient along the electrode by varying the geometry of the metal electrode. An oval shape seems to be a good compromise as proposed earlier by Schneider [Schneider 2000] and Flechsig [Flechsig 2004]. With these optimized parameters, we could reach a higher homogeneity of the temperature allocation which is shown in figure 16.

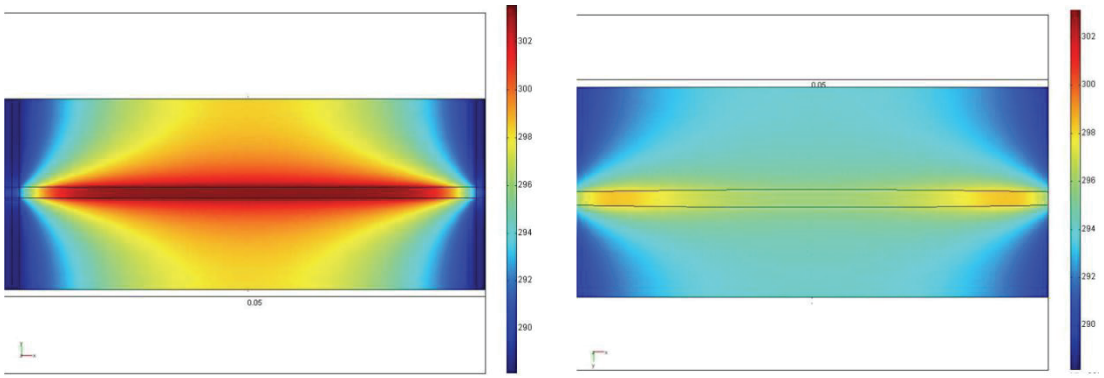

Figure 16: Optimized surface temperature of a heated electrode. Whereas the left image shows the behavior of the non optimized electrode the right image displays the surface temperature of the electrode with the optimized (oval) shape. (Publication IV)

Unfortunately the manufacturing of electrodes with the optimized parameters is quite complicated. Reducing a 5 mm long and 25 µm thick gold wire to both sides continuously with the received parameters is almost impossible. Even gold electrodes on printed circuit boards are only feasible with significant effort and at high costs. That is the reason why we decided to use another design for our latest 16-electrode array. Here we use a long gold electrode (several centimeters) which is fully covered with an isolating lacquer layer. Only a small spot in the middle is uncovered by laser ablation. We can assume that a constant temperature over the 50 µm spot is available here. This array has already been successfully applied in the detection of genetically modified maize as described in chapter 4.8.

4.4 Improvement of the Sensitivity: Electrode Material [Publication V & VI]

The sensitivity of an electrochemical sensor with regard to the limit of detection, is apart from the selectivity, the most important aspect for an application in DNA detection. There are several strategies to improve the sensitivity as mentioned above. We expected bismuth electrodes to form more stable alkanethiol SAMs as on gold. On the other side it should be possible to use more negative potentials and so other redox markers for the DNA detection. Unfortunately the affinity between bismuth and alkanethiol was that strong that no well ordered SAMs could be observed. Instead the formation of thiolate multilayers was found. Similar observations were described by Romann *et al*. [Romann 2007]. Sequence specific DNA detection was not possible with bismuth electrodes modified with an immobilized capture probe layer. That was why we decided to investigate whether new compositions of electrode materials could help to improve the limit of detection of DNA sensors. Mikkelsen *et al*. worked on several interesting publications dealing with alloy materials for electrochemical measurements [Mikkelsen 2000, Mikkelsen 2002a, Mikkelsen 2002b, Mikkelsen 2006, Skogvold 2008]. Especially the combination of a noble metal (like gold) with a small fraction of a compound with hydrogen overvoltage (like bismuth) seemed to be promising to build DNA sensors with alkanthiols and a wide working area to negative potentials. We expected to see the catalytic osmium peak at a gold-bismuth electrode if we can increase the potential window to more negative potentials as it is known for mercury or amalgam electrodes. Although we could recognize a wider potential window at the gold-bismuth electrode compared to a bare gold electrode, we were not able to detect the catalytic osmium signal.

We started with a self-made gold-bismuth (4% bismuth) alloy electrode. As to characterize the electrode material, we did some investigations into the electrochemical behavior of daunomycin (a typical DNA intercalator used as a drug in cancer therapy) compared to mercury, silver-mercury and gold-mercury electrodes. Adsorptive stripping voltammetry experiments showed benefits of the gold-bismuth electrode which can be found in the great linear concentration range up to 1 µM (shown in figure 17).

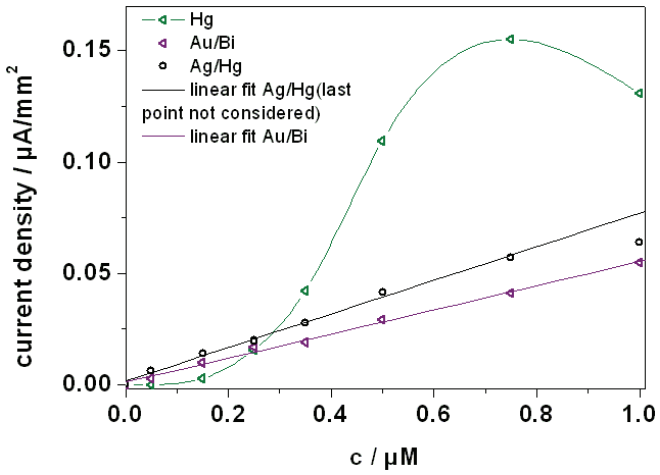

Figure 17: Adsorptive Stripping calibration plot for daunomycin at different electrode materials. Signals were recorded with differential pulse voltammetry in 200 mM acetate buffer (pH 5) with a deposition time of 120 s (deposition at -0.65 V). (Publication V, figure 7)

We also applied gold bismuth electrodes for the DNA detection. Here a capture probe was immobilized via gold-thiol chemisorption followed by incubation with an alkanthiol to form a self assembled monolayer. After a hybridization step with a complementary target sequence (labeled with [OsO_4(bipy)]) square wave voltammetric measurements were performed. Our experiments indicate a lower response on the gold-bismuth electrode than on the pure gold electrode (figure 18). So gold-bismuth electrodes do not seem to be the right way to increase the sensitivity of DNA sensors based on [OsO_4(bipy)].

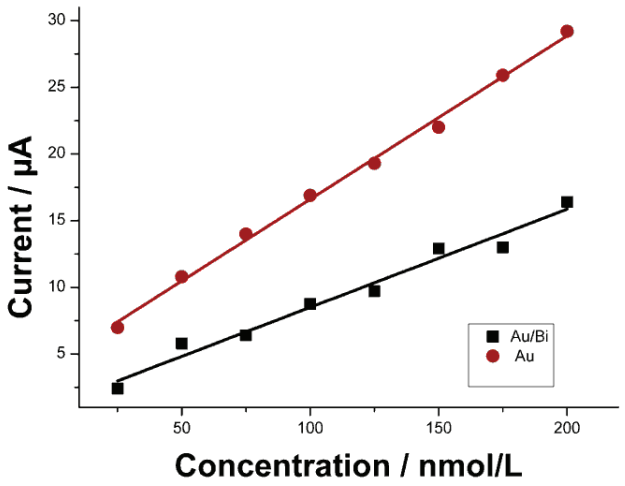

Figure 18: Calibration plot for DNA detection (targets labeled with [OsO4(bipy)]) at gold and gold/bismuth electrodes. Signals were obtained in 10 mM Tris-buffer (pH 7.5) containing 0.5 M Na_2SO_4. Hybridization was performed in a stirred solution for 15 minutes. (Publication VI, figure 4)

4.5 Improvement of the Sensitivity: Surface Modification [Publication VII]

Another strategy to increase the sensitivity of electrochemical sensors is the nano- and micro-structuring of the electrode surface so that the electro-active area grows whereas the outer geometric structure remains untouched. This holds great potential for the miniaturization of electrodes and especially electrode arrays. In our experiments we started with “simple” electroplating in cooperation with a research group at the Fraunhofer IZM Berlin. Galvanically controlled gold electroplating on freshly polished gold macro electrodes delivers well ordered structured surfaces. The structures were characterized by cyclic voltammetry in sulfuric acid and so the electro-active surface was determined by analyzing the gold oxide reduction peak. We then could increase the electro-active surface up to 700 % with optimized process conditions. Using a two step deposition (in the first step silver was deposited and in a second step gold was deposited on the silver surface) we could reach a 15-fold enhancement of the electro-active area (determined by cyclic voltammetry in sulfuric acid). Surface images of the gold and silver/gold modified electrodes are shown in figure 19.

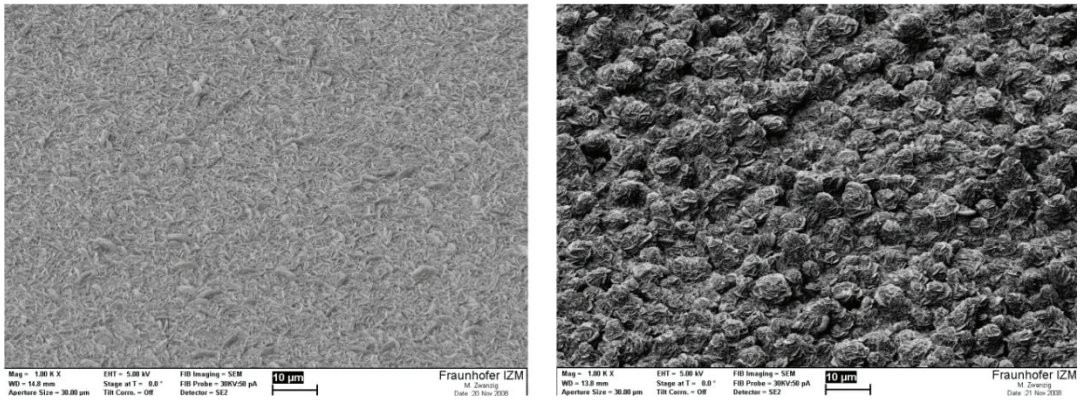

Figure 19: Surface images of gold and silver/gold modified electrodes. (Publication VII)

In further experiments the modified electrodes were tested regarding their suitability for DNA hybridization sensors. The immobilization of capture probes as well as the detection of complementary target sequences were carried out successfully. Even dehybridization steps and so the regeneration of the capture probe modified surface

could be performed effectively. The obtained hybridization signals were significantly higher on electrodes with surface modifications. The signals could be increased 4-fold at the gold modified electrode and 9-fold at the silver/gold electrode compared to the unmodified gold electrode. Although the hybridization signals with the complementary target could be increased non complementary targets do not cause a signal. The selectivity of the immobilized capture probe does not suffer from the surface modifications. These modifications are powerful tools to increase the sensitivity of DNA sensors, enhance the linear concentration range and lower the limit of detection. They will be useful for the miniaturization of electrochemical DNA arrays with µ-electrodes.

4.6 Detection of Polymerase Chain Reaction Products [Publication VIII]

The electrochemical detection of osmium labeled products (by means of protective strands) of symmetric polymerase chain reaction is only possible by using a sophisticated strategy. One primer has to be phosphorylized so that one single strand can be enzymatically degraded. The remaining single strand can be labeled with [OsO_4(bipy)] after having added a protective strand [Reske 2007]. The disadvantages of this procedure are the numerous steps necessary to create the single strand, necessity of a labeled primer and the necessity of sample purification/concentration after the enzymatic degradation and before the hybridization step. In order to improve the situation we decided to use an asymmetric polymerase chain reaction to create single stranded DNA directly. This is possible by adding one primer in a 10-fold excess whereas for symmetrical polymerase chain reactions the primers are added in equimolar concentrations. Even though the efficiency of an asymmetric polymerase chain reaction is not as good as in symmetric polymerase chain reaction this method is very well applicable to DNA detection. Although a certain amount of double stranded DNA ist produced during the polymerase chain reaction the product can be taken directly without any further purification to label it with [OsO_4(bipy)]. The double stranded DNA can not be labeled with the [OsO_4(bipy)] and so does not cause any signal. Only the single stranded product can be labeled after the addition of a protective strand. The labeled product delivers distinct electrochemical signals without any loss of selectivity. Relatively high signals can be achieved even after short hybridization times especially if the hybridization step is performed at an elevated temperature. This is shown in figure 20. Here the signal height can be doubled when the 30 min hybridization step is performed at 50 °C instead of 23 °C. Experiments to evaluate the optimum temperature for the hybridization displayed 50 °C to be the best hybridization temperature. Even small changes in the hybridization temperature to 45 °C or 55 °C result in a signal drop (about 30-35%). This behavior can be partially explained with the start of the melting curve as described earlier [Flechsig 2005b].

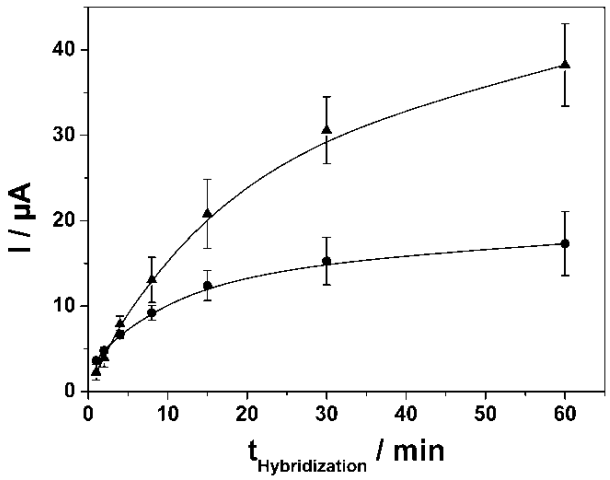

Figure 20: Dependency of the hybridization time and temperature to the electrochemical signal. Signals were recorded using square wave voltammetry. Hybridization was performed in a stirred solution containing 20 nM target DNA. The figure shows signals obtained at 23 °C (dot) and 50 °C (triangle) hybridization temperature. (Publication VIII, figure 3)

These results demonstrate a solution for a simple target amplification procedure which can be coupled with our osmium labeling procedure and the electrochemical detection. This will be useful for a micrufluidic device for DNA amplification coupled with sensitive DNA detection. Samples consisting of double stranded DNA can be partially converted to single stranded DNA and are then usable for the osmium labeling and electrochemical detection. Furthermore we could show the importance of an optimized hybridization temperature.

4.7 DNA-Detection without Target Labeling [Publication IX]

Time consuming target labeling procedures are necessary to use the DNA-detection methods described above. In a first step, the target sequence has to hybridize with an additionally added protective strand which usually takes two hours. In the second step, the formed partial double strand can be modified with [OsO_4(bipy)] on the single stranded part (non protected part). After a further reaction time of about two hours a dialysis is performed over night to remove the excess of [OsO_4(bipy)]. So all in all this labeling procedure takes about 14 hours before the first measurement can be performed. We wanted to reduce this time and developed a new DNA-detection method with [OsO_4(bipy)] labeled sequences. Instead of labeling the target sequence we decided to label a signaling oligo. The signaling oligo had the same sequence as the target DNA which should be detected. Protecting the signaling oligo sequence during the labeling procedure assures the possibility of hybridization. Only the five unprotected thymine nucleobases are labeled. These labeled signaling oligos are allowed to hybridize to the immobilized capture probe over night. Starting at a high electrochemical signal a significant decrease can be observed as soon as the unlabeled target DNA is added to the solution as shown in figure 21.

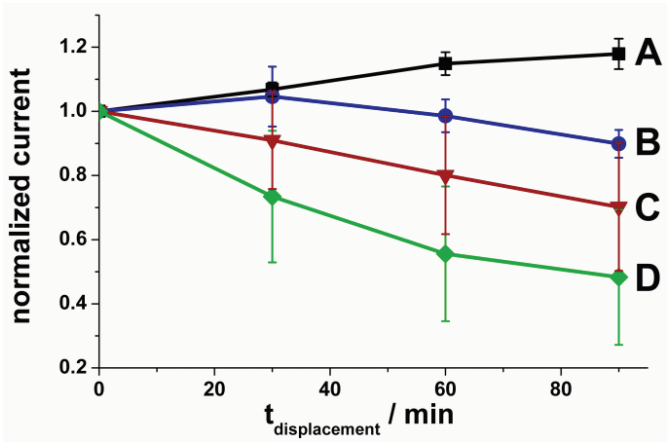

Figure 21: DNA-detection by means of signal oligo displacement. Square wave voltammetric signals obtained with signaling oligo concentration of 100 nM and hybridization temperature of 35 °C. (Publication VIII, figure 3). The curves show the response of the pre-hybridized electrodes to A) 0 nM target concentration, B) 100 nM non complementary target, C) 100 nM target with 1 base pair mismatch and D) 100 nM fully complementary target. (Publication IX, figure 4)

This method was proved to be able to distinguish between complementary and non complementary targets. With optimized conditions even the detection of a single base mismatch is possible within 60 minutes. We suppose this method to have a huge potential in diagnostics. As the time-consuming preparation of the electrode can be carried out in advance, the complete DNA detection can be performed in 30 to 60 min after the target sequence is available. Even the determination of concentration is possible as our results prove. This seems to indicate that there is a good possibility for a coupled DNA amplification and detection. The capture probe modified electrodes can be hybridized with the signaling oligo prior to the amplification step. As soon as the amplification starts the modified electrodes can detect the amount of produced target as no long reaction times are necessary.

4.8 Detection of Genetically Modified Maize [Publication X]

After evaluating the method of osmium labeling by means of protective strands in simple solutions with a negligible matrix or a simple matrix (polymerase chain reaction solution) we wanted to characterize this method in more complex solutions / which are more relevant in reality. We selected the detection of genetically modified maize as an example. We chose four capture probe sequences to detect a) the presence of the maize gene (2 capture probes), b) the presence of the genetically modification (1 capture probe) and to find out whether the genetically modification is inserted into the maize gene at the right position (1 capture probe). To simplify the experiment, we used synthesized oligonucleotides for our experiments. Nevertheless, several approaches were made in these experiments. It was the first time that we labeled more than one target sequence with [OsO_4(bipy)] by means of protective strands at the same time. We were able to perform the simultaneous labeling of -different sequences in the same solution. In the above mentioned publication (publication X) only four sequences are presented. Actually, we also carried out the experiments with five sequences, in that way that we added the fifth sequence as an indicator for a successful labeling procedure. Our experiments demonstrated the versatility of the [OsO_4(bipy)] labeling by means of protective strands. The selectivity does not suffer from the simultaneous labeling. Interferences between the different target sequences or protective strands were not observed when the sequences were chosen carefully. The discrimination between an artificial genetically modified (MON 810) maize sample and the non modified sample could be performed without problems as figure 22 shows.

The determination of the concentration was possible as well. We could also show that elevated temperatures during the hybridization step can improve the sensitivity as well as the selectivity in this kind of application. An explanation for this result can be found among other things in the reason that secondary structures, which DNA single strands form at low temperatures, are not stable at elevated temperatures. This is shown in publication X in figure 5.

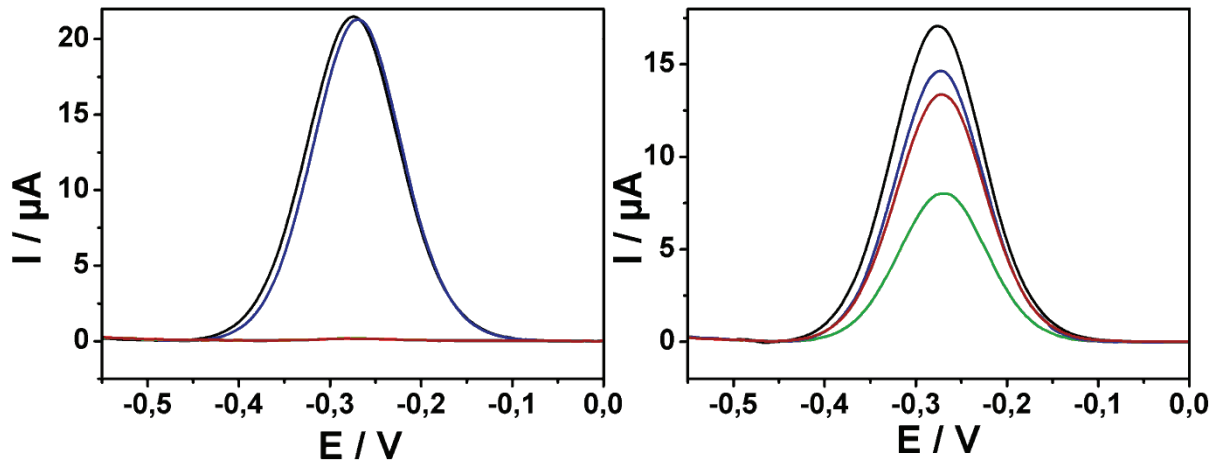

Figure 22: Detection of genetically modified maize. The figure displays the response to an artificial genetically non modified sample (left) and genetically modified sample (right). The blue and black signals represent the presence of maize, the red signal the presence of the genetical modification sequence and the green signal assures that the genetical modification is inserted at the right position into the maize gene. (Publication X)

Aiming at the realization of a simultaneous detection of several DNA sequences we developed a DNA-array with 16 electrodes. 16 electrodes are not that much for DNA arrays but in our case the electrodes are heatable. Each electrode is individually and independently addressable for electrochemical measurements and for the temperature control. We considered our technical expertise described above (Publication IV) for the design of the electrode. A picture of our 16-electrode array is shown in figure 23.

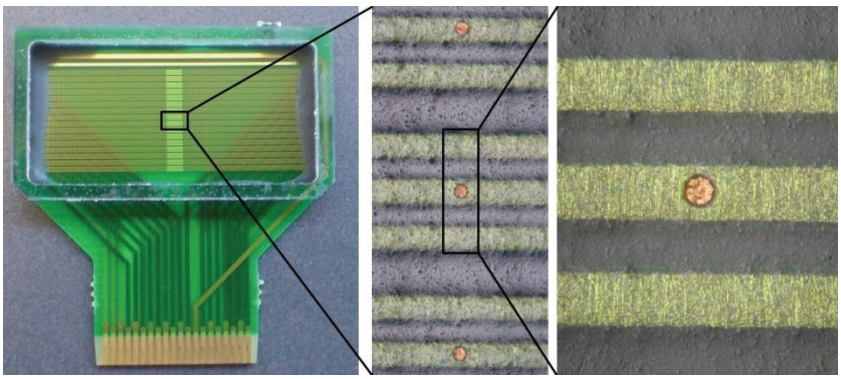

Figure 23: Heatable 16-electrode array. Each electrode (yellow spots) is indepently addressable for heating and measuring without influencing the other electrodes. The whole chip is covered with an isolating lacquer layer and only the small spots are laid open with laser ablation.

The necessary equipment for the temperature control was developed as well. The device, which allows us to address each electrode separately for the electrochemical measurement, was developed together with the company Friz Biochem. Our appropriate technique allowing us to heat the electrodes and perform measurements at the same time (inductive bridges, Publication I) was integrated. At the same time we developed a heating multiplexer on our own which allowed us to control the temperature on each electrode individually. Experiments with regard to the combination of all these components are conducted in our lab. The electrode array has already been tested for the detection of genetically modified maize. In 30 to 60 minutes it is possible to distinguish whether the sample was genetically modified or not as shown in figure 24. Although we only obtain very small signals, distinct results are obtained due to the low background level.

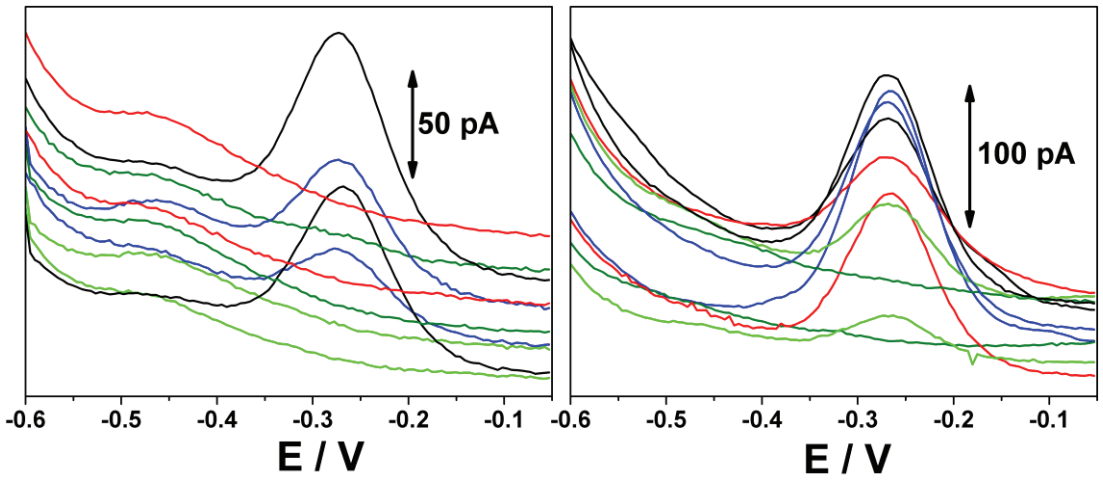

Figure 24: Voltammetric detection of genetically modified maize (16-electrode array). The signals were recorded using the heatable 16-electrode array (not all curves shown). In the left figure the signals obtained after a hybridization time of 60 minutes with an artificial maize sample are presented and B) shows the signals recorded after a hybridization time of 60 minutes with an artificial genetically modified maize sample. The blue and black signals represent the presence of maize, the red signals the presence of the genetical modification sequence and the light green signals assure that the genetical modification is inserted at the right position into the maize gene.The dark green lines are recorded on electrodes which were modified with mercaptohexanol only. (unpublished data)

4.9 Convective Polymerase Chain Reaction [Publication XI]

The detection of DNA in very low concentration is very important for e.g. diagnostic applications as described in chapter 3. One way to solve this challenge is the development of ultra sensitive DNA sensors. The other and in practical application more often used strategy is the combination with a preliminary polymerase chain reaction. In the polymerase chain reaction the target sequence is amplified exponentially so that regular DNA sensors can be used for the detection. To perform a polymerase chain reaction, special equipment (thermocycler) is necessary. In a thermocycler the sample is heated and cooled down in cycles to amplify the target DNA. This takes approximately two hours in conventional instruments. Some modern instruments can lower the time to about one hour. Some years ago Braun *et al*. introduced the “Junkyard PCR” [Braun 2004a, Braun 2004b, Braun 2002, Eisenstein 2006]. Braun *et al*. showed the successful amplification of DNA just by dipping heated wire into a solution for a polymerase chain reaction. This idea was first published by Hwang *et al*. [Hwang 2001]. Krishnan *et al*. described a simplified PCR in a Rayleigh-Benard convection cell [Krishnan 2002]. Our idea was to apply our heated wires (which we usually use as electrodes) for the DNA amplification. We started our experiments with a self-made setup of a small plastic tube where a small platinum wire was inserted close to the bottom. After the isolation we performed temperature calibration (chapter 2.3) to be able to control the polymerase chain reaction as precisely as possible. In our experiments we could demonstrate a successful amplification of target DNA even by carrying out asymmetric polymerase chain reaction. The product could be detected electrochemically after labeling with $[OsO_4(bipy)]$ as shown in figure 25. In the publication we compared our convective polymerase chain reaction with a conventional polymerase chain reaction. The convective polymerase chain reaction is not as powerful as a polymerase chain reaction performed in a themocycler but it was sufficient for a selective DNA detection. Even an asymmetric and convective polymerase chain reaction was possible and the product was electrochemical detectable. The equipment needed for the convective polymerase chain reaction is less expensive and smaller. It was the

first time that the convective polymerase chain reaction was coupled with the electrochemical detection. A significant discrimination between complementary and non complementary target was possible.

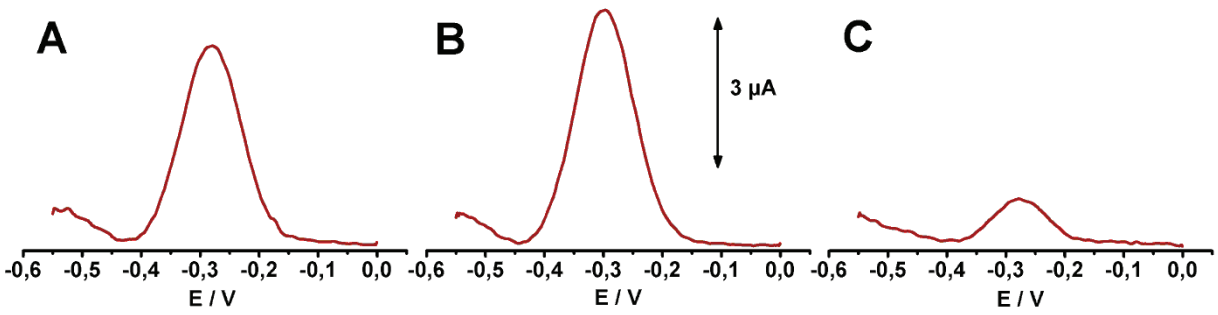

Figure 25: Voltammetric detection of the convective asymmetric PCR product. The graphs show the response of the (labeled) PCR product after A) 15 minutes and B) 30 minutes reaction time to a complementary capture probe. Figure C) displays the signal of the 30 minute product to a non complementary capture probe. Hybridization was performed for 30 minutes at 50 °C in a stirred solution. (Publication XI)

The current-time curve obtained from the electrochemical signals in dependency to the heating time (during the PCR) shows a similar behavior as known from real time polymerase chain reactions where fluorescence is detected. The amount of produced target in the convective polymerase chain reaction is not as high as if performed in a classical thermocycler. The reason for this could be found by mathematical simulations of the convective polymerase chain reaction. These simulations show the existence of several clearance volumes where the sample remains for a certain time without amplifying the DNA. The improvement of the cell design holds great promise to enhance the performance of the amplification by eliminating the clearance volumes. With enhanced performance the convective PCR presents a good method to be coupled with electrochemical detection of the amplified target sequence.

5. Summary and Outlook

Our research revealed dramatic potential for heated electrodes in bioanalytical chemistry. Due to our invention of the inductive bridge, symmetrical electrodes are no longer necessary for electrochemical measurements at electrically heated electrodes. We consider this new method to be the easiest way to realize measurements at heated electrodes reported yet. Neither expensive nor complicated instruments are required any more. Signal decreases due to electrode fouling / electrode blocking can be minimized effectively by combining heated electrodes and electrochemical pretreatment. This holds great potential for applications in long-term monitoring especially in hidden places where manual regeneration is connected to a high operating expense. Another interesting area of application for heated electrodes might be the use as a sensor in flow systems as also in high performance liquid chromatography. Beside the self cleaning properties, heatable electrodes can lower the limit of detection or even just allow detecting several substances. This complex will be explored in detail in a future project.

Improving the sensitivity in DNA-detection could be achieved by surface modification. Here our investigations have just started but first results are very promising for prospective developments. By means of further experiments we will characterize this type of modifications on micro electrodes and DNA arrays and apply them in DNA detection. Enzyme labeling provides another opportunity to enhance the sensitivity we are working on at the moment. In combination with heated electrodes this will help significantly to lower the limit of detection.

The detection of DNA with $[OsO_4(bipy)]$ by means of protective strands was investigated. A simple procedure for the detection of polymerase chain reaction products was developed. Using this asymmetric polymerase chain reaction allows direct labeling of the product without enzymatically treatment or further purification steps. Without labeling the target, our displacement procedure allows fast detection of DNA. Single base pair mismatches can be detected. The simultaneous labeling of four different DNA sequences was reported the first time. High selectivity was

demonstrated allowing the detection of a genetically modified maize sample and it was possible to distinguish between genetically not modified and genetically modified maize samples. Further investigations using our developed 16-electrode array for the simultaneous detection of DNA sequence are currently being performed. In the future we want to optimize the detection on this array in several aspects. Placing the temperature of each electrode individually will enable us to adjust stringent hybridization conditions at each electrode. This should allow us to discriminate between fully complementary targets and targets with one, two or three single base pair mismatches. Consequently, this will be a great improvement in comparison to other DNA arrays available where the hybridization temperature is the same for all electrodes. Measurements in more complicated matrices should be possible. Of course, this new method will be applied and examined with real samples of genetically modified maize. Beside the stringent hybridization conditions, the heated electrodes cause a temperature gradient and therefore convection. This fact should enable us to reduce the hybridization time significantly or if the time is kept constant, higher signals should be able to be achieved. In combination with surface modified electrodes and enzymatical labeling highly sensitive arrays should be possible. To improve the selectivity we want to add a further procedure after the hybridization detection is finished. By heating up the electrodes constantly and performing electrochemical measurements in this period, electrochemical melting curves can be recorded. This will provide us with additional data which helps to detect single base pair mismatches.

Instead of using ultra sensitive DNA sensors, the combination of polymerase chain reaction and DNA sensors is another promizing strategy. This combination is very powerful due to the exponential amplification during the polymerase chain reaction. As mentioned above, our heated electrodes allow us to perform a polymerase chain reaction without a thermocycler. In the future, we want to combine the DNA amplification with the DNA detection. Therefore, several capture probe modified electrodes will be needed near to the heated wire/metal-surface where the polymerase chain reaction takes place. Making use of our 16-electrode array will allow us to perform a real time polymerase chain reaction with an electrochemical detection. So, this might, hopefully, be a huge improvement because equipment with

optical detection used at present (for real time polymerase chain reaction) is expensive and bulky. Our technology will allow a significant miniaturization and will also lower the costs. To eliminate the labeling procedure after the DNA amplification we think of using labeled primers. Whereas [OsO_4(bipy)] labeled primers (by means of protected strands) could not be applied successfully to the polymerase chain reaction yet, we have promising results with commercially available ferrocene- or methyleneblue labeled primers as figure 26 shows (unpublished data). Even [OsO_4(bipy)] labeled primers could be successfully used if they were prepared by using magnetic beads to remove the protective strands.

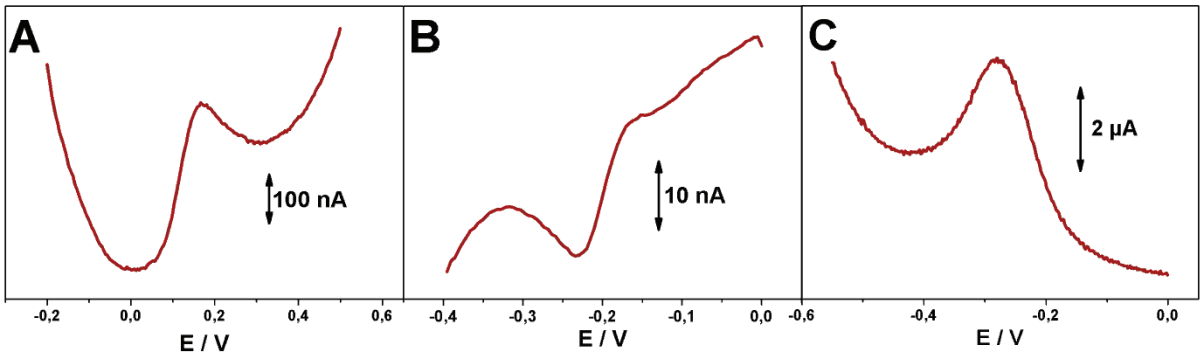

Figure 26: Voltammetric signals of PCR products obtained with labeled primers.The asymmetric PCR was performed with A) one ferrocene-labeled primer, B) one methylene-blue-labeled primer and C) one [OsO_4(bipy)] labeled primer. The signals were recorded using A) alternating current voltammetry, B) differential pulse voltammetry and C) square wave voltammetry. (unpublished data)

The measurements were performed using different voltammetric techniques. The reason for this was that with the [OsO_4(bipy)] labeled single strands mainly used square wave voltammetry we were not able to measure any signals with the methylene blue and ferrocene labeled primers. Here we used previous established methods known from the literature [Pänke 2007, Flechsig 2005b]. The obtained results are not directly comparable due to the different voltammetric methods used. Nevertheless these results show the successful target amplification and labeling during the polymerase chain reaction.

Figure 27 presents the signal-time curves obtained with the polymerase chain reaction products with labeled primers. As already indicated in figure 26, the product

obtained with the [OsO_4(bipy)] labeled primer delivers significant higher signals than the ferrocene or the methyleneblue labeled primer. Although different measuring techniques were used to obtain these results, it can be seen that the osmium labeled primer are most promising for low limits of detection and so further investigations will be performed with [OsO_4(bipy)] labeled primer.

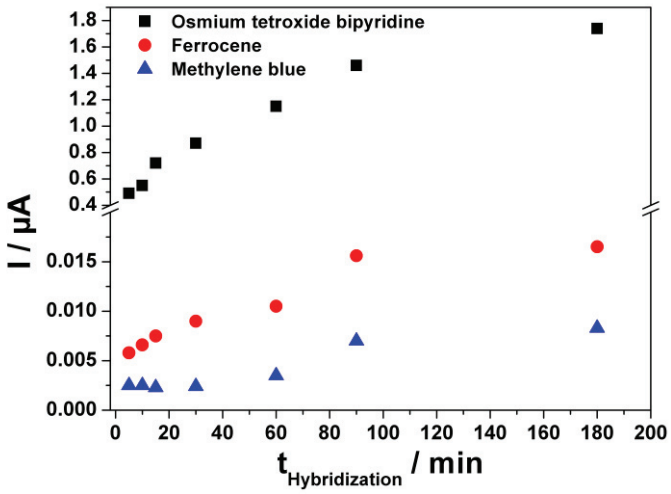

Figure 27: Voltammetric signals of PCR products obtained with labeled primers. The signals were recorded using alternating current voltammetry (osmium), differential pulse voltammetry (methylene blue) and square wave voltammetry (ferrocene) (unpublished data).

Another possibility is the use of labeled nucleobases that we will explore in the near future. To improve the amplification at heated electrodes we are currently working on two projects. In both projects the movement of the solution is not only caused by convection due to the temperature gradient. In one project the movement is caused by a miniaturized pump and by means of mechanical movement of the pump. The other project uses alternating current electro-hydrodynamic micro-pumps [Stubbe 2007] where the movement is caused without any mechanical moving parts. Both strategies are very promising as they will allow miniaturized systems for real time polymerase chain reaction with electrochemical detection at a low price.

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7. Publications

1. F. Wachholz, J. Gimsa, H. Duwensee, H. Grabow, P. Gründler, G.-U. Flechsig, *A compact and versatile instrument for radio frequency heating in non-isothermal electrochemical studies*, Electroanalysis 19 (2007) 535

2. H. Duwensee, T. Vázquez-Alvarez, G.-U. Flechsig, J. Wang, *Thermally induced electrode protection against biofouling*, Talanta 77 (2008) 1757

3. G.-U. Flechsig, H. Duwensee, J. Wang, *Verfahren zur elektrochemischen Behandlung einer Sensorvorrichtung*, Patentanmeldung Az. DE 10 2008 020 802.7 (2008)

4. N. Mahnke, A. Markovic, H. Duwensee, F. Wachholz, G.-U. Flechsig, U. van Rienen, *Numerically optimized shape of directly heated electrodes for minimal temperature gradients*, Sensors & Actuators B - Chemical 137 (2009) 363

5. H. Duwensee, M. Adamovski, G.-U. Flechsig, *Adsorptive stripping voltammetric detection of daunomycin at mercury and bismuth alloy electrodes*, Int. J. Electrochem. Sci. 2 (2007) 498

6. H. Duwensee, A.-E. Surkus, G.-U. Flechsig, *Detection of DNA-Hybridization on Gold-Bismuth (Au/Bi) Alloy Electrodes,* Poster contribution ESEAC 2008

7. F. Wachholz, H. Duwensee, R. Schmidt, M. Zwanzig, J. Gimsa, S. Fiedler, G.-U. Flechsig, *Template-free galvanic nano-structuring of gold electrodes for sensitive electrochemical biosensors,* Electroanalysis (2009) accepted

8. M. Mix, T. Reske, H. Duwensee, G.-U. Flechsig, *Electrochemical detection of asymmetric PCR products by labeling with osmium tetroxide*, Electroanalysis 21 (2008) 826

9. H. Duwensee, M. Jacobsen, G.-U. Flechsig, *Electrochemical competitive hybridization assay for DNA detection using osmium tetroxide-labelled signalling strands*, Analyst 134 (2009) 899

10. H. Duwensee, M. Mix, I. Broer, G.-U. Flechsig, *Electrochemical detection of modified maize gene sequences by multiplexed labeling with osmium tetroxide bipyridine,* Electrochem. Commun. (2009) accepted

11. H. Duwensee, M. Mix, M. Stubbe, J. Gimsa, M. Adler, G.-U. Flechsig, *Electrochemical Product Detection of an Asymmetric Convective Polymerase Chain Reaction,* Biosens. Bioelectron. (2009) submitted

Own Contribution

	Titel	Heiko Duwensee´s own Contribution
1	*A Compact and versatile instrument for radio frequency heating in non-isothermal electrochemical studies*	H.D. promoted the development of the heating generator together with H. Grabow. H.D. performed all experiments with the glassy carbon electrode. He contributed in writing and prepared the figures with the glassy carbon electrode.
2	*Thermally induced electrode protection against biofouling*	H.D. planned, performed and analyzed all experiments. He contributed in writing. Partly T. Vázquez-Alvarez assisted during the experiments and during the preparation of the figures.
3	*Verfahren zur elektrochemischen Behandlung einer Sensorvorrichtung*	H.D. contributed in writing and prepared the figures. The mentioned experiments were carried out by H.D.
4	*Numerically optimized shape of directly heated electrodes for minimal temperature gradients*	H.D. explained the assignment of tasks to the cooperating group which then performed the simulations. He contributed in writing and interpreting the received data.
5	*Adsorptive stripping voltammetric detection of daunomycin at Au/Bi and amalgam electrodes*	H.D. planned, performed and analyzed all experiments. He contributed in writing and prepared all figures.
6	*Detection of DNA hybridization with Au/Bi alloy electrodes*	H.D. planned, performed and analyzed all experiments. Partly S. Krüger assisted in the preparation of the electrodes and carried out some experiments. H.D. did all the writing and the preparation of the figures on his own.
7	*Template-free galvanic nano-structuring of gold electrodes for sensitive electrochemical biosensors*	H.D. performed and analyzed the cyclic voltammogramms together with F. Wachholz. H.D. performed most of the DNA measurements on his own. He contributed in interpreting the data, writing and he prepared some of the figures.
8	*Electrochemical detection of asymmetric PCR products by labeling with osmium tetroxide*	H.D. contributed in writing and in the interpretation of the results. He prepared some figures.
9	*Electrochemical competitive hybridization assay for DNA detection using osmium tetroxide-labelled signalling strands*	H.D. planned all experiments and assisted in the interpretation of the received data. He contributed in writing and created all figures.
10	*Electrochemical detection of modified maize gene sequences by multiplexed labeling with osmium tetroxide bipyridine*	H.D. planned all experiments and assisted performing the experiments and in the interpretation of the received data. He contributed in writing and created all figures.
11	*Electrochemical Product Detection of an Asymmetric Convective Polymerase Chain Reaction*	H.D. planned all experiments and performed most of the experiments. He assisted in the interpretation of the received data. He contributed in writing and created all figures.

Publication I

Falko Wachholz, Jan Gimsa, Heiko Duwensee, Harald Grabow, Peter Gründler, Gerd-Uwe Flechsig,

A compact and versatile instrument for radio frequency heating in non-isothermal electrochemical studies

Electroanalysis 19 (2007) 535

Full Paper

A Compact and Versatile Instrument for Radio Frequency Heating in Nonisothermal Electrochemical Studies

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Received: November 1, 2006
Accepted: December 15, 2006

Abstract
We describe a novel instrument and electrical circuit for sensitive electrochemical measurements at simultaneous direct electrode heating. The new measuring principle can be applied to working electrodes of various designs featuring two end contacts. In our experiments, the contacts were connected to a 100 kHz AC heating power supply and the potentiostat via the new inductor bridge circuit. A compact heating-generator housing contains all components necessary for sine wave generation as well as amplification and transformation of the heating power. The new arrangement yields high temperature cyclovoltammetric signals for the $[Fe(CN)_6]^{3-/4-}$ redox system with a noise level superior to the earlier symmetrically branched wire electrode designs. Noise and disturbances are dramatically suppressed especially for high resistance electrodes such as glassy carbon electrodes. Without a center contact, the working electrode design is greatly simplified. This opens new opportunities for the design of a great variety of heated electrodes that may be arranged in arrays or consist of materials with relatively high resistivity such as carbon and conducting polymers.

Keywords: Heated electrodes, Glassy carbon, Gold, Hot-wire electrochemistry, Inductor Bridge, Cyclic voltammetry

DOI: 10.1002/elan.200603757

1. Introduction

Temperature is an important adjustable parameter in electrochemical experiments apart from concentration, time, potential and current. Commonly, thermostated cells are employed to adjust the temperature in electrochemical experiments. Nevertheless, this approach brings about some problems. The whole cell including all electrodes and the bulk electrolyte must be heated. Delicate substances may decompose, purgeable compounds may vanish. The potential of the reference electrode shifts with changing temperature even though this effect may be prevented by separating the reference electrode from the bulk electrolyte by a salt bridge. Also, much energy is needed to reach and keep a high medium temperature or to adjust a defined temperature by heating or cooling, a process requiring a relatively long time. For these reasons, the above approach makes it hard to put "thermoelectrochemistry" into practice. Burstein and Moloney presented a technique for corrosion studies that they called "thermammetry", where the bulk solution is heated and cooled slowly by an external supply, while recording current vs. temperature at a constant potential [1]. Electrically heated electrodes have been proven to add great benefit to the analyses of different kinds of substances. Firstly, mass transport is generally enhanced by the micro-stirring effect caused by the thermal convection in the vicinity of the electrode surface. Secondly, kinetically sluggish reactions are accelerated. Thirdly, experiments at different temperatures can be conducted to distinguish different substances, and hence to increase the selectivity of the measurement. Several techniques with electrically heated electrodes have been developed during the last 40 years. In early experiments, the electrodes were heated indirectly, leaving the bulk solution temperature unaffected. In 1976 Harima et al. developed an indirectly heated gold film electrode [2]. Indirectly heated platinum electrodes have been applied to detect NADH while preventing electrode fouling [3], as well as to construct a heated glucoseoxidase biosensor [4]. Compton et al. employed microwave radiation to activate the electrode surface [5–7]. Even carbon electrodes can be heated by microwaves [8]. Baranski has demonstrated that a high frequency electrical heating current (0.1 to 2 GHz) can be applied to heat a thin solution layer close to the electrode surface without disturbing the electroanalytical signal detection [9].

Direct electrode heating in an electrolyte solution was first described by Ducret and Cornet in 1966 [10]. These authors used a thin platinum wire heated by AC in the audio-frequency range. Already at that time, it was evident that AC-heating is superior over DC-heating since the former avoids electrode polarization due to the iR-voltage drop along the length of the electrode. Nevertheless, even AC can generate an undesired polarization, causing a strong dis-

tortion of the voltammetric signals at lower frequencies. However, AC-heating avoids faradic processes. Gabrielli et al. [11, 12] presented preliminary results of 6 °C-temperature jump experiments with Pt wire electrodes in the ferro-/ferricyanide system using 250 kHz-AC heating. Adequate electrical filtering allowed to discriminate against AC contributions to the measuring signal. A similar arrangement was used in a preliminary experiment by Gründler et al. [13]. These authors used a 25 μm Pt wire with asymmetric contacting, i.e., the potentiostat was connected to one end of the wire. The results obtained by this simple setup were not satisfactory with the ferro-/ferricyanide redox couple. Even electrical filtering could not prevent an erroneous behavior of the cell.

Modern potentiostats show a fast reaction and may also correct for AC distortion from the heating currents. Generally, the magnitudes of the heating currents are much stronger than the measuring signal and may lead to a complete overmodulation of the potentiostat. Up to now, this problem was overcome by a symmetric electrode design [14, 15]. In this case, the potentiostat is connected to a symmetric center contact of the electrode. This approach allows for good results as long as the resistance of the electrode is not too high.

Calculations and IR-images show that both soldering end contacts of a directly heated electrode cause a temperature drop resulting in a gradient along the electrode axis [16]. In symmetric electrodes the center contact represents an additional heat sink making the design of temperature-gradient free electrodes even more difficult. In general, the gradients can be reduced in electrode shapes with narrowing ends or by partial coverage of the electrode surface [17]. Although experiments with carbon fibers were not successful [13], directly heated carbon paste electrodes could be successfully applied for DNA analysis [18, 19] and heavy metal detection [20] when the heating current was applied only during the accumulation step. For similar applications, Svancara et al. developed an indirectly heated "Groove Electrode" (EH-GrE) filled with carbon paste [21].

Applications of continuously heated wire electrodes include the voltammetric determination of dissolved oxygen [22] and organic substances that are characterized by a sluggish electrochemical response and/or sensitivity against long term exposure to increased temperature [23]. Stripping analysis has been performed for arsenic(V) at gold [24], copper, mercury and arsenic(III) at gold [25] and lead as PbO_2 at platinum [26] electrodes while heating the electrodes during both steps, the accumulation and the stripping steps. Moreover, heated wire electrodes were applied in non-aqueous solvents like THF [27] and for monitoring flow streams [28]. Cytochrome *c* was detected by a directly heated LTCC-electrode [29]. Recently, Lin et al. described electroluminescence at a directly heated symmetric wire electrode [30].

The term "Temperature Pulse Voltammetry" (in analogy to differential pulse voltammetry) [31 – 34] designates experiments where short heat pulses are applied to the electrode during a voltammetric scan. Also in these experiments, the current is measured before and at the end of each pulse. The detected signal is the difference of the two currents. In any case, this technique requires simultaneous measurements of the imposed temperature changes and the electrochemical signal.

General principles and applications of high-temperature electrochemistry have recently been reviewed [35] with a focus on heated electrodes [36]. Briefly, different approaches exist for heating the working electrode while leaving the bulk electrolyte (including reference and counter electrode) at ambient temperature. The following techniques attained the highest attention: 1) direct joule heating of the electrode body, 2) indirect joule heating by a separate heater inside the electrode substrate and 3) microwave activation.

Here we describe a novel instrument and electrical circuit allowing for simultaneous sensitive electrochemical measurements and direct electrical heating of a working electrode with only two end contacts. The 100 kHz AC heating power is applied to both contacts that are also connected to the potentiostat via an inductor bridge. The compact heating generator housing contains all components necessary for the sine wave generation as well as the amplification and transformation of the heating power [37, 38]. We present measurements with a novel directly heated glassy carbon electrode.

2. Experimental

2.1. Materials

All measurements were carried out in 0.1 M KCl-solution containing 5 mM $K_3[Fe(CN)_6]$ vs. a Ag/AgCl (3 M KCl) reference electrode and a glassy carbon counter electrode (both from Metrohm AG, Herisau, Switzerland) at a constant bulk solution temperature of 21 °C. The cyclic voltammogram parameters were: start potential 0.6 V, 1st vertex potential 0.6 V, 2nd vertex potential − 0.2 V; step potential 0.008 V; and scan rate 100 mV/s.

Potassium chloride, potassium hexacyanoferrate(III) and potassium hexacyanoferrate(II) were delivered by Fluka. The construction of the symmetrically connected 25 μm diameter gold wire electrode has been described elsewhere [24]. Briefly, 5 mm long gold wires were soldered to both sides of a $20 \times 20\ mm^2$ double-side printed circuit board. The two wires were bridging a $5 \times 10\ mm^2$ cut out section. The soldering contacts and copper tracks were isolated by means of a paraffin/polyethylene mixture. The new directly heated glassy carbon electrode is depicted in Figure 1.

2.2. Instrumentation

Potentiometry and cyclic voltammetry were performed by means of an AUTOLAB potentiostat PGSTAT20 (Eco Chemie, Utrecht, The Netherlands). The heating generator was assembled from parts delivered by Conrad Electronic SE (Hirschau, Germany).

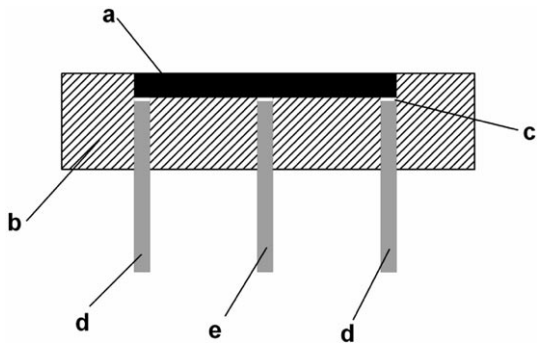

Fig. 1. Schematic representation of the new directly heated glassy carbon electrode, a) glassy carbon rod, b) epoxy resin body, c) silver epoxy glue, d) copper wire connection to the heating device and the optional inductor bridge, e) optional center-contact for a direct connection to the potentiostat.

The heating generator (Fig. 2) comprises the necessary circuitry such as the power supply, the waveform generator and the amplifier. It allows for a precise adjustment of the electrode temperature and features meters for heating current and frequency. The new component is a novel patent pending inductor/resistor bridge enabling the operation of symmetric and asymmetric electrodes (Fig. 3) [38]. We used inductors with 2.6 H and 9.2 Ω. The circuit blocks the AC heating current, while the measuring signal may pass undisturbed.

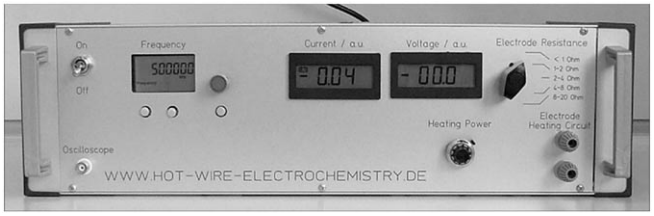

Fig. 2. The novel compact and easy to handle heating system.

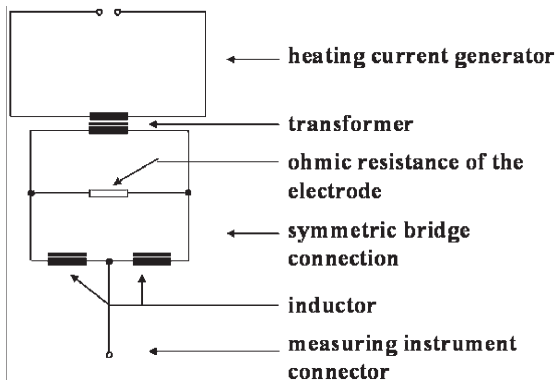

Fig. 3. Schematic drawing of the heating circuit including the novel symmetric inductor bridge connection used to block the AC heating current while leaving the measuring signal current undisturbed.

3. Results and Discussion

3.1. Temperature Calibration and Control

To adjust the temperature we performed temperature calibration experiments as described earlier [22, 36]. Briefly, the stationary temperature of all heated electrodes was measured by open circuit potentiometry using a 5 mM equimolar ferro-/ferricyanide solution in 0.1 M KCl. The temperature coefficient (– 1.56 mV/K) of this redox couple was used to calculate the temperature difference from the potential difference (vs. a reference electrode). Figure 4 displays an open circuit potentiogram of a temperature calibration experiment with a heated gold wire electrode. By adjusting various heating currents and plotting the temperature difference vs. current, we obtained temperature calibration functions. In the actual experiments the desired temperature was adjusted by adjusting the heating current.

Figure 5 exhibits the temperature calibration plot for the heated glassy carbon electrode. The maximum temperature

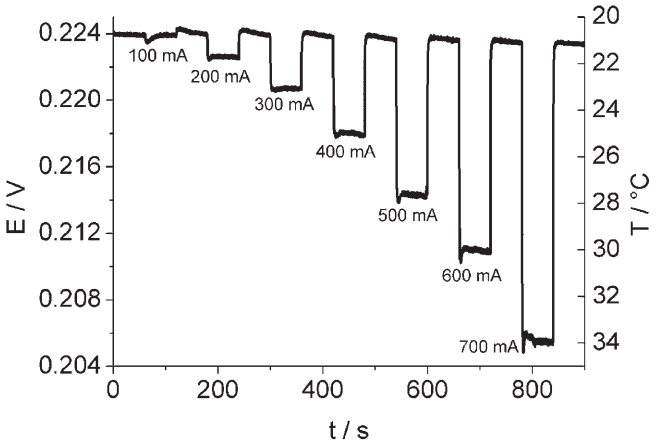

Fig. 4. Open circuit potentiometry temperature calibration of a gold-wire-electrode in a solution containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl vs. Ag/AgCl (3 M KCl) at 21° bulk solution temperature with the inductor bridge connection.

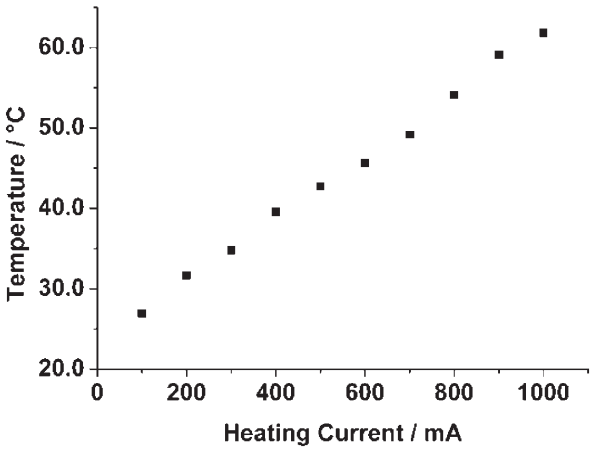

Fig. 5. Temperature calibration plot for the heated glassy carbon electrode measured under the same conditions as in Figure 4.

for this design is approx. 60 °C. At higher temperatures, the electrode center contacts were damaged.

3.2. Comparison of Asymmetric, Symmetric and Bridge Connection of the Working Electrode

3.2.1. Gold Wire Electrode

Starting in the early 1990s, Gründler et al. [13] used 25 μm Pt wire for an asymmetric heated electrode. The contacts at both ends of the wire were isolated from the electrolyte by silicon rubber. The potentiostat was connected to one end of the Pt-wire electrode. This simple asymmetric setup was tested with the ferro-/ferricyanide redox couple. Nevertheless, the results were unsatisfactory. Even with an efficient electrical filtering (low pass), the cell displayed an incorrect electrochemical behavior. The earlier used electro-mechanic plotters often were too slow to follow the disturbances caused by the heating AC (For a comparison of asymmetric with symmetric Pt wire electrodes see also [15, 22]).

Figure 6A depicts cyclic voltammograms (CVs) measured with an asymmetrically connected gold wire electrode at different heating currents representing different temperatures. Low heating currents up to 200 mA generating electrode temperatures up to 22 °C did not disturb the potentiostat-signal. However, a slightly higher heating current (300 mA) caused a faulty response. The expected signal increase at higher temperatures was not observed. Instead we observed a strong disturbing influence of the heating current and a complete failure of the electrochemical instrument at temperatures that were increased even further. In contrast, an almost full compensation of distortions caused by the AC heating current could be achieved when the voltammetric working electrode was equipped with a center contact connected to the working electrode input of the potentiostat [14, 15]. In this setup the AC generator was coupled to the electrode via a transformer floating vs. ground. The heating current generated an iR drop as before. However, with respect to the potentiostat's working electrode input, i.e., the symmetrical contact of the electrode, the two polarization voltages are equal in quantity and opposite in polarity at every moment. This has been explained earlier in more detail [14, 15]. Figure 6B depicts results of the same experiment as in Figure 6A for a symmetric connection of the heated Au-wire via a center contact. Here, the potentiostat is not affected by the heating current. We observed the advantageous effects that have been described earlier [13, 15, 22, 36]: briefly, due to enhanced mass transport (thermal convection and accelerated diffusion), the signal level is increasing with elevating electrode temperature. However, some visible noise could not be compensated. This effect illustrates the difficulties to construct an electrode with two perfectly symmetric branches. Even small disparities in length and, hence, ohmic resistance will cause the observed remnant signal noise. To demonstrate the ability of the novel inductor bridge

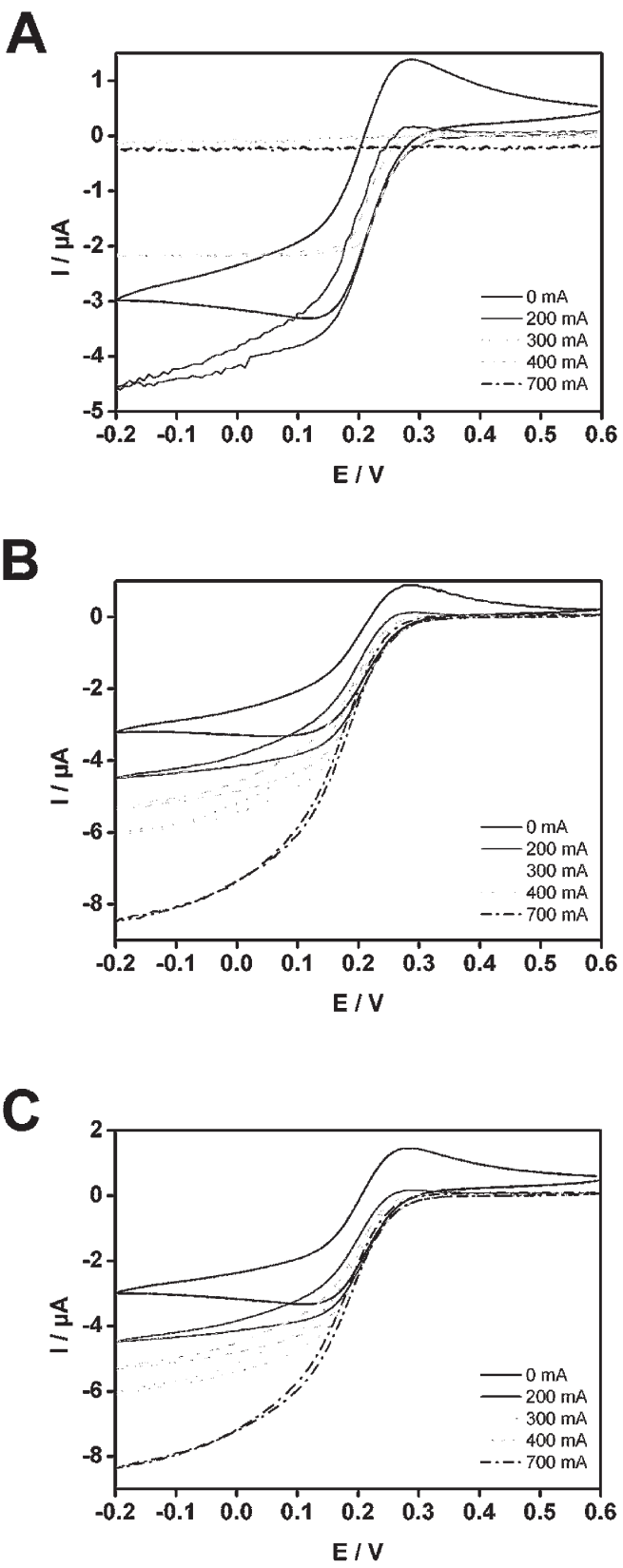

Fig. 6. Heating current effect during cyclic voltammetry for A) an asymmetrically connected one-piece gold wire, B) a symmetric two-piece gold-wire-electrode, and C) a one-piece gold-wire electrode connected via the inductor bridge. All measurements were performed in a solution containing 0.1 M KCl and 5 mM $K_3[Fe(CN)_6]$ vs. Ag/AgCl (3 M KCl) at 21° bulk solution temperature.

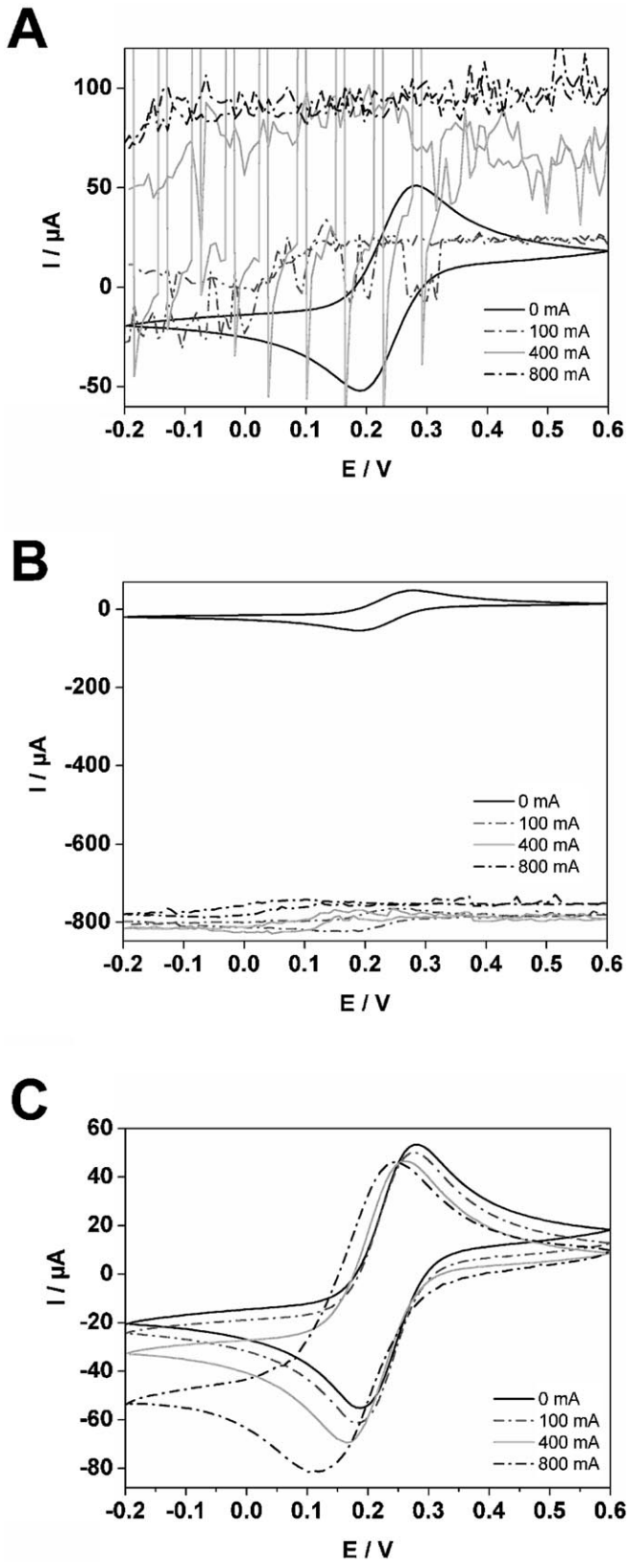

Fig. 7. The new inductor bridge drastically suppresses noise-effects for a heated glassy carbon electrode. A) Asymmetric connection, B) symmetric connection, C) connection via the new inductor bridge. Conditions as in Figure 6.

connection to fully suppress the heating current disturbances in the electrochemical signals, we connected the potentiostat via the bridge to both ends of the working electrode. Figure 6C indicates that all disturbing effects of the heating current can be eliminated by our new inductor bridge. A third center contact of the heated electrode is no longer required, significantly simplifying the electrode design.

3.2.2. Glassy Carbon Electrode

The efficiency of our new inductor bridge in suppressing noise and disturbances caused by the heating current is especially impressive for electrodes with high ohmic resistance such as glassy carbon electrodes. In these high resistance electrodes the voltage drop along the carbon rod generally causes serious problems with the potentiostat for both the asymmetric and the symmetric connection type. Figure 7A illustrates the disturbing effects of the heating current for the potentiostat being connected to one end of the glassy carbon rod only. The high voltage drop leads to severe overload and false readings of the electrochemical signals. This is also true in case of the symmetric connection as demonstrated in Figure 7B. The heavy noise and large off-sets of the "hot" CVs arise from problems with the imperfect symmetry of the center contact of the glassy carbon rod electrode. In the experiment the electrode had resistances of 30 Ohm and 35 Ohm between the center and the two end contacts. Figure 7C demonstrates the dramatic effect of the inductor bridge in eliminating noise and disturbances. Noise and off-sets fully vanished from the voltammetric signals. Due to the large electrode surface, the CVs are peak-shaped, even at high temperatures where convection leads to an accelerated mass transport. To reduce the convection effect we used a face-down arrangement of the glassy carbon electrode inside the measuring cell.

4. Conclusions

We could show that a simple inductor bridge can eliminate disturbing contributions of the heating current to the electrochemical signals in directly heated one-piece Au-wire or glassy carbon electrode setups. The new circuit yields cyclovoltammetric signals of ferric cyanide with a noise level superior to the earlier symmetrically branched electrode designs. This is especially true for heated glassy carbon electrodes and can be attributed to the ability of the inductor bridge to completely block high frequency signals, i.e., the heating current (100 kHz). Our simple circuit may open new opportunities to construct new types of heated electrodes or electrode arrays even with materials of high resistance.

5. Acknowledgements

The project was granted by Deutsche Forschungsgemeinschaft (DFG; FL 384/3-1). The technical assistance provided

by Carolin Lau in preparation of the heated glassy carbon electrode is gratefully acknowledged.

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Publication II

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Thermally induced electrode protection against biofouling

Talanta 77 (2008) 1757

Talanta 77 (2009) 1757–1760

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Thermally induced electrode protection against biofouling

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ARTICLE INFO

Article history:
Received 15 July 2008
Received in revised form 2 October 2008
Accepted 6 October 2008
Available online 17 October 2008

Keywords:
Heated electrode
Platinum wire electrode
Electrode fouling
Dopamine
Gelatin
Electrode conditioning

ABSTRACT

This paper demonstrates that a combined thermal and electrochemical conditioning step can greatly minimize electrode blocking. We detected 50 ppm dopamine after a blocking step in 1000 ppm gelatine solution. Only a treatment of the electrode at −1.5 V and 61.5 °C can reveal the voltammetric dopamine signals to 82%. The increase of the peak separation of the cyclic voltammograms obtained in 50 ppm dopamine is limited to 14%, whereas negative polarization (−1.5 V) alone leads to a 31% increase compared to 109% upon thermal and 105% without any conditioning. The positive effects can be addressed to an enforced reductive degradation and accelerated removal of the blocking agents. Also the formation of hydrogen bubbles might play a significant role. Thermo-electrochemical treatment holds great promise for electrochemical sensors and detectors which are applied for long-term monitoring of samples that contain blocking matrices.

1. Introduction

So called "electrode fouling" is a major hindrance for the practical application of electrochemical sensors at remote or hidden places. Such blocking of the electrode surface can occur in different ways. Real samples often come with a complex matrix that already contains blocking substances. The latter can also be produced by the electrode process itself such as oxidation of the analyte or certain matrix components. Such build-up of an inhibition layer often leads to severe suppressions and distortions of the current signals, along with irreproducible data.

One approach to minimize fouling is the use of alternate electrode materials like carbon nanotubes [1–3], anodically pretreated carbon fiber microelectrodes [4] or boron-doped diamond, which combine attractive electrochemical properties (stability and sensitivity) [5,6] and high resistance to deactivation via fouling [7] or dissolved oxygen [8]. Glassy carbon electrodes modified with single and multiwall carbon nanotubes show a decrease in the overvoltage for NADH detection along with minimal surface fouling during amperometric detection [2,9]. A chemical reversibility of the NADH oxidation reaction was achieved by modification of the electrode surface with poly(1,2-diaminobenzene) conducting nanotube coatings [10]. Baranski applied a hot micro-disk electrode for the determination of aniline, which is known to produce fouling effects and proposed to use electrode heating for cleaning [11]. In 2005 we proposed to heat the electrode during the voltammetric and amperometric detection of NADH in order to prevent electrode blocking due to oxidation products of the analyte [12]. Electrically heated electrodes have been successfully applied for the analysis of a variety of substances including dissolved oxygen [13] heavy metals [14,15], nucleic acids, carbon hydrates [16,17], and organic compounds [18,19]. Chen et al. reported about electroluminescence measurements at directly heated wire [20–22] and carbon paste electrodes [23–25]. Another interesting approach of Chen et al. is directly heated graphite electrodes and their application for the determination of trace riboflavin [26,27]. The temperature rise can also be achieved by means of microwaves [28–31]. Electrochemistry at elevated temperature has been reviewed [32,33].

Here, we present an effective pretreatment procedure which allows to measure trace amounts of dopamine following a blocking by high level of surface-active materials. High gelatin concentrations are used to illustrate the efficiency of pretreatment process.

2. Experimental

2.1. Reagents

All solutions were prepared using deionized water (PURELAB system, $R \geq 18.2\,M\Omega\,cm$). Square wave voltammetric and cyclic

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doi:10.1016/j.talanta.2008.10.011

voltammetric measurements were carried out in 100 mM phosphate buffer solution containing 50 ppm of dopamine vs. an Ag/AgCl (3 M KCl) reference electrode from CH Instruments Inc. (Austin, TX, USA) and a platinum counter electrode at a constant 24 °C bulk solution temperature. Dopamine hydrochloride, gelatin, potassium ferrocyanide, potassium ferricyanide and potassium chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). A pH 7.04 phosphate buffer (PB) was prepared using sodium phosphate (dibasic and monobasic), purchased from EMD (Gibbstown, NJ, USA). NaOH was used to adjust the buffer pH. Stock solutions of dopamine (1×10^4 μg/ml) and gelatin (1×10^3 μg/ml) in buffer were prepared daily.

2.2. Construction of the electrode

The used platinum wire electrode consisted of two 5-mm long pieces of platinum wires (50 μm in diameter) which were soldered carefully to both sides of a double printed circuit board (20 mm × 20 mm) which had a gap of 10 mm × 5 mm between the two copper contacts. Before the measurements the soldering contacts and the copper input leads were isolated using a mixture of paraffin/polyethylene and cleaned by glowing in air by applying a current. The construction of the used electrode has been described in detail previously [15].

2.3. Apparatus and procedures

2.3.1. Square wave voltammetry and cyclic voltammetry

All square wave voltammetric and cyclic voltammetric measurements were carried out with a PalmSens potentiostat (Ivium Technologies, Eindhoven, The Netherlands). These measurements were performed using a three-electrode measuring system containing a platinum wire electrode as working electrode (described above), a platinum wire as counter electrode and an Ag/AgCl (3 M KCl) reference electrode (CH Instruments Inc., Austin, TX, USA). In case of the cyclic voltammetry the measurements were started at −0.2 V with a step potential of 5 mV, and the second vertex potential was 0.5 V, the scan rate was 100 mV/s. Always the fifth scan was considered and is shown in Figs. 1 and 2(B).

The square wave voltammetry measurements were performed from +0 to +0.8 V with a step potential of 1 mV and pulses of 25 mV at 10 Hz.

2.3.2. Temperature calibration

We performed a temperature calibration using 1 mM equimolar ferro-/ferrycyanide in 20 mM potassium chloride by open circuit potentiometry with a μ-Autolab (Ecochemie, Utrecht, and The Netherlands). In these measurements we applied an alternating heating current with a frequency of 100 kHz which was delivered by a heating system that has been described previously [34,35]. Using the well known temperature coefficient for this redox couple (−1.56 mV/K) it is possible to calculate the electrode temperature from the measured potentials for each applied current. This procedure has also been described in more detail previously [13,14].

2.3.3. Passivation/cleaning and measuring procedure

To induce passivation of the electrode with gelatin, the electrode was dipped into a quiescent phosphate buffer solution 0.1 M (pH 7.04) containing 1000 ppm gelatin. After 5 min the electrode was removed, rinsed carefully with water and dipped into a phosphate buffer solution containing 50 ppm dopamine for the voltammetric measurements. To clean the electrode thermally (60 s at 61.5 °C), electrochemically (60 s, potential: −1.5 V), or electrochemically and thermally (60 s, potential: −1.5 V at 61.5 °C), a 60 s conditioning step has been performed immediately before the measurement.

3. Results and discussion

Fig. 1 displays the effect of three different conditioning procedures upon the cyclic voltammograms for 50 ppm dopamine. In all cases we initially performed a measurement on the clean electrode, followed by dipping it in a 1000 ppm gelatin solution for 5 min and repeating the voltammetric scan. In Fig. 1A we applied no cleaning step after the gelatin passivation. Fig. 1B depicts the cleaning effect of electrochemical conditioning at −1.5 V for 60 s. Although the original response could not be obtained, the dopamine signal is considerably better than without any conditioning. Thermal conditioning yields no improvement after the passivation (Fig. 1C). On the contrary, the signal of dopamine seemed even worse than without any cleaning. Only a combined thermo-electrochemical condition step of −1.5 V and 61.5 °C for 60 s leads to almost complete recovering of the dopamine signal as has been demonstrated in Fig. 1D. The improved resistance for fouling (vs. electrochemical activation alone) is more profound for a series of prolonged runs and exposures to gelatin (see additional data below).

Fig. 2 exhibits square wave and cyclic voltammetric curves of a series of repetitive cycles involving measurements of 50 ppm dopamine, a 5 min dipping in a 1000 ppm gelatin solution, and 60 s thermo-electrochemical conditioning steps at −1.5 V and 61.5 °C. Three cycles after 1, 4, and 7 cycles are displayed. The results of the full series of 12 cycles using thermal, electrochemical, and thermo-electrochemical cleaning procedures are depicted in Fig. 3. This series demonstrates the applicability of the new thermo-electrochemical cleaning procedure for long-term measurements. Only a combined 60 s conditioning at −1.5 V and 61.5 °C leads to a stable signal even after 12 repetitive passivation/cleaning cycles (Fig. 3d). Electrochemical conditioning alone is not sufficient for long-term measurements. Although after a few cycles a significant recovering of the dopamine signal is still observed (refer also to Fig. 1), a dramatic current suppression is observed after the fourth

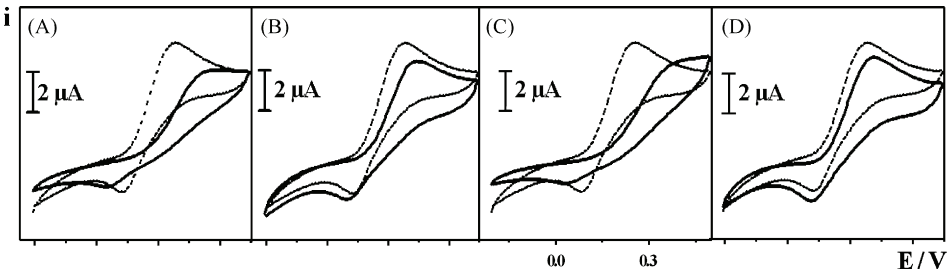

Fig. 1. Effect of different treatments: cyclic voltammetric response for 50 ppm dopamine in 100 mM phosphate buffer (pH 7.04) for a freshly cleaned platinum electrode (dotted line in all graphs). Before each of the other measurements the electrodes was dipped into a 1000 ppm gelatin solution for 5 min, rinsed and then dipped back in PB containing 50 ppm dopamine. Solid lines present the signal without any pretreatment (A), after an electrochemical pretreatment (60 s, potential: −1.5 V) (B), after a thermal pretreatment (60 s, at 61.5 °C) (C) and after a combination (thermal and electrochemical) pretreatment (60 s, potential: −1.5 V at 61.5 °C) (D).

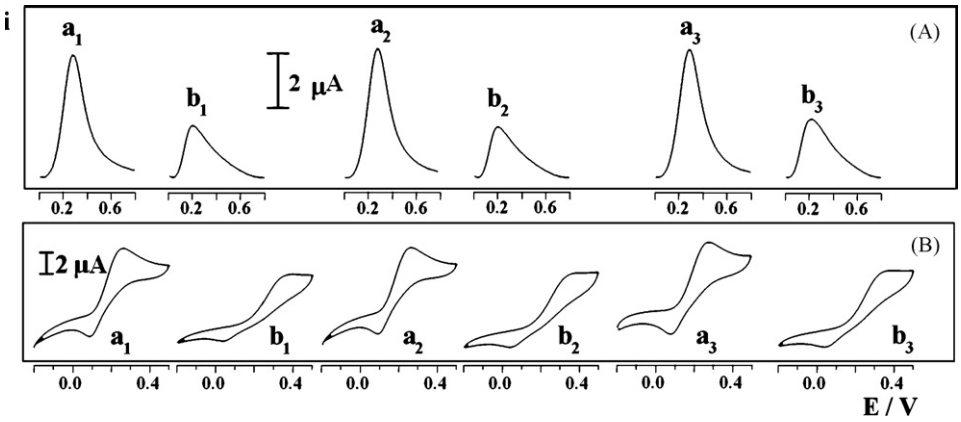

Fig. 2. Square wave voltammetry (A) and cyclic voltammetry (B) passivation and activation part of a series of 12 repetitive measurements (Ref. to Fig. 3). Out of these series the first (a_1, b_1), fourth (a_2, b_2) and seventh (a_3, b_3) measurements are presented. Between each measurement the electrode was dipped in 1000 ppm gelatin solution for about 5 min, rinsed and then dipped back in PBS containing 50 ppm dopamine. Measurements (b_n) have been conducted without any pretreatment. Measurements (a_n) have been conducted after the electrode was electrochemically and thermally pretreated (60 s at −1.5 and 61.5 °C).

cycle (Fig. 3b). Interestingly, the performance of a thermal treatment without polarization (Fig. 3c) is even slightly worse compared to measurements without any intermediate activation (Fig. 3a).

We observed a virulent gas bubble formation during the negative polarization (−1.5 V). These bubbles have been much smaller, if the electrode was simultaneously heated to 61.5 °C. At room temperature, the bubbles tended to grow and to stick on the electrode for a long time. We suppose the following effects of polarization and heating. The strongly negative potential together with the formed hydrogen *in statu nascendi* leads at first to reductive degradation of blocking substances at the electrode surface. Secondly, the formed hydrogen bubbles loosen blocking films and particles mechanically as they are formed beneath. Thirdly, the elevated temperature accelerates degradation reactions at the low potential and due to the formed hydrogen *in statu nascendi*. Fourthly, the microstirring effect around the heated electrode causes a strong mass transport and hence, removal of the potentially passivating compounds. Fifthly, adsorption of any compound at a solid surface is diminished with increasing temperature. The finding that only a combined thermo-electrochemical treatment yields a long-term anti-blocking effect means that reduction at negative potentials plays a crucial role and is dramatically enhanced by means of electrode heating (Fig. 3).

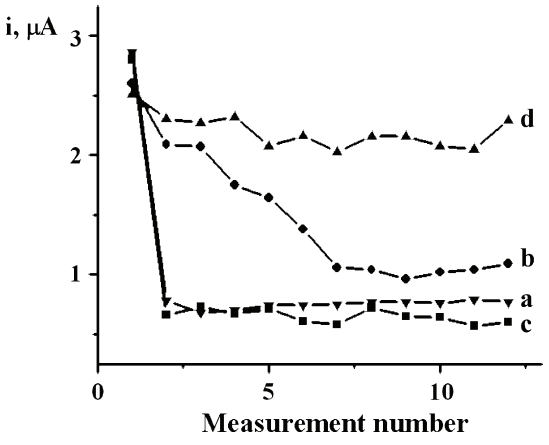

Fig. 3. Stability plots from 12 repetitive SWV measurements for 50 ppm dopamine in 100 mM phosphate buffer solution. Between each measurement the electrode was dipped in 1000 ppm gelatin solution for about 5 min, rinsed and then dipped back in PBS containing 50 ppm dopamine. Some measurements were taken out without any pretreatment (a), after electrochemical pretreatment (60 s, potential −1.5 V) (b), after thermal pretreatment (60 s, at 61.5 °C) (c) and after combined thermal and electrochemical pretreatment (60 s, potential: −1.5 V at 61.5 °C) (d).

Table 1
Anodic (E_{ap}) and cathodic (E_{cp}) cyclic voltammetric peak potentials, and peak separation ΔE_p observed in a 0.1 M phosphate buffer (pH 7.04) containing 50 ppm dopamine depending upon their respective conditioning parameters (all potentials were measured vs. Ag/AgCl (3 M KCl) reference electrode).

Electrode treatment	E_{ap}	E_{cp}	ΔE_p
Freshly glowed electrode	0.259	0.084	0.175
Passivation in 1000 ppm gelatine without conditioning	0.389	0.029	0.360
Passivation and thermal conditioning at 61.5 °C for 60 s	0.394	0.029	0.365
Passivation and electrochemical conditioning at −1.5 V for 60 s	0.294	0.064	0.230
Passivation and thermal-electrochemical conditioning at 61.5 °C and −1.5 V for 60 s	0.279	0.079	0.200

The positive effect of the new cleaning procedure is furthermore confirmed by the peak potentials of the cyclic voltammetric dopamine signals. Table 1 shows peak potentials and peak separations at the different cleaning conditions. Whereas the thermo-electrochemical treatment leads to a peak separation that is only slightly increased (by 25 mV), the electrochemical conditioning shows considerably higher increase in peak separation (55 mV) and hence, irreversibility due to the blocking gelatin film. The peak separations increase observed upon thermal (190 mV) or without any conditioning (185 mV) are dramatically worse.

4. Conclusions

The blocking effect of a 1000 ppm gelatin matrix component during the determination of 50 ppm dopamine can be dramatically reduced by means of a combined thermal and electrochemical conditioning step by means of a directly heated platinum wire electrode. The voltammetric dopamine signal can be maintained at ca. 82% of the initial value. A conventional electrochemical treatment alone yields only a limited cleaning effect for a few repetitive measurements. In long repetitive scan series the signal drops to ca. 39%. The blocking effect of 1000 ppm gelatin causes an immediate signal drop down to 29% of the initial value. Besides the peak currents also the peak separations can widely be stabilized by the new thermo-electrochemical conditioning; however, also the electrochemical conditioning preserves a relatively low peak separation.

The positive effects can be addressed to an enforced reductive degradation and accelerated removal of the blocking agents. Also the formation of hydrogen bubbles might play a significant role.

Thermo-electrochemical treatment holds great promise for unmodified electrochemical sensors and detectors which are applied for long-term monitoring of samples that contain blocking matrices. The improved resistance to surfactant interferences thus makes the new protocol very attractive for many direct electroanalytical applications in harsh environments, and obviates the need for protective membranes.

Acknowledgements

The research was granted by National Science Foundation (grant number CHE 0506529) and Deutsche Forschungsgemeinschaft (DFG; FL 384/2-3), H.D. was supported by Deutscher Akademischer Austausch Dienst (DAAD) and T.V. was supported by Louis Stokes AMP Bridges to the Doctorate Fellowship.

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Publication III

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Verfahren zur elektrochemischen Behandlung einer Sensorvorrichtung

Patentanmeldung Az. DE 10 2008 020 802.7 (2008)

Verfahren zur elektrochemischen Behandlung einer Sensorvorrichtung

Die Erfindung betrifft ein Verfahren nach dem Oberbegriff des Anspruchs 1, die Verwendung dieses Verfahrens nach Anspruch 18 und eine Sensorvorrichtung nach Anspruch 19.

Elektrochemische Detektoren und Sensoren haben in den letzten Jahrzehnten aufgrund ihrer guten Miniaturisierbarkeit und einfachen Datenerfassung und Datenauswertung immer mehr an Bedeutung gewonnen und werden jetzt in einer Vielzahl von Applikationen routinemäßig eingesetzt. Sie spielen z.B. in der Prozesskontrolle und der Online-Diagnostik eine wichtige Rolle.

Dabei kommt es aber abhängig von dem zu untersuchenden Analyten und der in der Probelösung vorhandenen Matrix schon nach kurzer Zeit zu einem Abfall des elektrochemischen Signals trotz gleichbleibender Konzentration des Analyten. Die Ursache liegt in der Ablagerung von unlöslichen oder zumindest stark adsorbierenden Substanzen an der Elektrodenoberfläche. Dadurch wird die Elektrodenreaktion stark behindert. Die blockierenden Substanzen können bereits in der Probenmatrix vorliegen oder im Verlauf der elektrochemischen Reaktion entstehen.

Um dieses Problem zu umgehen, kann die Elektrode vor jeder Messung mechanisch gereinigt werden, z.B. durch Polieren mit feinstem Aluminiumoxidpulver.

Eine andere Lösung besteht darin, die Elektrode elektrochemisch in der verwendeten Lösung zu reinigen. Dies kann z.B. durch das Anlegen einer positiven oder negativen Spannung für einen bestimmten Zeitraum von einigen Sekunden bis zu wenigen Minuten vor der Messung erfolgen. So wird gemäß der WO 2005/085828 A1 eine Arbeitselektrode anodisch gereinigt, wobei Verunreinigungen mittels Oxidation und /oder Hydratation entfernt werden. Auch die Kombination von positiver und negativer Spannung wurde schon erfolgreich angewendet (EP 1 452 858 A2, US 2005/0236280).

Die Selbstreinigung elektrochemischer Detektoren durch Pulsamperometrie ist beispielsweise in der HPLC verbreitet. Weitere Einsatzgebiete sind die Fließinjektionsanalyse und die Kapillarelektrophorese.

Durch diese Form der elektrochemischen Vorbehandlung kann die Signalstabilität in einigen Messlösungen verbessert werden, aber ein Abfall des Signals ist in den meisten Fällen immer noch zu beobachten, so dass nach relativ kurzer Zeit die Elektrode/der Sensor mechanisch gereinigt oder ersetzt werden muss.

Eine weitere Möglichkeit der Reinigung von Arbeitselektroden besteht in der Erwärmung der Elektrode, um Ablagerungen mittels Hitze zu entfernen (US 2002/0195340 A1).

Auch der Einsatz von Ultraschall, Mikrowellen oder Laserimpulsen zur Reinigung der Arbeitslektrode in der Messlösung ist bekannt, was aber die gesamte Messaparatur deutlich größer, komplizierter und teurer werden lässt.

Besonders interessante und vielversprechende Anwendungen für elektrochemische Sensoren sind in der medizinischen Onlinediagnostik direkt am bzw. im Patienten zu finden. Hier ist die gute Miniaturisierbarkeit elektrochemischer Sensoren besonders wichtig, weshalb große Systeme zur Reinigung der Elektrodenoberfläche nachteilig sind.

Bei diesem Einsatzgebiet ist das Problem der Blockierung der Elektrodenoberfläche und ein damit verbundenes Absinken des Signals besonders stark ausgeprägt. Die Ursache ist in der unspezifischen Absorption von Proteinen an der Elektrodenoberfläche zu finden. Um diese zu unterbinden, werden Membranen oder speziell modifizierte Elektrodenoberflächen wie z.B. Metallkomplexe eingebettet in ein Gel/Polymer vorgeschlagen. Neben der aufwändigen Herstellung solcher Beschichtungen ist auch stets die Gefahr gegeben, dass die metallhaltigen Oberflächen ausbluten und so z.B. Eisen, Platin oder Gold an den Körper und in die Analysenlösung abgeben.

Auch die kontinuierliche Abgabe von Stickstoffmonoxid vor der Arbeitselektrode wurde angewendet, um unspezifische Adsorption zu verhindern. Für bestimmte Anwendungen stehen dieser Lösung allerdings Sicherheitsbedenken entgegen.

Ein gleichzeitiges Heizen und elektrochemisches Messen ist ebenfalls aus der Literatur bekannt (Gründler und Flechsig, Microchimica Acta 154 (2006) 175; Voss et al., Electrochem Commun 1 (1999) 383; Wang et al., Talanta 50 (2000) 1205). Bisher wurden aber nur moderate Potentiale (beispielsweise von -1V bis +1.5 V vs. SCE) angelegt, wie sie typisch für elektrochemische Messungen sind. Zur Entfernung blockierender Substanzen genügt das im Allgemeinen nicht, weil diese Potentiale nicht zur Oxidation bzw. Reduktion derselben ausreichen. Daneben findet keine Gasentwicklung (Sauerstoff bzw. Wasserstoff) statt, die ein Ablösen anhaftender Schichten unterstützen könnte.

Der Erfindung liegt damit das Problem zugrunde, ein Verfahren bereitzustellen, mit welchem Sensorvorrichtungen elektrochemischer Messgeräte in-situ, d.h. in einer Messlösung, vor und/oder während jeder Messung behandelt, insbesondere gereinigt, werden können, so dass man ein über viele Messungen und sehr lange Zeit stabiles Messsignal erhält.

Diese Aufgabe wird durch die Bereitstellung eines Verfahrens mit den Merkmalen des Anspruchs 1, die Verwendung dieses Verfahrens nach Anspruch 18 und eine Sensorvorrichtung nach Anspruch 19 gelöst. Weitere vorteilhafte Ausgestaltungen ergeben sich aus den Unteransprüchen.

Zur Verfügung gestellt wird ein Verfahren zur elektrochemischen Behandlung einer für elektrochemische Messungen geeigneten Elektrode. Diese Elektrode wird auch als Arbeitselektrode bezeichnet. Die Elektrode kann Teil einer Sensorvorrichtung eines elektrochemischen Messgerätes sein, welches mindestens eine Elektrode umfasst.

Eine Referenzelektrode kann innerhalb der Sensorvorrichtung oder außerhalb derselben angeordnet sein.

Bei der Durchführung des erfindungsgemäßen Verfahrens wird die Elektrode erwärmt und gleichzeitig oder alternierend an die Elektrode ein gegenüber der Referenzelektrode negatives oder positives Potential angelegt.

Bei dem Verfahren kann das an die Elektrode angelegte negative oder positive Potential gegenüber einer Referenzelektrode bestimmt werden. Vorteilhafterweise wird an die Elektrode ein Potential von weniger als -1,0 V, bevorzugt weniger als -2,0 V, beispielsweise -2,5 V angelegt. Mit dem angelegten Potential wird eine reduzierende Reinigung bewirkt, die mit der Entwicklung von Gasbläschen aus molekularem Wasserstoff verbunden ist. Je niedriger der pH-Wert des Elektrolyten, desto weniger negativ kann das Potential gewählt werden.

Alternativ kann die Reinigung auch oxidativ unter Bildung von Sauerstoffbläschen erfolgen, wobei Potentiale über 1,5 V, insbesondere über 2 V, bevorzugt über 2,5 V verwendet werden können. Je höher der pH-Wert, desto niedriger kann das Potential gewählt werden. Vorteilhafterweise ist die Lösung dabei frei von Chlorid, Bromid und Jodid. Insbesondere Chlor und Brom, die bei hohen Potentialen entstehen, können Edelmetallelektroden angreifen.

Optional kann eine weitere Elektrode als Gegenelektrode zum Einsatz kommen, um die Referenzelektrode vom Elektrolysestrom zu entlasten und Potentialverschiebungen aufgrund des unkompensierten Widerstands der Elektrolytlösung zu vermeiden.

Die negative oder positive Polarisierung kann dabei so stark sein, dass es zur Gasentwicklung kommt. Dabei entstehen je nach Potential und Zusammensetzung des Elektrolyten durch dessen Elektrolyse Wasserstoff, Sauerstoff oder Halogene. Diese Prozesse führen in Kombination mit der erhöhten Elektrodentemperatur zu einer chemischen und mechanischen Lockerung der blockierenden Filme. Somit wird eine gute Reinigungswirkung auch bei stark absorbierenden bzw. blockierenden Substanzen erreicht, so dass das analytische Signal über einen langen Zeitraum konstant bleibt.

Das Verfahren ermöglicht eine Reinigung der Sensorvorrichtung direkt in der Messlösung. Damit ist es nicht länger erforderlich, die Elektrode aus der Messlösung zu entnehmen. Auch ist eine aufwändige Apparatur, die eine Anwendung im Körper des Patienten unmöglich macht, wie z.B. bei der Reinigung mittels Ultraschall, Mikrowellen oder Laserimpulsen, nicht länger notwendig.

Eine regelmäßige Erneuerung der Elektrode und eine damit verbundene erneute Kalibrierung entfallen bei Anwendung des vorliegenden Verfahrens ebenso wie die bisher regelmäßig durchzuführende mechanische Reinigung.

Soweit nicht anders angegeben, sind Potentiale jeweils gegen eine gesättigte Kalomelelektrode (SCE) gemessen.

Für die Elektrode (Arbeitselektrode) kommen vorteilhafterweise inerte Materialien zum Einsatz (Kohlenstoff bzw. Edelmetalle). Die Gegenelektrode besteht im Allgemeinen aus einem inerten Material (z.B. Platin oder Glaskohle).

Es ist von Vorteil, wenn die Erwärmung der Elektrode mittels eines durch die Elektrode fließenden Wechselstromes erfolgt. Vorteilhafterweise hat der Wechselstrom eine Frequenz von wenigstens 50 kHz und ist von seiner Stromstärke her so bemessen, dass eine Temperatur von mehr als 60 °C an der Elektrode erreicht wird. Bei Verwendung von Golddraht mit 25 µm Durchmesser als Material der Elektrode sind ca. 750 mA (eff.) nötig.

Es ist aber auch möglich, dass die Erwärmung der Elektrode durch ein nahe (bevorzugt mit einem Abstand von weniger als 0,5 mm) der Elektrode angeordnetes elektrisches Heizelement erfolgt, wobei das Heizelement bevorzugt galvanisch von der Elektrode getrennt ist. An das separate Heizelement wird vorteilhafterweise ein Gleich – oder Wechselstrom angelegt, wodurch eine Erwärmung desselben erfolgt.

Als Träger oder Substrat wird das elektrisch nicht leitfähige Material bezeichnet, welches der Sensoreinrichtung, die die Elektrode umfassen kann, physische Struktur

verleiht und die Elektrode trägt. Das separate Heizelement ist vorteilhafterweise im Substrat angeordnet, wobei die Menge der Materialien, aus denen das Substrat herstellbar ist, Keramik, Glas, Kunststoff und/oder einen Halbleiter umfasst. Keramik wird als Substrat bevorzugt, insbesondere LTCC (Low Temperature Cofired Ceramic).

In einer vorteilhaften Ausführungsvariante wird das an die Elektrode angelegte elektrochemische Potential pulsartig geändert und die Erwärmung der Elektrode erfolgt kontinuierlich.

In einer weiteren vorteilhaften Ausführungsform wird das an die Elektrode angelegte Potential kontinuierlich angelegt, wohingegen die Erwärmung der Elektrode pulsartig erfolgt. Hierdurch wird bei Pulsdauer von weniger als 100 ms ein kurzzeitiges Erhitzen bis über den Siedepunkt des die Elektrode umgebenden Mediums, des Elektrolyten, möglich. Die Überhitzung gelingt an glatten Elektrodenoberflächen viel leichter als an rauen. Nach Ende des Heizpulses löst sich die Überhitzung alsbald und verursacht Dampfblasenbildung. Dies wird kann gemäß einem Aspekt der Erfindung als „Dampfreinigungseffekt“ zusätzlich zu Potential und Heizung reinigungswirksam genutzt werden.

Es ist auch von Vorteil, wenn das an die Elektrode angelegte Potential pulsartig geändert wird und die Erwärmung der Elektrode ebenfalls pulsartig erfolgt, wobei die Pulsation von Potential und Erwärmung synchron oder asynchron erfolgt.

Für die Pulsheizung wird vorteilhafterweise eine direkt geheizte Elektrode verwendet, weil die Abkühlung und Erhitzung aufgrund der geringen thermischen Trägheit der Elektrode schnell erfolgen kann.

Die Pulsation entsteht durch einen Zyklus aus Heizpulsen von vorzugsweise unter 100 ms Dauer und Abkühlphase von bevorzugt weniger als 1 s. Die Potentialpulse können auch schneller aufeinander folgen.

Bevorzugt sind die bei der Wärmepulsation bewirkten Temperaturpulse kürzer als 1000 ms, insbesondere kürzer als 300 ms (Millisekunden).

Die Aufgabe der vorliegenden Erfindung wird auch durch die Verwendung des vorstehend beschriebenen Verfahrens in der Fließinjektionsanalyse, Hochdruckflüssigkeitschromatographie (HPLC), Kapillarelektrophorese, in der medizinischen Onlinediagnostik, zur Prozessüberwachung in der Industrieproduktion oder in Sensorsystemen zum Monitoring in der Umweltanalytik gelöst.

Das Verfahren ist vorteilhafterweise automatisiert. Dies ermöglicht eine selbsttätige Reinigung der Sensorvorrichtung während oder vor der Analyse.

Bevorzugt ist die Verwendung einer Elektrode als geheizte Mikroelektrode. Der Wärmeeintrag ist in dieser Ausführungsform, insbesondere bei gepulster Heizung, sehr klein, was eine Anwendung des Verfahrens direkt im Körper des Patienten z.B. im Rahmen von medizinischen Onlineüberwachungen ermöglicht.

Aufgrund der einfachen elektrischen Sensorstruktur sind der Miniaturisierung eines die beschriebene Elektrode enthaltenden Sensors kaum Grenzen gesetzt. Zusätzliche Komponenten für die Regelung der Temperatur der Arbeitselektrode sind nicht in der Sensorvorrichtung, sondern nur extern in einer Steuereinheit erforderlich. Vorteilhafterweise ist dazu die Sensorvorrichtung mit einer außerhalb der Sensorvorrichtung angeordneten Steuereinheit zur Temperaturregelung des Heizelementes verbunden.

Typische Anwendungsgebiete sind Detektoren in Fließsystemen (FIA), Detektoren in HPLC-Systemen, Medizinische Onlineüberwachung über mehrere Tage in Form von *in vivo* Sensoren und/oder *in vitro* Sensoren oder Detektoren in der Kapillarzonenelektrophorese.

Die Aufgabe der vorliegenden Erfindung wird auch durch eine Sensorvorrichtung mit den Merkmalen des Anspruchs 19 gelöst, welche mindestens eine Elektrode sowie ein elektrisches Heizelement umfasst, wobei das elektrische Heizelement in

räumlicher Nähe der Elektrode so angeordnet ist, dass eine Erwärmung des elektrischen Heizelements eine Erwärmung der Elektrode bewirkt.

Vorteilhafterweise liegt in der Sensorvorrichtung zwischen der Elektrode und dem Heizelement ein Wärmetransportmittel. Insbesondere ist bevorzugt, wenn dieses Wärmetransportmittel eine Silberschicht aufweist. Weiter bevorzugt ist ein Abstand zwischen der Elektrode und dem Heizelement von weniger als 0,5 mm.

Als Material der Elektrode sind mindestens ein Edelmetall und / oder Kohlenstoff bevorzugt.

Die Erfindung wird nachfolgend unter Bezugnahme auf die Figuren an mehreren Ausführungsbeispielen näher erläutert. Dabei zeigt

Fig. 1 den Aufbau eines Sensors mit erster Elektrode, Wärmetransportmittel, Heizmäander und Keramiksubstrat;

Fig. 2 ein LTCC-Element in der Draufsicht, unter Ansicht der einzelnen Schichten mit dessen Bestandteilen;

Fig. 3 ein LTCC-Element in der Seitenansicht;

Fig. 4a+b eine Platin-Drahtelektrode zur Dopaminbestimmung gemäß Beispiel 1;

Fig. 5 das Messergebnis einer Dopaminbestimmung mit der in Fig. 4 gezeigten Apparatur und Bestimmung mit cyclischer Voltammetrie;

Fig. 6 das Ergebnis wiederholter Dopaminbestimmungen mit der in Fig. 4 gezeigten Apparatur und Bestimmung mit Square Wave Voltammetrie und Cyclischer Voltammetrie;

Fig. 7 die Stabilität des Dopamin-Signals aus der Square Wave Voltammetrie bei 12 nacheinander durchgeführten Messungen;

Figur 1 zeigt als ein Ausführungsbeispiel der vorliegenden Erfindung eine indirekt geheizte Elektrode 1 innerhalb einer Sensorvorrichtung. Die Elektrode 1 ist als Goldschicht mit einem Durchmesser von 2 mm ausgeführt. Ein Wärmetransportmittel 2 ist als Silberschicht ausgeführt. Die Erwärmung der Elektrode 1 wird über ein Heizelement 3 herbeigeführt, wie beispielsweise einen Heizmäander 3. Die Elektrode 1 und Heizelement 3 sind gemeinsam in einem Substrat 5 untergebracht. Die erste Elektrode 1 wird über eine Kontaktbahn 6, hier aus Gold, elektrisch mit einer Lötkontaktplatte 7 verbunden.

Das erfindungsgemäße Verfahren bedient sich der Erwärmung der Elektrode 1, wobei ein negatives oder positives Potential an die Elektrode 1 angelegt wird. Dieses führt durch seine Reduktions- oder Oxidationswirkung zur Ablösung adsorbierter Schichten, gegebenenfalls auch unter Gasentwicklung an der Elektrode 1.

In einer bevorzugten Ausführungsform ist ein Heizmäander 3 weniger als 0,5 mm unterhalb einer in Gold ausgeführten Elektrode 1 (Durchmesser 2 mm) innerhalb eines Keramiksubstrats 5 untergebracht. Kontakte sind an den Lötkontaktplatten 7 an einer Unterseite des Sensorelements angebracht, um die Heizspannung 8 anzulegen.

Vorteilhafterweise ist das Heizelement 3 von der Elektrode 1 galvanisch getrennt und im Substrat 5 des Sensors angeordnet. Die galvanische Trennung ermöglicht die separate Zuführung von Heizstrom gleichzeitig zur Ansteuerung der Elektrode 1. Auch ist es von Vorteil, wenn die Sensorvorrichtung mit einer außerhalb der Sensorvorrichtung angeordneten Steuereinheit zur Temperaturregelung des Heizelementes 3 verbunden ist.

Figur 2 und 3 zeigen schematisch ein Ausführungsbeispiel einer erfindungsgemäßen Sensorvorrichtung. Dieses ist aus verschiedenen Schichten aufgebaut. Aus Gründen der Anschaulichkeit sind dabei in Figur 2 im Sensorelement übereinander liegende Schichten nebeneinander dargestellt. Eine erste Elektrode 1 hat eine Elektrodenfläche vom Durchmesser 2 mm und ist als Goldelektrode ausgeführt. Eine

Silberschicht 2 dient als Wärmetransportmittel. Direkt darunter ist ein Heizelement 3 angeordnet. Die elektrische Kontaktierung der Komponenten erfolgt über vertikale Kontaktbahnen 6 zur Lötkontaktplatte 7. Vertikale Kontaktbahnen 6 sind der Übersichtlichkeit halber in der Aufsicht (Fig. 2) nicht dargestellt. Figur 3 zeigt das Ausführungsbeispiel in der Seitenansicht.

Zur Ausführung des vorstehend beschriebenen Verfahrens wird die Elektrode 1 durch das Heizelement 3 erhitzt. Die Silberschicht 2 vermittelt dabei den Wärmetransport. Über die Lötkontaktplatte 7 werden dabei sowohl das Heizelement 3 als auch die Elektrode 1 kontaktiert. Die Erwärmung und gegebenenfalls Gasentwicklung an der Elektrode 1 führt zur Ablösung eventuell adsorbierten Materials und zur Regeneration der Elektrode auch bei hartnäckigen Verschmutzungen. Beispiele sind im Folgenden gegeben.

Figur 4 zeigt ein Ausführungsbeispiel einer Elektrode 1 als direkt geheizte Drahtelektrode. Dabei wird als Elektrode 1 ein Metalldraht 11 mit einem Wechselstrom (z.B. mit 50 kHz) über die in einem Glasrohr 9 geführten Leitungen 10 und die Kontakte 13 beheizt. Vorteilhaft ist dabei entweder die symmetrische Ausführung der Elektrode [Gründler und Flechsig, Principles and Analytical Applications of Heated Electrodes, Microchim. Acta 154 (2006) 175] oder die Verwendung einer der in der Patentschrift DE10 2006 006 347 B3 beschriebenen Einrichtungen. Referenz- und Gegenelektrode sind der Übersichtlichkeit halber in Fig. 4 nicht dargestellt.

Beispiel 1: Dopaminbestimmung in Gegenwart von Gelatine

Bestimmt wurde Dopamin an einer Elektrodenvorrichtung, wie sie in Figur 4 gezeigt ist. Diese umfasst als Elektrode 1 eine durch eine direkt angelegte Wechselspannung heizbare, 50 µm dicke Platin-Drahtelektrode 11. Dazu wurde der 5 mm lange und 50 µm dicke Platindraht 11 auf eine Leiterplatte 12 gelötet und die Lötstellen 13 sowie Zuleitungen 14 mit einer Wachsmischung isoliert.

Als Messmethoden wurden die cyclische Voltammetrie (Fig. 5) und Square Wave Voltammetrie (Fig. 6) gewählt. Es wurde jeweils mit einer Drei-Elektrodentechnik gemessen. Als Elektrode 1 wurde eine direkt heizbare Platinelektrode 11 gewählt. Gegen- und Referenzelektrode waren in der Nähe der Elektrode 1 angeordnet (5 mm entfernt). Die Messungen des Dopaminsignals wurden in 100 mM Phosphatpuffer pH 7.04 bei einem Dopamingehalt von 50 ppm durchgeführt. Um die Elektrode 1 gezielt zu blockieren, wird die Elektrode 1 für 300 s in 100 mM Phosphatpuffer mit 1000 ppm Gelatine gehalten und anschließend sorgfältig gespült.

Fig. 5 zeigt das Messergebnis einer Dopaminbestimmung mit der in Fig. 2 gezeigten Apparatur und Bestimmung mit cyclischer Voltammetrie. Untersucht wurde eine Lösung von 50 ppm Dopamin in 100 mM Phosphatpuffer bei pH 7. Das Signal der frisch gereinigten Elektrode 1 ist in allen Graphen gepunktet dargestellt. Vor jeder zweiten Messung wurde die Elektrode 1 absichtlich mit einem blockierenden Film versehen, indem sie für 300 s in eine Lösung von 1000 ppm Gelatine in 100 mM Phosphatpuffer pH 7 gestellt, gründlich gespült und dann zurück in die Dopamin-haltige Lösung gestellt wurde. Die durchgezogenen Linien geben die Signale A) ohne Vorbehandlung, B) nach elektrochemischer Vorbehandlung (-1.5 V für 60 s), C) nach thermischer Vorbehandlung (61,5 °C für 60 s) und D) nach kombinierter thermischer und elektrochemischer Vorbehandlung (-1.5V für 60 s bei 61.5 °C) wieder. Man erkennt, dass die Kombination von thermischer Behandlung und angelegtem Potential zu qualitativ und quantitativ deutlich verbesserter Reproduzierbarkeit der Ergebnisse der Messungen führt.

Fig. 6 zeigt das Ergebnis wiederholter Dopaminbestimmungen mit der in Fig. 2 gezeigten Apparatur und Bestimmung mit Square Wave Voltammetrie und Cyclischer Voltammetrie; Signale nach Aktivierung und Passivierung aus einer Serie von 12 Wiederholungen. Aus dieser Serie werden hier das erste (a_1, b_1), vierte (a_2, b_2) und siebte (a_3, b_3) Signal präsentiert. Zwischen jeder Messung wurde die Elektrode 1 für 300 s in eine Lösung von 1000 ppm Gelatine in 100 mM Phosphatpuffer pH 7 gestellt, gründlich gespült und dann zurück in die Dopamin-haltige Lösung gestellt. Einige Messungen wurden ohne Vorbehandlung durchgeführt (b).

Vor den Messungen (a) wurde die Elektrode 1 thermisch und elektrochemisch vorbehandelt (-1.5 V für 300 s bei 61.5 °C). Auch hier zeigt sich eine Verbesserung der Reproduzierbarkeit des Messergebnisses.

Fig. 7 zeigt die Stabilität des Dopamin-Signals (50 ppm in 100 mM Phosphatpuffer pH 7) aus der Square Wave Voltammetrie bei 12 nacheinander durchgeführten Messungen. Vor jeder Messung wurde die Elektrode 1 für 300 s in eine Lösung von 1000 ppm Gelatine in 100 mM Phosphatpuffer pH 7 gestellt, gründlich gespült und dann zurück in die Dopamin-haltige Lösung gestellt. Die Messungen wurden durchgeführt a) ohne Vorbehandlung, b) nach thermischer Vorbehandlung (61,5 °C für 60 s), c) nach elektrochemischer Vorbehandlung (-1.5 V für 60 s) und d) nach kombinierter thermischer und elektrochemischer Vorbehandlung (-1.5 V für 60 s bei 61.5 °C).

Beispiel 2: Phenolbestimmung

An rauen Oberflächen von Elektroden 1 bilden sich besonders leicht Dampfblasen, wobei die Überhitzung des Elektrolyten begrenzt wird.

Wenn als Elektrode 1 eine direkt geheizte Pt-LTCC-Elektrode eingesetzt wurde, wie sie in DE19842735 beschrieben ist, wurden bei einem Heizstrom von 4 A für 100 ms Spitzentemperaturen von 125 °C erreicht. Zwischen zwei Heizpulsen lag jeweils eine Sekunde Pause. In dieser Zeit bildeten sich Dampfbläschen und es war ein knisterndes Geräusch zu vernehmen. Diese Methode kann vorteilhaft zur Reinigung von Oberflächen von Elektroden 1 genutzt werden, wobei die sich bildenden Dampfblasen die blockierenden Schichten anheben. Bei Phenol und Naphthol wurden positive Effekte beobachtet, wenn gleichzeitig ein Potential von -1,5 V angelegt wurde. Beide Analyten sind dafür bekannt, im Verlaufe ihrer anodischen Bestimmung blockierende Filme zu bilden.

An Pt-Drahtelektroden kam es weniger leicht zur Bildung von Dampfblasen, obwohl auch hier schwache knisternde Geräusche vernehmbar waren.

Auch starke Wasserstoffentwicklung bei stark negativen Potentialen hat einen positiven Effekt, jedoch ist hierbei nachteilig, dass die gebildeten Wasserstoffbläschen nicht vollständig verschwinden, sondern leicht an der Elektrode 1 haften. Dampfbläschen bilden sich bei sinkender Temperatur zurück. Weiterhin ist hierbei von Nachteil, dass an der Gegenelektrode ebenfalls eine starke Elektrolyse auftritt, die je nach zersetztem Elektrolyt zu störenden Produkten führen kann.

Vorteilhaft erscheint daher die Kombination aus gemäßigtem Potential ohne Elektrolyse/Gasentwicklung und reversibler Dampfblasenbildung.

Beispiel 3

An einer indirekt geheizten Gold-LTCC-Elektrode als Elektrode 1 wurde ein dicker teerartiger Belag abgeschieden, indem eine Suspension von Multiwand-Kohlenstoffnanoröhrchen in DMF (Dimethlyformamid) auf der Oberfläche wiederholt eingedampft wurde. Dieser Belag ließ sich durch die üblichen Methoden wie elektrochemische Behandlung, Polieren und sogar mit Sandpapier nicht entfernen. Erfolgreich war erst eine kombinierte Behandlung aus kontinuierlicher Elektrodenheizung bei 80 °C und einem stark positiven Potential von 2,5 V. Der Elektrolyt war eine 1 M Schwefelsäure. Die Kombination aus Sauerstoffblasenbildung und heißer Elektrodenoberfläche führte zum Ablösen des Belags.

Bezugszeichenliste

1	Elektrode (Arbeitselektrode)
2	Wärmetransportmittel
3	Heizelement
4	Elektroden-Strom bzw. -Potential
5	Substrat
6	Kontaktbahn
7	Lötkontaktplatte
8	Heizspannung als Gleich- oder Wechselstrom
9	Glasrohr
10	Leitung
11	Platin-Drahtelektrode (Arbeitselektrode)
12	Leiterplatte
13	Kontakte
14	Wachsisolierte Zuleitung

Patentansprüche

1. Verfahren zur elektrochemischen Behandlung einer für elektrochemische Messungen geeigneten Elektrode,

   dadurch gekennzeichnet, dass

   die Elektrode (1) erwärmt wird und dass gleichzeitig oder alternierend an die Elektrode (1) ein negatives oder positives Potential angelegt wird.

2. Verfahren nach Anspruch 1, **dadurch gekennzeichnet,** dass an die Elektrode (1) ein Potential von weniger als -1 V oder mehr als 1,5 V, bevorzugt weniger als -2 V oder mehr als 2,5 V angelegt wird.

3. Verfahren nach Anspruch 1 oder 2, **dadurch gekennzeichnet,** dass das an die Elektrode (1) angelegte negative oder positive Potential gegenüber einer Referenzelektrode bestimmt wird.

4. Verfahren nach Anspruch 3, **dadurch gekennzeichnet,** dass als Referenzelektrode eine gesättigte Kalomelelektrode verwendet wird.

5. Verfahren nach mindestens einem der vorstehenden Ansprüche, **dadurch gekennzeichnet**, dass neben der Referenzelektrode mindestens eine weitere Elektrode als Gegenelektrode verwendet wird.

6. Verfahren nach mindestens einem der vorstehenden Ansprüche, **dadurch gekennzeichnet**, dass die Erwärmung der Elektrode (1) mittels eines durch die Elektrode (1) fließenden Wechselstromes erfolgt.

7. Verfahren nach Anspruch 6, **dadurch gekennzeichnet**, dass der Wechselstrom eine Frequenz von wenigstens 50 kHz hat und von seiner Stromstärke her so bemessen ist, dass eine Temperatur von mehr als 60 °C an der Elektrode (1) erreicht wird.

8. Verfahren nach mindestens einem der vorstehenden Ansprüche, **dadurch gekennzeichnet**, dass die Erwärmung der Elektrode (1) durch ein nahe der Elektrode (1) angeordnetes elektrisches Heizelement (3) erfolgt, wobei das Heizelement (3) galvanisch von der Elektrode (1) getrennt ist.

9. Verfahren nach Anspruch 8, **dadurch gekennzeichnet,** dass an das Heizelement (3) ein Gleich – oder Wechselstrom (8) angelegt ist.

10. Verfahren nach mindestens einem der Ansprüche 8 oder 9, **dadurch gekennzeichnet**, dass das Heizelement (3) in einem Substrat (5) der Elektrode (1) angeordnet ist.

11. Verfahren nach Anspruch 10, **dadurch gekennzeichnet**, dass das Substrat (5) Keramik, Glas, Kunststoff und / oder einen Halbleiter umfasst.

12. Verfahren nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet**, dass das an die Elektrode (1) angelegte Potential pulsartig geändert wird und die Erwärmung der Elektrode (1) kontinuierlich erfolgt.

13. Verfahren nach einem der Ansprüche 1 bis 11, **dadurch gekennzeichnet**, dass das an die Elektrode (1) angelegte Potential kontinuierlich angelegt ist und die Erwärmung der Elektrode (1) pulsartig erfolgt.

14. Verfahren nach einem der Ansprüche 1 bis 11, **dadurch gekennzeichnet**, dass das an die Elektrode (1) angelegte Potential pulsartig geändert wird und die Erwärmung der Elektrode (1) ebenfalls pulsartig erfolgt, wobei die pulsartige Änderung von Potential und Erwärmung synchron erfolgt.

15. Verfahren nach einem der Ansprüche 1 bis 11, **dadurch gekennzeichnet**, dass das an die Elektrode (1) angelegte Potential pulsartig geändert wird und die Erwärmung der Elektrode (1) ebenfalls pulsartig erfolgt, wobei die pulsartige Änderung von Potential und Erwärmung asynchron erfolgt.

16. Verfahren nach einem der Ansprüche 13 bis 15, **dadurch gekennzeichnet**, dass die Temperaturpulse kürzer als 1000 ms, insbesondere kürzer als 300 ms sind.

17. Verfahren nach Anspruch 16, **dadurch gekennzeichnet,** dass der Elektrolyt während oder nach jedem Temperaturpuls an der Oberfläche der Elektrode (1) pulsartig zum Sieden gelangt, so dass sich Dampfblasen an der Oberfläche der Elektrode (1) bilden.

18. Verwendung eines Verfahrens nach einem der vorhergehenden Ansprüche in der Fließinjektionsanalyse, Hochdruckflüssigkeitschromatographie (HPLC), Kapillarelektrophorese, in der medizinischen Onlinediagnostik, zur Prozessüberwachung in der Industrieproduktion oder in Sensorsystemen zum Monitoring in der Umweltanalytik.

19. Sensorvorrichtung umfassend mindestens eine Elektrode, zur Verwendung in einem Verfahren nach mindestens einem der Ansprüche 1 bis 17, **dadurch gekennzeichnet,** dass die Sensorvorrichtung mindestens ein elektrisches Heizelement (3) umfasst, wobei das elektrische Heizelement (3) in räumlicher Nähe der Elektrode (1) so angeordnet ist, dass eine Erwärmung des elektrischen Heizelements (3) eine Erwärmung der Elektrode (1) bewirkt.

20. Sensorvorrichtung gemäß Anspruch 19, **dadurch gekennzeichnet,** dass das elektrische Heizelement (3) näher als 0,5 mm der Elektrode (1) angeordnet ist, und/oder dass das elektrische Heizelement (3) in Form eines Heizmäanders (3) im Substrat (5) der Sensorvorrichtung angeordnet ist.

21. Sensorvorrichtung nach mindestens einem der Ansprüche 19 bis 20, **dadurch gekennzeichnet**, dass das Heizelement (3) von der Elektrode (1) galvanisch getrennt ist.

22. Sensorvorrichtung nach mindestens einem der Ansprüche 19 bis 21, **dadurch gekennzeichnet**, dass das Heizelement (1) in einem Substrat (5) der Elektrode (1) angeordnet ist.

23. Sensorvorrichtung nach mindestens einem der Ansprüche 19 bis 22, **dadurch gekennzeichnet,** dass die Sensorvorrichtung mit einer außerhalb der Sensorvorrichtung angeordneten Steuereinheit zur Temperaturregelung des Heizelementes (3) verbunden ist.

24. Sensorvorrichtung nach mindestens einem der Ansprüche 19 bis 23, **dadurch gekennzeichnet,** dass die Sensorvorrichtung zwischen der Elektrode (1) und dem Heizelement (3) ein Wärmetransportmittel (2) aufweist.

25. Sensorvorrichtung nach Anspruch 24, **dadurch gekennzeichnet,** dass das Wärmetransportmittel (2) eine Silberschicht aufweist.

26. Sensorvorrichtung mindestens einem der Ansprüche 19 bis 25, **dadurch gekennzeichnet,** dass der Abstand zwischen der Elektrode (1) und dem Heizelement (3) weniger als 0,5 mm beträgt.

27. Sensorvorrichtung nach mindestens einem der Ansprüche 19 bis 26, **dadurch gekennzeichnet,** dass das Material der Elektrode (1) mindestens ein Edelmetall und / oder Kohlenstoff umfasst.

Zusammenfassung

Die Erfindung betrifft ein Verfahren zur elektrochemischen Behandlung einer für elektrochemische Messungen geeigneten Elektrode, wobei die Elektrode erwärmt wird und gleichzeitig oder alternierend an die Elektrode ein negatives oder positives Potential angelegt wird. Weiterhin betrifft die Erfindung die Verwendung dieses Verfahrens in der Fließinjektionsanalyse, Hochdruckflüssigkeitschromatographie (HPLC), Kapillarelektrophorese, in der medizinischen Onlinediagnostik, zur Prozessüberwachung in der Industrieproduktion oder in Sensorsystemen zum Monitoring in der Umweltanalytik. Ebenso wird eine Sensorvorrichtung zur Verwendung in einem solchen Verfahren zur Verfügung gestellt, welche mindestens eine Elektrode (1) und mindestens ein elektrisches Heizelement (3) umfasst, wobei das elektrische Heizelement (3) in räumlicher Nähe der Elektrode (1) so angeordnet ist, dass eine Erwärmung des elektrischen Heizelements (3) eine Erwärmung der Elektrode(1) bewirkt.

(Figur 1)

FIG 1

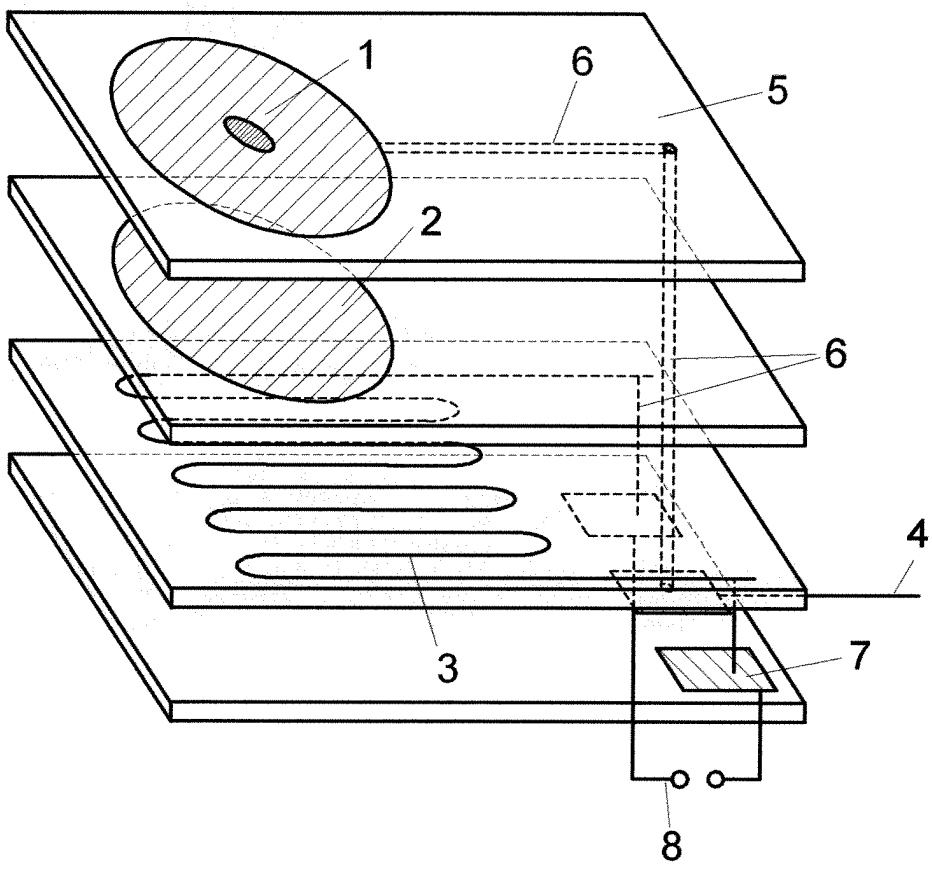


FIG 2

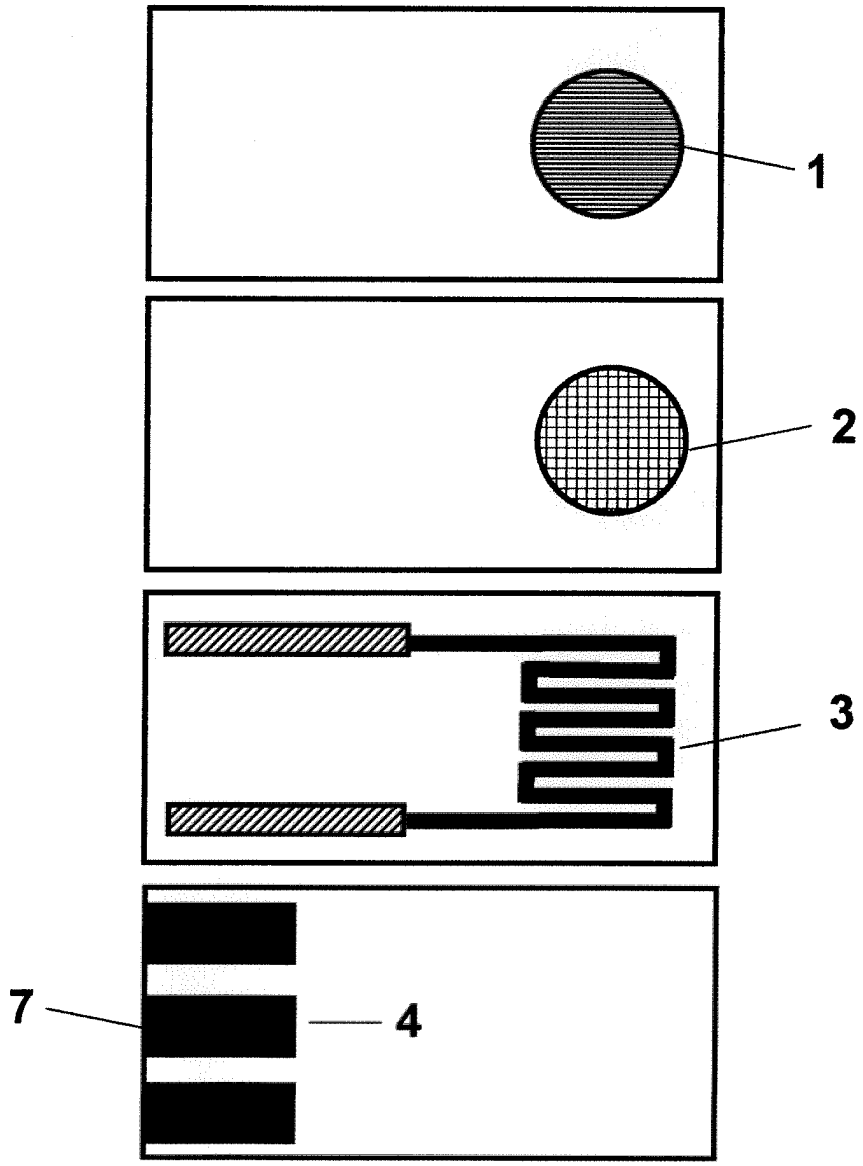


FIG 3

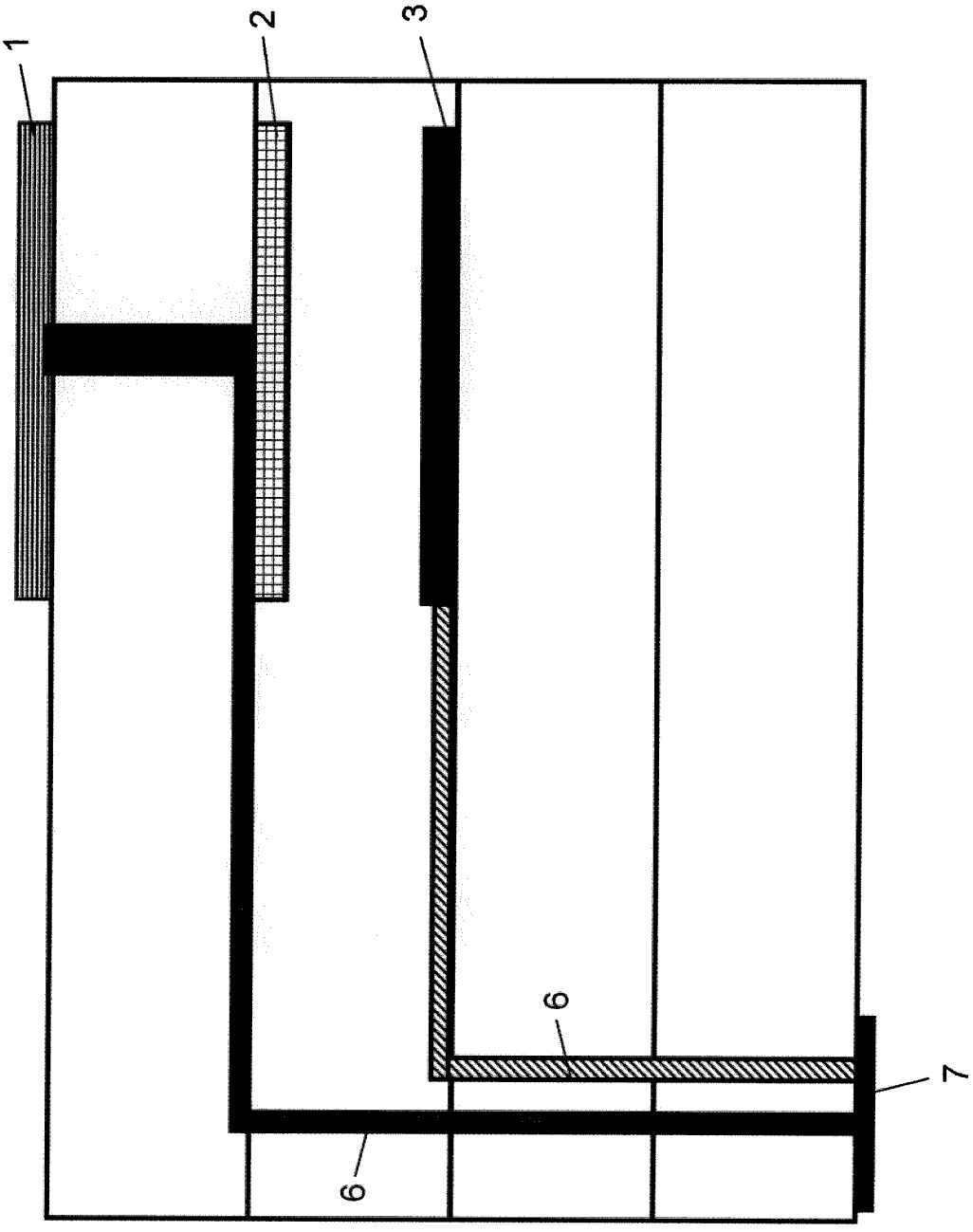


FIG 4a

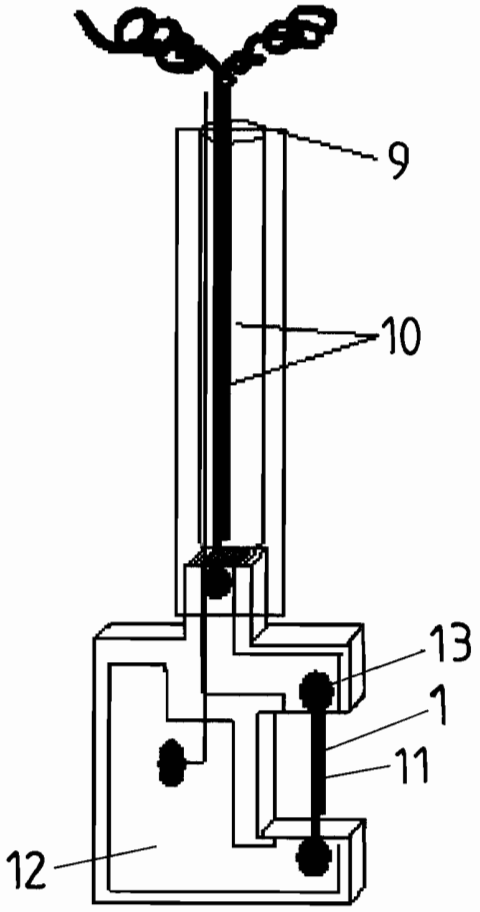


FIG 4b

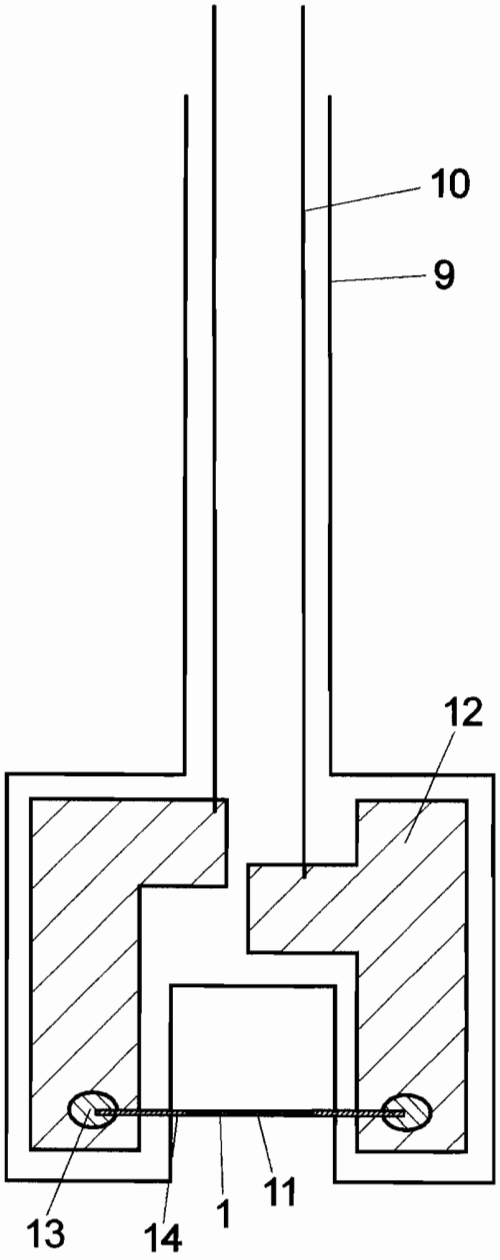


FIG 5

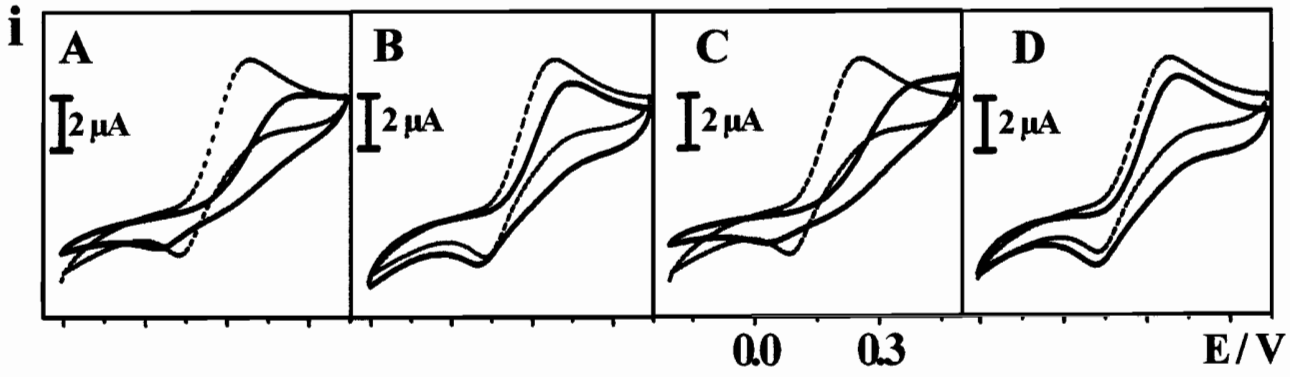


FIG 6

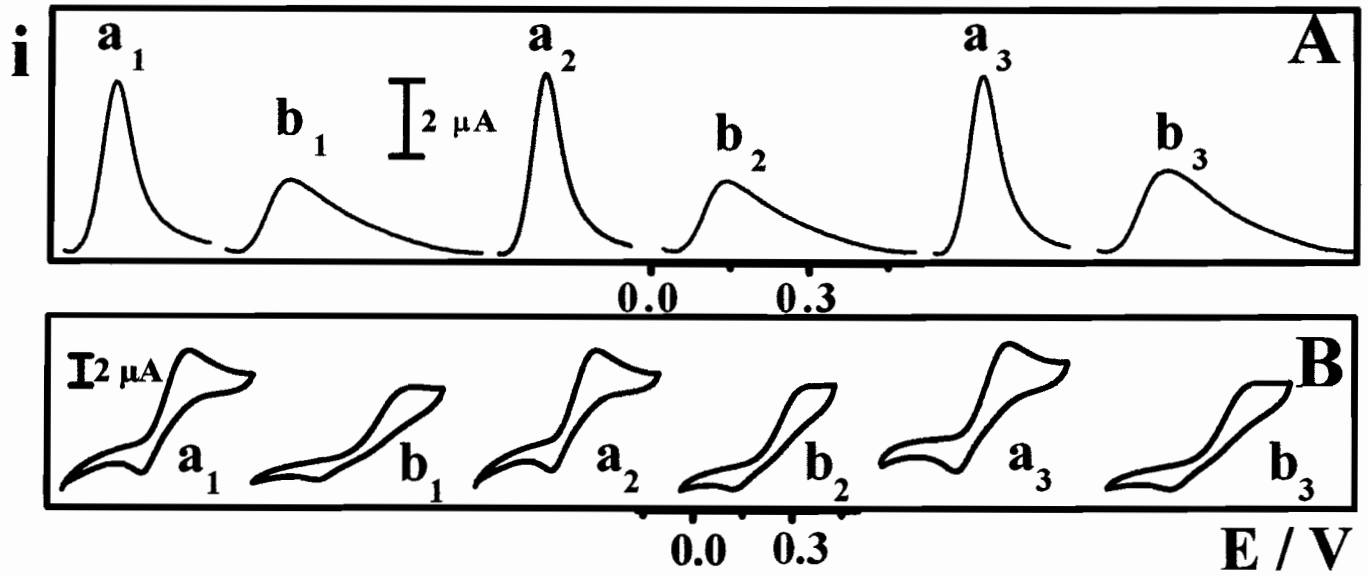


FIG 7

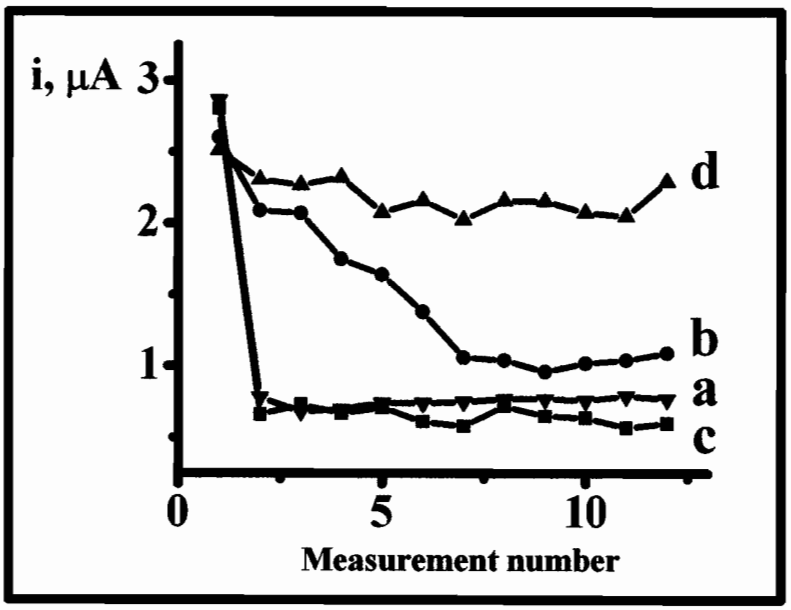


FIG 4a

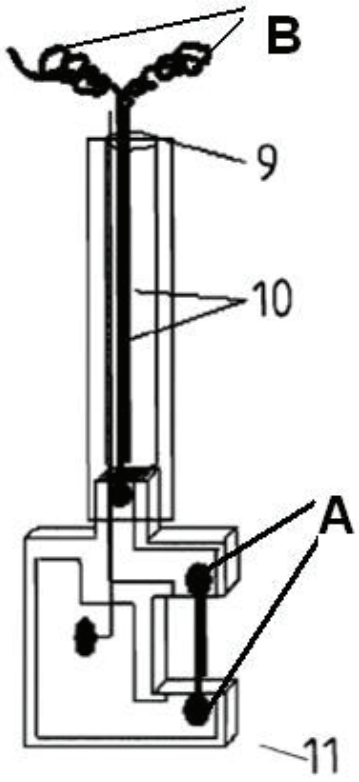

B
9
10
A
11

FIG 4b

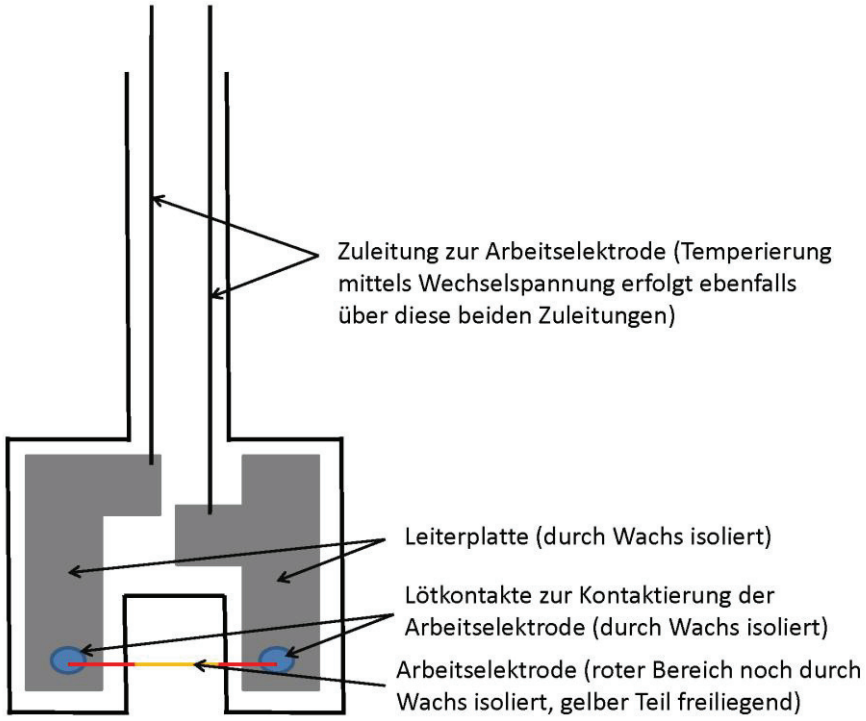

Zuleitung zur Arbeitselektrode (Temperierung mittels Wechselspannung erfolgt ebenfalls über diese beiden Zuleitungen)
Leiterplatte (durch Wachs isoliert)
Lötkontakte zur Kontaktierung der Arbeitselektrode (durch Wachs isoliert)
Arbeitselektrode (roter Bereich noch durch Wachs isoliert, gelber Teil freiliegend)

FIG 5

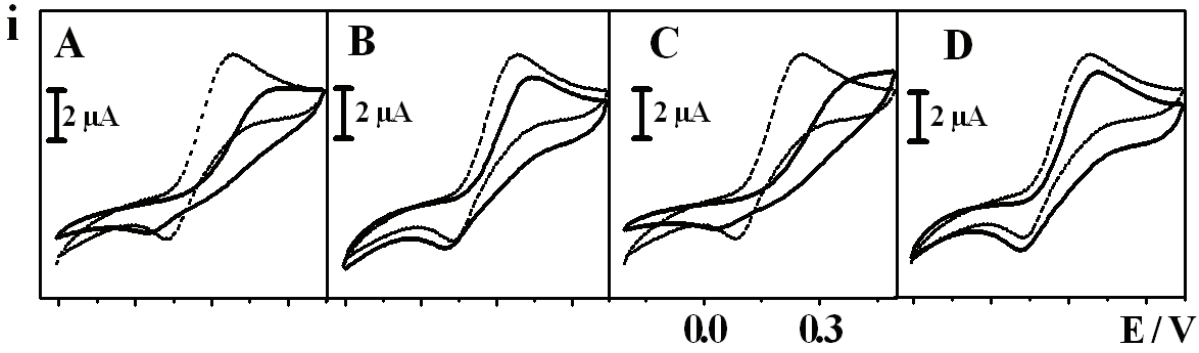

i
A
B
C
D
2 µA
2 µA
2 µA
2 µA
0.0
0.3
E / V

FIG 6

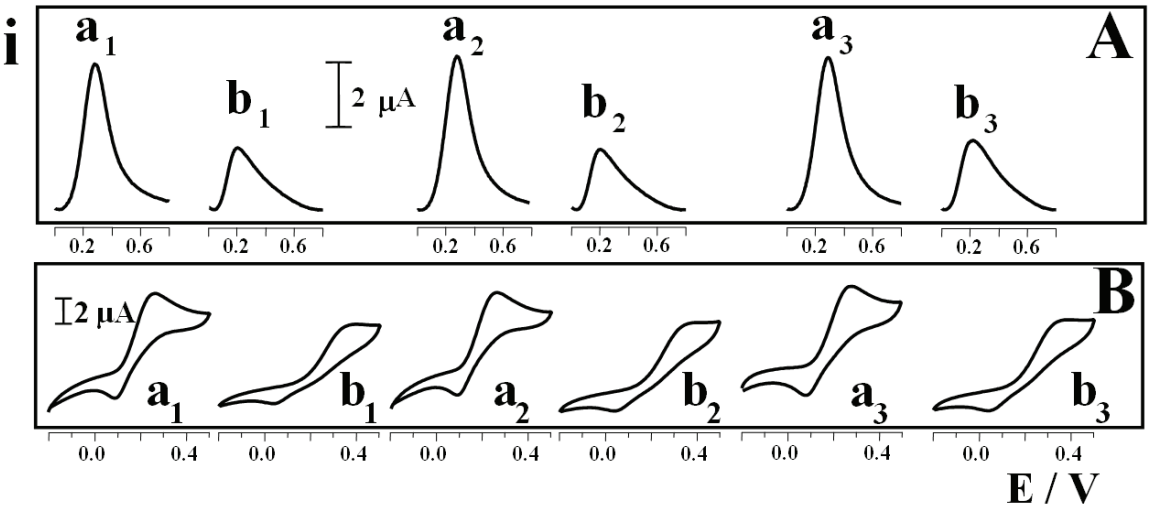
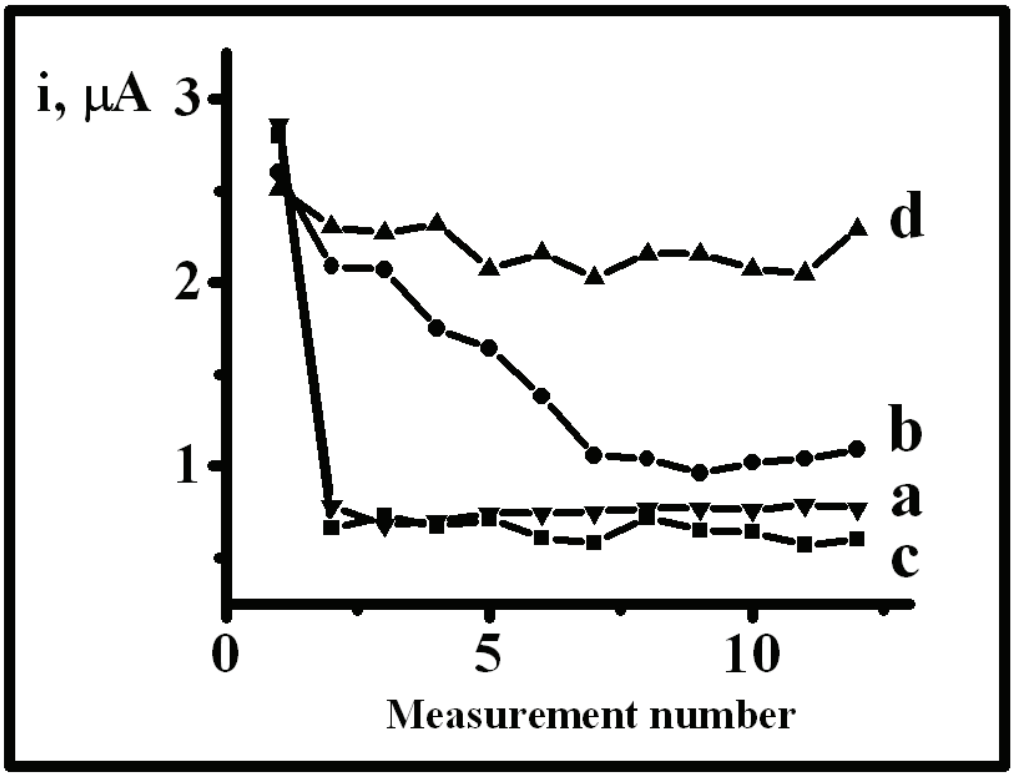

i
a1
b1
a2
b2
a3
b3
A
2 µA
0.2
0.6
2 µA
B
a1
b1
a2
b2
a3
b3
0.0
0.4
E / V

FIG 7



Publication IV

Nico Mahnke, Aleksandar Markovic, Heiko Duwensee, Falko Wachholz, Gerd-Uwe Flechsig, Ursula van Rienen,

Numerically optimized shape of directly heated electrodes for minimal temperature gradients

Sensors & Actuators B - Chemical 137 (2009) 363

Sensors and Actuators B 137 (2009) 363–369

Contents lists available at ScienceDirect

Sensors and Actuators B: Chemical

journal homepage: www.elsevier.com/locate/snb

Numerically optimized shape of directly heated electrodes for minimal temperature gradients

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ARTICLE INFO

Article history:
Received 29 September 2008
Received in revised form 17 November 2008
Accepted 19 November 2008
Available online 6 December 2008

Keywords:
Heated electrode
Numerical calculation
Thermal simulation
Temperature allocation

ABSTRACT

For electrochemical investigations at heated electrodes especially in the bioanalytical area a precise control of the surface temperature is necessary. Due to the problem of non-homogenous temperature allocation over the length of a directly heated electrode/conductor in electrochemical applications the possibility of changing its design in order to avoid such inhomogeneous effects was investigated. By mathematical simulations with the program Comsol Multiphysics©, concerning the temperature development along a conducting wire for different oval shapes depending on changing heating durations and current densities, the temperature gradient could be minimized. We could show the possibility to achieve an almost homogenous surface temperature by creating a special oval-shaped design for the electrode. The convection of surrounding media in the proximity of the conductor was not taken into account for the thermal simulation, which applies in particular for short-term pulse heating.

1. Introduction

During the last 15 years directly heated electrodes have attained considerable attention. They have been applied for a variety of analytical purposes. Gabrielli et al. [1,2] presented preliminary results of 6 °C temperature jump experiments performed at Pt wire electrodes using 250 kHz heating current and ferro/ferricyanide as a model analyte. Gründler et al. used electrically heated micro-wire electrodes [3] for the determination of lead [4], oxygen [5], and organic substances [6]. Later we detected mercury [7] by means of anodic stripping voltammetry. Hot-wire detectors can also be used in flow systems [8]. Experiments with carbon fibers were not successful [3]. However, directly heated carbon paste electrodes could be successfully applied for DNA analysis [9,10] and trace detection of cadmium, lead and zinc [11]. We further proposed to control DNA hybridization at heated gold wire electrodes that had been modified with a thiol-linked DNA probe layer [12].

Chen and co-workers described electroluminescence measurements at directly heated wire [13–15] and carbon paste electrodes [16–18]. Another interesting approach of Chen and co-workers is directly heated graphite electrodes and their application for the determination of trace riboflavin [19,20].

Gründler et al. introduced an electrochemical technique that they called "temperature pulse voltammetry (TPV)" [21,22]. Sampled voltammograms can be recorded on directly heated electrodes at temperatures far above the boiling point of the electrolyte; however, the critical state of that liquid can probably not be reached [23,24]. We proposed "temperature pulse amperometry" as simplified variant. Here, an amperometric measurement is conducted while short temperature pulses are being applied. The resulting peaks can be used as a concentration proportional signal. The detection of trace picric acid is possible this way [25].

Publications in the field of high-temperature electrochemistry have been reviewed [26–28]. In the recent years, investigations with electrically heated electrodes became more and more convenient due to improved instrumentation [29]. By using a filtering inductor bridge, the heating current (AC between 50 and 100 kHz, and ca. 1 A) can be separated from the electrochemical signal (1 nA to 10 μA) which leads to clear signals without any major disturbances [30,31].

However, most of the used electrodes (metal wires) still have the problem of a temperature gradient at the electrode surface due to different heat sinks in the middle and at the ends of an electrode. This temperature gradient is especially steep during short heat pulses. It is diminished during long-term heating in water, because of the cooling effect due to thermal convection [32]. In contrast to electrochemical experiments which usually provide only information about the average temperature of the surface, mathematical simulations give information about each point of the electrode surface. As could be assumed, it was evaluated that there is a signif-

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doi:10.1016/j.snb.2008.11.034

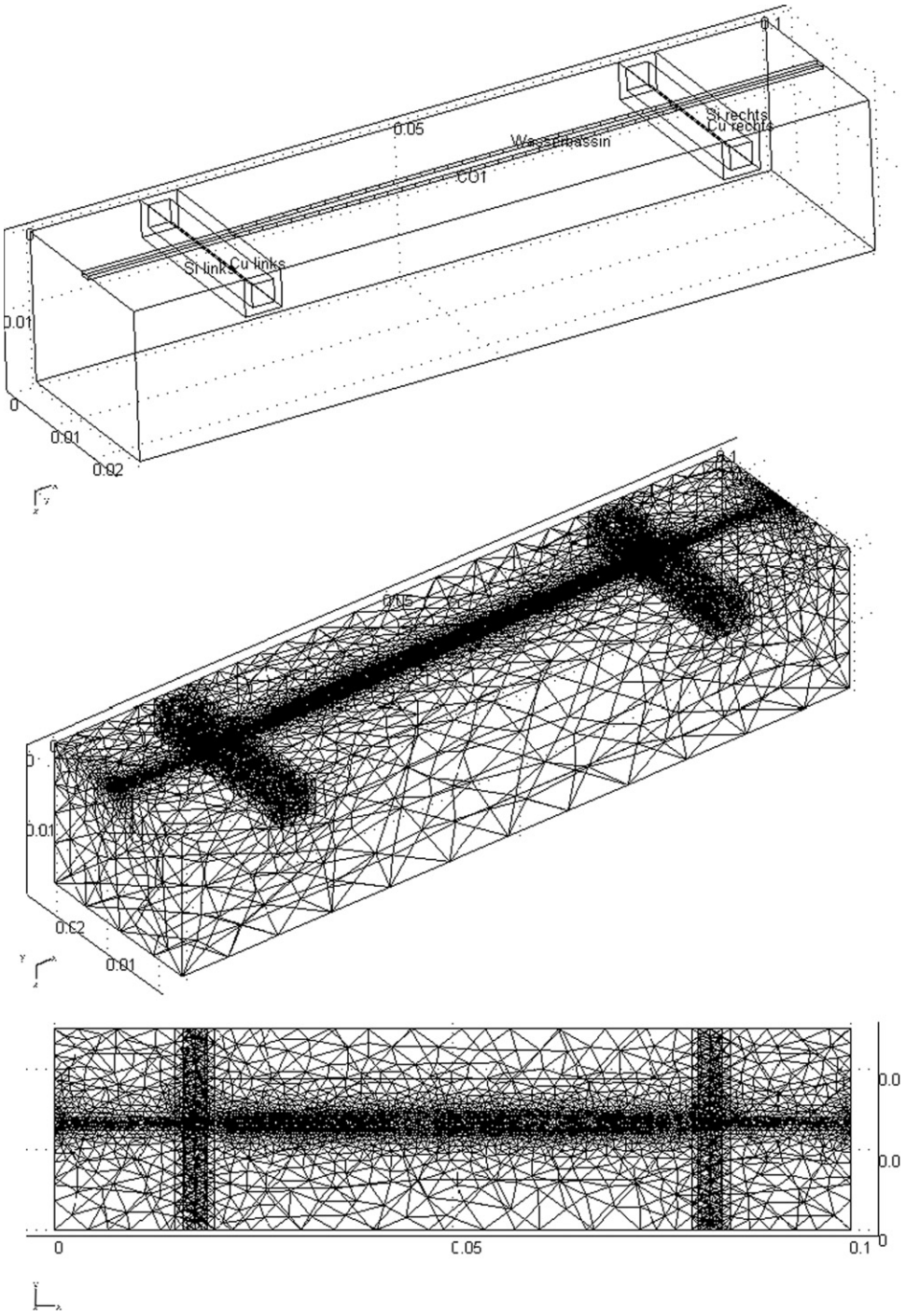

Fig. 1. Model of the electrode geometry (above) and the appropriate FEM discretization mesh (middle and below).

icant temperature gradient at electrodes of uniform cross-section. However, especially for the application of heated electrodes in bioanalysis a constant temperature at the electrode surface is absolutely necessary. Only in this way a precise control of the electrode reactions at the surface (e.g. the hybridization of two DNA-single strands) can be realized under stringent conditions. Immobilized enzymes are another important topic. There is only a small interval for the temperature optimum. If the temperature is too low, the enzyme reactivity is not that high and so the sensitivity of the sensor is low. But if the temperature is too high the denaturation process of the enzyme starts and the sensor cannot be used for long-term applications.

During the last decades the numerical simulations of temperature distribution advanced rapidly [33,34]. Here, we describe a simulation approach to design a directly heated electrode that almost completely eliminates the temperature gradient. This could lead to much more reliable and sensitive hot-wire sensors.

2. Simulation model

The main idea is to find the geometry of the electrode that compensates the difference in the heat sinks along the electrode. This optimization of the wire electrode design was done by numeri-

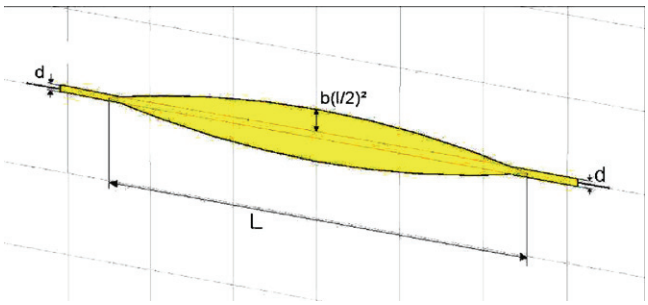

Fig. 2. Longitudinal profile of the sputtered gold conductor (x–y plane). The shape-coefficient b had to be optimized.

cal simulation experiments. For the simulation of the temperature development on the surface of the electrode we used the program Comsol Multiphysics©[35] which employs the finite element method (FEM) for discretization and solution of various differential equations on 3D geometries (Fig. 1).

The temperature development along the electrode is caused by the Joule or resistive heating which is described by the following simple expression:

$$P = UI = I^2R = \frac{\Delta Q}{\Delta t}. \quad (1)$$

The amount of Joule power P dissipated by a resistor R is, with the use of the ohmic law ($U = RI$; U—voltage, I—current), depending on the square of the current I flowing through. The time integral of the Joule power defines the amount of thermal energy Q dissipated by the resistor over the time t.

If the cross-section of the wire A is not constant, the current density J ($I = JA$) will also not be constant and so the heat development over the length of the wire should also not be constant. This simple assumption motivated the effort for numerical optimization of the electrode form in order to have as uniform as possible temperature distribution over the whole length of the electrode wire.

The whole set-up of the electrode around the wire including the wire itself and the surrounding material have been modeled with their electrical and thermal properties (Table 1).

It has been assumed that the electrode is made of gold wire and copper contacts sputtered on a silicon surface. Everything except the gold wire is encapsulated under a wax coating. The gold wire shown in Fig. 2 is in contact with the solution, for the simulation purposes we assumed water as a medium around the wire.

The simulation is a coupled electromagnetic and thermal simulation. The input source in the electromagnetic simulation is the applied current of 1 A. The pre-computed electric field components E_x, E_y and E_z from the electromagnetic simulation are the input for the thermal simulation. The appropriate boundary conditions for both types of simulations were applied. In the thermal simulation, movement of the surrounding medium around the wire, and therefore convective heat transport is neglected. This applies especially for short-term heating up to 200 ms [21,36]. At longer periods, thermal convection occurs and causes a negative feedback due to its cooling effect. This means the temperature gradients along the heated wire will decrease [32].

Since the conduction is the main mechanism for heat transportation in our model, the boundary condition of the gold wire is defined as "heat flux" given by the following equation:

$$n(k\Delta T) = h(T_{\text{inf}} - T) \quad (2)$$

The initial temperature of the surrounding in the thermal simulation is $T_{\text{inf}} = 293$ K and the heat conductivity of the gold amounts to $h = 317$ W/(m K). In order to get information for the temperature development on the wire surface over the time, the thermal simulation is discretized in time.

3. Results and discussion

First the temperature development along the electrode was simulated with a constant cross-section for a total time of 0.5 s, discretized in steps of 20 ms. Fig. 3 shows the temperature allocation change along a linear conductor within 0.5 s (B). A corresponding 3D plot for the time of 100 ms (A) as well as a 2D plot for the same time (C) is also shown in the same figure. A huge temperature gradient from the middle to both ends of the conductor was obtained for a constant cross-section wire. While heat is produced from electric energy evenly along the heated conductor, contact sites at both ends are effective heat sinks. Thus we tried to minimize the temperature gradient by using different shapes for the electrode. According to Eq. (1), the local heat development along the conductor $\Delta Q/\Delta t$ is a consequence of the electric current I flowing through the cross-section of the electrode. The change of the electrode cross-section is effectively changing the local resistivity along the current path. Hence, if a constant current applied, changing the resistivity along the current path enables the control of the heat development in different sections along the electrode. This eventually results in a control of the temperature gradient along the electrode. Having a locally larger cross-section, the resistivity and the heat development will be locally lower. To compensate for the heat sinks at both ends of the wire, we introduced small cross-section here, and bigger cross-section in the middle of the wire. As a result of such a consideration, an oval shape of the electrode seemed to be the best way to get more homogeneous temperature allocation along the whole lengths of the wire or screen-printed layer.

For the optimization of the electrode's geometry a model equation:

$$y = -bx^2 + d + \left(\frac{L}{2}\right)^2 b \quad (3)$$

was adopted. By varying the coefficient b, the shape in the x–y plane could be adjusted which had an effect on the temperature allocation along the heated wire. Now, less heat is produced in the middle which compensates for the effect of the heat sinks.

As shown in Fig. 4A–C, the conductor is more or less at the same temperature over its whole lengths. This becomes especially apparent by comparing the calculated graphs for the temperature gradients in Figs. 3B and 4B. The maximal temperature difference between the coolest and the hottest point could be diminished from

Table 1
Relevant material parameters for the materials used in the simulation model.

Physical properties	Gold	Copper	Silicon	Water
Density (kg/m^3)	19,320	8920	2330	0.001[a]
Specific heat capacity (J/(kg K))	128	385	700[a]	4187
Electric conductivity (S/m)	45.2×10^6	58×10^6	2.54×10^{-4}	[b]
Thermal conductivity (W/(m K))	317	401	148	0.597[c]

[a] Applies for $T = 25$ °C.
[b] Electric conductivity of water was not considered.
[c] Applies for $T = 20$ °C.

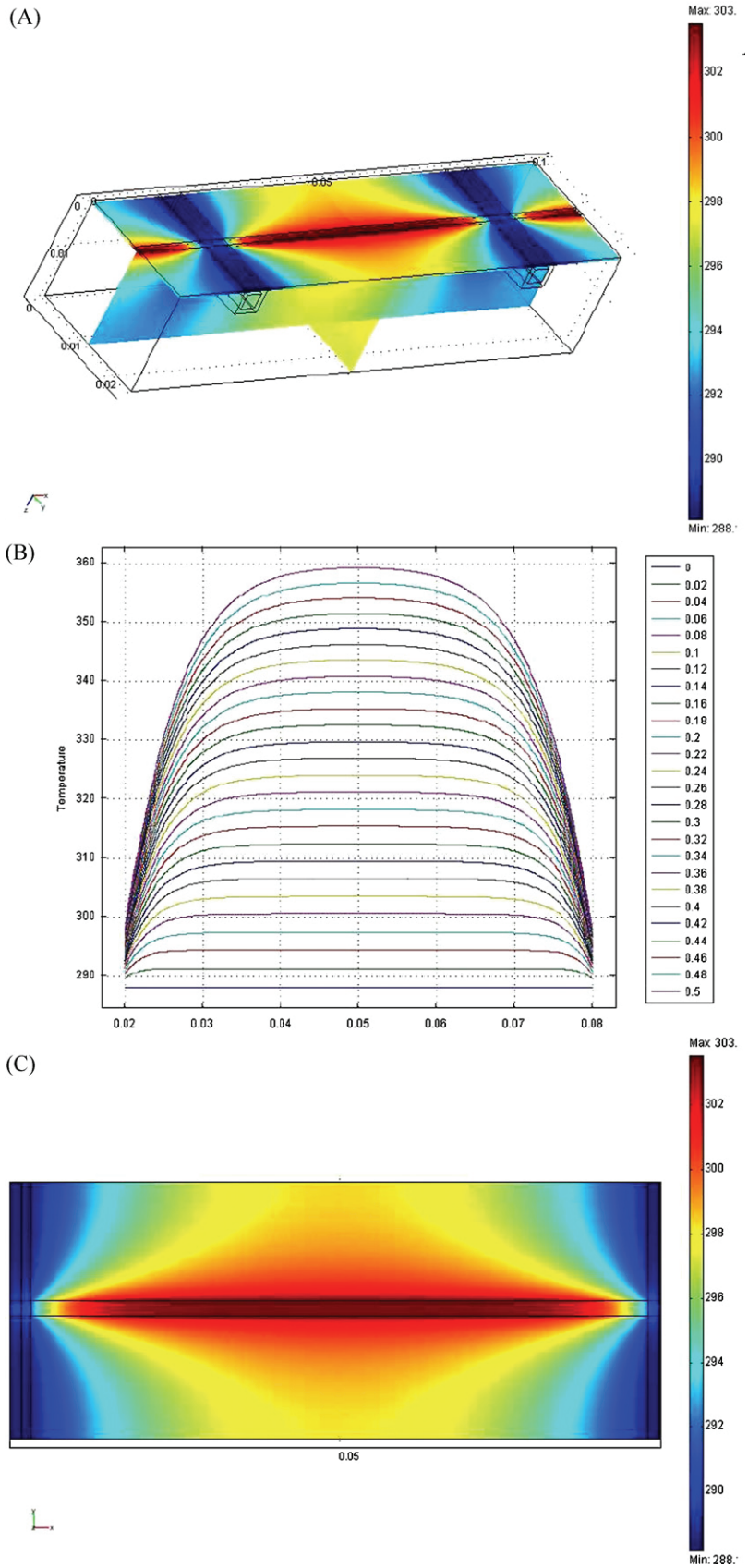

Fig. 3. (A–C) Temperature allocation along a linear sputtered conductor on an insulating substrate; huge temperature gradient between both ends; (A/C) 3D/2D-plot for 100 ms heating duration, (B) plot of the temperature development for the duration of at least 500 ms.

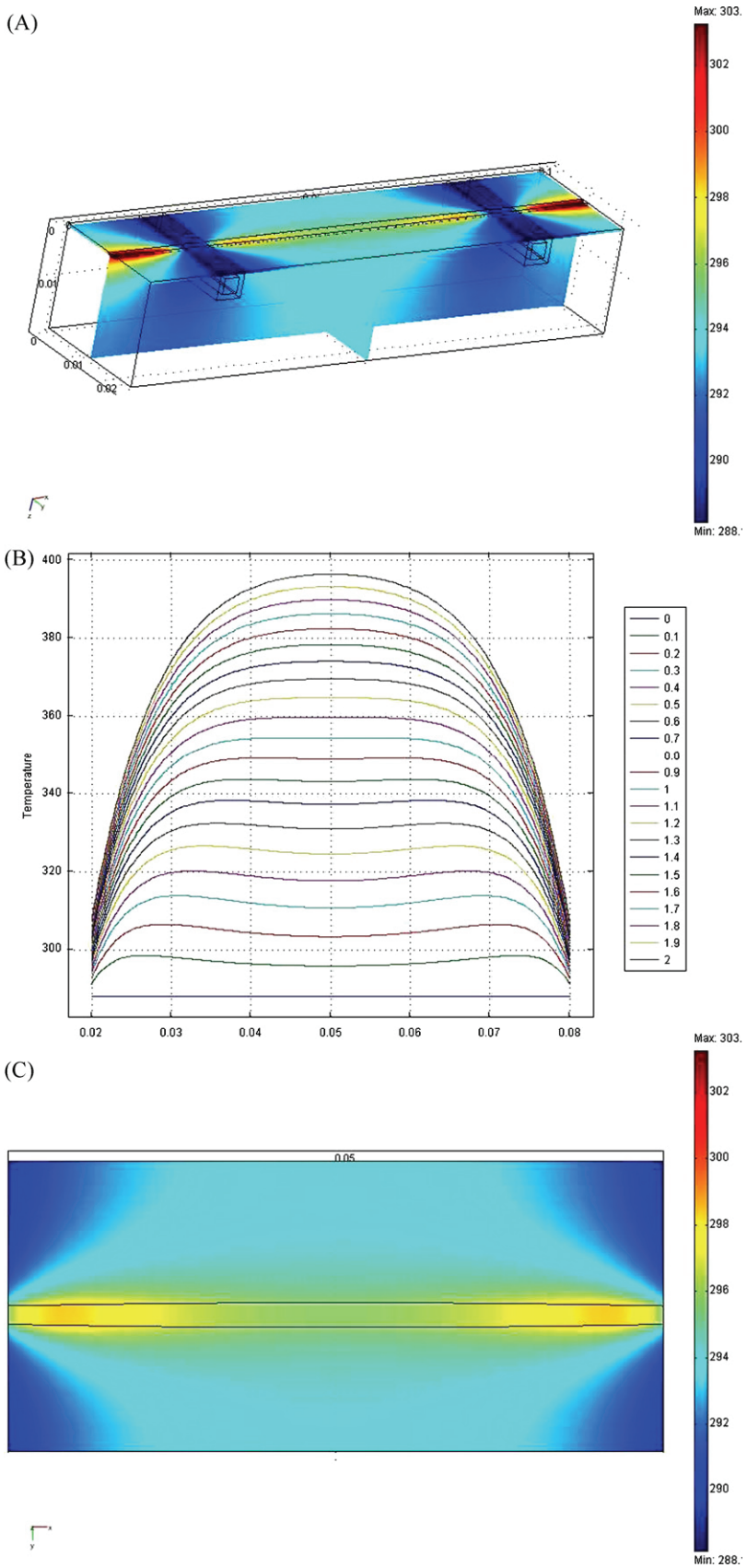

Fig. 4. (A–C) Nearly constant temperature allocation along the conductor after optimizing the "wire" by fitting the shape-coefficient *b*; (A/C) 3D/2D-plot for 100 ms heating duration and (B) plot of the temperature development for the duration of at least 2 s.

ca. 62 to 30 K at a heating time of 0.5 s. For a certain temperature of such as 360 K, this means an enhancement of uniformity (±1 K) from ca. 30% (Fig. 3B) to 50% (Fig. 4B) of the whole length of the heated wire or layer. The longer heating time results from the lower overall resistance of the oval wire or layer and can be compensated by higher heating current.

Concerning a uniform temperature allocation along the wire for the time period of 100 ms (e.g. for temperature pulse voltammetry), the optimal value of b was found to be 0.05.

These results of the calculated temperature allocation along sputtered conductors led us to an optimized shape for a completely new designed electrode type.

4. Conclusions

The mathematical simulations proved our estimation of a non-constant temperature at the surface of directly heated electrodes. To minimize the temperature gradient, an oval shape (rather than cylindrical or other shapes with a constant cross-section) would be indeed a significant improvement. Further improvement of the oval shape considering more complicated functions would require considerably more computing resources and will be performed in the future.

Acknowledgements

The authors want to thank the Federal State of Mecklenburg-Vorpommern (Landesexzellenzförderung) and the Deutsche Forschungsgemeinschaft (DFG) for financial support.

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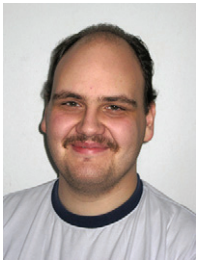
Biographies



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Publication V

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Adsorptive stripping voltammetric detection of daunomycin at mercury and bismuth alloy electrodes

International Journal of Electrochemical Science 2 (2007) 498

Int. J. Electrochem. Sci., 2 (**2007**) 498 - 507

International Journal of
ELECTROCHEMICAL
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Adsorptive stripping voltammetric detection of daunomycin at mercury and bismuth alloy electrodes

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Received: 25 April 2007 / *Accepted:* 17 May 2007 / *Published:* 1 June 2007

We investigated the analytical response of daunomycin, an effective drug used for cancer chemotherapy, at different types of electrodes. The following five different materials for the electrodes have been compared: hanging mercury drop, gold, gold/bismuth, gold/mercury and silver mercury. Especially at the hanging mercury drop electrode and also at the silver/mercury and gold/mercury amalgam electrode, nicely shaped cyclic voltammograms with nearly no peak separation could be found whereas both the gold and the gold/bismuth electrode showed a significant peak separation. The linear dependency to the scan rate of the signal of all the electrodes is a hint for adsorption rather than diffusion control. In adsorptive stripping voltammetry it could be shown that especially the gold/bismuth alloy (4 % Bi) has a great linear calibration range of daunomycin, whereas the linear range at the hanging mercury drop electrode is surprisingly limited. The reason for the advantageous properties of the gold/bismuth alloy in adsorptive stripping analysis can probably be addressed to sufficiently strong and particularly constant adsorption at low daunomycin concentrations at the electrode surface. Hence, the gold/bismuth electrode can be recommended for adsorptive stripping voltammetry of trace amounts of daunomycin.

Keywords: Daunomycin, adsorptive stripping voltammetry, hanging mercury drop electrode, gold bismuth alloy electrode, silver amalgam, gold amalgam

1. INTRODUCTION

Electrochemical stripping analysis is a very important and widely used tool for the trace determination of various elements and compounds. Major advantages include low detection limits coupled with good selectivity and reproducibility due to the in situ pre-concentration step. This is especially true for adsorptive stripping voltammetry (AdSV), where the analyte is deposited on the working electrode by adsorption. However, an electrode material is needed providing not only good adsorption of the analyte but also a stable and reproducible electrode surface. The most commonly used working electrode material in this method is mercury. The advantages of the hanging mercury

drop electrode (HMDE) include renewable surface and high hydrogen overpotential. Thus, it is possible to study reactions of compounds that otherwise would be hidden by reduction of water. Due to toxicity of mercury, several approaches were supposed to replace the mercury by other materials such as carbon, gold etc., but none of these materials was found to be an acceptable replacement for common mercury electrodes. Nevertheless, there is high interest in new alternative electrode materials with similar properties especially for on-site environmental monitoring.

Bismuth provides several advantageous properties in electrochemical analysis. Compounds of Bi(III) adsorb on platinum electrodes and can catalyze the oxidation of glucose [1]. Wang et al. [2] introduced the bismuth film electrode (BiFE) representing an attractive alternative to mercury film electrodes. They demonstrated that bismuth films, deposited on various electrode substrates, offer well-defined, undistorted and highly reproducible signals of heavy metals. High hydrogen overvoltage, favourable signal-to-background ratio, and excellent resolution and reproducibility are sometimes superior to those of common mercury electrodes. Besides, bismuth is an environmentally unconcerned non-toxic element. All bismuth electrodes provide a wide potential window of -1.2 to -0.2 V vs. SCE. It allows for stripping detection of most metals that can be determined also at mercury electrodes. Examples include anodic stripping of zinc, cadmium, lead, and tin [2,3,4,5,6,7,8,9,10], as well as adsorptive stripping of nickel [11], uranium [12], aluminium [13], and cobalt [14,15]. Organic compounds such as organic nitro compounds have been determined by means of differential pulse voltammetry and amperometry [16]. The bismuth electrode can also be modified with a Nafion film to minimize the interferences of surfactants during the voltammetric determination of metal ions like lead and cadmium in some real samples [17]. Bismuth electrodes are less prone to interferences by dissolved oxygen in non-deaerated solutions, often still yielding a flat baseline [2,11].

In case of the preparation of a BiFE for AdSV application, external plating is recommended. However, non-complexed Bi(III) ions very easily form insoluble hydroxo-compounds. Due to this risk of hydrolysis, only acidic media can be considered as plating solutions for the bismuth film preparation. The application of bromide containing plating solution and the use of strongly acidic media (1 mol/L HCl) allows using higher concentrated Bi(III) solution (0.02 mol/L) and minimizing the risk of their hydrolysis [15].

Furthermore, the carbon paste electrode (CPE) can be used as a substrate for the preparation of BiFE [5]. However, the electrochemical activity of the BiFE depends on the structure of the electrode surface, which is not ideal with CPEs for external plating of a film. AFM studies of a bismuth-modified carbon paste electrodes have recently been published [18]. The bulk phase modification of carbon paste with Bi_2O_3 is very efficient and useful for the stripping voltammetric determination of Cd and Pb [19].

Only a few contributions report about the bismuth bulk electrode. Pauliukaite et al. [20] investigated the cathodic behaviour of polycrystalline metal bismuth electrodes during the voltammetric detection of cadmium(II) and 2-nitrophenol. The characteristics of the BiBE under anodic conditions, i.e., at bismuth surface coated with a thin conductive Bi_2O_3 film, were examined by testing potassium hexacyanoferrate(III) and hexaammineruthenium(III) chloride. Daunomycin has been analyzed by means of adsorptive stripping voltammetry on a bismuth bulk electrode [21]. We recently reported about self-assembled monolayers of alkanethiols on bismuth [22]. A general problem

arising during the application of massive bismuth electrodes is corrosion and formation of oxide films on the surface. Hence, the electrode surface has often to be renewed by polishing. Electroanalysis on bismuth electrodes has been reviewed [23,24,25].

Mikkelsen and Schrøder found that small addition of bismuth (about 4 %) can improve the analytical applicability of electrode materials with low hydrogen overvoltage such as gold [26,27] and silver [28,29]. Such electrode materials combine advantages of both bismuth and noble metal electrodes. They are less prone to corrosion. The alloy made of gold and 4 % bismuth for example has been found to be applicable in both the negative (detection of cadmium and iron) and positive (detection of mercury) potential range [30].

Amalgam electrodes have been introduced by Mikkelsen and Schrøder [28,31], and by Yosypchuk and Novotny [32], independently. Solid amalgams provide most of the positive properties found on mercury. However, they still contain considerable amounts of the toxic element mercury. Organic [33,34] and inorganic [31,35,36] substances have been analyzed on amalgam electrodes. Nucleic acids have also been detected on amalgam electrodes [37]. Analytical methods basing on amalgam electrodes have been reviewed [38].

Daunomycin (DM), an anthracycline antibiotic, is an effective drug used for cancer chemotherapy. In electroanalytical chemistry it is also widely used as an indicator of DNA [39]. Cytostatic activity of anthracyclines like daunomycin has been known for over 20 years, but it has not been resolved, by which mechanism they exert the cytostatic action. Apparently the DM molecule intercalates into GC base pairs of DNA or binds to nucleotides and subsequently the synthesis of DNA and RNA is inhibited. Ultrafast electron-transfer reactions between DM and base guanine were found. The reduced DM radicals can catalyze super oxide formation from dioxygen which then triggers redox cycling. Thus, molecular oxygen also plays a partial role in the drug action. The most important side effect of anthracycline antibiotics is their cardio toxicity at high doses. Patients taking these drugs are at increased risk of arrhythmias, irreversible cardio myopathy and the risk of congestive heart failure. For that reason it is very important to monitor the drug level. A traditional assay for the detection of these compounds is HPLC (High Performance Liquid Chromatography) with fluorescence detection [40]. We found that daunomycin strongly adsorbs on bismuth electrodes, which can be used for trace detection of this compound by means of AdSV [21].

This contribution reports for the first time about the application of gold-bismuth alloys for adsorptive stripping voltammetry. As a model analyte, daunomycin has been determined at different electrode materials including Au/Bi (4 % Bi), Ag/Hg, Au/Hg, and the hanging mercury drop electrode (HMDE).

2. EXPERIMENTAL PART

2.1. Materials

All measurements were carried out in 50 mM phosphate buffer solution (pH 7) or 200 mM acetate buffer solution (pH 5). During the measurements with the Autolab, we applied an Ag/AgCl (3 M KCl) reference electrode (from Meinsberg Sensortechnik GmbH, Meinsberg, Germany) and a

laboratory-made platinum counter electrode. For the measurements with the polarograph, we used an Ag/AgCl (3 M KCl) reference electrode und a platinum counter electrode (delivered by Metrohm AG, Filderstadt, Germany).

The chemicals potassium dihydrogenphosphate, potassium hydrogenphosphate, potassium acetate, silver powder and daunomycin were purchased from Fluka. The used gold/bismuth rod was delivered by MaTeck GmbH, Jülich, Germany. The used purified water (18 MOhm, TOC < 2 ppb) was prepared by means of an SG Ultra Clear water system (SG Water GmbH, Barsbüttel, Germany).

2.2. Instrumentation

The measurements with the gold/bismuth electrode (Au/Bi), the silver/mercury electrode (Ag/Hg) and the gold/mercury electrode were carried out with an Autolab PGSTAT 20 (Eco Chemie, Utrecht, Netherlands). All experiments at the hanging mercury drop electrode were performed by means of a 797 VA Computrace (Metrohm AG, Filderstadt, Germany).

2.3. Preparation of the electrodes

2.3.1. Gold-bismuth electrode

A gold rod containing 4 % bismuth was delivered by Mateck GmbH (Germany, Jülich). We machined a rod with 3 mm in diameter. A 10 mm diameter PTFE rod with a 3 mm hole was used as housing for the gold-bismuth rod. The connection has been realised by a copper bolt inside the PTFE rod. The detailed construction of this type of electrode has been described earlier [21].

2.3.2. Gold mercury electrode

The formerly described (indirectly heatable) LTCC (Low Temperature Cofired Ceramics) sensor, but here with a gold instead of a platinum electrode [41,42], was dipped for 30 s into mercury. With the thin mercury and gold amalgam film on it, the electrode was left for one hour to allow further amalgamation and then polished carefully with slurry consisting of alumina powder (first 0.3 micron, then 0.05 micron) and water.

2.3.3. Silver amalgam electrode

To build a silver amalgam electrode, we followed an earlier procedure [32], and firstly drilled a 3 mm hole into a 10 mm diameter PTFE rod. There has been a connection made of copper inside this rod forming the base of the hole. Secondly, we filled the hole with silver powder and pressed it. On the top of the compressed powder we put a drop of mercury and left the electrode for 24 h. Finally we removed the liquid mercury and ground the electrode with fine sandpaper.

Before each measurement, the electrodes were firstly ground on very fine (grit #1000) wet sandpaper. Secondly the electrodes were polished with alumina slurry in two steps (first 0.3 micron, then 0.05 micron, both from Bühler, Uzwil, Switzerland).

3. RESULTS AND DISCUSSION

3.1. Cyclic voltammetry

As depicted in Fig. 1, we applied cyclic voltammetry to study the affinity of daunomycin to adsorb on different electrode materials.

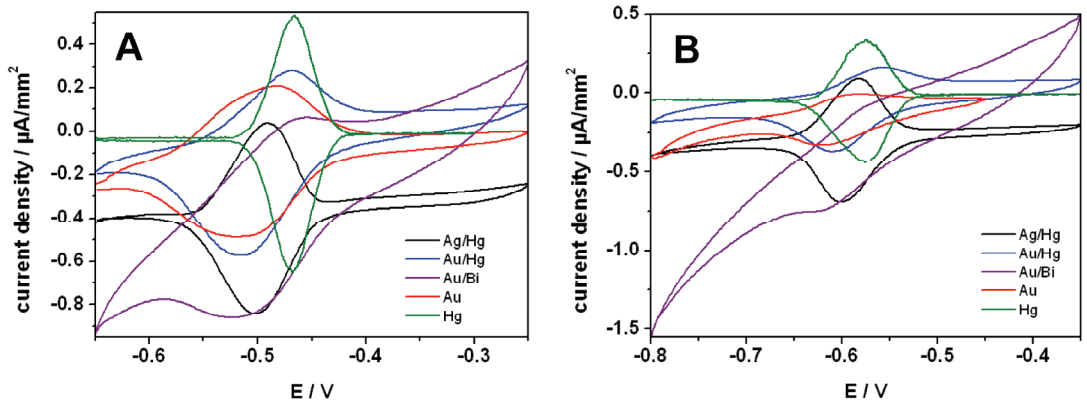

Figure 1. Cyclic voltammograms of 10 µM daunomycin on different electrode materials in A) 200 mM acetate buffer at pH 5.0 and B) 50 mM phosphate buffer at pH 7.0; scan rate 200 mV/s.

The hanging mercury drop electrode is well known to provide a perfect and easily renewable surface but also to strongly adsorb many organic substances. We found a nearly perfect cyclic voltammogram of adsorbed daunomycin on mercury. Here, the peak potentials of the cathodic and the anodic scan have been equal in both acetate and phosphate buffer. As one would expect following a general rule, the peak potentials are more positive in the acetate buffer at lower pH. The silver amalgam electrode exhibits almost the same behaviour. This can be attributed to a thorough amalgamation of the silver powder which served as a base for the amalgam formation. In contrast, both the gold amalgam and the gold electrodes show a significant peak separation, which can be attributed to a considerable contribution of analyte diffusion. Amalgamation at the LTCC gold layer electrode is probably not as efficient as compared to the silver amalgam electrode. Therefore, adsorption is not as strong at these types of gold electrodes. The peak shapes are deformed to a significant degree. This is particularly true for the gold-bismuth alloy electrode in phosphate buffer. Another possibility for such peak separation is kinetic hindrance of the electrode reaction. In earlier studies [21], a bismuth bulk electrode exhibited a cyclic voltammetric behaviour of daunomycin that now can be compared with the silver amalgam electrode. Obviously, a small percentage of bismuth in an alloy with gold is not sufficient to meet all the positive properties of the pure bismuth electrode.

We also studied the effect of the scan rate. Dependence of the cyclic voltammetric peak currents upon the scan rate is displayed in Figure 2.

A linear relation between peak currents and scan rate is a hint for reactions of an adsorbed or otherwise deposited substance. Diffusion controlled electrode processes would, in contrast, yield a linear function to the square root of the scan rate. We found a linear dependence for all types of

electrodes studied, particularly for gold, gold-bismuth, silver amalgam, and gold amalgam; and a somewhat strange behaviour of the HMDE.

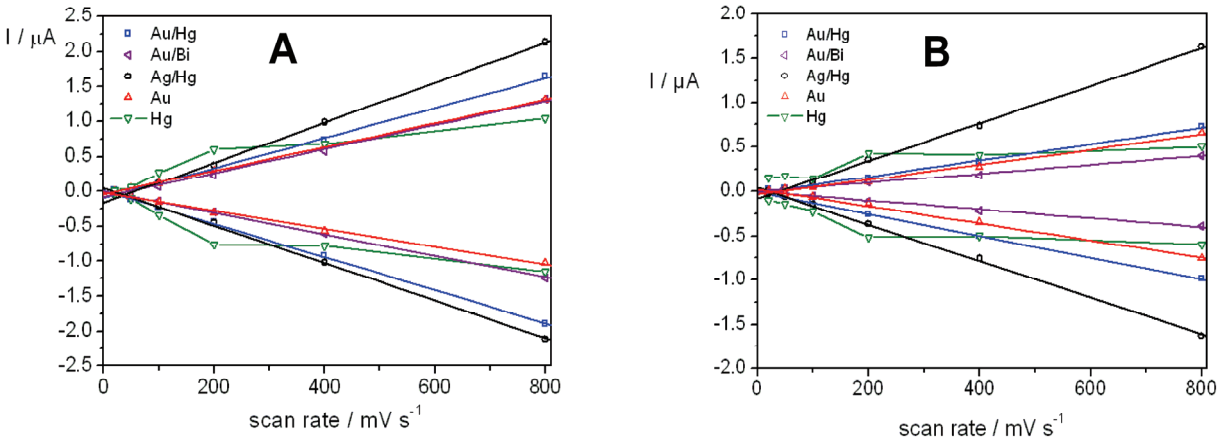

Figure 2. Effect of the scan rate upon cyclic voltammetric signals of daunomycin in A) acetate buffer and B) phosphate buffer. Other conditions as in Fig. 1.

3.2. Adsorptive stripping voltammetry

Figure 3 depicts effect of deposition time on a mercury drop electrode. The background has been very constant during all experiment, since the drop has been renewed every time. At 60 s deposition time and above, we observed nearly constant stripping signals indicating a saturated mercury surface.

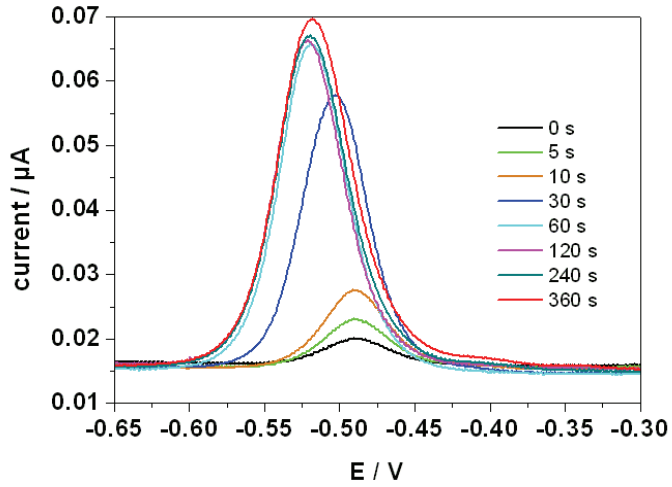

Figure 3. Effect of the deposition time upon DPV adsorptive stripping detection of 10 µM daunomycin in 200 mM acetate buffer at pH 5.0 on a HMDE; deposition potential -0.65 V, scan rate 10 mV/s, pulse amplitude 25 mV.

Figure 4 exhibits the influence of the deposition time upon the adsorptive stripping DPV signals of daunomycin at a silver amalgam electrode. Here, a slightly changing background current has been observed, a typical disadvantage of solid metal electrodes. We observed saturation beginning at 240 s deposition time. This is significantly longer than on mercury, probably due to significant lower adsorption of daunomycin compared to pure mercury.

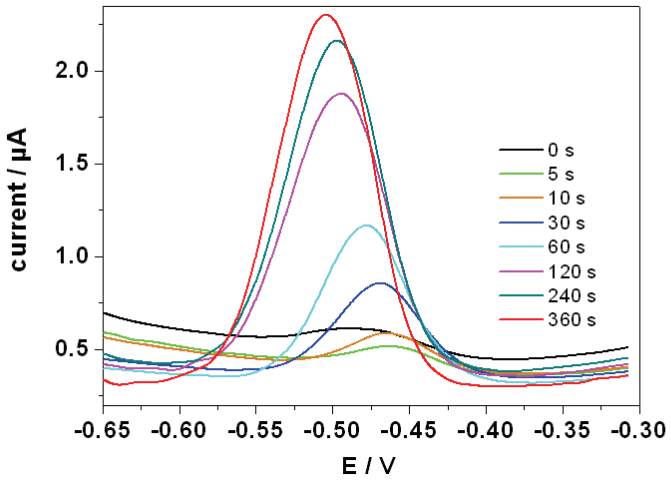

Figure 4. Effect of the deposition time upon DPV adsorptive stripping detection of daunomycin on a silver amalgam electrode; conditions as in Fig. 3.

Figure 5 displays the effect of the deposition time upon the adsorptive stripping DPV signals of daunomycin at a gold-bismuth electrode. In contrast to cyclic voltammetry, DPV provides well shaped stripping peaks on this electrode material. Although the background changed even more during one series compared to the silver amalgam electrode, all signals could be easily processed. Saturation has not been observed even at 360 s deposition time.

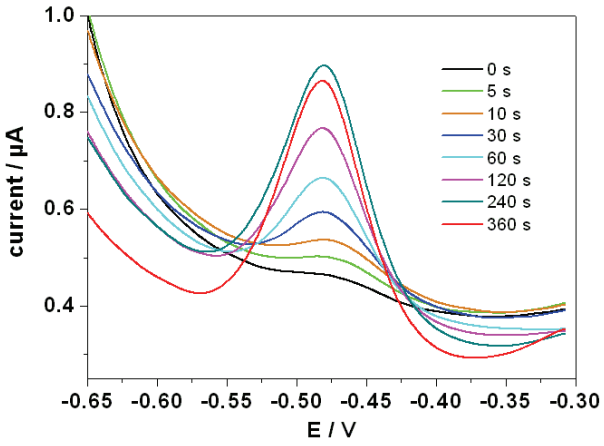

Figure 5. Effect of the deposition time upon DPV adsorptive stripping detection of 10 µM daunomycin in 200 mM acetate buffer on a gold-bismuth electrode; other conditions as in Fig. 3.

Figure 6 illustrates the effect of deposition time on the adsorptive stripping DPV signals of daunomycin on four different electrodes. Whereas gold amalgam and mercury electrodes showed saturation at 30 and 60 s, respectively, the stripping voltammetric signals on silver amalgam and gold-bismuth electrodes has been increasing in the whole range between 0 and 360 s deposition time.

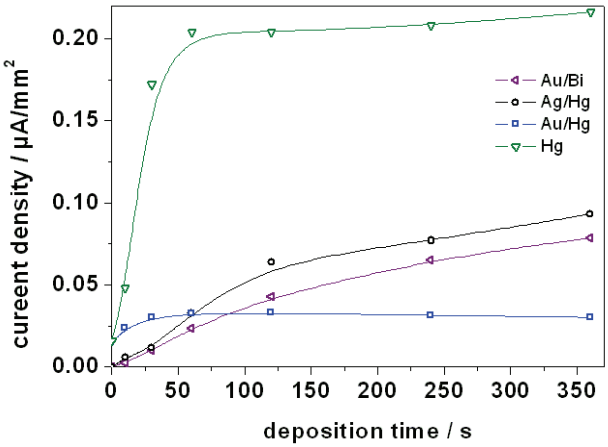

Figure 6. Effect of the deposition time upon DPV adsorptive stripping detection of 10 µM daunomycin in 200 mM acetate buffer at pH 5.0 on several electrodes; other conditions as in Fig. 3.

Figure 7 depicts calibration plots of daunomycin on three different electrodes. The very strong adsorption on mercury limits the linear range significantly. The sigmoidal shape of the calibration curve obtained at the HMDE can probably be addressed to the formation of daunomycin multi layers during the deposition step. At the lowest concentrations, a relative weak adsorption has been visible, whereas the surface seemed saturated at concentration above 700 nmol/L. The silver amalgam electrode has exhibited a linear range up to 750 nmol/L. Only the gold-bismuth electrode yielded linearity in the whole range between 0 and 1000 nmol/L daunomycin.

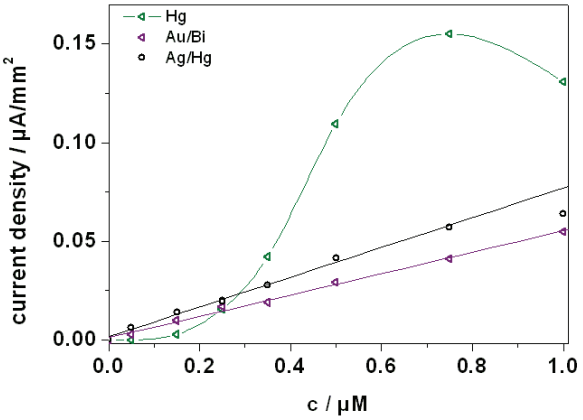

Figure 7. Calibration plots of DPV adsorptive stripping detection of daunomycin in 200 mM acetate buffer at pH 5.0 on several electrodes; deposition time 120 s, other conditions as in Fig. 3.

4. CONCLUSIONS

We could demonstrate the applicability of all five electrode materials for the detection of daunomycin in phosphate and acetate buffer solution. Especially the gold/bismuth electrode is well applicable due to the great linear concentration range. The strongest adsorption has been found for the hanging mercury drop electrode, which causes a serious drawback in regard of lacking linearity of the calibration plot. Silver amalgam and gold amalgam electrodes perform better than mercury but still show effects of saturation due to strong adsorption. Despite the large background in the cyclic voltammograms, the gold/bismuth electrode seems to be a very promising electrode material for the routine analysis of daunomycin. This is supported by the easy handling of the Au/Bi electrodes and its mixture of nontoxic metals.

ACKNOWLEDGEMENT
The authors are grateful to the Deutsche Forschungsgemeinschaft for financial support (FL 384/2-1, 2-3)

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Publication VI

Heiko Duwensee, Annette Enrica Surkus, Gerd-Uwe Flechsig

Detection of DNA-Hybridization on Gold-Bismuth (Au/Bi) Alloy Electrodes

Poster contribution ESEAC 2008

DETECTION OF DNA-HYBRIDIZATION ON GOLD-BISMUTH (Au/Bi) ALLOY ELECTRODES

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INTRODUCTION

The most common techniques for the detection of DNA-hybridization are optical detection methods. In the last years a variety of electrochemical methods have been proposed. Low costs, simple handling and high efficiency are the main advantages of the electrochemical approach. Gold electrodes are often used for the detection of DNA-hybridization events. However, due to the typical potential range of gold only a few redox-active labels are usable. In this case osmiumtetroxide bipyridine [OsO_4(bipy)] is widely used but the catalytic peak (-1.1 V) can not be detected on gold electrodes because of their low hydrogen overvoltage. There is a lot of scientific research dealing with other electrode materials like bismuth [1, 2]. Bismuth is not toxic and it offers a high hydrogen overvoltage and adsorption affinity. However, the disadvantage of pure bismuth is the relatively small corrosion resistivity. This should be improved considerably in combination with gold as a Au/Bi alloy [3, 4], while preserving the wide potential window. First investigations with Au/Bi alloy electrodes (96% Au / 4% Bi) demonstrated this assumption. After SAM-formation the hybridization with an [OsO_4(bipy)]-labelled target was carried out. In addition, the influence of the target concentration and the duration of hybridization were examined using voltammetric methods.

RESULTS

Preparation of the Au/Bi electrodes:

The gold/bismuth alloy (96% Au, 4% Bi) was delivered by Mateck GmbH. We machined a rod with 3 mm diameter. A PTFE rod (10 mm diameter, with a 3 mm hole at one end) was used as the housing for the gold-bismuth rod. From the other side a copper bolt has been inserted to realise the contact. The other gold/bismuth electrode (65% Au, 35% Bi) was constructed in the same way.

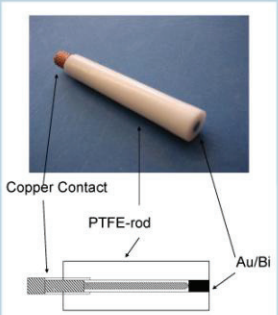


Figure 1: **Gold-bismuth electrode**

Preparation of the electrodes:

Before the measurements the electrodes had been polished with alumina powder. The second step was the incubation with the DNA probe (5´-AAG TCC TTG AGC TGA TTT TAC TGT ATT TTT TTT TTT TTT [Dithio]$_3$-3´) at a concentration of 30 µM for 10 h. After 1 h in 1 mM aqueous mercaptohexanol solution the baselines were measured. The hybridization was performed by dipping the electrode into 1 ml stirred target solution. After each measurement the electrodes were dehybridized by dipping into a stirred solution of deionized water at 50 °C for 60 s. The labelling procedure of the target has been earlier described in more details [5].
Target: 5´-TTT TTA ATA CAG TAA AAT CAG CTC AAG GAC TT-3´
Protective strand: 5´-CAG TGT TTG ACC TGA TTT TAC TGT CTT-3´

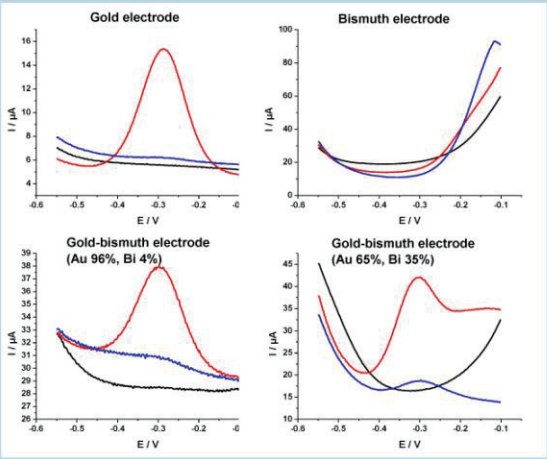
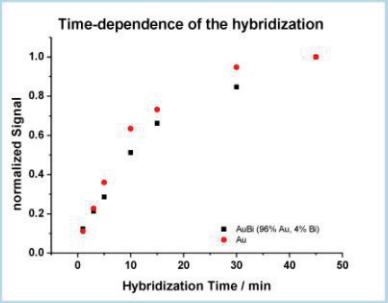


Figure 2:
Square wave voltammetric signals at different electrodes
black = probe in 10 mM Tris-buffer containing 0.5 M Na_2SO_4 at pH 7.5
red = after 15 min hybridization in 50 nM target
blue = after dehybridization

Figure 3:
Time dependence of the hybridization
The signals are normalized to the signal obtained after 45 min hybridization. The used Au/Bi electrode had a composition of 96 % gold and 4 % bismuth.
All measurements were performed using square wave voltammetry (200 Hz, 2 mV step potential, 40 mV amplitude) in 10 mM Tris-buffer containing 0.5 M Na_2SO_4 at pH 7.5. During the hybridization step the electrode was dipped into a stirred 100 nM target solution containing 10 mM Tris-buffer and 0.5 M Na_2SO_4 at pH 7.5



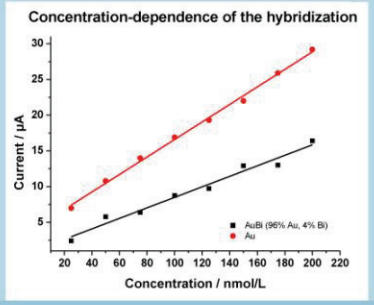


Figure 4:
Concentration dependence of the hybridization
The used Au/Bi electrode had a composition of 96% gold and 4% bismuth.
All measurements were performed using square wave voltammetry (200 Hz, 2 mV step potential, 40 mV amplitude) in 10 mM Tris-buffer containing 0.5 M Na_2SO_4 at pH 7.5.
During the hybridization step the electrode was dipped for 15 min into a stirred target solution containing 10 mM Tris-buffer and 0.5 M Na_2SO_4 at pH 7.5

SUMMARY

The gold and the gold-bismuth (96:4) electrodes show almost the same behaviour although the alloy electrode has a little lower response and the overall background seems higher. On the pure bismuth bulk electrode it was not possible to detect DNA-hybridization. A promising composition seems to be the gold-bismuth (65:35) electrode. DNA-hybridization could be detected but it is not the typical signal of a pure gold electrode. It is in general more difficult to operate bismuth-rich electrodes in thiol-containing solutions as shown by E. Lust et al. [6]. The gold-rich electrodes will be characterized more detailed within the next months.

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Acknowledgement
The authors want to thank the Federal State of Mecklenburg-Vorpommern (Landesexzellenzförderung) and the Deutsche Forschungsgemeinschaft (DFG) for financial support.

Publication VII

Falko Wachholz, Heiko Duwensee, Ralf Schmidt, Michael Zwanzig, Jan Gimsa, Stefan Fiedler, Gerd-Uwe Flechsig

Template-free galvanic nano-structuring of gold electrodes for sensitive electrochemical biosensors

Electroanalysis, submitted

Template-free galvanic nano-structuring of gold electrodes for sensitive electrochemical biosensors

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Abstract

Gold nano-structuring of smooth gold disk-electrodes has been performed by electrodeposition. FE-SEM images indicate a strongly enlarged electrochemically active surface of the modified electrodes. This could be approved by the gold oxide reduction peak in cyclic voltammetry. The electrodes were also applied for sequence-specific DNA detection. For this, complementary single stranded capture probes were immobilized on the electrodes and consecutively hybridized with complementary and non-complementary [OsO_4(bipy)]-labelled DNA targets. We found that intermediate dehybridization steps did not decrease the sensitivity for the specific target. Moreover, the surface modification increased the voltammetric signal by an approx. factor of 9. Further, an enhanced linear calibration range was observed. The presented simple nano-structuring process holds great promise for many electrochemical sensor applications.

Keywords

Nano-structuring, Osmium tetroxide bipyridine, DNA hybridization, Gold electrode, FE-SEM, Voltammetry

1 Introduction

Biosensors on the basis of self-assembled monolayers (SAMs) are widely used. Especially gold as a substrate for SAMs shows very strong affinity to thiol-modified probe molecules. While miniaturised electrodes provide many advantages such as parallelization and microelectrode behaviour, the smaller active surface leads to lower analytical signals, which are prone to electronic interferences. Therefore, the enlargement of the electrochemically active electrode surface is desirable. Several approaches for micro- or nano-structuring of electrode surfaces have been proposed during the last couple of years.

Carbon nanotubes are reported to greatly increase voltammetric signals of carbon paste electrodes [1]. Also metal electrode surfaces can be modified by depositing carbon nanotubes, which is useful for many analytical purposes [2]. Carbon nanotube fibre microelectrodes were prepared and characterized due to their performance regarding electroanalytic oxidation of analytes via dehydrogenase-mediated reactions using NAD^+ as a mediator [3]. The enhancement of the active electrode area by modifying the surface with cell imprints in polyurethanes or porous gold with latex imprints was also reported [4,5]. A very efficient method to enhance the performance of cell-based diagnostic assays is the lithographic modification with focused laser radiation and interference lithography creating magnetic nano-dots or regular structures [6-9]. Direct electrochemical deposition of gold or gold containing particles is an elegant possibility to obtain well defined and highly ordered structures with a tremendous effect on the electroactive surface. Common techniques often require the use of nano-structured templates when using three-electrode arrangements [10], high-frequency impedance [11] or direct electrodepositing methods [12,13]. Template-free electrochemical deposition was carried out by French et al. using bipotentiostatic growing of gold on self-made platinum disc electrodes [14,15].

Capture probe-modified electrodes can be used to detect the hybridization with complementary DNA target strands. Some reported procedures for the electrochemical

recognition can be performed using indicator molecules that are (a) covalently attached to the target, (b) covalently attached to a signalling/ reporter probe, (c) interacting with the negatively charged phosphate groups, or (d) intercalating into the base stack. Another possibility is to take advantage of the intrinsic electrochemical activity of the DNA, e.g. the oxidation of guanine [16–18]. The capture probe strands can be immobilized on magnetic beads [19,20] or as self-assembled monolayers on metal surfaces [21–27]. Methods of electrochemical hybridization detection were reviewed a number of times [28–32]. We already reported the application of covalently attached redox markers together with thiol-linked probe oligonucleotides on heated gold electrodes [33,34]. For this purpose the complex [OsO_4(bipy)] can be utilized as an electrochemically reversible covalent DNA-label [35]. It reacts with the pyrimidine bases thymine, uracil and cytosine [36]. In this way, labelling of DNA-strands of any length can be easily performed in the laboratory which is an important advantage of this approach. Palecek et al. demonstrated that osmium tetroxide complexes can be used as sensitive and selective probes for single stranded and damaged DNA [37–40]. The "two surface strategy" allows to conduct the steps of Os(VIII)-labelling and probe hybridization on magnetic beads, while the detection can be performed on mercury or carbon electrodes [41–43]. As known from earlier biochemical applications [44], also protective oligonucleotides can be used to label target strands with [OsO_4(bipy)] without affecting their ability for hybridization reactions [45-47]. Fojta et al. reported the use of protective oligonucleotides for the preparation of osmium tetroxide-labelled reporter strands by magnetic separation [48]. Recently, Havran et al. and Bartosik et al. demonstrated the ability of [OsO_4(bipy)] to detect DNA damage as well as unpaired thymine residues [49,50].

In this work, we present one possibility for a dramatic increase in active electrode area by direct template-free galvanic electroplating of gold and silver with a minimum of complexity. The modified electrodes were characterised using cyclic voltammetry in sulphuric acid and

performing hybridization experiments with [OsO_4(bipy)]-labelled DNA target strands on thiol-linked probe SAMs.

2 Experimental

2.1 Materials

2,2'-Bipyridine was delivered by Merck and osmium tetroxide as a 2 % aqueous solution by Fluka. TRIS buffer (10 mM tris(hydroxymethyl)-aminomethane, 0.5 M Na_2SO_4 both from Fluka) was adjusted to pH 7.5 using sulphuric acid. Single stranded 20-mer DNA (ssDNA) was purchased from FRIZ Biochem GmbH (Munich, Germany). Ultrapure water (>18 MΩ cm, TOC < 2 ppb) was prepared with an Ultra Clear TOC system (SG Water Systems, Germany) comprising ultra filtration and UV oxidizing modules. All chemicals were of analytical grade. Buffer solutions, beakers and pipette tips were autoclaved prior to use. Chemicals and solutions used for silver and gold plating were of technical grade [51].

2.2 Galvanic deposition method

The deposition of gold nano-structures was performed with a potentiostat / galvanostat PGSTAT 12 from Metrohm (Switzerland), silver plating with a HP 6177c DC (Hewlett Packard, Germany) current source. The PGSTAT 12 was used in its galvanostatic mode together with a platinum anode in a two-electrode setup. Electroplating of both silver and gold was performed using cyanide-based plating solution with a magnetic stirrer at 100 rpm at room temperature. Current densities in the range of 15 - 30 mA/cm² in a strong alkaline solution were applied for the silver plating process. Gold plating was performed with 1 – 5 mA/cm² in neutral pH range [51]. The active surface and consequently the real current density depends basically on the surface profile and changes after 3D-nano-structure plating. The electroplating was performed directly on polished gold disc electrodes with 3 mm diameter,

preplated with a 2 µm grain refined gold layer from a commercially available soft gold bath (Puramet; Doduco company).

2.3 Electrode surface evaluation

The surface profiles of all galvanic layers were documented in a FE-SEM (Zeiss CrossBeam 1540 XB) in the secondary electron mode and the inlens mode at magnifications 1000x and 5000x with a view angle of 45 degree. The electrical contact was provided by the backside connector. An aluminium foil was used to protect the electrode body.

2.4 Gold surface determination experiments

The accessible active gold surface was determined with a PalmSens potentiostat (Palm Instruments BV, The Netherlands) by cyclovoltammetry (CV) in 0.5 M sulphuric acid [13,47,52] . The 20 ml electrochemical cell comprised an Ag/AgCl (3 M KCl) reference and a platinum counter electrode (Metrohm). The measurements were carried out at room temperature (22 °C) and analysed using the PalmSens software.

2.5 Electrochemical detection of [OsO_4(bipy)]-labelled oligonucleotides

A 27-mer thiol-linked DNA-oligonucleotide with a 15 thymine bases spacer between linker and detection sequence (5´-AAGTCCTTGAGCTGATTTTACTGTATTTTTTTTTTTTTTTT[Dithio]3-3´) was immobilized on the freshly plated electrodes overnight. After a mercaptohexanol treatment (1 h in 100 mM aqueous solution), the voltammetric baselines were recorded. A 5 µl droplet of [OsO_4(bipy)]-labelled target solution of a given concentration was placed at the electrode surface to allow for hybridization in a water saturated atmosphere for one hour. The modification and hybridization procedure has been described earlier in full detail [46,47].

Dehybridization was performed by incubation in deionised water at 50 °C for 60 s. The sequence MON 810 (5´-TTTTTGTTGTCCATGGCCGCTTGGTATCT-3´) was used as a noncomplementary target. This strand was labelled with [OsO_4(bipy)] after hybridization with the protective strand (5´-AGATAGCAAGCGGCCATGCAGAAGAA-3´). All square-wave voltammetric (SWV) experiments were performed with a PGSTAT 12 (Metrohm, Switzerland). The 20 ml electrochemical cell comprised an Ag/AgCl (3 M KCl) reference (Sensortechnik Meinsberg GmbH, Germany) and a self-made platinum counter electrode. All voltammetric experiments were performed at room temperature (22 °C). All peak values were determined using a GPES 4.9 software and graphically plotted with Origin 8 software.

3 Results and Discussion

Polished gold electrodes used in our experiments did not show a completely flat surface at the micrometer scale (data not shown). The main reason for this could be the ductility and the low hardness (less than 100 HV) of gold. Since silver disk electrodes As shown below, the active surface of the used gold electrodes is dramatically enlarged by template-free gold electroplating. This effect can be further enhanced by consecutive Ag/Au plating. Figure 1, I-A and B show FE-SEM images of fine-gold nano-structure crystals plated on smooth gold surfaces. In contrast, images II-A and B show structures obtained by consecutive silver/gold electroplating. Fine, regular structures, with a shape reminding on shark teeth, were obtained by plating gold galvanically onto the gold disk electrode. We designate these electrodes as "ST-AuE" in the following sections. Consecutive silver/gold electroplating leads to relatively large microstructures of silver covered by crystallized gold nano-particles. The resulting structures bear a striking resemblance to cabbage and were designated "CAB-AgAuE" in the following.

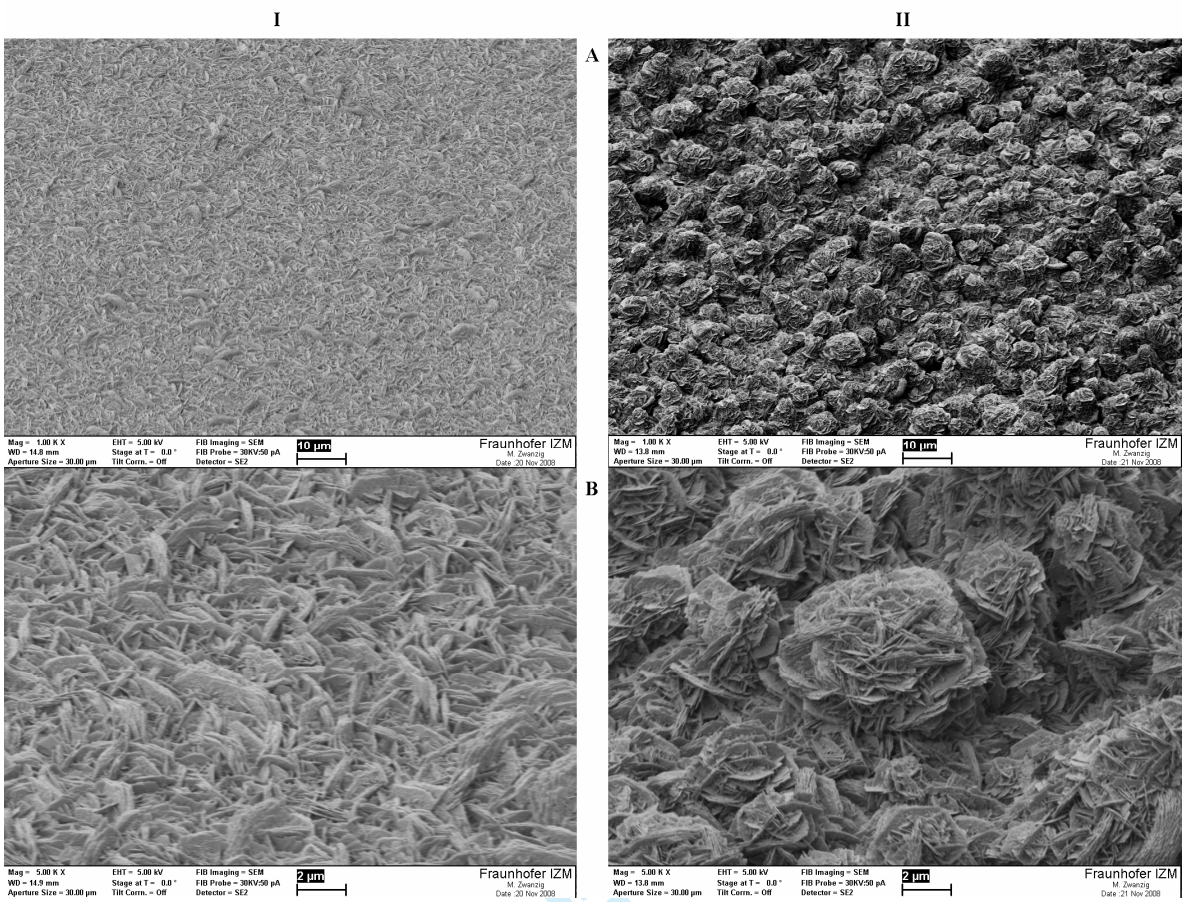

Fig. 1. FE-SEM micrographs of I) a ST-AuE and II) CAB-AgAuE in (A) thousand fold and (B) five thousand fold magnification; The shown nano-crystals of gold with their definite orientation cause a dramatic enhancement of the available active gold surface. A completely different base-structure can be achieved if the electrode is first plated with silver followed by galvanic gold nano-structuring (II-A; II-B).

Figure 2 displays cyclovoltammetric measurements in 0.5 M sulphuric acid using either an ST-AuE (A) or a CAB-AgAuE (B). As can be seen, the integrated gold oxide reduction peak at 0.88 V, and hence, the active area of the electrode, increases dramatically. The average increases are approx. 7-fold for the ST-AuE and 15-fold for the CAB-AgAuE with respect to the "normal" gold disk electrode,

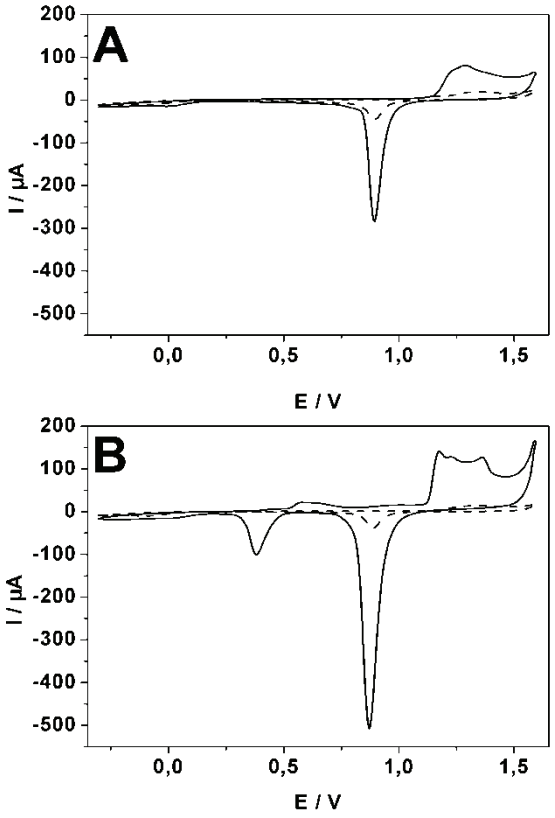

Fig. 2. Cyclic voltammograms in 0.5 M H_2SO_4; step potential 5 mV, scan rate 0.1 V s^{-1}; dashed line – before modification, full line – with (A) ST-AuE and (B) CAB-AgAuE.

A small reduction peak can be observed at 0.38 V in Fig. 2B which is related to silver. The silver oxidation (presumably at approx. 1.17 V) can be hardly distinguished from the gold oxidation signals. These findings indicate pinholes in the gold layer covering the silver structures. Also areas with uncovered silver crystals may exist between the cabbage-like structures as depicted in figure 1 (II-A and B). However, this will not be a problem for electrochemical scans in the negative potential range as demonstrated below. Szamocki et al. achieved an about 30 times greater surface by depositing 7½ layers of hollow spheres of macroporous gold within about 25 minutes [13]. Considering the shorter deposition time of 15 minutes and the lower complexity of the ingredients, our simple electroplating method is a good compromise of cost and usability. Further optimization of the electroplating parameters current density and deposition time may provide even better results in the enlargement of the electrochemically active surface.

For an independent confirmation of the tremendous increase in the active electrode area, DNA-hybridization experiments with surface-confined capture probes were performed.

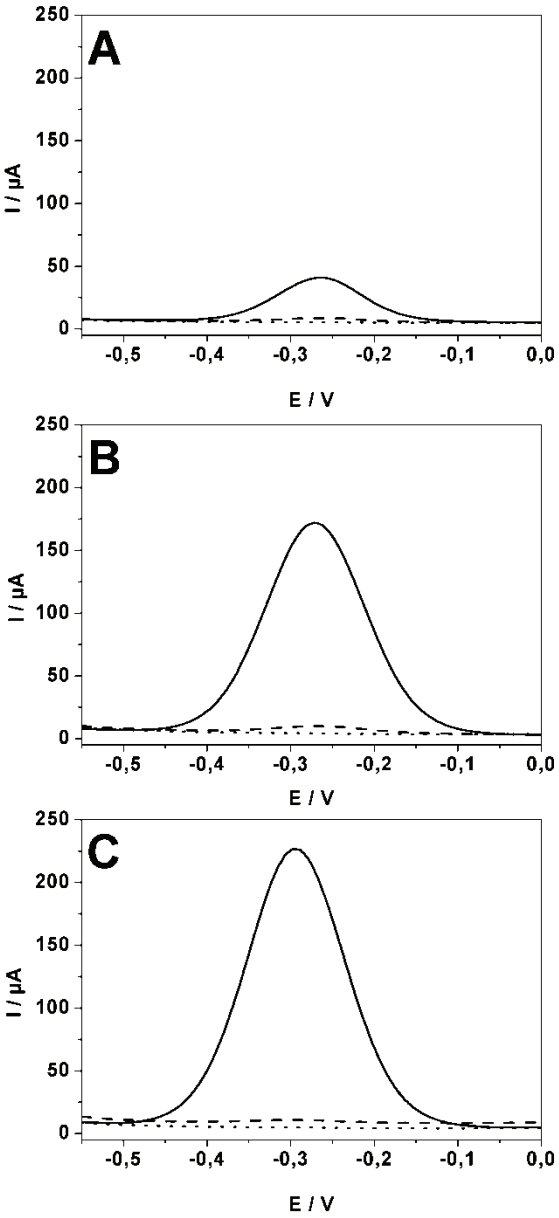

Fig. 3. Square-wave voltammograms of DNA-hybridization events; step potential 1.95 mV, amplitude 40 mV, frequency 200 Hz; dotted – baseline, solid – hybridization, dashed – dehybridization; hybridization with 625 nM [OsO_4(bipy)]-labelled target for 1 h; A) plain gold disk electrode, B) ST-AuE, C) CAB-AgAuE.

Figure 3 shows square-wave voltammetric signals of the osmium labelled target strands. An electrochemical peak current of 40 µA was obtained with the plain gold disk electrode only

modified with the thiol-linked capture probes (Fig. 3A). Dehybridizing in 50 °C warm water gave a signal of about 5 µA. This indicates good regeneration capability and corresponds with former observations reporting a remaining signal height of about 10% on this type of probe-modified gold electrodes after dehybridization [47].

In contrast, an ST-AuE yields an approx. 4-fold enhanced hybridization signal (Fig. 3B). Moreover, the thermal dehybridization step is strongly improved as indicated by a remaining 6% SWV signal. These effects are even more pronounced for the CAB-AgAuE. These nanostructures result in a 9-fold increase (Fig. 3C) compared with the plain probe-modified gold disk electrode. The hybridization/dehybridization signal ratio further decreased below 5%. This can possibly be explained by an improved contact of the hot water with the upper layers of the nanostructured electrodes. The nanostructures are almost completely surrounded by hot water diminishing the cooling effect of the gold disk beneeth.

The greatly improved hybridization signals can be explained by an enlarged active electrode surface able to carry more capture probes. The enlarged surface also results in a larger linear calibration range as illustrated in Figure 4.

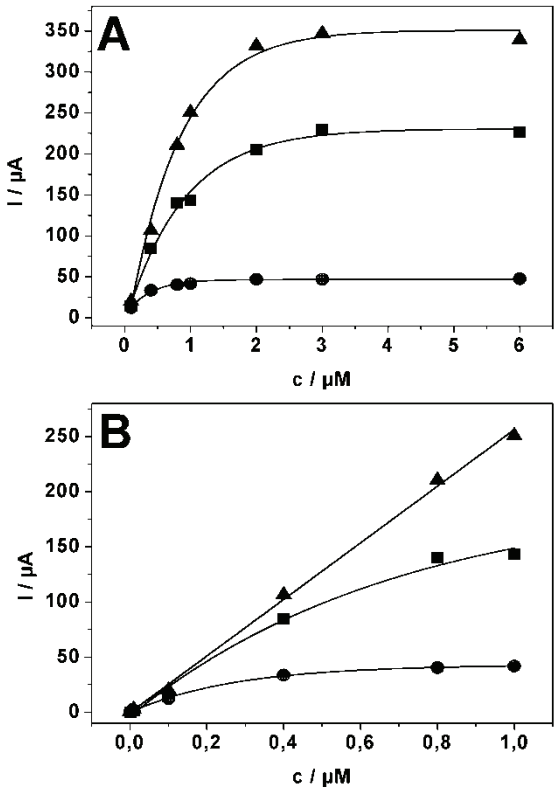

Fig. 4. Concentration calibration of [OsO_4(bipy)]-labelled ssDNA on plain Au disk (circles), ST-AuE (squares), and CAB-AgAuE (triangle) for concentration ranges of A) 0.1 to 6 µM and B) 0.001 to 1 µM.

The target concentration was varied between 0.1 and 6 µM (Fig. 4A). For the CAB-AgAuE (triangle), a linear relation could be observed up to a target concentration of 1 µM. The ST-AuE (squares) and plain Au disk electrode (circles) were already saturated at target concentrations of 2 and 1 µM, respectively. This is a further indication for a tremendous surface increase achieved by the consecutive silver/gold plating method. Even at lower concentrations between 0.001 and 1.0 µM (Fig. 4B), the CAB-AgAuE shows an almost linear behaviour, whereas the calibration range of the plain Au disk is limited to approx. 0.2 µM target concentrations.

The detection limit improved from 83 nM for the plain gold disk electrode and 89 nM for the ST-AuE to 64 nM for the CAB-AgAuE (graphically determined following DIN 32645; see figure 5).

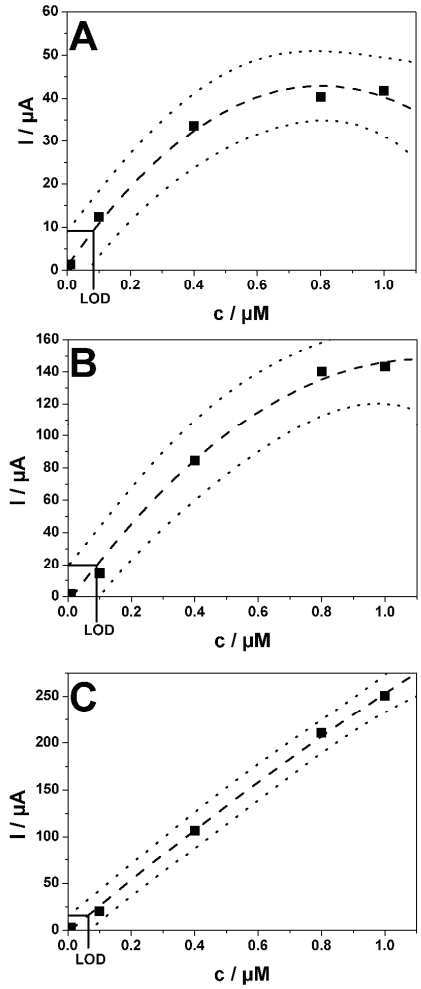

Fig. 5. Determination of the limit of detection (LOD) following DIN 32645 for the bare gold electrode (A), St-AuE (B) and CAB-AgAuE (C).

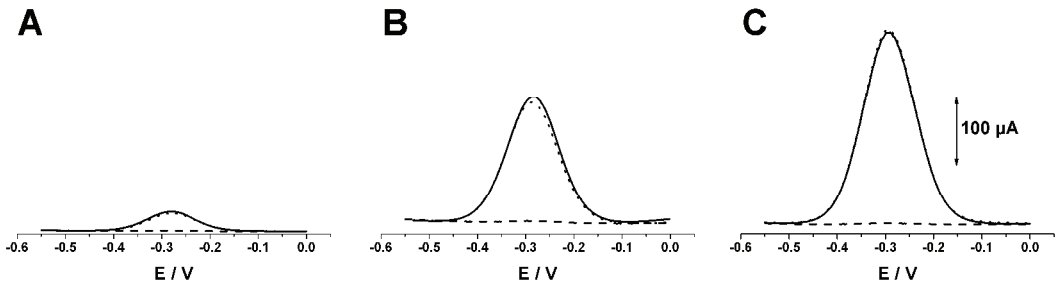

Fig. 6. Selectivity of 30 min-hybridizations of three differently structured electrodes in 1 µmol/l target solutions. Measured signals were recorded for the hybridization with a complementary $[OsO_4(bipy)]$-labelled target (solid), followed by the hybridization with a non-complementary target MON 810 (dashed), and another hybridization with the complementary target (dotted) on a smooth Au disk electrode (A), ST-AuE (B), and CAB-AgAuE (C), respectively.

Hybridization experiments were performed with labelled complementary and non-complementary targets to verify the selectivity of the nanostructured probe-modified electrodes. As a first step, the immobilized capture probe was hybridized for 30 min with a 1 µM complementary target (solid line). After a dehybridization step, the non-complementary target MON 810 (1 µM) was allowed to hybridize for 30 min with the capture probe (dashed line). Almost no nonspecific response was observed. To assure that the immobilized capture probe was still able to selectively detect the complementary single strand, we allowed the dehybridized electrode to again hybridize with the 1 µM complementary target solution for 30 min. The experiments verified the high selectivity of the immobilized capture probe. Two dehybridization steps and hence, thermal treatment did not result in a decrease of the sensitivity. The surface modification resulting in the increase of the voltammetric signals did obviously not affect the selectivity. Even the more complex CAB-AgAuE did not suffer from unspecific binding.

4 Conclusions

We conducted template-free galvanic formation experiments of nanostructures on gold disk electrodes. A high increase in the electrochemically active surface was observed as verified by cyclic voltammetry of gold oxide reduction. The highest increase was achieved by consecutive plating of silver and gold. The active electrode area increased up to 15-fold compared with the plain Au disk.

Experiments with $[OsO_4(bipy)]$–labelled ssDNA targets confirmed the great increase in the accessible gold surface. A higher coverage with capture probe strands (with respect to the geometric area), and hence, higher sensitivity of electrochemical DNA hybridization could be achieved. The LOD obtained with the silver/gold modified disk electrode is significantly enhanced. Selectivity and reproducibility are very good for all three types of working electrodes.

Our template-free formation of nanostructured electrode surfaces holds great promise for future electrochemical biosensor applications and in particular for the parallel detection of multiple analytes on microelectrode arrays.

Acknowledgement

The authors are grateful to the Deutsche Forschungsgemeinschaft (grant # FL 384/4-2, 7-1, 8-1) and to the State of Mecklenburg-Vorpommern (Excellence Funding Programme) for financial support.

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Publication VIII

Maren Mix, Thomas Reske, Heiko Duwensee, Gerd-Uwe Flechsig

Electrochemical detection of asymmetric PCR products by labeling with osmium tetroxide

Electroanalysis 21 (2008) 826

Full Paper

Electrochemical Detection of Asymmetric PCR Products by Labeling with Osmium Tetroxide

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Received: October 13, 2008
Accepted: December 25, 2008

Abstract
Single stranded DNA-targets from asymmetric polymerase chain reaction (PCR) of a sequence of the gram positive, spore forming bacterium *Clostridium acetobutylicum* were detected by square-wave voltammetry after labeling with osmium tetroxide bipyridine and hybridization with DNA capture probes immobilized on gold electrodes. The asymmetric PCR, performed with a 10-fold excess of the forward-primer, was used without any further purification for hybridization with protective strands and covalent labeling with osmium tetroxide bipyridine. Square-wave voltammetric signals of 20 nmol/L targets were significantly higher at 50 °C compared with 23 °C hybridization temperature. A fully noncomplementary protective strand yielded thoroughly modified targets unable for further hybridization. Coupling this with thermal discrimination opens new opportunities for sequence specific DNA detection.

Keywords: Asymmetric polymerase chain reaction, PCR, Square-wave voltammetry (SWV), Osmium tetroxide bipyridine, Covalent DNA label, Electrochemical hybridization detection

DOI: 10.1002/elan.200804493

1. Introduction

In the recent decade one could observe a dramatically increased publishing activity in the area of electrochemical DNA detection. It was shown that the detection of hybridization of two complementary DNA single strands (probe and target) could not only be achieved by classic molecular biological methods such as Northern or Southern Blots or optical microarrays but also by nucleic acid-modified electrochemical biosensors. The electrochemical transduction can be performed by molecules that are either covalently attached to the target, interacting with the negatively charged phosphate groups or intercalating into the base stack. It is also possible to make use of the intrinsic electrochemical activity of the DNA, e.g., the oxidation of guanine [1].

Most common for the immobilization of probe strands are magnetic beads and self-assembled monolayers [2]. We recently reported about the application of covalently attached redox markers together with thiol-linked probe oligonucleotides on heated gold electrodes [3].

In other publications the electrochemical detection of PCR-products was performed with methylene blue [4, 5] or sandwich hybridization with an alkaline phosphatase conjugate on biotinylated signaling probes [6]. One of the problems of hybridization detection basing on intercalators is that only the (often small) difference of the electrochemical response before and after target binding has to be taken as the hybridization signal. The methods of electrochemical hybridization detection have been reviewed many times [7 – 15].

The complex $[OsO_4(bipy)]$ can be utilized as an electrochemically reversible covalent DNA-label. It reacts rapidly with the pyrimidine base thymine and less quickly with cytosine [16] forming a diester of osmic(VI) acid in well-known manner under oxidation of the C-C-double bond in the pyrimidine ring. These reactions have been investigated for about 25 years by Palecek et al. [17 – 20]. One of the main advantages of this approach is that the labeling of DNA-strands of any length can be easily performed in the laboratory. However, only pyrimidine bases of single strands can be labeled this way. Such Os-modified DNA strands on the other hand are unable to form double strands, i.e. hybridization with probe oligos is no longer possible. Until now, the steps of Os(VIII)-labeling, probe hybridization, and electrochemical detection have been conducted in solution and at separate surfaces, respectively, especially by means of magnetic beads [21 – 25]. Since only ss-DNA can be labeled by means of $[OsO_4(bipy)]$, protective partially complementary oligonucleotides (which can be replaced later by the fully complementary capture probe) have to be used for the labeling process. Protective oligonucleotides are known from biochemical applications, where, e.g., DNA strands have to be protected against cleavage by nucleases [26]. Recently, we reported about how protective oligonucleotide strands can be utilized for labeling thymine-containing nucleic acids by means of $[OsO_4(bipy)]$ without loosing their ability for

Table 1. DNA sequences, the mismatches in the protective strand are highlighted.

Designation	Length	Sequence
Fragment	136	5′-CTTACAGGAATTGTTTTGGGACTTGCAAGGGCTT-TTGGAGAGGCATTAGCAGTT-CAAATGGTAATAGGTA-ATACAGTAAAATCAGCTCAAGGACTTTTTTCTCCTAC-TAC-CACTTTGACAAGCGTACTTACAATGG −3′
F-protector	27	5′-AAG**AG**C**A**TGAGCTGATTTTA**G**TG**A**ATT-3′
F-probe	27	5′-AAGTCCTTGAGCTGATTTTACTGTATT-TTTTTTTTTTTTTTT[Dithio]$_3$−3′
P1-protector	32	5′-GCCTCTTTATAGTTATCAGGATCCATTTTTGG-3′

hybridization reactions with immobilized oligonucleotide capture probes [27 – 29].

The electrochemical detection of DNA-sequences amplified by PCR (polymerase chain reaction) has been described in a couple of publications in the last few years. In most cases the template is amplified by symmetric PCR. Before any electrochemical hybridization measurement with the PCR product can be performed, a denaturation step is necessary to separate the double strand (produced during the PCR) into two single strands. We recently reported on the treatment of symmetric PCR products with Lambda Exonuclease to remove one DNA strand [29].

We think it is favorable if electrochemical signals are only obtained upon positive detection, and especially if a blank sample does not yield any response. This seems to be much more reliable than other procedures described earlier delivering either a decrease of the signal in case of a positive detection [30], or an increase of a signal which is already present without any target DNA [31]. Furthermore, time-consuming cleaning steps are often needed [32, 33]. Cost is another concern to be considered. Expensive, though frequently used, materials include magnetic beads, biotinylated primers, digoxigenin-labeled sequences or antibodies [34 – 36]. A promising detection protocol is symmetric PCR with ferrocene-labeled dUTP as suggested by Hsing et al. [37]. However, the synthesis of the ferrocene-labeled dUTP seems to be time-consuming and complicated. Another possibility is the detection of hybridization based on an enzyme-linked assay [38].

There are only a few reports on the electrochemical detection of asymmetric PCR-products [39, 40]. To our knowledge, these methods suffer from some of the problems mentioned above or do not have desirable detection limits. The main problem here is limited efficiency of asymmetric PCR.

Here, we demonstrate that osmium tetroxide bipyridine can also be applied to detect the products of asymmetric polymerase chain reactions on gold electrodes modified with capture probes. The used model sequence was randomly chosen from a bacterium used in molecular biology as a model organism for non human-pathogenic Clostridia. Usually, PCR amplicons are obtained in form of double strands. By raising the concentration of one primer, the (now asymmetric) PCR yields ssDNA besides dsDNA. This mixed product was used in the experiments without further purification.

2. Experimental

2,2′-Bipyridine was delivered by Merck and osmium tetroxide as a 2% aqueous solution was obtained from Fluka. All DNA oligo nucleotides (*probes, targets and protector strands*) were delivered by Friz Biochem GmbH (Munich, Germany). The "Tris-buffer" (containing 10 mmol/L tris-(hydroxymethyl)-aminomethane (Fluka) and 0.5 mol/L sodium sulfate (Fluka)) was adjusted to pH 7.5 using sulfuric acid. The latter was used instead of HCl to avoid chloride, which is suspected to form various gold chlorides on the working electrode's surface disturbing formation/durability of SAMs. Table 1 lists the sequences of all DNA-strands together with their length and designations used in this paper. The *F-protector* oligonucleotides were designed to have 5 mismatches (underlined in Table 1) to facilitate their later displacement by the immobilized *F-probe*. Highly purified water (18 MΩ cm, TOC < 2 ppb) prepared by means of a system form SG Water (Barsbüttel, Germany) was used throughout.

2.1. Preparation of SAM-Modified Gold Electrodes

First, the gold disk electrode (Metrohm, Switzerland) was polished using corundum 0.3 μm (Buehler) and rinsed thoroughly with water. It was then electrochemically treated by means of 25 voltammetric cycles between −0.2 und +1.65 V at 100 mV/s in 0.5 M sulfuric acid. The electrode was rinsed with water before one droplet of 15 μL *F-probe* solution (30.3 μmol/L) was placed onto the electrode surface in order to form the capture probe SAM. After 16 h at 10 °C in a water-saturated atmosphere, the electrode was rinsed and immersed in 1 mmol/L aqueous mercaptohexanol solution for one hour.

2.2. Hybridization and Dehybridization Procedure

For the hybridization step, the probe-SAM-modified gold disk electrode was dipped into a beaker containing the target solution. The solution was stirred constantly using a magnetic stirrer. Heating of the sample solution was conducted by means of a water bath. After the hybridization, the electrode was rinsed with Tris-buffer.

Following the electrochemical measurement, the electrode was regenerated by a dehybridization step. To retrieve the probe-SAM back, the electrode was immersed in 50 °C deionized water for 60 s.

2.3. Electrochemical Measurements

For all electrochemical procedures, an Autolab PSTAT10 (Ecochemie, Utrecht, The Netherlands) controlled by a PC with GPES 4.8 software and an electrode stand inside a Faraday cage was used together with a Ag/AgCl (3 mol/L KCl) reference electrode and a glassy carbon counter electrode (all from Metrohm AG, Herisau, Switzerland). The parameters for square-wave voltammetry were 200 Hz and 40 mV amplitude. The scan was performed from −0.55 to 0.0 V. All measurements were performed at room temperature in Tris-buffer. The peak-shaped signals were smoothed by means of a Savitzky and Golay filter (level 3) and baseline-corrected (moving average, peak width 0.03).

2.4. PCR Conditions

A synthetic 136-base-DNA strand (sequence from the genome of *Clostridium acetobutylicum* [1855609–1855744]) was used as the template. The asymmetric PCR was performed with 100 ng of template DNA, 0.4 μM dNTPs, 1 μM of the primer C.aceto F and 0.1 μM C.aceto R (Table 2), and 2.5 U Sawady-Taq-DNA-Polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany) in 50 μL reaction volume according to the manufacturer's instructions. In case of the symmetric PCR an equal amount of the primers was used (0.5 μM). 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 gelelectrophoresis 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.

Table 2. Primers.

Sequence: 5′-3′		
C.aceto F	CTTACAGGAATTGTTTTGGGA	forward primer
C.aceto R	CCATTGTAAGTACGCTTGTCA	reverse primer

2.5. Modification of Fragment Oligonucleotide (Target)

For the modification, 200 μL of PCR-solution (containing the single target strand *Fragment*) were used without further purification. The *Fragment* at its maximum concentration (ca. 20 nmol/L) and the protective strand *F-Protector* (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-A-Lyzer MINI Dialysis Units, 3500 MWCO (Rockford, IL, USA) at 4 °C for 19 hours. After the dialysis, the purified solution (containing the osmium-labeled target *Fragment*) was used for the hybridization experiments.

3. Results and Discussion

Figure 1 displays gel electrophoretic analysis of symmetric (lanes 2 and 3) and asymmetric (lanes 4 and 5) PCR products. While classic symmetric PCR provides an optimal yield of the double stranded amplicon, asymmetric PCR is usually prone to limited efficiency. However, this variant also yields ssDNA, which is visible on the gel above the dsDNA. Excess primers can be found right below the dsDNA bands. The DNA ladder can be applied only for dsDNA, since ssDNA (primers and products) shows a different running behavior in the gel. The single strands can be used directly for further modification or hybridization steps. Here, we used the asymmetric PCR products for labeling with osmium tetroxide bipyridine. As described earlier [27], the single stranded target DNA is first hybridized with a protective strand in order to preserve an unmodified recognition site for later capturing by immobilized probes. The latter event has been detected by square-wave voltammetry as depicted in Figure 2. While a blank capture probe layer does not show any voltammetric response (Fig. 2a), we found a large reversible peak at −0.29 V (vs. Ag/AgCl | 3 mol/L KCl) for the hybridization of the immobilized capture probe with the $[OsO_4(bipy)]$-modified asymmetric PCR product (Fig. 2b, c). This peak is caused by the reversible redox couple osmium (IV/VI) according to earlier findings of Lukasova et al. [18], as well as results of Crowell et al. and Meites [41, 42]. The reduction of Os(VIII) happens at 0 V and the reduction of Os(IV) to Os(III) at ca. −1.1 V. We found that the voltammetric response can be doubled if hybridization solution temperature is increased from 23 to 50 °C. In contrast, we observed a much smaller peak upon hybridization of the same PCR product after protection with the noncomplementary *P1-protector* (Fig. 2d, e). In this case, a stringent hybridization temperature (50 °C) leads to a diminished contribution of the thoroughly labeled and, hence, nonspecific DNA. The latter is discriminated only during hybridization with the protective strand. By using a noncomplementary protective

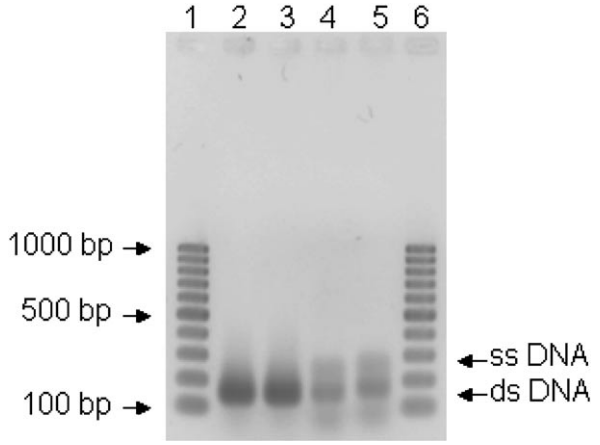

Fig. 1. 2% [w/v] agarose gel stained with Gelred (Cambridge BioScience, UK); lane 1 + 6: Gene Ruler 100 bp DNA Ladder (0.3 μg), lane 2 + 3: 2 μL symmetric PCR-product (136 bp-fragment), lane 4 + 5: 5 μL asymmetric PCR-product (136 bp-fragment in lower band and 136 b-fragment in higher band)

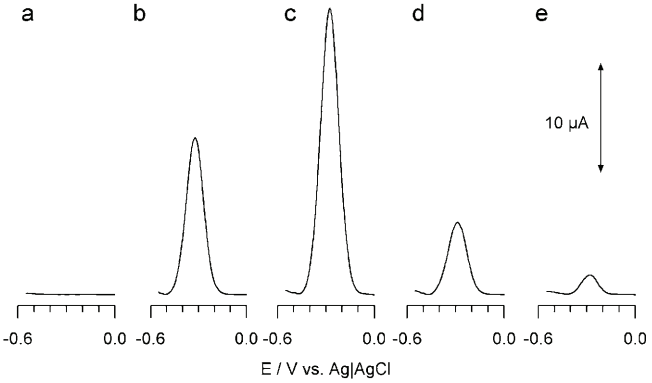

Fig. 2. Square-wave voltammetric signals at gold disk electrodes in Tris-buffer with immobilized probe strands, a) *F-probe* not hybridized at 23 °C, b) *F-probe* 15 min hybridized with 20 nmol/L [OsO_4(bipy)]-labeled *Fragment* at 23 °C, c) *F-probe* 15 min hybridized with 20 nmol/L [OsO_4(bipy)]-labeled *Fragment* at 50 °C, d) *F-probe* 15 min hybridized with 20 nmol/L [OsO_4(bipy)]-labeled *Fragment* at 23 °C, modification of the Fragment with noncomplementary protecting strand *P1-protector* and (e) *F-probe* 15 min hybridized with 20 nmol/L [OsO_4,bipy]-labeled *Fragment* at 50 °C, modification of the Fragment with noncomplementary protecting strand *P1-protector*; frequency 200 Hz; amplitude 40 mV; stirring during the hybridization step at 1000 rpm.

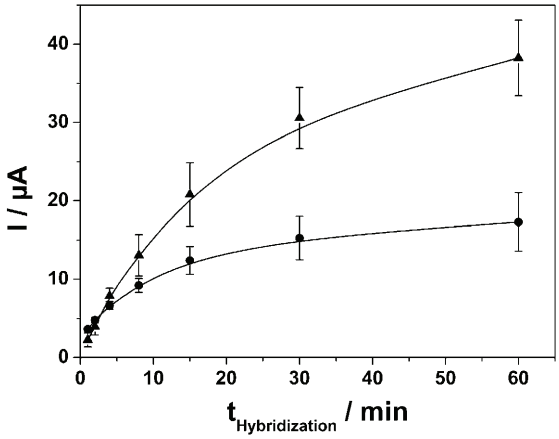

Fig. 3. Effect of the hybridization time of immobilized *F-Probe* with 20 nmol/L target (*Fragment*) at a) 50 and b) 23 °C hybridization temperature. The error bars indicate the standard deviation of 3 independent hybridization-dehybridization series, each performed with a newly prepared probe-SAM. Other conditions were the same as in Figure 2.

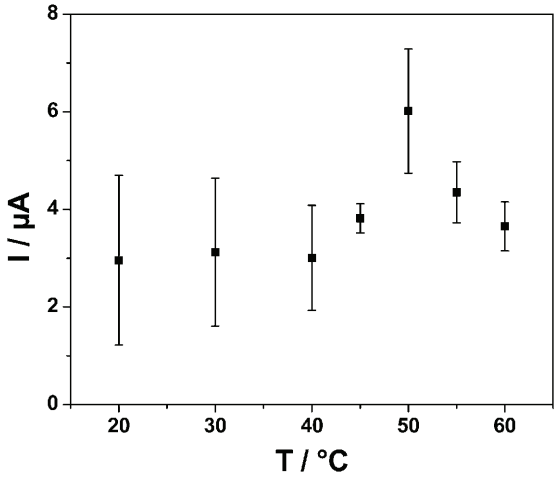

Fig. 4. Dependence of the square-wave voltammetric signals on the hybridization temperature, *F-Probe*-SAM 5 min hybridized with 20 nmol/L target (*Fragment*). The error bars indicate the standard deviation of 3 independent PCR runs and hybridization-dehybridization series, each performed with a newly prepared probe-SAM. Other conditions were the same as in Figure 2.

strand, the protection widely fails and causes labeling of the entire target strands (i.e. pyrimidine bases therein) leaving no intact recognition sites.

Figure 3 displays the effect of hybridization time upon the square-wave voltammetric response of the [OsO_4(bipy)]-label at 23 and 50 °C. Whereas a beginning saturation of the capture probe layer can be observed after 30 min. at 23 °C, the hybridization process does not appear finished at 50 °C, even after 60 min. Capture probe availability and hence, hybridization efficiency seems significantly better at elevated hybridization temperature due to dissolving of secondary structures of the capture probe and an accelerated reaction between capture probe and target. Obviously, the saturation level is at least twice as high comparing 50 and 23 °C hybridization experiments. The effect is similar to what we have found earlier when we treated symmetric PCR products with Lambda Exonuclease to remove one DNA strand [29].

Figure 4 exhibits the effect of hybridization temperature upon the voltammetric signals of the [OsO_4(bipy)]-label following a 5 min. hybridization period. Even after this short hybridization time, good signal height of several μA is achieved. The observed temperature effect is comparable to

longer hybridization periods (Fig. 3). Above 50 °C, a signal decrease is observed, presumably due to double strand melting (the melting temperature is 55 °C according to oligonucleotide calculation programs). This indicates that 50 °C is indeed the optimal stringent hybridization temperature. Although the absolute signal level of different PCR runs and different hybridization – dehybridization series is subject to fluctuations as indicated by the error bars in Figure 4, the average signals as well as the trend in each series are well reproducible. The reason for these fluctuations can be found in variations of the surface coverage with probe strands. This can be compensated by calibration. Furthermore, the simple symmetric and asymmetric PCR protocols are known as qualitative amplification methods. They are not well suited for quantification. Future development will lead to quantitative real-time PCR with electrochemical detection.

4. Conclusions

Asymmetric PCR products contain single stranded target strands, which can easily be modified using covalent [OsO_4(bipy)]-labels. Protective strands preserve a recognition site of the target for later hybridization with immobilized capture probes on gold electrodes. The redox couple osmium (IV/VI) of the [OsO_4(bipy)]-labels yields large reversible square-wave voltammetric signals. Discrimination of noncomplementary DNA is possible even by hybridization with the protective strand. This opens a route towards double discrimination by consecutive stringent hybridization with both protective and capture probes allowing selective determination of DNA sequences even in presence of a large excess of fully noncomplementary or mismatched DNA.

5. Acknowledgements

The authors are grateful to the Deutsche Forschungsgemeinschaft (DFG projects FL 384/4-2, 7-1, and 8-1) and to the State of Mecklenburg-Vorpommern (Excellence Funding Program) for financial support.

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Publication IX

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Electrochemical competitive hybridization assay for DNA detection using osmium tetroxide-labelled signalling strands

Analyst 134 (2009) 899

Electrochemical competitive hybridization assay for DNA detection using osmium tetroxide-labelled signalling strands

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Received 30th October 2008, Accepted 17th February 2009
First published as an Advance Article on the web 4th March 2009
DOI: 10.1039/b819283a

In this report, we present sequence-specific DNA detection by means of a competitive hybridization assay with osmium tetroxide-labelled signalling strands. The labelling of the signalling strands has been performed using protective strands to preserve the recognition site of these single strands for hybridization with the immobilized capture probes. At optimized measuring conditions and especially assay temperature, we could detect the presence of 25 nM target DNA within 30 minutes, whereas the non-complementary target sequence did not yield any signal. The latter was observed as a decrease in square-wave voltammetric response of the signalling probes. Single base mismatches could be detected at a stringent 35 °C electrolyte temperature. Moreover, the concentration dependency of the signal was investigated. A time-consuming labelling procedure of the target, as typically used before, is not necessary. Upon application of the new protocol, there is no need for handling osmium(VIII) compounds during sample treatment. The signalling strands containing Os(VI) are prepared separately and can be stored over several months.

Introduction

Nucleic acids can be detected electrochemically in several ways. One possibility is to modify target nucleic acids with covalent labels such as osmium tetroxide complexes and ferrocene that deliver large reversible electrochemical signals. Palecek *et al.* demonstrated that osmium tetroxide complexes with organic ligands such as bipyridine could be utilized as versatile tools for studying nucleic acid electrochemistry.[1–4] The complex [OsO_4(bipy)] oxidizes the C=C double bond in the pyrimidine bases thymine, uracil and cytosine forming a glycol diester of osmic(VI) acid.[5,6] This reaction can be utilized for fast and easy labelling of DNA strands in the laboratory. Osmium tetroxide bipyridine-modified DNA single strands are unable to form double strands. Therefore, sequence-specific DNA detection by means of osmium tetroxide labels has been conducted in two steps at separate surfaces, including (a) hybridization at magnetic beads and (b) adsorptive stripping on mercury, carbon or amalgam electrodes. This approach is called a 'two surface strategy'.[7–11]

Another possibility is to use protective oligonucleotide strands for labelling thymine-containing nucleic acids with [OsO_4(bipy)] while preserving their ability for hybridization with immobilized probes.[12,13] Protective oligonucleotides coupled with magnetic separation have also been used for the preparation of osmium tetroxide-labelled reporter strands.[14] We have recently demonstrated that [OsO_4(bipy)] and protective strands can also be applied to detect the products of polymerase chain reactions (PCR).[15,16] Furthermore, we demonstrated that tRNA can be labelled with osmium tetroxide complexes.[17]

It is generally desirable to avoid additional steps during sample preparation. Signalling probes covalently modified with electroactive moieties have been introduced to avoid modification of the target strands. Such competitive assays are widely used in immunoassays with antibodies or aptamers. This approach also works with nucleic acids. The unlabelled target strand, which is complementary to the probe strand, will displace the electroactive signalling strand (Fig. 1), and hence, a decrease of voltammetric response is detected and taken as the analytical signal.[18–23] Oligonucleotides with electroactive labels[24] or enzyme labels producing electroactive substances[25] can also be used as reporter probes in a sandwich-like assay. In this case, two recognition sites at the target strand are needed, one for the capture probe, and the other for the reporter probe. Hence, two hybridization events have to take place in order to observe any electrochemical response. The different approaches of

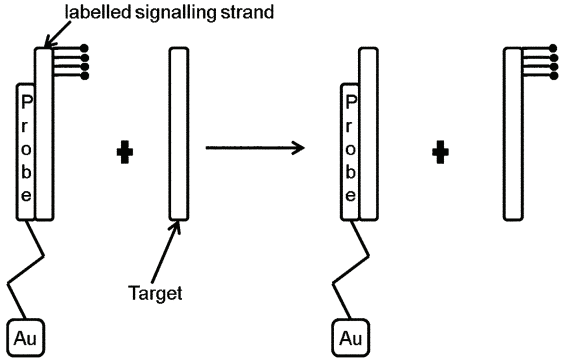

Fig. 1 Scheme of the competitive assay at the surface of a gold electrode. The thiol-linked capture probes form a self-assembled monolayer (SAM). Only the surface-confined signalling strands yield the observed square-wave voltammetric peaks.

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electrochemical sequence-selective DNA detection including commercial solutions have been carefully reviewed by Mascini and co-workers.[26]

Here, we show how osmium tetroxide-labelled signalling probes can be used for the sequence-specific detection of DNA on capture probe-modified gold electrodes by means of a competitive assay without the need to modify the target strands.

Experimental

Reagents and instrumentation

Most of the chemicals were obtained from Fluka (Germany, Taufkirchen). Only sodium sulfate was purchased from Riedel-de-Haën (Germany, Taufkirchen) and ethanol was delivered by AppliChem (Germany, Darmstadt). The used oligonucleotides (HPLC-purified) were obtained from Friz Biochem (Germany, Neuried):

Target/signalling oligo: 5′-TTTTTAATACAGTAAAATCA-GCTCAAGGACTT-3′;

Target with 1 mismatch: 5′-TTTTTAATACAGTAAAAT-CA**C**CTCAAGGACTT-3′;

Capture probe: 5′-AAGTCCTTGAGCTGATTTTACTG-TATTttttttttttttttt[Dithio]$_3$-3′;

Protective strand: 5′-CAGTGTTTGACCTGATTTTACTG-TCTT-3′.

The probe strand contained 15 thymine bases as a spacer to allow high hybridization efficiency on the electrode surface.

All electrochemical measurements were performed in a 3-electrode system using a μ-Autolab (Metrohm AG, Filderstadt, Germany). The used reference electrode was a Ag/AgCl-(3 M KCl) reference electrode (Metrohm) and the counter electrode was self-made using a platinum wire (1 mm in diameter). A modified (see below) gold disc electrode (Metrohm, 3 mm in diameter, 7.07 mm^2 area) has been used as the working electrode. All measurements have been performed in a thermostated jacket electrochemical cell (Metrohm). Temperature was controlled by means of a cryostat type CC2 (Huber, Offenburg, Germany)

Preparation of the signalling oligo. The labelling of a single DNA strand with [OsO_4(bipy)] by means of protective strands has been described in detail earlier.[12,15,27] To label the signalling oligo, we used the following procedure. In the first step, 100 μl unlabelled signalling oligo solution and 100 μl protective strand solution (each 100 μM in 'Tris-buffer' (10 mM tris(hydroxymethyl)aminomethane buffer solution containing 0.5 M Na_2SO_4 at pH 7.5)) were mixed together. After shaking the solution vigorously for 10 s, it was kept at room temperature for 3 hours. Then, 137 μl 10 mM [OsO_4(bipy)] were added to label the five unprotected 5′-terminal thymine bases and left again for 3 hours at room temperature. This way, the recognition site of the signalling strand remained unmodified. To remove the excess of [OsO_4(bipy)], the solution was divided into 4 fractions and each fraction was given into a dialysis cell (Slide-A-Lyser Mini Dialysis Units, USA, Rockford). Dialysis was performed against 500 ml Tris-buffer overnight. In the last step, the purified solution from the dialysis cells was filled up to 1600 μl with Tris-buffer.

Preparation of the electrode. Before each measurement, the electrode was firstly polished carefully for 60 seconds using alumina powder (Buehler, Germany, Düsseldorf) and rinsed thoroughly with deionised water (18 MΩ cm, TOC < 2 ppb, prepared with a system from SG Water, Germany, Barsbuettel). Secondly the electrode was cleaned electrochemically by using cyclic voltammetry (25 scans, −0.3 to 1.6 V, scan rate 100 mV/s) in 0.5 M sulfuric acid. After rinsing the electrode with water and ethanol, the electrode was dried under a stream of argon. In the next step, a 4 μl drop of the probe solution (30 μM in Tris-buffer) was pipetted onto the top of the cleaned electrode. This volume was sufficient to ensure complete coverage. After 3 h at room temperature in a water-saturated atmosphere, the electrode was dipped into a solution of 1 mM 11-mercapto-1-undecanol in ethanol overnight to minimize both non-specific adsorption and double layer capacity. After rinsing the electrode with ethanol and water, the electrode was dipped into deionised 50 °C water for 60 s for removing non-specifically adsorbed DNA. Afterwards, the electrode was dipped into a solution of 1 mM 6-mercapto-1-hexanol in water to completely erect the probe strands, which had partially folded in the alcoholic medium. After 1 hour in the 1 mM 6-mercapto-1-hexanol solution, the electrode was removed, rinsed with water and ethanol, dried under a steam of argon and then placed in a 100 nM signalling oligo solution in Tris-buffer overnight. These working electrodes modified with a dsDNA-SAM of capture probes and labelled signalling strands were used for hybridization experiments with the target strands. A similar protocol has been reported earlier by Hartwich and co-workers.[22]

Measurements. The electrochemical measurements were performed using square-wave voltammetry (SWV) with the following parameters, unless otherwise stated: start potential −0.6 V, end potential 0 V, step potential 15 mV, amplitude 45 mV, frequency 20 Hz, scan rate 290 mV/s. For the blank measurements, the electrodes were used in the blank solution containing the signalling oligo. The competitive hybridization assay was conducted by adding a variable amount of target to the solution containing the signalling oligo and the measurements were started immediately. The signalling oligo had to be present in the sample solutions in order to prevent non-specific signal decrease. All modified electrodes were used only once to investigate the process of the competitive replacement of the signalling oligo by the target. After the voltammetric measurements, the electrode was dipped into 50 °C water for 60 s to dehybridize the signalling oligo/target. Then the electrode was cleaned as described above.

Results and discussion

Fig. 2 displays the square-wave voltammetric response of a gold electrode modified with a SAM of capture and signalling probes before and after target hybridization. No signals are observed on bare gold electrodes and SAMs of mercaptohexanol or capture probes alone. The maximal voltammetric response is obtained after hybridization with an [OsO_4(bipy)]-modified signalling strand (Fig. 2D). The peak at *ca.* −0.28 V can be addressed to osmium(IV/VI) in the complex with bipyridine and thymine glycol. In the presence of the target strand (*i.e.* the analyte), the

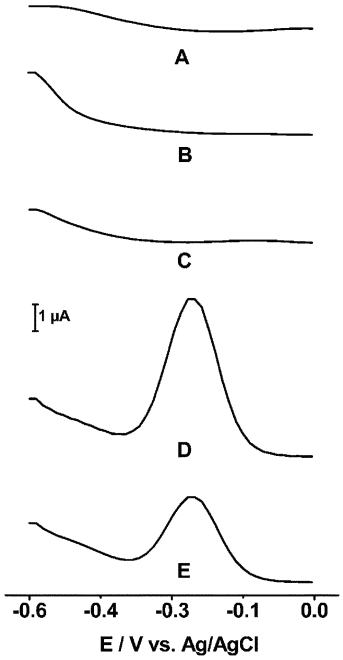

Fig. 2 SWV signals at a (A) bare gold electrode, (B) gold electrode with SAM (6-mercapto-1-hexanol), (C) gold electrode with immobilized probe SAM, (D) gold electrode with SAM of immobilized probe and 6-mercapto-1-hexanol after hybridization with the signalling oligo, (E) gold electrode with immobilized probe and hybridized signalling oligo after 90 min displacement in 100 nM target solution. All measurements were conducted at room temperature in Tris-buffer containing 100 nM signalling oligo.

signalling strand is replaced to a certain degree and hence the response is declining. This way, any modification of the target is not necessary. This permits fast, cheap and easy sequence-specific DNA detection.

Fig. 3 exhibits the effect of assay time and repetitive square-wave voltammetric scans upon the response of the $[OsO_4(bipy)]$-modified signalling strands hybridized with the immobilized capture probes. A decline is observed whenever a potential of −0.5 V is applied between the measurements (Fig. 3A–C). The complex with osmium(IV) does not seem to be stable. We assume that the osmium(IV) complex is prone to hydrolysis, which leads to loss of the covalent label. In contrast, if no potential is applied after each scan, the voltammetric response stabilizes slightly above the initial value (Fig. 3D). This indicates that the osmium(VI) complexes are stable over a long time, which corresponds with the previous experiences. After leaving the probe-modified electrode in the signalling oligo solution overnight, we expected the hybridization process to reach a steady state. Nevertheless you can see a small increase of the signal (Fig. 3D). We suppose that this is due to a small movement of the SAM structure as described before[28] caused by the first electrochemical measurements. Such movement permits more signalling oligos to hybridize with the immobilized probe leading to the small observed increase. DNA modified with the complex $[Os^{VI}O_4(bipy)]$ can be stored for months in the fridge without losing the covalent labels.

Fig. 4 demonstrates the displacement effect at the capture probe-modified gold electrode if 100 nM of the complementary target is added to 100 nM signalling probe solution (Fig. 4D). A 50% equilibrium response can be expected due to equal occupancy of both signalling probe and unmodified target. This state is seemingly approached after a 90 min assay time. In contrast, 100 nM non-complementary strands lead to only 10% decrease of the voltammetric signal after 90 min (Fig. 4B). Even 30 min suffice for a significant discrimination between fully and non-complementary DNA. A strand with a single base mismatch yields a 30% decreased response, which makes it distinguishable from both non-complementary and target strand (Fig. 4C). However, the discrimination between the target and 1-base-mismatch is more affected by the comparably large standard deviation. Only repetitive measurements allow observation of a statistically significant difference. These relatively large standard deviations result from the manual preparation of the probe SAMs. Whereas measurements within a single series are well reproducible, we find a considerable statistical deviation between series measured in repetition with newly prepared probe SAMs. We expect a great improvement of this situation, if the working

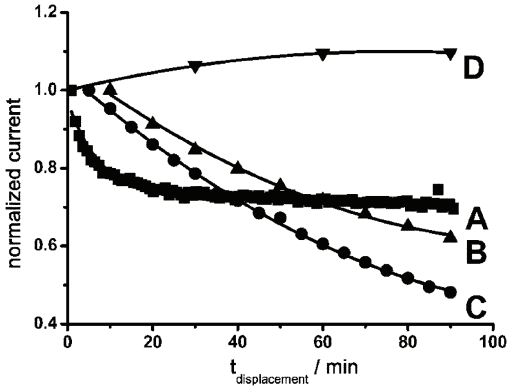

Fig. 3 Normalized SWV response of the probe-modified gold electrode in Tris-buffer containing 100 nM signalling oligo. The measurements were performed at 20 °C in (A) 1 min, (B) 10 min, (C) 5 min and (D) 30 min intervals. Between the measurements, a standby potential of −0.5 V was applied, except for (D).

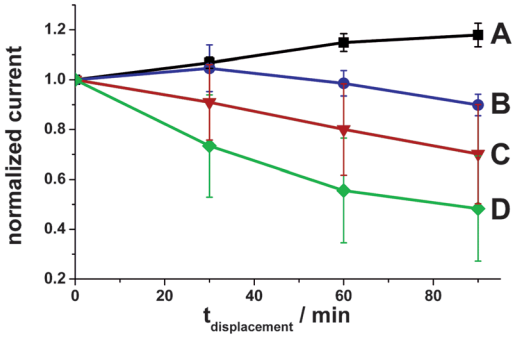

Fig. 4 Effect of displacement time upon the normalized SWV response of blank (A), 100 nM non-complementary target (B), 100 nM 1-base-mismatched target (C), and 100 nM complementary target (D). All measurements have been performed in 10 mM Tris-buffer with 100 nM signalling oligo at 35 °C. The error bars represent the standard deviation of measurements obtained in 3 independent series, each with a freshly prepared probe SAM.

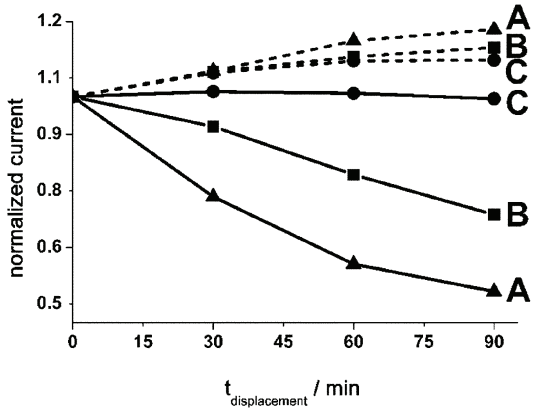

Fig. 5 Effect of displacement time upon the normalized SWV response of blank (dashed lines) and 100 nM target (solid lines) at (A) 35 °C, (B) 20 °C and (C) 5 °C. All measurements were performed in Tris-buffer containing 100 nM signalling oligo.

electrodes are automatically cleaned and modified (*e.g.* spotter device, electrode array).

These results show that unmodified target DNA sequences can be detected specifically on gold electrodes by means of immobilized capture probes under the displacement of [OsO_4(bipy)]-modified signalling probes.

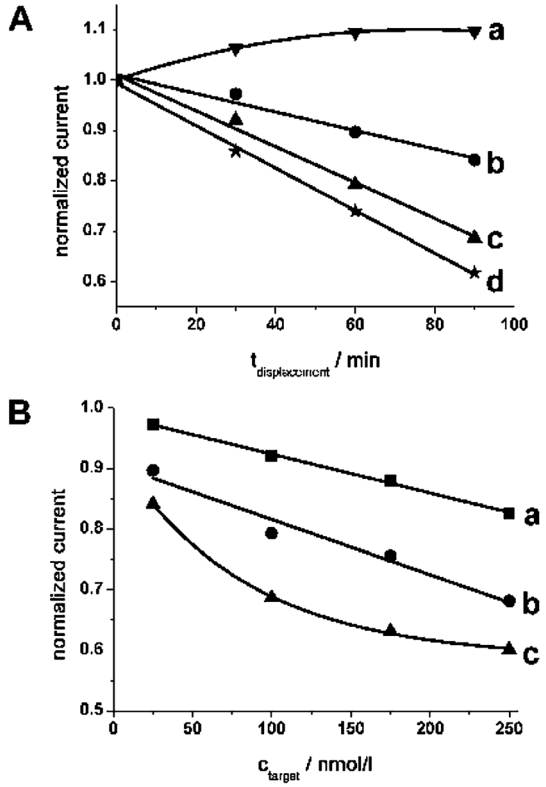

Fig. 6 Effect of (A) displacement time upon the normalized SWV signals of blank (a), 25 nM (b), 100 nM (c), and 250 nM (d) target, and (B) target concentration upon the normalized SWV signals following 30 min (a), 60 min (b) and 90 min (c) hybridization with the capture probe. All measurements were performed at 20 °C in Tris-buffer with 100 nM signalling oligo.

Fig. 5 shows how temperature affects the displacement process. The 50% equilibrium is reached faster if the experiment is performed at 35 instead of 20 °C bulk electrolyte temperature. At 5 °C, however, no significant displacement is observed (Fig. 5C). This corresponds to earlier findings where the normal hybridization of capture probes and covalently labelled target strands was dramatically affected by temperature.[29]

Fig. 6 exhibits the effect of target concentration upon the voltammetric response of the [OsO_4(bipy)]-modified signalling strand. The signal decrease at 3 different target concentrations is displayed in Fig. 6A. Whereas a 50% equilibrium can be expected at 100 nM, which is equimolar to the signalling probe, 25 nM target should lead to a smaller and 250 nM to a higher decrease, respectively. However, the final state could not be reached at room temperature within the 90 min assay time. Linear calibration plots can be obtained if the displacement time is 60 min or less (Fig. 6B).

Conclusions

Signalling probe strands which are covalently labelled with the complex [OsO_4(bipy)] allow the detection of low concentrations of target DNA. Discrimination of single base mismatches is possible. We think that this approach of sequence-specific DNA detection is very useful for several reasons. Firstly, the modification of the signalling oligo can be performed in every laboratory. No expensive instruments are needed and additionally, the chemicals are cheap. Secondly, the prepared electrodes can be stored in the fridge for weeks. As soon as the sample with the target DNA is available, the electrodes can be used directly and without any further preparation (*e.g.* labelling of the target DNA). Thirdly, we think it is advantageous if the end user does not need to handle osmium(VIII) compounds in the millimolar concentration range. The labelled signalling strands, and thus, the ready-to-use biosensors contain only trace amounts of osmium(VI).

Acknowledgements

The authors are grateful to the Deutsche Forschungsgemeinschaft (DFG) for financial support (projects FL 384/7-1 and 8-1)

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Publication X

Heiko Duwensee, Maren Mix, Inge Broer, Gerd-Uwe Flechsig

Electrochemical detection of modified maize gene sequences by multiplexed labeling with osmium tetroxide bipyridine

Electrochemical Communications (2009) submitted

Electrochemistry Communications xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Electrochemistry Communications

journal homepage: www.elsevier.com/locate/elecom

Electrochemical detection of modified maize gene sequences by multiplexed labeling with osmium tetroxide bipyridine

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ARTICLE INFO

Article history:
Received 5 March 2009
Received in revised form 15 May 2009
Accepted 19 May 2009
Available online xxxx

Keywords:
Assorted labeled targets
Square-wave voltammetry, SWV
Osmium tetroxide bipyridine
Covalent DNA-label
Electrochemical hybridization detection
Genetically modified maize

ABSTRACT

In this report we demonstrate an approach for the electrochemical detection of four sequences from maize and genetically modified (GM) maize by means of square-wave voltammetry (SWV). After multiplexed labeling with osmium tetroxide bipyridine ([OsO_4(bipy)]), the target oligonucleotides are hybridized with a complementary DNA capture probe immobilized on gold electrodes. The multiplexed labeling was performed by mixing the four target strands with the respective oligonucleotides 80% homologous to the central target recognition sequences in order to protect the latter from binding of [OsO_4(bipy)] to its thymine or cytosine residues. All components were added to the same solution. No significant decreases in SWV hybridization signals were observed after such multiplexed labeling of up to four target strands in the same reaction batch. Obtained voltammetric signals were significantly higher at 50 °C compared to 25 °C hybridization temperature and very low response was observed for non-complementary strands. Multiplexed labeling with osmium tetroxide bipyridine holds great promise for the development of simple and effective voltammetric detection protocols for GM organisms.

1. Introduction

Over the past decade, electrochemical detection of nucleic acid hybridization has become a common tool besides classical molecular biological methods such as Southern or Northern blots. The electrochemical transduction can be performed using covalent labels, intercalators or ions interacting electrostatically with the DNA. Exploiting the electrochemical activity of the DNA is also possible.

Electrochemical detection of DNA sequences can be performed such as by using self-assembled monolayers of capture probes on electrode surfaces [1–6] or hybridization at magnetic beads coupled with AdSV ("two surface strategy") [7,8]. Our group reported about [OsO_4(bipy)] as an electrochemically reversible covalent DNA-label together with thiol-linked probe oligonucleotides on gold electrodes [9].

Other approaches for the electrochemical hybridization detection of PCR products or oligonucleotides include methylene blue [10,11] or sandwich hybridization with an alkaline phosphatase conjugate on biotinylated signaling probes [12]. The different methods of electrochemical hybridization detection have been reviewed several times [13–18]. The complex [OsO_4(bipy)] can be utilized as an electrochemically reversible covalent DNA-label as described by Palecek et al. [19–22]. It reacts with the pyrimidine bases [23] under oxidation of the C–C-double bond in the pyrimidine ring forming a diester of osmic(VI) acid. This modification can be easily accomplished with single-stranded oligonucleotides or PCR products by formation of a central double strand with a protective partly homologous oligonucleotide as we have described earlier [9,25,26]. Besides single-stranded DNA and PNA, also tRNA can be labeled with osmium tetroxide [27]. Also PNA can be modified with this osmium complex, allowing stripping detection at the low pM level [24]. The temperature influence upon the hybridization process using protective strands has been characterized earlier [28]. "Multicolor labeling" of different target strands is possible using osmium tetroxide complexes with different tertiary amine ligands, which influence the electrochemical potential of the resulting covalent osmium(VI)-labels [29].

Here, we describe a simple approach for multiplexed covalent labeling of target oligonucleotides representing four sequences designed to discriminate between natural and genetically modified maize.

2. Experimental

2.1. Material

2,2′-Bipyridine was delivered by Merck and osmium tetroxide was obtained as a 2% aqueous solution from Fluka. All DNA oligonucleotides (*probes, targets and protector strands*) were delivered by

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1388-2481/$ - see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.elecom.2009.05.037

Friz Biochem (Neuried, Germany). The "tris-buffer" contained 10 mmol/l tris-(hydroxymethyl)-aminomethane and 0.5 mol/l sodium sulfate and was adjusted to pH 7.5 using sulfuric acid. The sequences of all DNA-strands together with their length and designations are given in Table 1. All *protector* oligonucleotides contain 3 to 5 mismatches (underlined in Table 1) to ease their later displacement by the immobilized probes.

2.2. Modification of target oligonucleotides

The *target oligonucleotides* and the 80% homologous protective strands were mixed in equimolar ratio and left for two hours at room temperature to allow hybridization. The probe SSIIb detects the starch synthase gene IIb and the probe ivrp detects the invertase gene of maize therefore serving as a positive control for the detection of maize. The probe CRY detects the existence of the cryIa/b transgene within the sample and the probe 810 detects the existence of the transgene at the MON 810 specific insertion locus in the maize genome. By choosing these sequences one can (a) detect the presence of maize (SSIIb and ivrp), (b) detect the presence of a specific transgene cryIa/b (CRY) and (c) detect the presence of the specific event Mon 810 (810) [30]. The sequences are given in Table 1.

The dialysis procedure, the preparation of SAM-modified gold electrodes, the hybridization and dehybridization procedures, and the electrochemical measurements have been described in more detail earlier [9].

3. Results and discussion

Fig. 1A and B displays the effect of the hybridization temperature upon SWV signals of the capture probe ivrp hybridized with either the complementary ivrp target or the non-complementary 810 target. In this figure as in all other figures where hybridization signals are displayed as voltammetric responses, representative peaks were chosen that were closest to the average value of three individual experiments. While the response of both strands is visible after room temperature hybridization (Fig. 1A), the signal of ivrp is about 10-times higher than the non-complementary strand. At optimized 50 °C hybridization temperature, the non-specific response is completely suppressed (Fig. 1B). Similar optimized conditions were used in previous work [26]. This demonstrates not only the selectivity of the probes to their respective targets (as it is displayed further in Fig. 2), but also the influence of optimized temperature. The latter does not only increase the selectivity, but also the sensitivity of the sensor. We found the signal height to be approximately four times higher at 50 °C compared to room temperature. Further temperature optimization is displayed also in Fig. 1 (C and D). It depicts calibration plots for the targets 810 and ivrp at 40 °C (C) and 50 °C (D) hybridization temperature in three separate calibration experiments each with separately prepared target, probe and electrode. 10 nM target could be detected in both cases. The signal increase when going from 40 °C to 50 °C was 2-fold and 4-fold in case of 810 and ivrp, respectively. The lower signal of ivrp at 40 °C is probably due to stable secondary structures of the probe ivrp at lower temperatures. Other reasons for increased signals at elevated hybridization temperature include enhanced mass transport due to diffusion and thermal microstirring [31]. At 40 °C, linear calibration ranges could be found up to 50 and 100 nM for 810 and ivrp, respectively (Fig. 1C). At 50 °C, however, an almost linear calibration range could be observed between 25 and 200 nM for both targets (Fig. 1D). Although coefficient of determination r^2 was better for a squared calibration function compared to a linear one, the difference between the residual sums of squares was not significant (*F*-test, not shown). These findings indicate how important an individually optimized hybridization temperature is. Reproducibility (as indicated by error bars) was found to be good even if comparing responses of independent repetitive measurement series each obtained with a newly prepared probe SAM. Similar high standard deviations have been observed before [26].

Fig. 2 exhibits how the four different capture probes ivrp, SSIIb, 810 and CRY (Table 1) respond to the four different targets in separate experiments. In Fig. 2A, the capture probe ivrp yields only response after hybridization with the full matching target ivrp. All other sequences are non-specific and fully suppressed. In comparison, the three other capture probes show full selectivity only with certain non-specific strands; however, in all cases, the non-specific response is greatly reduced due to the optimized 50 °C hybridization temperature. In these cases, all targets had been protected with their special protective strands during the modification with osmium tetroxide bipyridine.

In Fig. 3, it is demonstrated how a sample containing different target strands can be investigated using different capture probe-modified electrodes. Error bars indicate standard deviations of three independent repetitive experiments with different targets, probes and newly prepared probe SAMs. Probe ivrp only yields large response when the mixed sample contains the ivrp target sequence (Fig. 3A). Mixed samples containing mixed non-specific 810, SSIIb and CRY sequences yield much smaller signals (10 to 30 times smaller). Similar applies to probe 810 as displayed in Fig. 3B. Only target 810 gave a large response while signals of the three mixed non-specific sequences have been greatly suppressed.

Furthermore, we demonstrate in Fig. 3C how genetically modified maize sequences can be discriminated from the wild type sequence by means of four working electrodes, each modified with another kind of capture probes. Probe 810 and probe CRY, which are designed for indicating the genetic modification, yielded only minor response in presence of the wild type sequences ivrp and

Table 1
Sequences of deoxyoligonucleotides.

Designation	Length	Sequence
Probe ivrp	29 + 15 a	5′-CACGTGAGAATTTCCGTCTACTCGAGCCT- aaaaaaaaaaaaaa[Dithio]3-3′
Target ivrp	29 + 5 t	5′-tttttAGGCTCGAGTAGACGGAAATTCTCACGTG-3′
Protector ivrp	29	5′-CAGCTGAGAATTTCCGTCTAGTGCAGCCT-3′
Probe SSIIb	25 + 15 a	5′-AGCAAAGTCAGAGCGCTGCAATGCA-aaaaaaaaaaaaaa[Dithio]3-3′
Target SSIIb	25 + 5 t	5′-tttttTGCATTGCAGCGCTCTGACTTTGCT-3′
Protector SSIIb	25	5′-ACGAAAGTCAGAGCGCTCGAATGGA-3′
Probe CRY	26 + 15 a	5′-AGATACCAAGCGGCCATGGACAACAA- aaaaaaaaaaaaaa[Dithio]3-3′
Target CRY	26 + 5 t	5′-tttttTTGTTGTCCATGGCCGCTTGGTATCT-3′
Protector CRY	26	5′-AGATAGCAAGCGGCCATGCAGAAGAA-3′
Probe 810	23 + 15 a	5′-AACATCCTTTGCCATTGCCCAGC- aaaaaaaaaaaaaa[Dithio]3-3′
Target 810	23 + 5 t	5′-tttttGCTGGGCAATGGCAAAGGATGTT-3′
Protector 810	23	5′-AACATCCTTTGGCATTCCCCACg-3′

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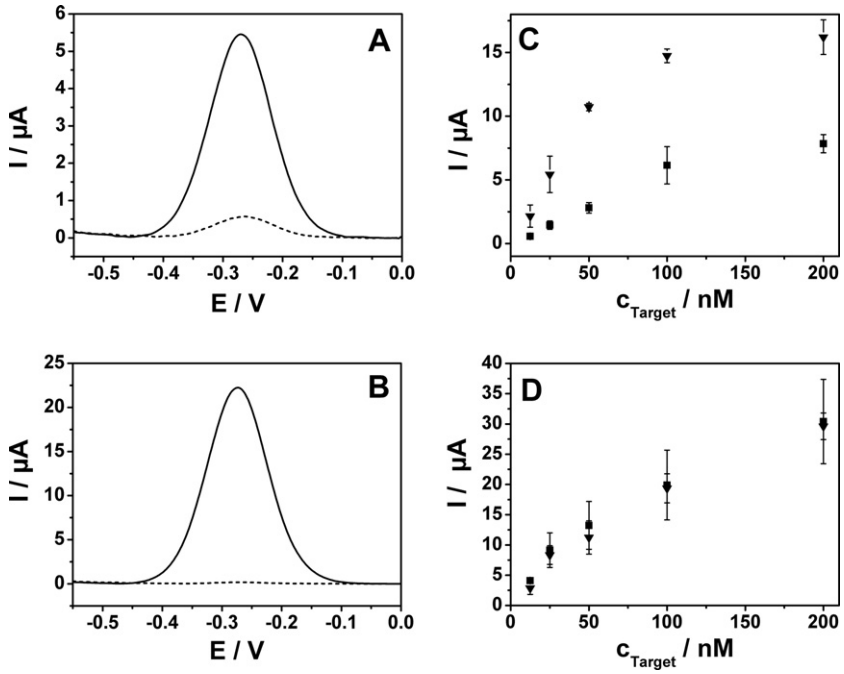

Fig. 1. Influence of temperature and target concentration during the hybridization step: Hybridization signal of probe ivrp after 15 min in 200 nM complementary target ivrp (solid line) and 200 nM non-complementary target 810 (dash line) at (A) 25 and (B) 50 °C; target ivrp with probe ivrp (triangles) and target 810 with probe 810 (squares) at (C) 40 and (D) 50 °C; covalent labeling of targets with [OsO_4(bipy)], SWV in tris-buffer at 200 Hz and 40 mV amplitude. The error bars indicate standard deviations of three independent repetitive measurement series, each obtained with a newly prepared probe SAM.

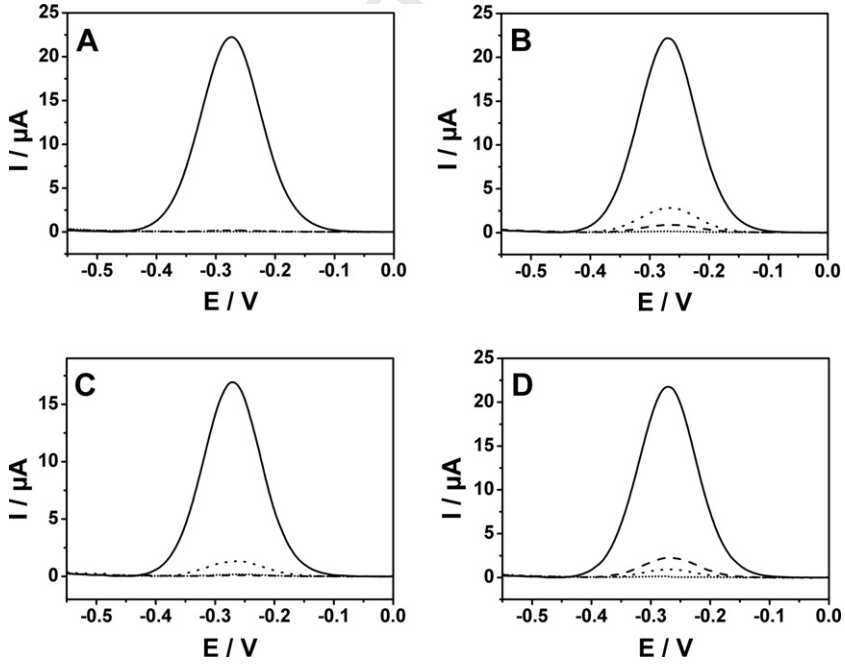

Fig. 2. Sequence selectivity of the immobilized probes: Signals of the different targets ivrp, 810, CRY and SSIIb after separate [OsO_4(bipy)]-modification and hybridization with the immobilized probes (A) ivrp, (B) SSIIb, (C) 810 and (D) CRY. Solid lines indicate the complementary target according to the probe, dashed line, short dashed line and dotted line indicate the different non-complementary targets; Hybridization was performed at 50 °C in a 200 nM target solution in tris-buffer for 15 min. Other conditions as in Fig. 1.

SSIIb (white bars). Samples containing all four GM maize sequences yielded significantly higher response with the probes 810 and CRY allowing for discrimination from the wild type. The presence of any maize in the sample was confirmed by the large response of the control probes ivrp and SSIIb. In contrast, all probes exhibited large signals when all targets were present in the sample as it would be true for GM maize (gray bars). The hybridization signals decreased by approximately 40–75% (compared to single targets or double targets) when all four targets are labeled together. The largest signal drop was observed for probe 810,

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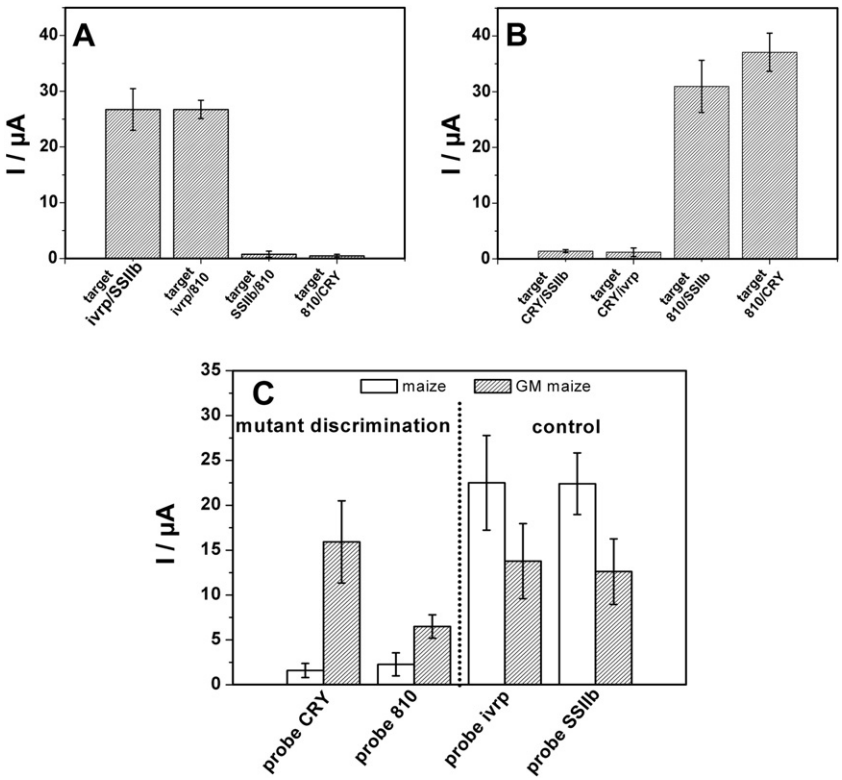

Fig. 3. Multiplexed labeling of mixed targets in one beaker and detection of sequences from genetically modified maize or wild type samples: Hybridization of surface-confined probes with mixed target solutions: (A) probe ivrp with targets ivrp/810 or targets ivrp/SSIIb as well as mixed samples containing targets SSIIb/810 and 810/CRY (B) probe 810 with the targets 810/CRY, 810/SSIIb and the targets CRY/ivrp and CRY/SSIIb; (C) hybridization of probes ivrp, 810, CRY and SSIIb with mixed double targets containing only ivrp/SSIIb to detect maize (white bars) and with mixed targets containing ivrp/810/CRY/SSIIb to detect GM each target concentration 200 nM in tris-buffer, 15 min hybridization at 50 °C, the error bars indicate standard deviations of independent repetitive measurement series, each obtained with a newly prepared probe SAM. Other conditions as in Fig. 1.

the smallest for probe CRY. Such signal decrease can probably be explained by interferences between the different target and protective strands that are present during the labeling and hybridization processes and may form secondary structures and conjugates. Nevertheless, we are still able to distinguish reliably between maize and GM maize. Our results confirm that multiplexed labeling with [OsO_4(bipy)] is possible. This will be useful to screen for genetic modifications of maize by means of four different capture probes and protective strands. Whereas multiplexed labeling of different targets with the same electroactive marker allows for selective detection on an electrode array, a multicolor label may allow detection of different targets on the same electrode [29]. The combination of both approaches will be interesting for highly parallelized electrochemical detection of many DNA sequences in one sample.

4. Conclusions

We demonstrated that four different target oligonucleotides can be simultaneously modified with [OsO_4(bipy)] in the same vessel (using four different protective strands) without major losses in the electrochemical hybridization signals on probe-modified Au electrodes. Reliable discrimination between maize and GM maize was not affected. Furthermore, we have shown that for the utilized sequences from maize and genetically modified maize, the hybridization temperature was crucial for optimal hybridization signals due to denaturation of secondary structures of the different probes. We consider this a promising approach for future electrochemical biosensor arrays designed to discriminate between natural and genetically modified plants.

Acknowledgements

The authors are grateful to the Deutsche Forschungsgemeinschaft (DFG) for financial support (Grants FL 384/4-2, 7-1, 8-1).

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Publication XI

Heiko Duwensee, Maren Mix, Marco Stubbe, Jan Gimsa, Marcel Adler, Gerd-Uwe Flechsig

Electrochemical Product Detection of an Asymmetric Convective Polymerase Chain Reaction

Biosensors and Bioelectronics (2009) submitted

Electrochemical Product Detection of an Asymmetric Convective Polymerase Chain Reaction

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Abstract

For the first time, we describe the application of heated microwires for an asymmetric convective polymerase chain reaction (PCR) in a modified tube with a low volume. The partly single-stranded product was labeled with the electrochemically active compound osmiumtetroxide bipyridine using protective strands. The labeled product could be successfully detected at a gold electrode modified with a complementary single stranded capture probe. Our simple thermo-convective PCR yielded electrochemically detectable fragments after only 5 to 10 min. Nevertheless, the total product yield was approx. half the amount of the classical thermocycler PCR. Numerical simulations describing the thermally driven convective PCR explain the received data. Discrimination between complementary capture probes and non-complementary capture probes was performed using square wave voltammetry.

Keywords

Heated electrode, asymmetric PCR, thermal simulation, fluid flow simulation, DNA-Detection

Introduction

The polymerase chain reaction (PCR) is a powerful tool for the detection of germs, genetic diseases or genetically modified plants in molecular biology. Several different approaches have been described to miniaturize the PCR system, besides the commonly used thermocyclers. One approach are microfluidic chips with external pumps for the fluid movement [Li et al., 2006; Kricka et al., 2003; Chou et al., 2002]. We describe a much simpler thermo-convective PCR where the fluid movement is induced by a temperature gradient. The general possibility was first described in a patent by Hwang et al. [2001]. Later on, modified types of convective PCR were described in the literature [Krishnan et al. 2002; Agrawal et al., 2007; Hennig et al., 2005; Wheeler et al., 2004; Braun et al., 2004a; Chen et al., 2004; Braun, 2004b]. Braun et al. [2003] used fluorescence measurements to determine the amount of amplified DNA. Theoretical considerations and numerical simulations showed the presence of dead zones, explaining the relatively long time needed for the amplification by the convective approach [Allen et al., 2009].

Electrochemical applications with directly heated microwires have attained considerable attention during the last decade. Heated wire electrodes were used for several analytical purposes. Gründler et al. introduced the “hot-wire-electrochemistry” with a symmetric working-electrode contact as well as the temperature-pulse voltammetry (TPV). In these approaches, noble metal wire electrodes (mostly 25 µm in diameter) were used [Kirbs, Gründler et al., 1996]. Determinations of arsenic(V) and mercury [Wang et al., 1999] at

gold [Gründler et al., 1998], as well as lead on platinum electrodes [Zerihun et al., 1996a], were reported. Hot wire detectors were also applied in flow systems [Wang et al., 2000a]. We suggested thermal control of DNA hybridization at heated gold wire electrodes which were modified with a thiol-linked DNA probe layer [Flechsig et al., 2005].

Chen et al. used electroluminescence instead of the electrochemical detection [Lin et al., 2006; Lin et al., 2007a; Lin et al., 2007b] in combination with carbon paste electrodes [Lin et al., 2008; Chen et al., 2007a; Chen et al., 2007b]. They applied directly heated graphite electrodes for the determination of trace riboflavin [Sun et al., 2007; Wu et al., 2007]. The temperature field around a heated microwire has been the subject of several theroretical and empirical studies [Gründler, 1996; Frischmuth, 1998; Schneider, 2000; Beckmann, 2000].

A good overview on high-temperature electrochemistry is given in: [Wildgoose et al., 2004; Gründler et al., 2006]. In recent years, investigations at electrically heated electrodes are becoming more popular [Flechsig et al. 2009]. One reason is the development of the suitable equipment making electrochemical measurements with heated electrodes easier [Wachholz et al., 2007b]. A main focus of this research is the detection of DNA.

Nevertheless, the advantages of the technique are promising for a broad variety of other detectable compounds. The reaction temperature of adsorption or hybridization processes can be adjusted without heating the whole working solution. This is of advantage when thermo-sensitive components are employed. The convection induced by punctual heating results in a mass transport of all reaction-components as we could

show by numerical simulation. Experimentally, we could show the promotion of the PCR within a PCR-tube with a heated platinum wire by this increased mass transport.

Osmium tetroxide bipyridine is a versatile covalent label for nucleic acids containing pyrimidine bases [Palecek et al., 1981; Palecek et al. 1983, Lukasova et al., 1982; Lukasova et al., 1984]. The C-C-double bond in the pyrimidine ring is oxidized and the osmium complex added forms a glycol ester of osmic(VI)acid. This label yields high and reversible voltammetric signals [Palecek, 1992]. Intact double stranded DNA does not react with the compound. On the other hand, a thoroughly modified single strand cannot hybridize with its complementary strand. Therefore, protective strands have been introduced to protect a certain recognition site within the target strand [Fojta et al., 2007; Flechsig et al, 2007]. Since the [OsO4(bipy)] reacts only with single strands, it is necessary to modify the common PCR protocols. Asymmetric PCR with one primer in large excess yields single stranded as well as double stranded PCR products, which can be modified with [OsO4(bipy)] followed by hybridization with surface-confined capture probes and voltammetric detection [Mix et al.; 2009].

In this work, we demonstrate for the first time that an asymmetric convective PCR can be conducted in a modified PCR-tube with a built-in heated microwire. The resulting PCR-product could be detected electrochemically by a probe-modified gold disk electrode after labeling with the electrochemically active [OsO4(bipy)].

Experimental

2,2'-Bipyridine was delivered by Merck and osmium tetroxide as a 2 % aqueous solution was obtained from Fluka. All DNA oligo-nucleotides (probes, targets and protector strands) were delivered by Friz Biochem GmbH (Munich, Germany). The "Tris-buffer" (containing 10 mmol/l tris-(hydroxymethyl)-aminomethane (Fluka) and 0.5 mol/l sodium sulfate (Fluka)) was adjusted to pH 7.5 using sulfuric acid. Table 1 lists the sequences of all DNA-strands together with their length and designations used in this paper. The protector oligonucleotides were designed to have 5 mismatches (underlined in Tab. 1) to facilitate their later displacement by the immobilized *F-probe*. Highly purified water (18 MΩ cm, TOC < 2 ppb) was prepared by a system of SG Water (Barsbüttel, Germany) and used in all experiments.

TABLE 1

Preparation of the PCR-E-Cup

A 0.2 ml-PCR-tube was punctured twice close to its bottom. A 25 µm diameter platinum wire was inserted through the holes and the ends were contacted by soldering. To seal the holes completely the wired tube was inserted into a 1.5 ml-E-Cup filled with epoxy after the top 1.5 cm of the E-Cup was cut off. The epoxy was left for hardening for at least 24 hours. A temperature calibration of each PCR-E-Cup was performed with an Autolab PSTAT10 (Ecochemie, Utrecht, The Netherlands) controlled by a PC with GPES 4.8 software in a solution containing equimolar 5 mmol/l ferry-/ferrocyanide and 0.1 mol/l KCl vs. Ag/AgCl (3 mol/l KCl) at 50 °C bulk solution temperature. The parameters for

zero current potentiometry were an interval time of 0.2 s and a maximum measurement time of 1999 s. The heating current was applied with a heating device developed in our group before [Wachholz et al., 2007]. The resulting current was measured with a multimeter. The current difference was divided by -1.56 mV/K, the temperature coefficient for the equimolar ferry-/ferrocyanide system and added to 50 °C to achieve the effective temperature of the wire.

Thermoconvective PCR with the PCR-tube

The asymmetric PCR was performed with 100 ng of template DNA, 0.4 µmol/l dNTPs, 1 µmol/l of the primer C.aceto F and 0.1 µmol/l C.aceto R (Tab. 1), and 2.5 U Sawady-Taq-DNA-Polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany) in 50 µl reaction volume. The PCR-tube was filled with the PCR-reaction solution which was overlaid with a 40 µl mineral oil seal to avoid evaporation. It was placed in a waterbath at $T_{ext} = 50°C = 323.15\,K$. An approximate temperature of $T_{heat} = 89°C = 362.15\,K$ was adjusted according to the temperature calibration by AC-heating for 0, 5, 10, 15, 30 and 45 minutes, respectively. After that, the PCR-tube was incubated for another 30 minutes in the waterbath at T_{ext} to complete the PCR.

Preparation of SAM-modified gold electrodes

The gold disk electrode (Metrohm, Switzerland) was polished using corundum 0.3 µm (Buehler) and thoroughly rinsed with water. After this, the electrode was electrochemically treated with 25 voltammetric cycles between -0.2 und +1.65 V at 100 mV/s in 0.5 mol/l sulfuric acid and again rinsed with water. A droplet of 15 µl *probe* solution (30.3 µmol/l) was placed onto the electrode surface in order to form the capture

probe SAM. After 16 h at 10 °C in a water-saturated atmosphere, the electrode was rinsed and immersed in 1 mmol/l aqueous mercaptohexanol solution for one hour.

Hybridization and dehybridization procedure

The probe-SAM-modified gold disk electrode was dipped into a beaker containing the target solution for the hybridization step for 30 minutes. The sample solution was stirred constantly using a magnetic stirrer and heated in a water bath. After the hybridization the electrode was rinsed with Tris-buffer. The electrode was regenerated by a dehybridization step following the electrochemical measurement. To retrieve the probe-SAM back, the electrode was immersed in 50 °C deionized water for 60 s.

Electrochemical measurements

An Autolab PSTAT10 (Ecochemie, Utrecht, The Netherlands) controlled by a PC with GPES 4.9 software and an electrode-stand inside a *Faraday* cage was used for all electrochemical procedures. An Ag/AgCl (3 mol/l KCl) and a glassy carbon electrode were used as reference and counter electrodes, respectively (all from Metrohm AG, Herisau, Switzerland). The square-wave voltammetry was conducted at 200 Hz and an amplitude of 40 mV in a scan-range from -0.55 to 0.0 V. All measurements were performed at room temperature in Tris-buffer. The peak-shaped measuring signals were smoothed with a level 2 Savitzky-Golay filter and baseline-corrected (moving average, peak width 0.03).

Conditions of control PCR and agarose gelelectrophoresis

A synthetic 136-base-DNA strand (sequence from the genome of *Clostridium acetobutylicum* [1855609-1855744]) was used as template. The asymmetric PCR was performed with 100 ng of template DNA, 0.4 µmol/l dNTPs, 1 µmol/l of the primer C.aceto F and 0.1 µmol/l C.aceto R (Tab. 1), and 2.5 U Sawady-Taq-DNA-Polymerase (Peqlab Biotechnologie GmbH, Erlangen, Germany) in a reaction volume of 50 µl according to the manufacturer's instructions. For the symmetric PCR an equal primer concentration of 0.5 µmol/l was used. For the PCRs a thermocycler (Hybaid PCR Sprint Thermal Cycler, Thermo Scientific) was used with the following parameters: 1 x 94 °C, 2 min; 30 x (94 °C, 30 s, 57 °C, 45 s, 72 °C, 30 s); 1 x 72 °C, 7 min. The agarose gelelectrophoresis was performed with a 2 % [w/v] agarose gel in 1x TAE-buffer (40 mmol/l tris-(hydroxymethyl)-aminomethane, 20 mmol/l glacial acetic acid, 1 mmol/l 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 using the DNA-ladder (Fermentas GmbH, St. Leon-Roth, Germany) as a comparison.

Modification of amplified Fragment oligonucleotide (Target)

For modification, 100 µl of asymmetric PCR-solution from the PCR-E-Cup (containing the amplified single target strand *Fragments*) were used without further purification. The *Fragments* were mixed at their maximum concentrations (~ 10 nmol/l) and 1 µl of the

protective strand *F-Protector* (10 µmol/l in the hybridization solution) 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 to achieve a final concentration of 2 mmol/l of [OsO_4(bipy)] 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 with a Slide-A-Lyzer MINI Dialysis Units, 3500 MWCO (Rockford, IL, USA) at 4 °C for 19 hours. After dialysis, the purified solution (containing the osmium-labeled target *Fragment*) was used for the hybridization experiments.

FEM-simulation

In our experiments, a heated microwire drove the thermo-convective flow of the PCR-solution. The temperature distribution and the resulting fluid flow inside the small PCR-tube were considered by FEM-simulation employing the General Heat Transfer and the Incompressible Navier-Stokes modules of the FEM-software Comsol Multiphysics 3.4.

The following material parameters were used. For polypropylene (PCR-tubes) and the epoxy resin $k = 0.22\,Wm^{-1}K^{-1}$, $C_p = 1680\,Jkg^{-1}K^{-1}$, $\rho = 906\,kgm^{-3}$ and $k = 0.252\left(0.118 + 0.88\left(T - T_0\right)\right)Wm^{-1}K^{-1}$, $C_p = 1520\,Jkg^{-1}K^{-1}$, $\rho = 1150\,kgm^{-3}$, respectively. For air: $k = 0.261\,Wm^{-1}K^{-1}$, $C_p = 717\,Jkg^{-1}K^{-1}$, $\rho = 1293\,kgm^{-3}$, mineral oil: $k = 0.15\,Wm^{-1}K^{-1}$, $C_p = 2130\,Jkg^{-1}K^{-1}$, $\rho = 800\,kgm^{-3}$, the aqueous PCR solution: k = -0.719416+(7.2455·10^{-3}T) - (9.37413·10^{-6}T^2)$Wm^{-1}K^{-1}$, C_p=207872.047528-(3031.74) + (18.0348T^2)-(535876·10^{-2}T^3)+(7.95093·10^{-5}T^4)-(4.71045·10^{-8}T^5)$Jkg^{-1}K^{-1}$, ρ=233.172+ (6.76474T)-

$(0.0187743T^2)+(1.56896\cdot10^{-5}T^3)\,kgm^{-3}$, $\alpha = 9.14\cdot10^{-4} + 5.7\cdot10^{-2}e^{\left(-1.4\cdot10^{-2}T\right)}$, and platinum-heating wire: $k = 71.6\,Wm^{-1}K^{-1}$, $C_p = 133\,Jkg^{-1}K^{-1}$, $\rho = 21450\,kgm^{-3}$. The parameters for every material were included as temperature dependent values if possible, otherwise fixed values were used [Lide, 1998; Assael et al., 2008].

For the 3-dimensional geometry shown in figures 4 and 5 a mesh of 162002 degrees of freedom was generated. The equation used in the heat transfer module for the solid bodies is $\nabla\cdot\left(-k\nabla T\right) = Q$. For the fluids the convective heat transfer was applied resulting in $\nabla\cdot\left(-k\nabla T\right) = Q - pC_p u\cdot\nabla T + n\left[\nabla u + \left(\nabla u\right)^T - \frac{2}{3}\left(\nabla\cdot u\right)I\right]:\nabla u$ with $n\left[\nabla u + \left(\nabla u\right)^T - \frac{2}{3}\left(\nabla\cdot u\right)I\right]:\nabla u \equiv \sum_{i,j} n\left[\nabla u + \left(\nabla u\right)^T - \frac{2}{3}\left(\nabla\cdot u\right)I\right]_{i,j}\nabla u_{i,j}$, the contraction of the two tensors. Lateral external boundaries of the big PCR-tube were set to the water bath temperature $T_{ext} = 323.15\,K$. The top boundaries were assumed to be thermally insulated because of their contact to air. The boundaries of the heating wire were assumed to be $T_{heat} = 362.15\,K$ and all other to continuity. In the Incompressible Navier-Stokes module the equations $\rho u\cdot\nabla u = \nabla\left[-\rho I + n\left(\nabla u + \left(\nabla u\right)^T\right)\right] + F$ with F being the convective force, and $\nabla\cdot u = 0$ were used to solve the fluid-flow problem inside the small PCR-tube (for variables please see table 2). This module was only applied inside the PCR solution in the small PCR-tube in order to minimize the solving time. The boundary conditions were set to no-slip conditions at the tube boundaries and to slip condition at the solution-to-oil boundary.

best place for TABLE 2

Results and Discussion

Figure 1 displays the gelelectrophoretic analysis of the symmetric PCR in the specifically designed PCR-tube with a heated wire (lanes 2-7) after 0, 5, 10, 15, 30 and 45 min of heating in comparison to the classical symmetric PCR performed in a thermocycler (lanes 8-12). For calculation of the efficiency of the wire-heated PCR-tubes, the PCR-samples from the thermocycler were removed after 10 (lane 8), 15 (lane 9), 20 (lane 10), 25 (lane 11), and 30 cycles (lane 12). The comparison of the bands intensities clearly shows the lower efficiency of the thermo-convective PCR in the wire-heated tubes.

best place for FIGURE 1

Because of the lower PCR-efficiency, also lower detection signals were expected. We combined three different PCR samples from each heating duration for the modification with [OsO_4(bipy)] as described in section 2.2. Results are shown for the hybridization of the complementary *F-probe* and the non-complementary *MON810-probe* with the *Fragment* (Fig. 2). Sequence specific target detection could be achieved as shown in (A) and (B). The complementary target generates a signal of approximately 2 µA (Fig. 2A) whereas with the non-complementary target only a signal of about 0.6 µA (Fig. 2B) is obtained. The blank value represents the signal obtained with the probe-modified Au-electrode without heating the PCR solution in the wire-modified tube (Fig.2C). As expected, the asymmetric PCR product obtained with the conventional thermocycler provides the largest signals (about 3.5 µA, Fig. 2D). This indicates that the efficiency of the asymmetric PCR is only slightly affected by the thermo-convective mode. All peaks

are caused by the reversible redox couple osmium(IV/VI) in the covalent label of glycyl osmate.

best place for FIGURE 2

Figure 3 presents the dependency between the heating time of the thermo-convective PCR and the electrochemically obtained signal. The error bars represent the deviation observed in three different PCR batches with three different probe-modified Au-electrodes. With increasing heating times growing electrochemical signals were obtained. The strong increase between 10 and 15 minutes was reproducible. These findings are in very good accordance with results reported by Braun et al. [2003] who used fluorescence detection. We suppose that in this period of time the reaction is in its exponential amplification period that ends after about 15 minutes. Accordingly, a sigmoidal behavior, typical for real-time PCR is observed. This finding will be a focus of future work.

best place FIGURE 3

Consideration of the experimental PCR and the FEM-simulation results suggests a possible reason for the lower signal from the heated-wire tubes. Obviously, the circulating PCR-solution does not experience the optimal temperature sequence used in the classical PCR (Fig. 4). The hottest area in the centre is surrounded by medium with a temperature decreasing to T_{ext} at the external boundaries. These results correspond to our gelelectrophoresis results as well as to the lower product yield in the electrochemical

measurements observed with the PCR-E-Cup compared to the classical PCR (Fig. 1). The resulting electrochemical signals were approximately two times lower (Fig. 2A and D). This is the result of the suboptimal mass transport in the PCR-E-Cup as shown in Fig. 4.

best place FIGURE 4

The PCR solution cycles through different temperature ranges driven by the thermal convection of the medium. Two large vortexes with their central axes oriented parallel to the heating wire are observed. They generate wake space in their centers close to the heating wire. Spaces with a very low convection are also observed at the upper surface of the PCR-solution as well as at the bottom of the tube (Fig. 4). Obviously, the temperature sequence for denaturation, primer binding and elongation is suboptimal in comparison to a cycle in the classical PCR, reducing the PCR-product yield.

Conclusions

We could demonstrate that the convective PCR in our simple PCR-E-Cup yielded electrochemically detectable fragments after only 5 to 10 min. Nevertheless, the total product yield was significantly lower in the convective PCR compared to the classical thermocycler PCR. The electrochemical signals of the classical PCR were only twice as high than the product concentration from the convective PCR at a time saving of 1 h using the convective PCR. We suppose that the reasons for the lower PCR-product yield

of the thermo-convective PCR are the suboptimal thermal sequence in the convective mass-flow and wake spaces that are largely excluded from the convections.

These properties can be nicely investigated by numerical models. Likewise, numerical calculations can be used to optimize thermo-convective PCR-systems, e.g. by variations of the length, orientation, or localization of the heated wire.

Acknowledgement

The authors want to thank the Federal State of Mecklenburg-Vorpommern (Landesexzellenzförderung) and the Deutsche Forschungsgemeinschaft (DFG) for financial support.

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Tables

Table 1: DNA sequences, the mismatches in the protective strand are highlighted

Designation	Length	Sequence
Fragment	136	5'- CTTACAGGAATTGTTTTGGGACTTGCAAGGGCTT- TTGGAGAGGCATTAGCAGTTCAAATGGTAATAGGT A- ATACAGTAAAATCAGCTCAAGGACTTTTTTCTCCTA C-TACCACTTTGACAAGCGTACTTACAATGG -3'
F-protector	27	5´-AAG**AG**C**A**TGAGCTGATTTTA**G**TG**A**ATT-3´
F-probe	27	5´-AAGTCCTTGAGCTGATTTTACTGTATT- TTTTTTTTTTTTTTT[Dithio]3-3´
MON810-*probe*	26	5`-AGATACCAAGCGGCCATGGACAACAA- aaaaaaaaaaaaaa[Dithio]3-3`
Forward primer C.aceto F	21	5`-CTTACAGGAATTGTTTTGGGA-3`
Reverse primer C.aceto R	21	5`-CCATTGTAAGTACGCTTGTCA-3`

Table 2: Summary of variables and input values for the 3-dimesional FEM-model

Symbol	Variable	Remarks
k	thermal conductivity	T-dependent (see Ref. 38, data sheet)
C_p	heat capacity	T-dependent (see Ref. 38, data sheet)
Q	heat source	$Q = 0$
T	temperature	Solved by general heat transfer module
T_{ext}, T_{heat}	external and heating wire temperature	$T_{ext} = 323.15\,K$, $T_{heat} = 362.15\,K$
ρ	density	T-dependent (see Ref. 38, data sheet)
g	gravity acceleration	$g = 9.81\,ms^{-1}$
α	thermal coefficient of expansion	see Ref. 38, data sheet)
F	convective force	$F = \alpha\,\rho\,g\left(T - T_{ext}\right)$
n	normal unit vector	-
I	identity matrix or unit diagonal matrix	-

Figures

Figure 1

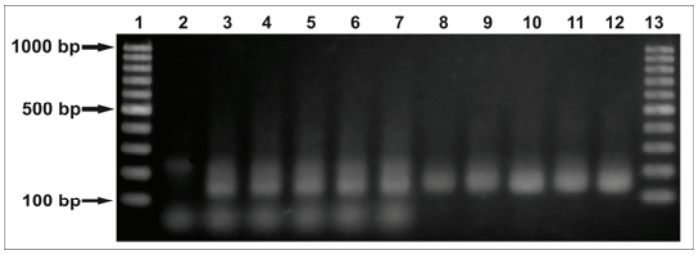

Figure 2

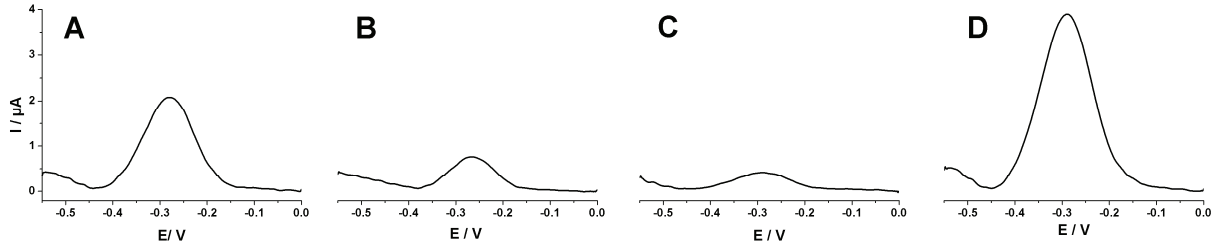

Figure 3

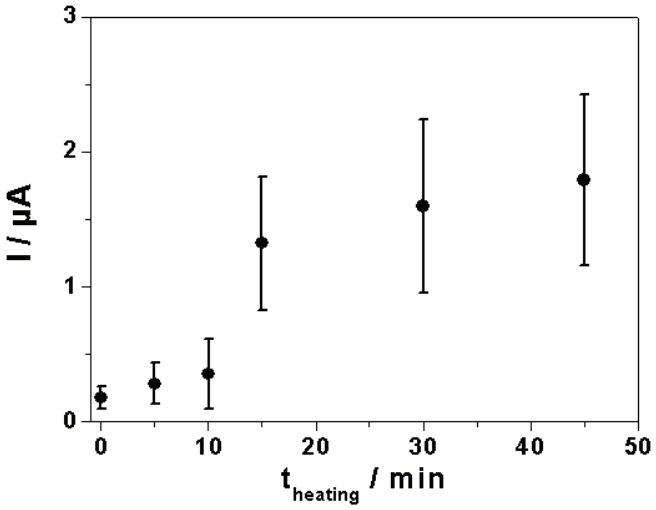

Figure 4

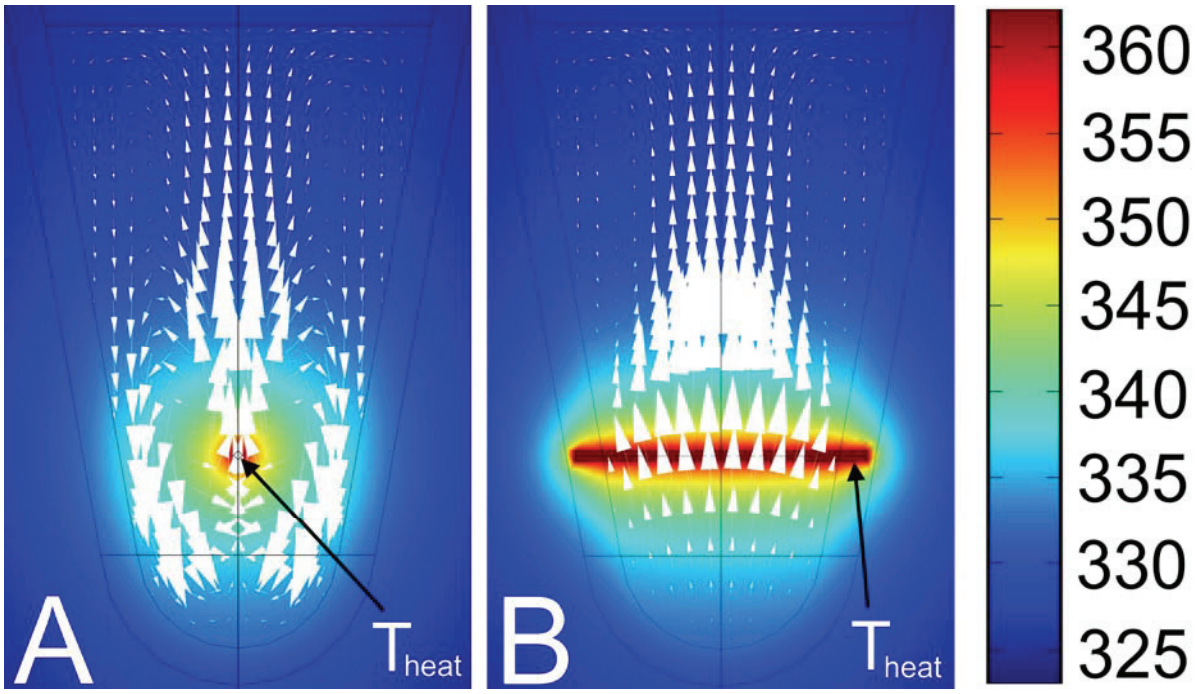

Figure Captions

Fig 1: 2 % [w/v] agarose-gel stained with Gelred (Cambridge BioScience, UK); lanes 1 + 13: Gene Ruler^{Tm} 100 bp DNA Ladder (0.1 µg); lanes 2-7: PCR with wire-heated PCR-tube, heating times: 0 min (lane 2), 5 min (lane 3), 10 min (lane 4), 15 min (lane 5), 30 min (lane 6), and 45 min (lane 7). Lanes 8-12: cycler-PCR after number of cycles: 10 (lane 8); 15 (lane 9), 20 (lane 10), 25 (lane 11), and 30 (lane 12).

Fig 2: Square-wave voltammetric signals at gold disk electrodes in Tris-buffer with immobilized probe strands; (A) F-probe with fragment (30 min heating), (B) non-complementary probe MON810 with fragment (30 min heating), (C) blank value F-probe with PCR-solution (not heated), (D) F-probe with fragment (produced in thermocycler); SWV conditions: frequency 200 Hz; amplitude 40 mV; During the hybridization step the solution was stirred by a magnetical stirrer at 20 °C.

Fig 3: Square-wave voltammetric signals at gold disk electrodes in Tris-buffer after 15 min of hybridization. $t_{heating}$ represents the onset of solution heating by the platinum wire; frequency 200 Hz; amplitude 40 mV. During the hybridization step the solution was stirred by a magnetical stirrer.

Fig. 4: temperature distribution and velocity field inside the small PCR-tube with views along (A) and perpendicular (B) to the heating wire. T_{heat} is the hottest area and marks the position of the heating wire. The directions and the lengths of the arrows represent the direction and velocity of the convection in the PCR-solution (longer arrow correspond to higher velocity).

Publications

Journal contributions

1. F. Wachholz, J. Gimsa, H. Duwensee, H. Grabow, P. Gründler, G.-U. Flechsig, *A compact and versatile instrument for radio frequency heating in non-isothermal electrochemical studies*, Electroanalysis 19 (2007) 535

2. H. Duwensee, M. Adamovski, G.-U. Flechsig, *Adsorptive stripping voltammetric detection of daunomycin at mercury and bismuth alloy electrodes*, International Journal of Electrochemical Science 2 (2007) 498

3. H. Duwensee, T. Vázquez-Alvarez, G.-U. Flechsig, J. Wang, *Thermally induced electrode protection against biofouling*, Talanta 77 (2009) 1757

4. N. Mahnke, A. Markovic, H. Duwensee, F. Wachholz, G.-U. Flechsig, U. van Rienen, *Numerically optimized shape of directly heated electrodes for minimal temperature gradients*, Sensors & Actuators B - Chemical 137 (2009) 363

5. M. Mix, T. Reske, H. Duwensee, G.-U. Flechsig, *Electrochemical detection of asymmetric PCR products by labeling with osmium tetroxide*, Electroanalysis 21 (2008) 826

6. H. Duwensee, M. Jacobsen, G.-U. Flechsig, *Electrochemical competitive hybridization assay for DNA detection using osmium tetroxide-labelled signalling strands*, Analyst 134 (2009) 899

7. H. Duwensee, M. Mix, I. Broer, G.-U. Flechsig, *Electrochemical detection of modified maize gene sequences by multiplexed labeling with osmium tetroxide bipyridine*, Electrochemistry Communications (2009) accepted

8. F. Wachholz, H. Duwensee, R. Schmidt, M. Zwanzig, J. Gimsa, S. Fiedler, G.-U. Flechsig, *Template-free galvanic nanostructuring of gold electrodes for sensitive electrochemical biosensors*, Electroanalysis (2009) accepted

9. H. Duwensee, M. Mix, M. Stubbe, J. Gimsa, M. Adler, G.-U. Flechsig, *Electrochemical Product Detection of an Asymmetric Convective Polymerase Chain Reaction,* Biosensors and Bioelectronics (2009) submitted

Patents

1. G.-U. Flechsig, H. Duwensee, J. Wang, *Verfahren zur elektrochemischen Behandlung einer Sensorvorrichtung*, Patentanmeldung Az. DE 10 2008 020 802.7 (2008)

Poster Contributions

1. H. Duwensee, C. Lau, P. Gründler, *Nicht-isotherm arbeitender Maltose-Biosensor zur Parallelbestimmung von Maltose neben Glucose*, GDCh Annual Meeting 11-14 September (2005), Düsseldorf, Germany

2. H. Duwensee, C. Lau, P. Gründler, *Non-isothermal working maltose-biosensor for thermal determination of maltose next to glucose*, International Conference on Electrochemical Sensors, 13-18 November (2005), Mátrafüred, Hungary

3. H. Duwensee, M. Adamovski, G.-U. Flechsig, *Adsorptive Stripping Voltammetric Detection of Daunomycin at Bismuth/Gold and Amalgam Electrodes*, International Conference on Electroanalysis ESEAC, 11-15 June (2006), Bordeaux, France

4. H. Duwensee, F. Wachholz, G.-U. Flechsig, *Elektrochemische Mehrfachdetektierung von DNA-Hybridisierungen mittels eines selektiv heizbaren Elektrodenarrays*, National Meeting ELACH 7, 19-21 September (2006), Waldheim, Germany

5. F. Wachholz, H. Duwensee, G.-U. Flechsig, *Neuartiges Instrument für das direkte und indirekte Heizen von Elektroden*, National Meeting ELACH 7, 19-21 September (2006), Waldheim, Germany

6. H. Duwensee, F. Wachholz, N. Mahnke, A. Markovic, U. van Rienen, G.-U. Flechsig, *Thermal Behaviour of Heated Electrodes*, International Conference on Electroanalysis ESEAC, 16-19 June (2008), Prague, Chech Republic

7. H. Duwensee, A.-E. Surkus, G.-U. Flechsig, *Detection of DNA-Hybridization on Gold-Bismuth (Au/Bi) Alloy Electrodes*, International Conference on Electroanalysis ESEAC, 16-19 June (2008), Prague, Chech Republic

8. M. Mix, H.Duwensee, I. Broer, G.-U. Flechsig, *Electrochemical Detection of Genetically Modified Plants by Means of Heated Electrodes*, ACS Spring Meeting, 22-26 March (2009), Salt Lake City, USA

Oral Presentations

1. H. Duwensee, F. Wachholz, G.-U. Flechsig, *Brückenschaltung für direkt elektrisch geheizte Elektroden*, Bunsen Colloqium, 2. February (2007), Dresden, Germany

2. M. Mix, T. Reske, H. Duwensee, G.-U. Flechsig, *Electrochemical Detection of PCR-Products by Labelling with Osmium Tetroxide*, Satellite Symposium to ESEAC, 19-22 June (2008), Brno, Czech Republic

3. F. Wachholz, H. Duwensee, G.-U. Flechsig, *Enhancement of Amperometric Signals by Means of Heated Sensors*, International Conference on Electroanalysis ESEAC, 16-19 June (2008), Prague, Chech Republic

4. H. Duwensee, G.-U. Flechsig, *Temperature in Electrochemistry*, Univerity Joseph Fourier / CNRS, 3. September (2008), Grenoble, France

Ich versichere hiermit an Eides statt, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Rostock, 28.4.2009

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