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**NOVEL APPROACHES IN ELECTROCHEMICAL
DETERMINATION OF XENOBIOTIC COMPOUNDS
AND IN STUDY OF THEIR INTERACTION WITH DNA**

**NOVÉ PŘÍSTUPY PŘI ELEKTROCHEMICKÉM
STANOVENÍ CIZORODÝCH LÁTEK A STUDIU JEJICH
INTERAKCE S DNA**

Ph.D. Thesis

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Mgr. Andrea Hájková

Supervisor: Doc. RNDr. Vlastimil Vyskočil, Ph.D.

Supervisor-consultant: Prof. RNDr. Jiří Barek, CSc.

I declare that all the results which are used and published in this Ph.D. Thesis have been obtained by my own experimental work and that all the ideas taken from work of others are properly referred to in the text and the literature survey. I am conscious that the prospective use of the results, published in this Ph.D. Thesis, outside the Charles University in Prague is possible only with a written agreement of this university.

I also declare that neither this Ph.D. Thesis nor its significant part has been submitted in any form for another degree or diploma at any university or other institution of tertiary education.

In Prague, 28. 6. 2016

Mgr. Andrea Hájková

This Ph.D. Thesis was experimentally carried out in the period from 2012 till 2016 at the Charles University in Prague, Faculty of Science, Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry. During this period, the research visit in the laboratory of Prof. Dr. Frank-Michael Matysik at the University of Regensburg, Faculty of Chemistry and Pharmacy, Institute of Analytical Chemistry, Chemo- and Biosensors, Regensburg, Germany, was completed, too. Some experiments were also carried out in cooperation with Ing. Bohdan Josypčuk, Ph.D., from the J. Heyrovský Institute of Physical Chemistry of the Academy of Sciences of the Czech Republic, v.v.i.

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Abstract

Presented Ph.D. Thesis is focused on the development of analytical methods applicable for determination of selected xenobiotic compounds and for monitoring DNA damage they can induce. The main attention has been paid to the development and testing of non-toxic electrode materials for preparation of miniaturized electrochemical devices and novel electrochemical DNA biosensors.

2-Aminofluoren-9-one (2-AFN) was selected as a model environmental pollutant, which belongs to the group of hazardous genotoxic substances. Its carcinogenic and mutagenic effects may represent a risk to living and working environment. 2-AFN has one oxo group, where the cathodic reduction occurs, and one amino group, where the anodic oxidation occurs.

The voltammetric behavior of 2-AFN in the negative potential region was investigated at a mercury meniscus modified silver solid amalgam electrode (m-AgSAE) representing a non-toxic and more mechanically robust alternative to mercury electrodes. This working electrode was subsequently used for the development of a newly designed miniaturized electrode system (MES), which has many benefits as the possibility of simple field measurements, easy portability, and the measurement in sample volume 100 μ L. Moreover, a glassy carbon electrode (GCE) was used for further investigation of electrochemical transformations of 2-AFN in both cathodic and anodic potential regions and its determination. All the newly developed voltammetric methods were successfully applied on model samples of drinking and river waters.

In this Ph.D. Thesis, highly sensitive electrochemical techniques were also employed for investigating and monitoring DNA damage. An electrochemical DNA biosensor, based on the GCE and low-molecular-weight double-stranded DNA (dsDNA) from salmon sperm immobilized onto the electrode surface, was developed and characterized using modern advanced imaging techniques. Subsequently, a combination of several electrochemical techniques (direct and indirect) was used for investigation of the interaction between various xenobiotic compounds and dsDNA. Detrimental effects of selected derivatives of fluorene (2-AFN, 2-aminofluorene, 2-acetylaminofluorene, and 2,7-diaminofluorene) and hydroxyl radicals were studied. Hydroxyl radicals were generated electrochemically on the surface of a boron-doped diamond electrode and chemically (via the Fenton's reaction and the auto-oxidation of Fe(II)).

Abstrakt

Předložená disertační práce je zaměřena na vývoj analytických metod pro stanovení vybraných cizorodých látek a ke sledování poškození DNA, které mohou způsobit. Hlavní pozornost byla věnována vývoji a testování netoxických elektrodových materiálů pro přípravu miniaturizovaných elektrochemických zařízení a nových elektrochemických DNA biosenzorů.

2-Aminofluoren-9-on (2-AFN) byl vybrán jako modelový polutant životního prostředí, který patří do skupiny nebezpečných genotoxických látek. Jeho karcinogenní a mutagenní účinky mohou představovat riziko jak pro životní, tak pro pracovní prostředí. 2-AFN má oxo skupinu, kterou lze elektrochemicky redukovat, a aminoskupinu, kterou lze elektrochemicky oxidovat.

Voltametrické chování 2-AFN v negativní oblasti potenciálu bylo zkoumáno na rtuťovým meniskem modifikované stříbrné pevné amalgamové elektrodě (m-AgSAE), která představuje netoxickou a mechanicky stabilní alternativu ke rtuťovým elektrodám. Tato pracovní elektroda byla následně použita pro vývoj nově navrženého miniaturního elektrodového systému (MES), který má mnoho výhod, jako je možnost jednoduchého měření v terénu, snadná přenosnost a měření ve 100 μ L objemu vzorku. Také elektroda ze skelného uhlíku (GCE) byla použita pro další výzkum elektrochemického chování 2-AFN a jeho stanovení v katodické i anodické oblasti potenciálů. Všechny nově vyvinuté voltametrické metody byly úspěšně použity na modelové vzorky pitné a říční vody.

V této disertační práci byly vysoce citlivé elektrochemické techniky použity také pro vyšetřování a sledování poškození DNA. Elektrochemický DNA biosenzor, který je založen na GCE a na nízkomolekulární dvouřetězcové DNA (dsDNA) z lososích spermii, jež je imobilizována na povrch elektrody, byl vyvinut a charakterizován pomocí moderních zobrazovacích technik. Kombinace několika elektrochemických metod (přímé i nepřímé) byla použita pro zkoumání interakce mezi různými cizorodými látkami a dsDNA. Byly sledovány škodlivé účinky vybraných derivátů fluorenu (2-AFN, 2-aminofluoren, 2-acetylaminofluoren a 2,7-diaminofluoren) a hydroxylových radikálů. Hydroxylové radikály byly generovány elektrochemicky na povrchu borem dopované diamantové elektrody a chemicky (Fentonova reakce a auto-oxidace ionty Fe^{2+}).

Key Words:

Genotoxic Fluorene Derivatives
Mercury Meniscus Modified Silver Solid Amalgam Electrode
Miniaturized Electrode System
Glassy Carbon Electrode
Electrochemical DNA Biosensor
DNA Damage
Hydroxyl Radicals
Fenton's Reaction
Boron-Doped Diamond Electrode

Klíčová slova:

Genotoxické deriváty fluorenu
Rtuťovými meniskem modifikovaná stříbrná pevná amalgamová elektroda
Miniaturizovaný elektrodový systém
Elektroda ze skelného uhlíku
Elektrochemický DNA biosenzor
Poškození DNA
Hydroxylové radikály
Fentonova reakce
Borem dopovaná diamantová elektroda

List of Symbols and Abbreviations

2-AAF	2-acetylaminofluorene
2-AF	2-aminofluorene
2-AFN	2-aminofluorene-9-one
2,7-DAF	2,7-diaminofluorene
FN	fluorene-9-one
α	significance level
γ	concentration of dsDNA
AcB	acetate buffer
AdSDPV	adsorptive stripping differential pulse voltammetry
AE	auxiliary electrode
AgA-PE	silver amalgam paste electrodes without pasting liquid
AgSA-PE	silver amalgam paste electrode with an organic pasting liquid
APAHs	amino polycyclic aromatic hydrocarbons
BB	borate buffer
BDDE	boron-doped diamond electrode
BiF-AgSAE	bismuth film electrode utilizing silver solid amalgam substrate
BR	Britton-Robinson
c	molar concentration
CFE	carbon film electrode
CPE	carbon paste electrode
CV	cyclic voltammetry
DCTP	direct current stripping polarography
DCV	direct current voltammetry
DME	dropping mercury electrode
DNA	deoxyribonucleic acid
DPAdSV	differential pulse adsorptive stripping voltammetry
DPP	differential pulse polarography
DPV	differential pulse voltammetry
dsDNA	double-stranded deoxyribonucleic acid
dsDNA/GCE	double-stranded DNA modified glassy carbon electrode
DW	drinking water
E_{acc}	accumulation potential
E_{act}	activation potential
E_{ads}	adsorption potential

EDTA	ethylenediaminetetraacetic acid
EIS	electrochemical impedance spectroscopy
E_p	peak potential
$E_{reg,1}$	more positive regeneration potential
$E_{reg,2}$	more negative regeneration potential
G	guanosine
GCE	glassy carbon electrode
HMDmE	hanging mercury drop minielectrode
IARC	International Agency for Research on Cancer
I_a	anodic peak
I_c	cathodic peak
I_F	fluorescence intensity
I_p	peak current
L_Q	limit of quantification
m-AgSAE	mercury meniscus modified silver solid amalgam electrode
MES	miniaturized electrode system
MF-AgSAE	mercury film electrode utilizing silver solid amalgam substrate
n	number of measurements
p_A	peak of adenosine
p-AgSA-CE	polished silver solid amalgam composite electrode
p-AgSAE	polished silver solid amalgam electrode
PAHs	polycyclic aromatic hydrocarbons
PB	phosphate buffer
p_G	peak of guanosine
PF	theoretical preconcentration factor
pH	negative of the decadic logarithm of the activity of hydronium ions
pH^*	resulting pH of the aqueous-methanolic solution
p_{TC}	peak of thymidine and cytidine
$R^2 (r^2)$	coefficient of determination
R_{CT}	charge transfer resistance
RE	reference electrode
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSD	relative standard deviation
RSS	relative sulfur species
RW	river water
S_0	signal response before interaction dsDNA with damaging agent

S_1	signal response after interaction dsDNA with damaging agent
SCAgAE	solid amalgam minielectrode based on a single silver amalgam crystal
SPE	solid phase extraction
SPCE	screen-printed carbon electrode
S_{rel}	relative biosensor response
SWCNT	single-walled carbon nanotube
SWV	square wave voltammetry
t_{acc}	accumulation time
t_{act}	activation time
t_{ads}	adsorption time
UV	ultraviolet part of the spectrum
ν	scan rate
VIS (or Vis)	visible part of the spectrum
WE	working electrode

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1. INTRODUCTION

This Ph.D. Thesis was elaborated at the UNESCO Laboratory of Environmental Electrochemistry in Prague. Its scientific activities are focused on a long term research in the field of highly sensitive and selective electrochemically methods for monitoring and determination of biologically active compounds important from the environmental, medicinal, pharmaceutical, and toxicological point of view, with a special emphasis placed on the development of new types of electrochemical sensors and materials for their preparation. In the last five years, our attention has been paid to the development of electrochemical DNA biosensors which can be used for investigation of interactions between xenobiotic compounds and DNA and for detection of DNA damage. **The Ph. D. Thesis presented is based on following seven scientific publications [1-7] which are attached as Appendix parts I – VII (Chapters 6 – 12). To distinguish the references referring to these publications in entire text of this Ph.D. Thesis, corresponding numbers in square brackets are in bold and underlined.**

- [1] **A. Hájková**, J. Hraníček, J. Barek, V. Vyskočil, Voltammetric Determination of Trace Amounts of 2-Aminofluoren-9-one at a Mercury Meniscus Modified Silver Solid Amalgam Electrode. *Electroanalysis*, 25, 1 (2013): 295–302.
- [2] **A. Hájková**, V. Vyskočil, B. Josypčuk, J. Barek, A Miniaturized Electrode System for Voltammetric Determination of Electrochemically Reducible Environmental Pollutants. *Sensor. Actuat. B: Chem.*, 227 (2016): 263–270.
- [3] **A. Hájková**, V. Vyskočil, J. Barek, Combined Miniature Electrode System with Silver Amalgam Electrode. *Czech National Utility Model* (Number 28234) (2015).
- [4] V. Vyskočil, **A. Hájková**, Novel Electrochemical DNA Biosensors as Tools for Investigation and Detection of DNA Damage, in *Trends in Bioelectroanalysis*, ed. F.-M.

- Matysik (Cham: Springer International, 2015): published online (doi:10.1007/11663_2015_5002).
- [5] **A. Hájková**, J. Barek, V. Vyskočil, Voltammetric Determination of 2-Aminofluoren-9-one and Investigation of Its Interaction with DNA on a Glassy Carbon Electrode. *Electroanalysis*, 27, 1 (2015): 101–110.
- [6] **A. Hájková**, V. Vyskočil, J. Barek, Ch. Iffelsberger, F.-M. Matysik, Electrochemical Investigation of DNA Damage Induced by Genotoxic Derivatives of Fluorene: *prepared for submission*.
- [7] **A. Hájková**, J. Barek, V. Vyskočil, Electrochemical DNA Biosensor for Detection of DNA Damage Induced by Hydroxyl Radicals: *prepared for submission*.

This Ph.D. Thesis has been submitted as a contribution to the ever growing efforts of environmental analysis. Currently, apart from the sensitive determination of various xenobiotic compounds, their transformation in the body is also very important and desirable research topic. Xenobiotic compounds can significantly damage biomolecules and thus induced serious diseases. Therefore, the clarification of damaging mechanisms occurring upon the interaction with DNA is highly demanded, too.

Polycyclic aromatic hydrocarbons (PAHs) and their derivatives (nitro, amino, oxo, and hydroxy derivatives) constitute an important class of compounds ubiquitous in both the living and working environment [8]. They are well-known environmental chemical carcinogens and/or mutagens frequently causing all types of pollution (air, water, soil, and sediments) [8,9]. Therefore, analytics and monitoring of environmental pollutants and the development of highly sensitive analytical methods for their determination are among the most demanding tasks in environmental analysis nowadays [10,11]. Electroanalytical methods play a useful role in this field because they have merits of simplicity, rapidity, high sensitivity, good compatibility with biological samples, and they are inexpensive from the point of view of both investment and running costs [9].

Besides PAHs, reactive oxygen species (ROS) also belong among xenobiotic compounds which cause damage to cell structures (including lipids and membranes, proteins and nucleic acids) when their levels increase dramatically [12,13]. This event is called oxidative stress [14,15]. Hydroxyl radicals are one of the most ROS that induce lesions in DNA [15].

The effects of xenobiotic compounds in living organisms may range from mild discomfort to serious diseases such as cancer. Many genetic diseases are caused by relatively small changes in the DNA structure [16]. Detection of specific mutations in DNA sequences and studies of supramolecular interactions of DNA with various dangerous organic compounds are one of the most important research areas of bioanalytical chemistry [17]. A complex diagnostic approach in the detection of DNA damage (introduced by Labuda *et al.* [13,18] and further developed and improved in this Ph.D. Thesis) represents a novel model for *in vitro* classification of potentially genotoxic chemicals (environmental pollutants, pesticides, conventional or newly synthesized drugs, *etc.*). It is based on a set of criteria that determine whether and under which conditions the investigated agent causes damage to DNA. Electrochemical DNA biosensors, realized by immobilizing an oligonucleotide sequence on a suitable electrode surface, are simple to assemble and can provide reliable results in this research [4,17,19,20].

With respect to above mentioned facts, the first aim of this Ph.D. Thesis was the development of sensitive electroanalytical methods for the determination of a selected genotoxic amino and oxo derivative of fluorene, namely **2-aminofluoren-9-one** (2-AFN). 2-AFN was determined at a **mercury meniscus modified silver solid amalgam electrode** (m-AgSAE) [1], which was subsequently used for the development of a **newly designed miniaturized electrode system** (MES) [2,3], and at a **glassy carbon electrode** (GCE) [5].

The second aim of this Ph.D. Thesis was to employ highly sensitive electrochemical techniques for monitoring DNA damage and for characterization of detrimental effects of selected xenobiotic compounds: (i) genotoxic derivatives of fluorene, namely **2-aminofluorene** (2-AF), **2-acetylaminofluorene** (2-AAF), **2,7-diaminofluorene** (2,7-DAF) [6], and 2-AFN [5], and (ii) **hydroxyl radicals** [7]. For this purpose, a novel type of **electrochemical DNA biosensor based on a GCE** (dsDNA/GCE biosensor) was developed, characterized, and successfully applied [4-7].

1.1 References

- [1] **A. Hájková**, J. Hraníček, J. Barek, V. Vyskočil, Voltammetric Determination of Trace Amounts of 2-Aminofluoren-9-one at a Mercury Meniscus Modified Silver Solid Amalgam Electrode. *Electroanalysis*, 25, 1 (2013): 295–302.

- [2] **A. Hájková**, V. Vyskočil, B. Josypčuk, J. Barek, A Miniaturized Electrode System for Voltammetric Determination of Electrochemically Reducible Environmental Pollutants. *Sensor. Actuat. B: Chem.*, 227 (2016): 263–270.
- [3] **A. Hájková**, V. Vyskočil, J. Barek, Combined Miniature Electrode System with Silver Amalgam Electrode. *Czech National Utility Model* (Number 28234) (2015).
- [4] V. Vyskočil, **A. Hájková**, Novel Electrochemical DNA Biosensors as Tools for Investigation and Detection of DNA Damage, in *Trends in Bioelectroanalysis*, ed. F.-M. Matysik (Cham: Springer International, 2015): published online (doi:10.1007/11663_2015_5002).
- [5] **A. Hájková**, J. Barek, V. Vyskočil, Voltammetric Determination of 2-Aminofluoren-9-one and Investigation of Its Interaction with DNA on a Glassy Carbon Electrode. *Electroanalysis*, 27, 1 (2015): 101–110.
- [6] **A. Hájková**, V. Vyskočil, J. Barek, Ch. Iffelsberger, F.-M. Matysik, Electrochemical Investigation of DNA Damage Induced by Genotoxic Derivatives of Fluorene: *prepared for submission*.
- [7] **A. Hájková**, J. Barek, V. Vyskočil, Electrochemical DNA Biosensor for Detection of DNA Damage Induced by Hydroxyl Radicals: *prepared for submission*.
- [8] K. Srogi, Monitoring of Environmental Exposure to Polycyclic Aromatic Hydrocarbons. *Environ. Chem. Lett.*, 5, 4 (2007): 169–195.
- [9] V. Vyskočil, J. Barek, Electroanalysis of Nitro and Amino Derivatives of Polycyclic Aromatic Hydrocarbons. *Curr. Org. Chem.*, 15, 17 (2011): 3059–3076.
- [10] B. A. M. Bandowe, J. Sobocka, W. Wilcke, Oxygen-Containing Polycyclic Aromatic Hydrocarbons (OPAHs) in Urban Soils of Bratislava, Slovakia: Patterns, Relation to PAHs and Vertical Distribution. *Environ. Pollut.*, 159, 2 (2011): 539–549.
- [11] J. Namieśnik, Modern Trends in Monitoring and Analysis of Environmental Pollutants. *Pol. J. Environ. Stud.*, 10, 3 (2001): 127–140.
- [12] A. A. Ensafi, N. Kazemnadi, M. Amini, B. Rezaei, Impedimetric DNA-Biosensor for the Study of Dopamine Induces DNA Damage and Investigation of Inhibitory and Repair Effects of Some Antioxidants. *Bioelectrochemistry*, 104 (2015): 71–78.
- [13] L. Hlavatá, K. Beníková, V. Vyskočil, J. Labuda, Evaluation of Damage to DNA Induced by UV-C Radiation and Chemical Agents Using Electrochemical Biosensor Based on Low Molecular Weight DNA and Screen-Printed Carbon Electrode. *Electrochim. Acta*, 71 (2012): 134–139.
- [14] X. L. Wang, R. Chen, L. Sun, Z. Y. Yu, Study on the Antioxidant Capacities of Four Antioxidants Based on Oxidizing Guanine in a Composite Membrane. *Int. J. Electrochem. Sci.*, 9, 12 (2014): 6834–6842.
- [15] M. F. Barroso, C. Delerue-Matos, M. Oliveira, Electrochemical Evaluation of Total Antioxidant Capacity of Beverages Using a Purine-Biosensor. *Food Chem.*, 132, 2 (2012): 1055–1062.
- [16] V. Vyskočil, J. Labuda, J. Barek, Voltammetric Detection of Damage to DNA Caused by Nitro Derivatives of Fluorene Using an Electrochemical DNA Biosensor. *Anal. Bioanal. Chem.*, 397, 1 (2010): 233–241.
- [17] V. Vyskočil, M. Blašková, **A. Hájková**, E. Horáková, Z. Krejčová, K. Stávková, J. Wang, Electrochemical DNA Biosensors – Useful Diagnostic Tools for the Detection of Damage to DNA

-
- Caused by Organic Xenobiotics, in *Sensing in Electroanalysis*, eds. K. Vytřas, K. Kalcher, I. Švancara (Pardubice: University Press Centre, 2012), 141–162.
- [18] G. Ziyatdinova, J. Labuda, Complex Electrochemical and Impedimetric Evaluation of DNA Damage by Using DNA Biosensor Based on a Carbon Screen-Printed Electrode, *Anal. Methods*, 3, 12 (2011): 2777–2782.
- [19] M. Mascini, I. Palchetti, G. Marrazza, DNA Electrochemical Biosensors. *Fresen. J. Anal. Chem.*, 369, 1 (2001): 15–22.
- [20] J. Labuda, V. Vyskocil, DNA/Electrode Interface, Detection of Damage to DNA Using DNA-Modified Electrodes, in *Encyclopedia of Applied Electrochemistry*, eds. G. Kreysa, K. Ota, R. Savinell, R. (New York: Springer Science+Business Media, 2014), 346–350.

2. XENOBIOTIC COMPOUNDS

2.1 Genotoxic Derivatives of Fluorene

Fluorene is one of the polycyclic aromatic hydrocarbons (PAHs), which is widely distributed in various parts of the environment [1,2]. Fluorene, a tricyclic PAH with two benzene rings fused to a cyclopentane ring, is a typical by-product of coal-conversion and energy-related industries and is commonly found in vehicle exhaust emissions, crude oils, motor oils, coal and oil combustion emissions [2,3]. This xenobiotic compound and its derivatives are the major environmental concern associated with petroleum and oil spills, waste incineration, and industrial effluents. Fluorene and its derivatives have many applications in industry, since they are used as base materials for dyes and optical brightening agents. Some of their characteristics, such as light and temperature sensitivities, heat resistance, conductivity, and corrosion resistance, are used in application in the areas of thermo and light sensitizers, luminescence chemistry, spectrophotometric analysis, and molecular chemistry [3]. Fluorene has posed a great problem for environmental and occupational medicine because significant carcinogenic and genotoxic effects have been associated with its exposure [4-6].

Two amino derivatives of fluorene, belonging to the group of amino polycyclic aromatic hydrocarbons (APAHs), namely **2-aminofluorene** (2-AF) and **2,7-diaminofluorene** (2,7-DAF), one oxygenated amino derivative of polycyclic aromatic hydrocarbons (oxy-APAHs), namely **2-amino-9-fluorenone** (2-AFN), and a metabolite of 2-AF, namely **2-acetylaminofluorene** (2-AAF) (see Fig. 2-1), are investigated in this Ph.D. Thesis. Originally, these compounds were developed as pesticides, but they were never introduced as pesticides because toxicity tests showed that they are potent liver carcinogens [7,8]. Their highly potent carcinogenicity and mutagenicity became the focus of a very large research

effort to uncover the molecular origin of their tumorigenic effect [8,9]. They have become model compounds for genetic toxicity testing [7,9,10].

2-AF is present in synthetic fuels [11] and has been shown to be carcinogenic to liver, urinary bladder, colon, lung, also in circulating leukocytes (lymphocytes and monocytes) and other tissues of variety of species [11-14]. 2-AF should be acetylated by *N*-acetyltransferase to form 2-AAF and then metabolized by host enzymes to initiate carcinogenesis in target tissues [13]. 2-AAF is a liver carcinogen [11,15]. 2-AF and 2-AAF are oxidized by cytochrome P450 to *N*-hydroxy-aminofluorene and *N*-hydroxy-*N*-acetylaminofluorene, which can covalently bind to DNA and thus caused its damage [15].

2,7-DAF is an amino derivative of fluorene, which has two amino group and acts both as photoreductant and as photoinitiator [16]. 2,7-DAF interacts with DNA and is thus classified as a carcinogen and a mutagen [17].

2-AFN is a genotoxic amino and oxo derivative of fluorene [18]. Its occurrence in the environment is associated mainly with processing and purification of the natural gas in gas plants [19,20]. Its fluorescent properties can be used for the investigation of biological environment as a fluorescent probe [21]. The genotoxic effect of 2-AFN was investigated and the formation of a DNA–2-AFN complex was confirmed [9,18].

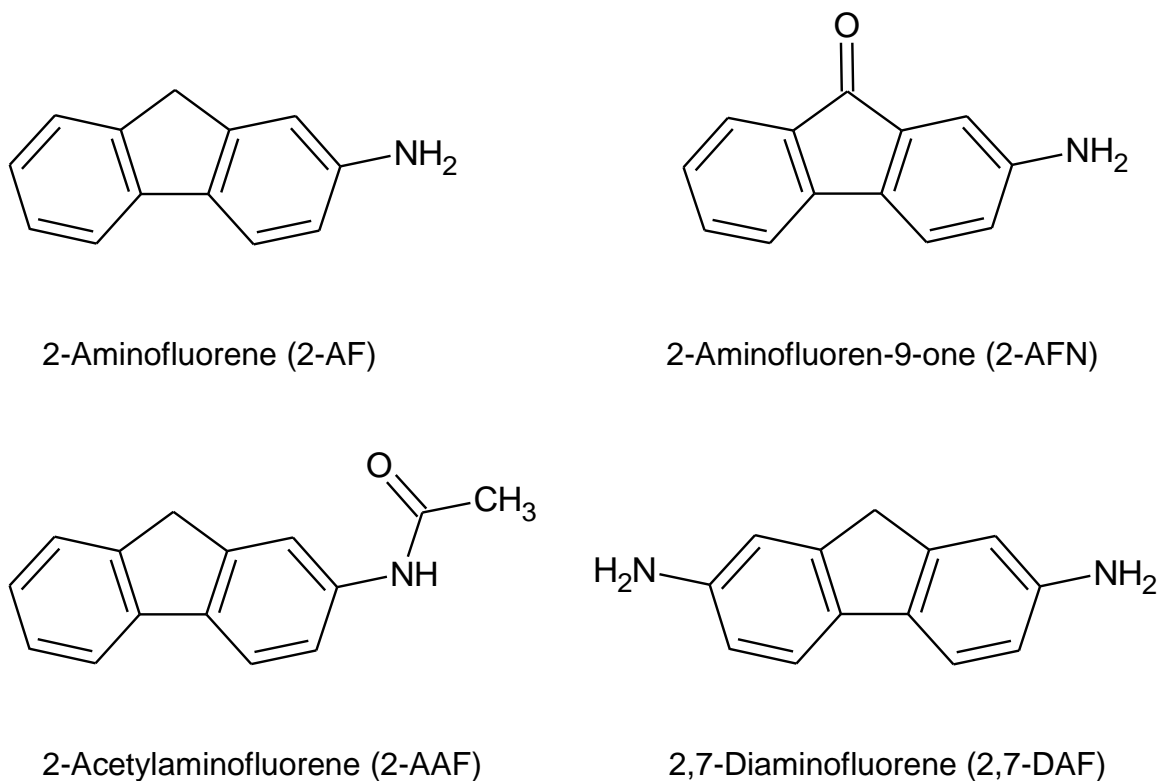


Fig. 2-1 Structural formulae of investigated compounds.

2.2 Reactive Oxygen Species

Reactive oxygen species (ROS), such as superoxide radicals ($O_2^{\bullet-}$), hydroxyl free radicals (OH^{\bullet}), hydrogen peroxide (H_2O_2), and so on, can be generated in organisms via normal aerobic metabolism [22,23] or by exogenous ways (such as by carcinogenic compounds or by ionizing radiations) [24]. On one hand, they can assist the immune system, mediate cell signaling, and be essential in apoptosis. They have important physiological effects on transcription factors and cell proliferation and differentiation [22]. On the other hand, excess ROS are mainly of damage to cell structures (including lipids and membranes, proteins and nucleic acids) [25-27]. In human tissues of patients, they can cause disorders, such as cellular death or aging, and many diseases as arteriosclerosis [22,28], cardiovascular disease, hepatitis, cystic fibrosis, Parkinson's and Alzheimer's diseases, rheumatoid arthritis, brain ischemia, *etc.* [29-31]. The *in vivo* oxidation of DNA has the central role in mutagenesis, carcinogenesis, and various diseases involving inflammatory processes [25,26]. ROS induce the oxidative stress in DNA. Oxidative stress is an imbalance between prooxidant and antioxidant reactions, which causes disruption of their redox mechanisms [29]. These radicals cause oxidation of the DNA bases and deoxyribose, which leads to a release of the bases and an interruption of the phosphodiester bonds. A variety of modifications at DNA level (including base and sugar lesions, strand breaks, DNA-protein cross-link, and base free sites) can be detected [24,28,32].

Hydroxyl radicals are one of the most reactive radical species that induce lesions in DNA [24] by abstracting hydrogen atoms or by direct adduct formation with nucleobases [33]. They can be produced by the Fenton's reaction, UV light, simple ozonation, sonolysis, and other similar processes [30]. They are able to non-selectively destroy most organic and organo-metallic contaminants (giving their dehydrogenated or hydroxylated derivatives) until their complete mineralization, *i.e.*, their conversion into CO_2 , water, and inorganic ions [34].

The superoxide anion radical is the most abundant radical in biological systems resulting from the univalent reduction of oxygen [35]. It is formed in almost all aerobic cells and it is the most abundant species generated by several enzymatic and non-enzymatic pathways in mammalian tissues, leading to unfavorable alteration of biomolecules, including DNA [36].

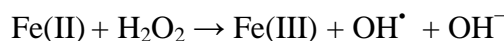
In this Ph.D. Thesis, **hydroxyl radicals** and **superoxide radicals** causing DNA damage are investigated (presented in the Appendix VII) [37]. Hydroxyl radicals were generated

electrochemically on the surface of a boron-doped diamond electrode (BDDE) and chemically (via the Fenton's reaction or the auto-oxidation of Fe(II)).

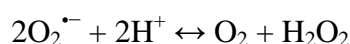
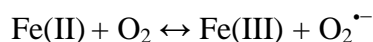
Hydroxyl radicals are generated from water discharge on the BDDE surface in acid, neutral, or alkaline media [23,34] using the electrochemical anodic oxidation process at high potentials applied [38]:



Moreover, hydroxyl radicals can be produced through the Fenton's reaction induced by H_2O_2 and transition metal ions (Fe(II), Cu(II), *etc.*) [27]. It depends on the molar ratio of the Fenton's reagents (Fe: H_2O_2 :EDTA) and on the solution pH. In this reaction, Fe(II) is oxidized to Fe(III) by H_2O_2 and free hydroxyl radicals are produced [24]:



Superoxide radicals were generated from the reaction between Fe(II) and molecular oxygen. It is process of auto-oxidation of Fe(II). Subsequently, the superoxide radicals are dismutated to H_2O_2 which can serve as the precursor of hydroxyl radical in the Fenton's reaction. These reactions are as follows [39,40]:



2.3 References

- [1] M. P. Kolomytseva, D. Randazzo, B. P. Baskunov, A. Scozzafava, F. Briganti, L.A. Golovleva, Role of Surfactants in Optimizing Fluorene Assimilation and Intermediate Formation by *Rhodococcus Rhodochrous* VKM B-2469. *Bioresource Technol.*, 100, 2 (2009): 839–844.
- [2] M. Grifoll, M. Casellas, J. M. Bayona, A. M. Solanas, Isolation and Characterization of a Fluorene-Degrading Bacterium: Identification of Ring Oxidation and Ring Fission Products. *Appl. Environ. Microb.*, 58, 9 (1992): 2910–2917.
- [3] K. Chupungars, P. Rerngsamran, S. Thaniyavarn, Polycyclic Aromatic Hydrocarbons Degradation by *Agroclybe* sp. CU-43 and Its Fluorene Transformation. *Int. Biodeter. Biodegr.*, 63, 1 (2009): 93–99.
- [4] G. Falco, J. L. Domingo, J. M. Llobet, A. Teixido, C. Casas, L. Muller, Polycyclic Aromatic Hydrocarbons in Foods: Human Exposure Through the Diet in Catalonia, Spain. *J. Food Prot.*, 66, 12 (2003): 2325–2331.
- [5] J. Yan, L. Wang, P. P. Fu, H. T. Yu, Photomutagenicity of 16 Polycyclic Aromatic Hydrocarbons from the US EPA Priority Pollutant List. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.*, 557, 1 (2004): 99–108.

- [6] A. C. Olsson, J. Fevotte, T. Fletcher, A. Cassidy, A. 't Mannetje, D. Zaridze, N. Szeszenia-Dabrowska, P. Rudnai, J. Lissowska, E. Fabianova, D. Mates, V. Bencko, L. Foretova, V. Janout, P. Brennan, P. Boffetta, Occupational Exposure to Polycyclic Aromatic Hydrocarbons and Lung Cancer Risk: A Multicenter Study in Europe. *Occup. Environ. Med.*, 67, 2 (2010): 98–103.
- [7] S. Dutta, Y. Li, D. Johnson, L. Dzantiev, C. C. Richardson, L. J. Romano, T. Ellenberger, Crystal Structures of 2-Acetylaminofluorene and 2-Aminofluorene in Complex with T7 DNA Polymerase Reveal Mechanisms of Mutagenesis. *Proc. Natl. Acad. Sci. U.S.A.*, 101, 46 (2004): 16186–16191.
- [8] Z. Gu, A. Gorin, B. E. Hingerty, S. Broyde, D.L. Patel, Solution Structures of Aminofluorene [AF]-Stacked Conformers of the *Syn* [AF]-C₈-dG Adduct Positioned Opposite dC or dA at a Template-Primer Junction. *Biochemistry*, 38, 33 (1999): 10855–10870.
- [9] R. H. Heflich, R. E. Neft, Genetic Toxicity of 2-Acetylaminofluorene, 2-Aminofluorene and Some of Their Metabolites and Model Metabolites. *Mutat. Res-Rev Genet.*, 318, 2 (1994): 73–174.
- [10] N. German, S. Armalis, J. Zima, J. Barek, Voltammetric Determination of Fluoren-9-ol and 2-Acetamidofluorene Using Carbon Paste Electrodes. *Collect. Czech. Chem. Commun.*, 70, 3 (2005): 292–304.
- [11] S. S. Lin, Ch. F. Hung, Ch. C. Ho, Y. H. Liu, H. C. Ho, J. G. Chung, Effects of Ellagic Acid by Oral Administration on *N*-Acetylation and Metabolism of 2-Aminofluorene in Rat Brain Tissues. *Neurochem. Res.*, 25, 11 (2000): 1503–1508.
- [12] P. Adris, K. T. Chung, Metabolic Activation of Bladder Procarcinogens, 2-Aminofluorene, 4-Aminobiphenyl, and Benzidine by *Pseudomonas Aeruginosa* and Other Human Endogenous Bacteria. *Toxicol. In Vitro*, 20, 3 (2006): 367–374.
- [13] K. H. Lu, K. H. Lue, T. C. Hsia, M. C. Chou, K. L. Lin, J. G. Chung, Oral Administration of Paclitaxel Affects the Distribution and Metabolism of 2-Aminofluorene in Various Tissues of Sprague-Dawley Rats. *Phytomedicine*, 12, 8 (2005): 577–587.
- [14] H. M. Kuo, H. J. Ho, P. D. L. Chao, J. G. Chung, Quercetin Glucuronides Inhibited 2-Aminofluorene Acetylation in Human Acute Myeloid HL-60 Leukemia Cells, *Phytomedicine*, 9, 7 (2002): 625-631.
- [15] M. E. Lee, P. Urso, Suppression of T Lymphocyte Proliferation to Antigenic and Mitogenic Stimuli by Benzo(alpha)pyrene and 2-Aminofluorene Metabolites. *Immunopharm. Immunot.*, 29, 3-4 (2007): 425–438.
- [16] L. Balan, M. Jin, J. P. Malval, H. Chaumeil, A. Defoin, L. Vidal, Fabrication of Silver Nanoparticle-Embedded Polymer Promoted by Combined Photochemical Properties of a 2,7-Diaminofluorene Derivative Dye. *Macromolecules*, 41, 23 (2008): 9359–9365.
- [17] S. Armalis, N. Novikova, E. Kubiliene, J. Zima, J. Barek, Voltammetric Determination of 2-Aminofluorene and 2,7-Diaminofluorene Using Carbon Paste Electrode. *Anal. Lett.*, 35, 9 (2002): 1551–1559.
- [18] M. Zeisig, L. Moller, ³²P-HPLC Suitable for Characterization of DNA Adducts Formed *In Vitro* by Polycyclic Aromatic Hydrocarbons and Derivatives. *Carcinogenesis*, 16, 1 (1995): 1–9.
- [19] B. Edler, C. Zwiener, F. H. Frimmel, Particle Beam LC/MS Screening of Polar Compounds of Contaminated Ground Water Samples from a Former Gas Plant. *Fresen. J. Anal. Chem.*, 359, 3 (1997): 288–292.

- [20] P. A. Kosian, E. A. Makynen, P. D. Monson, D. R. Mount, A. Spacie, O. G. Mekenyan, G. T. Ankley, Application of Toxicity-Based Fractionation Techniques and Structure–Activity Relationship Models for the Identification of Phototoxic Polycyclic Aromatic Hydrocarbons in Sediment Pore Water. *Environ. Toxicol. Chem.*, 17, 6 (1998): 1021–1033.
- [21] I. V. Muthu Vijayan Enoch, M. Swaminathan, Fluorimetric Study on Molecular Recognition of β -Cyclodextrin with 2-Amino-9-fluorenone. *J. Fluoresc.*, 16, 4 (2006): 501–510.
- [22] X. L. Wang, R. Chen, L. Sun, Z. Y. Yu, Study on the Antioxidant Capacities of Four Antioxidants Based on Oxidizing Guanine in a Composite Membrane. *Int. J. Electrochem. Sci.*, 9, 12 (2014): 6834–6842.
- [23] S. C. B. Oliveira, A. M. Oliveira-Brett, *In Situ* DNA Oxidative Damage by Electrochemically Generated Hydroxyl Free Radicals on a Boron-Doped Diamond Electrode. *Langmuir*, 28, 10 (2012): 4896–4901.
- [24] M. F. Barroso, C. Delerue-Matos, M. B. P. P. Oliveira, Electrochemical Evaluation of Total Antioxidant Capacity of Beverages Using a Purine-Biosensor. *Food Chem.*, 132, 2 (2012): 1055–1062.
- [25] A. A. Ensafi, N. Kazemnadi, M. Amini, B. Rezaei, Impedimetric DNA-Biosensor for the Study of Dopamine Induces DNA Damage and Investigation of Inhibitory and Repair Effects of Some Antioxidants. *Bioelectrochemistry*, 104 (2015): 71–78.
- [26] A. A. Ensafi, M. Amini, B. Rezaei, Assessment of Genotoxicity of Catecholics Using Impedimetric DNA-Biosensor. *Biosens. Bioelectron.*, 53 (2014): 43–50.
- [27] J. J. Zhang, B. Wang, Y. F. Li, W. L. Jia, H. Cui, H. S. Wang, Electrochemical Study on DNA Damage Based on the Direct Oxidation of 8-Hydroxydeoxyguanosine at an Electrochemically Modified Glassy Carbon Electrode. *Electroanalysis*, 20, 15 (2008): 1684–1689.
- [28] H. Y. Xiong, Y. Wang, X. H. Zhang, Y. Ye, S. F. Wang, Evaluation of Antioxidative Capacity via Measurement of the Damage of DNA Using an Electrochemical Biosensor and an Ionic Liquid Solvent. *Microchim. Acta*, 176, 3-4 (2012): 479–484.
- [29] S. C. B. Oliveira, A. M. Olivieira-Brett, Boron Doped Diamond Electrode Pre-Treatments Effect on the Electrochemical Oxidation of dsDNA, DNA Bases, Nucleotides, Homopolynucleotides and Biomarker 8-Oxoguanine. *J. Electroanal. Chem.*, 648, 1 (2010): 60–66.
- [30] I. Gualandi, D. Tonelli, A New Electrochemical Sensor for OH Radicals Detection. *Talanta*, 115 (2013): 779–786.
- [31] H. Y. Xiong, Y. Chen, X. H. Zhang, H. S. Gu, S. F. Wang, An Electrochemical Biosensor for the Rapid Detection of DNA Damage Induced by Xanthine Oxidase-Catalyzed Fenton Reaction. *Sensor. Actuat. B:Chem.*, 181 (2013): 85–91.
- [32] A. H. Kamel, F. T. C. Moreira, C. Delerue-Matos, M. G. F. Sales, Electrochemical Determination of Antioxidant Capacities in Flavored Waters by Guanosine and Adenine Biosensors. *Biosens. Bioelectron.*, 24, 4 (2008): 591–599.
- [33] A. Baumann, W. Lohmann, S. Jahn, U. Karst, On-Line Electrochemistry/Electrospray Ionization Mass Spectrometry (EC/ESI-MS) for the Generation and Identification of Nucleotide Oxidation Products. *Electroanalysis*, 22, 3 (2010): 286–292.
- [34] A. Hmani, Y. Samet, R. Abdelhedi, Electrochemical Degradation of Auramine-O Dye at Boron-Doped Diamond and Lead Dioxide Electrodes. *Diam. Relat. Mat.*, 30 (2012): 1–8.

-
- [35] M. F. Barroso, C. Delerue-Matosa, M. B. P. P. Oliveira, Electrochemical DNA-Sensor for Evaluation of Total Antioxidant Capacity of Flavours and Flavoured Waters Using Superoxide Radical Damage. *Biosens. Bioelectron.*, 26, 9 (2011): 3748–3754.
- [36] G. Waris, K. Alam, Immunogenicity of Superoxide Radical Modified-DNA: Studies on Induced Antibodies and SLE Anti-DNA Autoantibodies. *Life Sci.*, 75, 22 (2004): 2633–2642.
- [37] **A. Hájková**, J. Barek, V. Vyskočil, Electrochemical DNA Biosensor for Detection of DNA Damage Induced by Hydroxyl Radicals: *prepared for submission*.
- [38] N. Rabaaoui, M. E. K. Saad, Y. Moussaoui, M. S. Allagui, A. Bedoui, E. Elaloui, Anodic Oxidation of *o*-Nitrophenyl on BDD Electrode: Variable Effects and Mechanisms of Degradation. *J. Hazard. Mater.*, 250 (2013): 447–453.
- [39] J. M. Kim, B. N. Oh, J. H. Kim, S. K. Kim, Effect of pH on the Iron Autooxidation Induced DNA Cleavage. *Bull. Korean Chem. Soc.*, 33 (2012): 1290–1296.
- [40] L. A. Loeb, E. A. James, A. M. Waltersdorff, S. J. Klebanoff, Mutagenesis by the Autoxidation of Iron with Isolated DNA. *Proc. Natl. Acad. Sci. U.S.A.*, 85, 11 (1988): 3918–3922.

3. SENSORS AND BIOSENSORS

3.1 Sensors Based on Silver Amalgam

Due to increasing fears of liquid mercury toxicity during the last decades, which has resulted in the “mercuophobia”, a certain attention is paid to the development of working electrodes containing no or minimum amount of liquid mercury [1,2]. More than ten years have elapsed since the introduction of silver solid amalgam electrodes in voltammetric analysis of electrochemically reducible organic compounds [3], which are environmentally friendly in electrochemical analyses. Since their introduction, these electrodes have gone through an extensive development and there exists many types of amalgam electrodes [4]. Solid amalgam electrodes are based on dental amalgam and amalgam prepared from various metals [5]. They can be polished, film modified, meniscus modified, composite, paste with pasting liquid, and paste without pasting liquid [4]. A polished silver solid amalgam electrode (p-AgSAE) and a **mercury meniscus modified silver solid amalgam electrode** (m-AgSAE) were the first variants applicable not only for sensitive voltammetric determinations, but for amperometric detection in flowing systems (HPLC and FIA) as well [4,6]. Subsequently developed types were: polished silver solid amalgam composite electrodes (p-AgSA-CE) [4], which under certain circumstances behave as an array of microelectrodes, silver amalgam paste electrodes with an organic pasting liquid (AgSA-PE) [7], silver amalgam paste electrodes without pasting liquid (AgA-PE) [4]. And very recently developed types were: mercury (MF-AgSAE) and bismuth (BiF-AgSAE) film electrodes utilizing a silver solid amalgam substrate [8] and solid amalgam minielectrodes based on a single silver amalgam crystal (SCAgAE) [5]. Preparation, general characterization [4], and the wide field of analytical applications of these amalgam electrodes has been reviewed [4,9].

In this Ph.D. Thesis, m-AgSAE (Fig. 3-1) was used for sensitive determination of **2-aminofluoren-9-one** (2-AFN) (described in the Appendix I) [10]. A good mechanical stability, simple handling, and regeneration including an electrochemical pretreatment of the

electrode surface are among the main advantages of the m-AgSAE [2,8]. It represents electrochemically the most similar alternative to mercury electrodes due to comparable cathodic potential window, low noise, and relatively high sensitivity [5,11,12]. The additional benefit is its mechanical robustness that allows its application in liquid flow systems [6,11,12]. The main problem connected with the use of any solid electrode, the surface of which is not renewed in the course of voltammetric measurement, is the passivation of the electrode surface by components of the analyzed solution or by products of the electrode reaction. This problem is usually overcome by regular mechanical or electrochemical cleaning of the electrode surface. The mechanical regeneration of the m-AgSAE can be realized by polishing or by covering the electrode surface with a new mercury meniscus (amalgamation). An electrochemical activation of the electrode can be done by imposition of a highly negative potential. The potential -2200 mV for 5 min in 0.2 mol L^{-1} KCl solutions is commonly applied for the m-AgSAE. This procedure is repeated after each amalgamation, before starting the work after every pause longer than one hour or when the performance of the electrode deteriorates. Using the periodically inserted high and low potential steps, the m-AgSAE can be easily and quickly electrochemically regenerated in measured solution between individual measurements. Regeneration lasting about 30 s before each measurement is carried out by periodical switching every 0.1 s between potentials at least 100 mV more negative than the potential of amalgam dissolution ($E_{\text{reg},1}$) and at least 100 mV more positive than the potential of hydrogen evolution ($E_{\text{reg},2}$). The optimum values of $E_{\text{reg},1}$ and $E_{\text{reg},2}$ are usually found experimentally as the values leading to most stable signal values in repeated measurements [7,13,14].

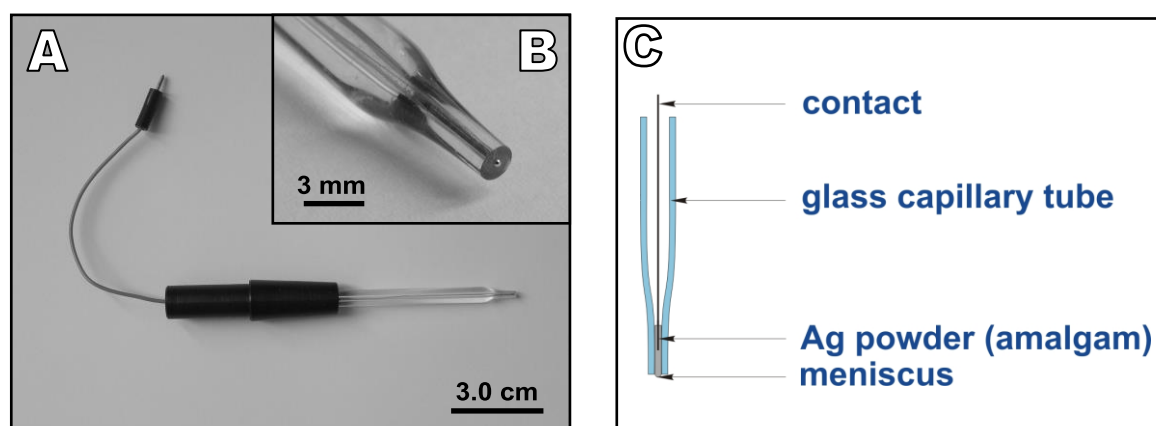


Fig. 3-1 (A) Mercury meniscus modified silver solid amalgam electrode (m-AgSAE), (B) detailed picture of meniscus, and (C) scheme of this electrode.

Attractiveness of electroanalytical techniques consists mainly in variability, simplicity, and rapidity of analysis, high sensitivity, easy automatization, good compatibility with biological samples, and the possibility of miniaturization of instrumentation [14,15]. Due to the unique advantages of microsize, low cost, ease of fabrication, and flexibility in design, miniaturized systems are a highly hot topic and gain maturity in modern analytical chemistry in the latest several years [15,16]. These miniaturized systems can then be used in mobile analytical chemistry, allowing transport of the measuring device itself and execution analysis at the point of sampling or the preparation of the sensor directly into a tissue of a living organism [15]. They have been applied in a variety of areas, including biochemical assays, immunoassays, biosensors, clinical diagnostics, environmental monitoring, biochemistry, proteomics, and cell biology [17,18]. The easy portability of the miniaturized systems and possibility to perform the analysis direct on site (applications outside the laboratory environment) intercept problems with the collection of a sample and its transport to the laboratory, which are time consuming, expensive, and can affect the analysis accuracy, since the analyte speciation can often be changed quickly as a result of chemical, biological, and physical reactions [15,19,20]. Moreover, a low consumption of chemicals and samples is a result of a pointed electrochemical detection in a small volume/space [20,21].

A newly designed miniaturized electrode system (MES) (Fig. 3-2) was introduced and described for the first time in the Appendix II [22]. It is a compact three-electrode system consisting of a silver amalgam working electrode, a silver/silver chloride reference electrode, and a platinum auxiliary electrode. This MES was used for voltammetric determination of electrochemically reducible organic compounds. It has many advantages such as the possibility of simple field measurements without the need to carry fragile electrodes (*e.g.*, the glass ones), its easy portability, when it is connected to a small-size electrochemical analyzer and a notebook or tablet, and the possibility of high-throughput and automatic measurements (using a microtiter plate as a system of electrochemical cells) in small sample volumes (μL).

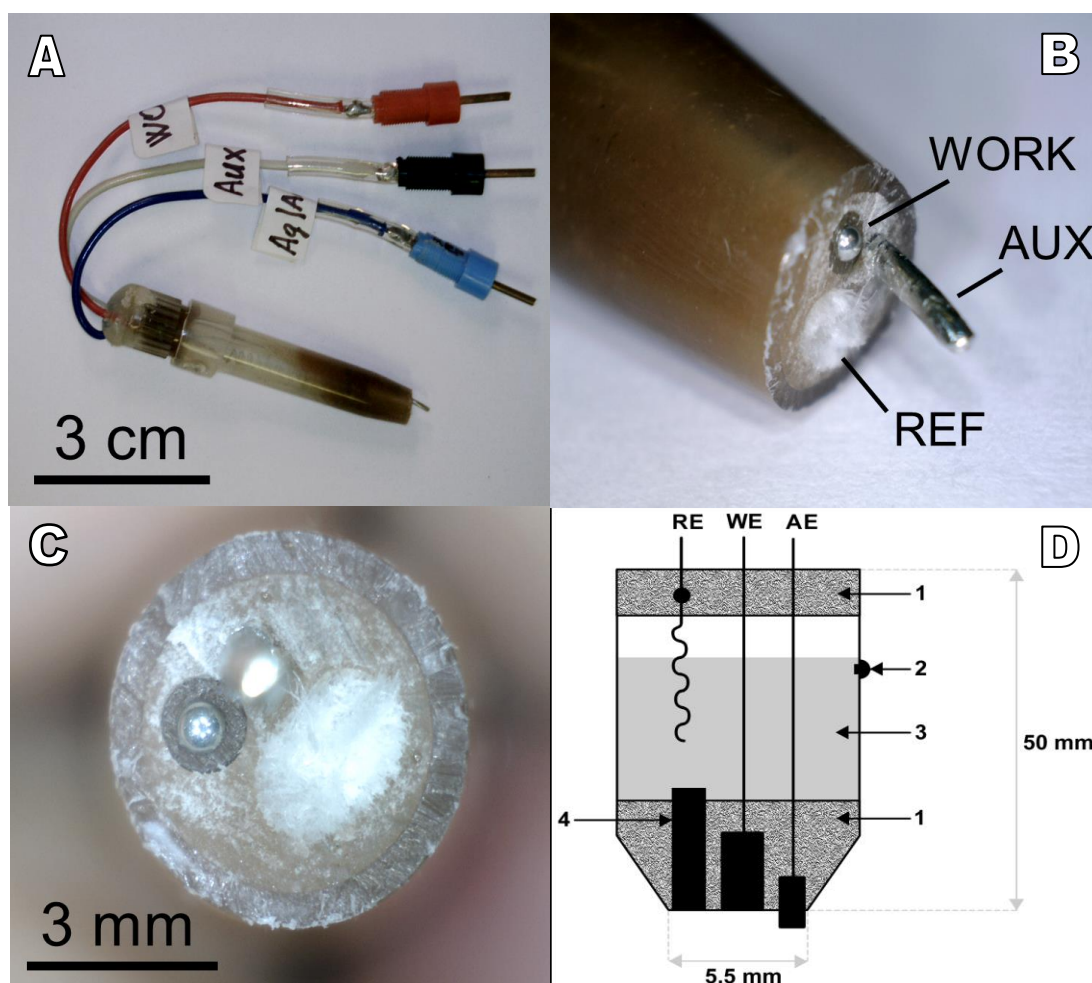


Fig. 3-2 (A) The miniaturized electrode system (MES) with mercury meniscus modified silver solid amalgam electrode, (B) and (C) detailed pictures of three electrodes of the MES, and (D) scheme of the MES.

Following voltammetric techniques have been used in this Ph.D. Thesis: **DC voltammetry** (DCV), **differential pulse voltammetry** (DPV), and **differential pulse adsorptive stripping voltammetry** (DPAdSV). For these techniques, a potentiostat inserts a potential to an electrode and the resulting current flowing through the electrochemical cell is registered. They are active techniques because the applied potential forces a change in the concentration of an electroactive species at the electrode surface by electrochemical reduction or oxidation. Voltammetric techniques include high sensitivity with a very large useful linear concentration range (10^{-12} to 10^{-1} mol L⁻¹). DCV and DPV have limit of detection about 10^{-5} mol L⁻¹ to 10^{-7} mol L⁻¹. DPAdSV belongs to the most sensitive voltammetric technique with limit of detection about 10^{-12} mol L⁻¹ [23,24]. Adsorptive stripping techniques employ

the electrochemical accumulation/preconcentration of the analyte onto the electrode surface and thus become one of the most sensitive electroanalytical techniques [9].

3.2 Sensors and DNA Biosensors Based on Carbon

Solid electrodes based on carbon materials are very popular and commonly used in electroanalysis for their broad potential window. They can be used in both negative and positive potential regions. Their advantages are low background current, low cost, chemical inertness, and suitability for various sensing and detection applications [9,24]. Carbon electrodes are made of various carbonaceous materials such as graphite of spectral purity, glassy carbon, graphite powder with liquid or solid binders, carbon fibers, and highly oriented pyrolytic graphite [23]. Well-known types of carbon electrodes are a **glassy carbon electrode** (GCE) [25,26], a carbon paste electrode (CPE) [27,28], and a carbon film electrode (CFE) (e.g., a microcrystalline natural graphite–polystyrene composite film electrode) [29].

In this Ph.D. Thesis, GCE was used for determination of 2-AFN (presented in the Appendix V) [26]. GCE is frequently used as an inexpensive electrode material with excellent electrical and mechanical properties, wide potential range, extreme chemical inertness, high resistance to acid attack, impermeability to gases and relatively reproducible performance [30]. Possibilities of chemical or biological modification of the GCE are very popular. The modification of the GCE can be, for example, by polymer films [31], cyclodextrins [32], various nanowires [33] and nanoparticles [34]. GCE is a transducer part of many various biosensors (e.g., immunosensors [35], glucose biosensors [36], and DNA biosensor [37]).

Electrochemical DNA biosensors have been successfully used for a number of applications including monitoring DNA damage, studies of the supramolecular interactions of DNA with various organic xenobiotic and genotoxic compounds (carcinogens, mutagens, toxins, drugs, *etc.*), and also for the detection of specific mutations in DNA sequences [38,39]. They are realized by immobilizing an oligonucleotide sequence on a suitable electrode surface. DNA solution can be dropped onto a bare electrode and left to evaporate to dryness for several hours (usually overnight) in order to obtain a DNA layer stable during subsequent use of the biosensor in solution [39]. This limiting step in the preparation of the biosensor can be successfully replaced using a potentiostatic deposition of DNA. The whole preparation of these biosensors takes no more than five minutes, which represents a significant shortening of the preparation time in comparison to procedures making use of an air-drying a DNA solution on an electrode surface [26,37]. Electrochemical DNA biosensors

are fast, simple, and reproducible instruments for measuring the analyte–DNA interactions [29]. They represent an attractive solution in many different fields of applications such as rapid monitoring of pollutant agents or metals in the environment, investigation and evaluation of DNA–drug interaction mechanisms, detection of DNA damage in clinical diagnosis, or detection of specific DNA sequences in human, viral, and bacterial nucleic acids [40,41].

DNA is a very important biomolecule that has an essential role in the life process because it carries hereditary information and instructs the biological synthesis of proteins and enzymes through the process of replication and transcription of genetic information in living cells [40,42,43]. It consists of two antiparallel polynucleotide chains formed by monomeric nucleotide units. Each nucleotide is formed by three types of chemical components: a phosphate group, a sugar called deoxyribose, and four different nitrogen bases. The phosphate-deoxyribose sugar polymer represents the DNA backbone. The cellular genetic information is coded by the purine bases, adenine (A) and guanine (G), and the pyrimidine bases, cytosine (C) and thymine (T), as a function of their consecutive order in the chain. The two strands of nucleotides are twisted into a double helix, held together by hydrogen bonds between the A=T and G≡C bases of each strand [40].

Serious DNA damage can be induced by a variety of physical or chemical agents occurring in the environment or generated in the organisms as by-products of metabolism [44]. Binding of small molecules to DNA occurs primarily in three modes: (i) electrostatic interactions with the negative-charged nucleic sugar–phosphate structure, (ii) binding interactions with two grooves of DNA double helix, and (iii) intercalation of planar condensed aromatic ring systems between the stacked base pairs of native DNA [45,46]. Oxidative attack against DNA is caused by reactive oxygen species (ROS). They oxidize the DNA bases and deoxyribose, which leads to a release of the bases and an interruption of the phosphodiester bonds. A variety of modifications at DNA level, including base and sugar lesions, strand breaks, DNA–protein cross-links, and base free sites, can be detected [47,48]. Damage to DNA may then result in critical disturbances in the cell life, including mutations or malignant transformations, and have serious impacts on the human health [49]. Therefore, efficient techniques to detect DNA damage are sought, among which electroanalytical methods hold an important position [50].

In this work, **an electrochemical DNA biosensor based on the GCE** was prepared by electrochemical adsorption of low-molecular-weight double-stranded DNA (dsDNA) from salmon sperm onto the electrode surface (dsDNA/GCE biosensor) (described in the Appendix

IV and V [26,37]. The dsDNA/GCE biosensor was characterized and used for research of DNA damage caused by xenobiotic compounds (2-AFN [26], 2-aminofluorene (2-AF), 2-acetylaminofluorene (2-AAF), 2,7-diaminofluorene (2,7-DAF) [51], and hydroxyl radicals [52]).

DCV and DPV have been used in this Ph.D. Thesis for determination of 2-AFN in positive and negative potential range on the GCE. In a comprehensive electrochemical biosensing approach, a combination of several electrochemical detection techniques has been applied. The interaction between derivatives of fluorene (2-AFN [26], 2-AF, 2-AAF, and 2,7-DAF [51]) and dsDNA was investigated by DPV performed at the bare GCE when both dsDNA and one of the fluorene derivatives were present in measured solution. At the dsDNA/GCE biosensor, two detection modes were used: (i) direct electrochemical method based on monitoring the oxidation of DNA bases (utilizing square-wave voltammetry (SWV)) and (ii) indirect electrochemical method using the DNA-specific redox active indicator $[\text{Fe}(\text{CN})_6]^{4-/3-}$ (utilizing cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS)) [37,50].

3.3 References

- [1] V. Vyskočil, T. Navrátil, P. Polášková, J. Barek, Voltammetric Determination of Genotoxic Nitro Derivatives of Fluorene and 9-Fluorene Using a Mercury Meniscus Modified Silver Solid Amalgam Electrode. *Electroanalysis*, 22, 17-18 (2010): 2034–2042.
- [2] D. Deýlová, V. Vyskočil, J. Barek, Voltammetric Determination of 2-Amino-6-nitrobenzothiazole at Two Different Silver Amalgam Electrodes. *Electrochim. Acta*, 62 (2012): 335–340.
- [3] L. Novotný, B. Yosypchuk, Solid Silver Amalgam Electrodes. *Chem. Listy*, 94, 12 (2000): 1118–1120.
- [4] B. Yosypchuk, J. Barek, Analytical Applications of Solid and Paste Amalgam Electrodes. *Crit. Rev. Anal. Chem.*, 39 (2009): 189–203.
- [5] A. Daňhel, V. Mansfeldová, P. Janda, V. Vyskočil, J. Barek, Crystalline Silver Amalgam – A Novel Electrode Material. *Analyst*, 136, 18 (2011): 3656–3662.
- [6] A. Daňhel, K. K. Shiu, B. Yosypchuk, J. Barek, K. Pecková, V. Vyskočil, The Use of Silver Solid Amalgam Working Electrode for Determination of Nitrophenols by HPLC with Electrochemical Detection. *Electroanalysis*, 21, 3-5 (2009): 303–308.
- [7] A. Daňhel, B. Yosypchuk, V. Vyskočil, J. Zima, J. Barek, A Novel Paste Electrode Based on a Silver Solid Amalgam and an Organic Pasting Liquid. *J. Electroanal. Chem.*, 656, 1-2 (2011): 218–222.
- [8] D. Deýlová, B. Yosypchuk, V. Vyskočil, J. Barek, Voltammetric Determination of 4-Nitrophenol and 5-Nitrobenzimidazole Using Different Types of Silver Solid Amalgam Electrodes – A Comparative Study. *Electroanalysis*, 23, 7 (2011): 1548–1555.

- [9] J. Barek, J. Fischer, T. Navrátil, K. Pecková, B. Yosypchuk, J. Zima, Nontraditional Electrode Materials in Environmental Analysis of Biologically Active Organic Compounds. *Electroanalysis*, 19, 19-20 (2007): 2003–2014.
- [10] **A. Hájková**, J. Hraníček, J. Barek, V. Vyskočil, Voltammetric Determination of Trace Amounts of 2-Aminofluoren-9-one at a Mercury Meniscus Modified Silver Solid Amalgam Electrode. *Electroanalysis*, 25, 1 (2013): 295–302.
- [11] O. Yosypchuk, J. Karásek, V. Vyskočil, J. Barek, K. Pecková, The Use of Silver Solid Amalgam Electrodes for Voltammetric and Amperometric Determination of Nitrated Polyaromatic Compounds Used as Markers of Incomplete Combustion. *Sci. World J.*, (2012): 1–12.
- [12] B. Yosypchuk, L. Novotný, Electrode of Nontoxic Solid Amalgams for Electrochemical Measurements. *Electroanalysis*, 14, 24 (2002): 1733–1738.
- [13] J. Barek, J. Fischer, T. Navrátil, K. Pecková, B. Yosypchuk, Silver Solid Amalgam Electrodes as Sensors for Chemical Carcinogens. *Sensors*, 6, 4 (2006): 445–452.
- [14] V. Vyskočil, J. Barek, Electroanalysis of Nitro and Amino Derivatives of Polycyclic Aromatic Hydrocarbons. *Curr. Org. Chem.*, 15, 17 (2011): 3059–3076.
- [15] X. L. Xu, S. Zhang, H. Chen, J. L. Kong, Integration of Electrochemistry in Micro-Total Analysis Systems for Biochemical Assays: Recent Developments. *Talanta*, 80, 1 (2009): 8–18.
- [16] R. Szamocki, A. Velichko, C. Holzapfel, F. Mucklich, S. Ravaine, P. Garrigue, N. Sojic, R. Hempelmann, A. Kuhn, Macroporous Ultramicroelectrodes for Improved Electroanalytical Measurements, *Anal. Chem.*, 79, 2 (2007): 533–539.
- [17] P. S. Dittrich, K. Tachikawa, A. Manz, Micro Total Analysis Systems – Latest Achievements and Trends. *Anal. Chem.*, 78, 12 (2006): 3887–3907.
- [18] J. West, M. Becker, S. Tombrink, A. Manz, Micro Total Analysis Systems – Latest Achievements. *Anal. Chem.*, 80, 12 (2008): 4403–4419.
- [19] T. S. Le, P. Da Costa, P. Huguet, P. Sizat, F. Pichot, F. Silva, L. Renaud, M. Cretin, Upstream Microelectrodialysis for Heavy Metals Detection on Boron Doped Diamond. *J. Electroanal. Chem.*, 670 (2012): 50–55.
- [20] A. Jang, Z. W. Zou, K. K. Lee, C. H. Ahn, P. L. Bishop, Potentiometric and Voltammetric Polymer Lab Chip Sensors for Determination of Nitrate, pH and Cd(II) in Water, *Talanta*, 83, 1 (2010): 1–8.
- [21] I. Palchetti, G. Marrazza, M. Mascini, New Procedures to Obtain Electrochemical Sensors for Heavy Metal Detection, *Anal. Lett.*, 34, 6 (2001): 813–824.
- [22] **A. Hájková**, V. Vyskočil, B. Josypčuk, J. Barek, A Miniaturized Electrode System for Voltammetric Determination of Electrochemically Reducible Environmental Pollutants, *Sensor. Actuat. B: Chem.*, 227 (2016): 263–270.
- [23] A. M. Bond, R. G. Compton, D. A. Fiedler, G. Inzelt., H. Kahlert, in *Electroanal. Methods*, ed. F. Scholz (Berlin: Springer International, 2002).
- [24] J. Wang, in *Analytical Electrochemistry*, 3rd ed. (Hoboken: John Wiley & Sons, 2006).
- [25] B. R. Kozub, N. V. Rees, R. G. Compton, Electrochemical Determination of Nitrite at a Bare Glassy Carbon Electrode; Why Chemically Modify Electrodes? *Sensor. Actuat. B: Chem.*, 143, 2 (2010): 539–546.

- [26] **A. Hájková**, J. Barek, V. Vyskočil, Voltammetric Determination of 2-Aminofluoren-9-one and Investigation of Its Interaction with DNA on a Glassy Carbon Electrode. *Electroanalysis*, 27, 1 (2015): 101–110.
- [27] I. Švancara, K. Kalcher, A. Walcarius, K. Vytřas, in *Electroanalysis with Carbon Paste Electrodes* (CRC Press: Taylor & Francis Group, 2012).
- [28] H. Dejmková, L. Houšková, J. Barek, J. Zima, Utilization of Carbon Paste Electrodes for the Voltammetric Determination of Chlortoluron. *Electroanalysis*, 25, 6 (2013): 1529–1534.
- [29] V. Vyskočil, J. Barek, Voltammetric DNA Biosensor Based on a Microcrystalline Natural Graphite–Polystyrene Composite Transducer. *Procedia Chem.*, 6 (2012): 52–59.
- [30] J. Zavázalová, M. E. Ghica, K. Schwarzová-Pecková, J. Barek, Ch. M. A. Brett, Carbon-Based Electrodes for Sensitive Electroanalytical Determination of Aminonaphthalenes. *Electroanalysis*, 27, 7 (2015): 1556–1564.
- [31] P. S. Dorraji, F. Jalali, Differential Pulse Voltammetric Determination of Nanomolar Concentrations of Antiviral Drug Acyclovir at Polymer Film Modified Glassy Carbon Electrode. *Mater. Sci. Eng.*, 61 (2016): 858–864.
- [32] M. Zhang, H. T. Zhao, X. Yang, A. J. Dong, H. Zhang, J. Wang, G. Y. Liu, X. C. Zhai, A Simple and Sensitive Electrochemical Sensor for New Neonicotinoid Insecticide Paichongding in Grain Samples Based on β -Cyclodextrin-Graphene Modified Glassy Carbon Electrode. *Sensor. Actuat. B: Chem.*, 229 (2016): 190–199.
- [33] H. Huang, R. Nie, Y. Song, Y. Ji, R. Guo, Z. Liu, Highly Sensitive Electrochemical Sensor for Tulobuterol Detection Based on Facile Graphene/Au Nanowires Modified Glassy Carbon Electrode. *Sensor. Actuat. B: Chem.*, 230 (2016): 422–426.
- [34] N. S. K. Gowthaman, S. Kesavan, S. A. John, Monitoring Isoniazid Level in Human Fluids in the Presence of Theophylline Using Gold@platinum Core@shell Nanoparticles Modified Glassy Carbon Electrode. *Sensor. Actuat. B: Chem.*, 230 (2016): 157–166.
- [35] M. Zhu, Y. Tang, Q. Wen, J. Li, P. Yang, Dynamic Evaluation of Cell-Secreted Interferon Gamma in Response to Drug Stimulation Via a Sensitive Electro-Chemiluminescence Immunosensor Based on a Glassy Carbon Electrode Modified with Graphene Oxide, Polyaniline Nanofibers, Magnetic Beads, and Gold Nanoparticles. *Microchim. Acta*, 183, 5 (2016): 1739–1748.
- [36] M. Zhen-Zhen, Y Hui-Cheng, W. Zhao-Yang, W. Yan, X. Fu-Bing, A Highly Sensitive Amperometric Glucose Biosensor Based on a Nano-Cube Cu₂O Modified Glassy Carbon Electrode. *Chin. J. Anal. Chem.*, 44, 5 (2016): 822–827.
- [37] V. Vyskočil, **A. Hájková**, Novel Electrochemical DNA Biosensors as Tools for Investigation and Detection of DNA Damage, in *Trends in Bioelectroanalysis*, ed. F.-M. Matysik (Cham: Springer International, 2015): published online (doi:10.1007/11663_2015_5002).
- [38] V. Vyskočil, M. Blašková, **A. Hájková**, E. Horáková, Z. Krejčová, K. Stávková, J. Wang, Electrochemical DNA Biosensors – Useful Diagnostic Tools for the Detection of Damage to DNA Caused by Organic Xenobiotics, in *Sensing in Electroanalysis*, eds. K. Vytřas, K. Kalcher, I. Švancara (Pardubice: University Press Centre, 2012), 141–162.

- [39] V. Vyskočil, J. Labuda, J. Barek, Voltammetric Detection of Damage to DNA Caused by Nitro Derivatives of Fluorene Using an Electrochemical DNA Biosensor. *Anal. Bioanal. Chem.*, 397, 1 (2010): 233–241.
- [40] V. C. Diculescu, A. M. Ch. Paquim, A. M. O. Brett, Electrochemical DNA Sensors for Detection of DNA Damage. *Sensors*, 5, 6-10 (2005): 377–393.
- [41] M. Mascini, I. Palchetti, G. Marrazza, DNA Electrochemical Biosensor. *Fresenius J. Anal. Chem.*, 369, 1 (2001): 15–22.
- [42] S. Rauf, J. J. Gooding, K. Akhtar, M. A. Ghauri, M. Rahman, M. A. Anwar, A. M. Khalid, Electrochemical Approach of Anticancer Drugs–DNA Interaction. *J. Pharmaceut. Biomed.*, 37, 2 (2005): 205–217.
- [43] A. M. Oliveira-Brett, J. A. P. Piedade, L.A. Silva, V. C. Diculescu, Voltammetric Determination of All DNA Nucleotides. *Anal. Biochem.*, 332, 2 (2004): 321–329.
- [44] J. Labuda, V. Vyskočil, DNA/Electrode Interface, Detection of Damage to DNA Using DNA-Modified Electrodes, in *Encyclopedia of Applied Electrochemistry*, eds. G. Kreysa, K. Ota, R. Savinell, R. (New York: Springer Science+Business Media, 2014), 346–350.
- [45] M. Fojta, Electrochemical Sensors for DNA Interactions and Damage. *Electroanalysis*, 14, 21 (2002): 1449–1463.
- [46] L. Wei, J. Borowiec, L. Zhu, J. Zhang, Electrochemical Investigation on the Interaction of Diclofenac with DNA and Its Application to the Construction of a Graphene-Based Biosensor. *J. Solid State Electrochem.*, 16, 12 (2012): 3817–3823.
- [47] M. F. Barroso, C. Delerue-Matos, M. B. P. P. Oliveira, Electrochemical Evaluation of Total Antioxidant Capacity of Beverages Using a Purine-Biosensor. *Food Chem.*, 132, 2 (2012): 1055–1062.
- [48] A. H. Kamel, F. T. C. Moreira, C. Delerue-Matos, M. G. F. Sales, Electrochemical Determination of Antioxidant Capacities in Flavored Waters by Guanine and Adenine Biosensors. *Biosens. Bioelectron.*, 24, 4 (2008): 591–599.
- [49] E. Paleček, M. Fojta, M. Tomschik, J. Wang, Electrochemical Biosensors for DNA Hybridization and DNA Damage. *Biosens. Bioelectron.*, 13, 6 (1998): 621–628.
- [50] M. Fojta, A. Daňhel, L. Havran, V. Vyskočil, Recent Progress in Electrochemical Sensors and Assays for DNA Damage and Repair. *Trends Anal. Chem.*, 79 (2016): 160–167.
- [51] **A. Hájková**, V. Vyskočil, J. Barek, Ch. Iffelsberger, F.-M. Matysik, Electrochemical Investigation of Damage Induced by Genotoxic Derivatives of Fluorene: *prepared for submission*.
- [52] **A. Hájková**, J. Barek, V. Vyskočil, Electrochemical DNA Biosensor for Detection of DNA Damage Induced by Hydroxyl Radicals: *prepared for submission*.

4. RESULTS AND DISCUSSION

4.1 Sensors Based on Silver Solid Amalgam

4.1.1 Mercury Meniscus Modified Silver Solid Amalgam Electrode

Very recently, highly sensitive polarographic and voltammetric methods for the determination of 2-aminofluorene (2-AFN) have been developed (in the framework of my Bachelor and Diploma Thesis projects) using traditional mercury electrodes (namely dropping mercury electrode (DME) and hanging mercury drop minielectrode (HMDmE) [1]. At the DME, there were used direct current fast polarography (DCTP) with the limits of quantification (L_Q) $0.5 \mu\text{mol L}^{-1}$ and differential pulse polarography (DPP) ($L_Q = 0.1 \mu\text{mol L}^{-1}$). Direct current voltammetry (DCV), differential pulse voltammetry (DPV), and differential pulse adsorptive stripping voltammetry (DPAdSV) at a hanging mercury drop electrode (HMDE) were used, too. The L_Q s were $0.2 \mu\text{mol L}^{-1}$ (for DCV), $0.1 \mu\text{mol L}^{-1}$ (for DPV), and $0.004 \mu\text{mol L}^{-1}$ (for DPAdSV) [1,2].

For comparison of electrochemical behavior and determination of 2-AFN at mercury electrodes [1,2], 2-AFN has been also investigated using a mercury meniscus modified silver solid amalgam electrode (m-AgSAE) (attached in the Appendix I) [3]. The m-AgSAE, representing a non-toxic and more mechanically robust alternative to mercury electrodes, was used for sensitive determination of 2-AFN. The electrochemical behavior of 2-AFN was investigated using DCV and DPV. The influence of the pH on the DCV and DPV curves of $100 \mu\text{mol L}^{-1}$ 2-AFN at the m-AgSAE has been examined in a mixture of methanol and the Britton-Robinson (BR) buffer with pH values of 2.0 to 13.0 (1:9, v/v). For 2-AFN, one well-shaped cathodic voltammetric peak was observed in the whole pH range. It corresponds to the two-electron reduction of the carbonyl group to the hydroxyl group [3,4]. The second peak can be observed at more negative potentials. The peak potential (E_p) value of the first peak was shifted towards more negative potentials with increasing pH (in the pH range from 2.0 to

9.0). On the other hand, in the pH range from 10.0 to 13.0, the E_p values were pH-independent. Detailed description of reduction processes at the m-AgSAE and corresponding DC and DP voltammograms are presented in ref. [3]. The polarographic reduction of relatively few fluorene derivatives containing nitro and/or oxo groups has been reported in the literature [5-8].

The development of DC and DP voltammetric methods for the determination of 2-AFN at the m-AgSAE (described in the Appendix I) [3] included optimization of the pH and composition of the supporting electrolyte and further characterization of the analytical parameters (repeatability, linear dynamic range, and L_Q). Optimal medium, consisting of a 1:9, v/v mixture of a methanol and BR buffer pH 4.0, was chosen for determination of 2-AFN because there were the highest and best-developed voltammetric peaks observed. Furthermore, optimum regeneration potentials were found. With regeneration step prior to each measurement, RSD of 20 consecutive DCV and DPV determinations of 2-AFN ($c = 100 \mu\text{mol L}^{-1}$) was found to be 0.48% and 0.93%, respectively. Regeneration potentials $E_{\text{reg},1} = 0 \text{ mV}$, $E_{\text{reg},2} = -1200 \text{ mV}$ were used for determination of 2-AFN for DCV, DPV, and DPAdSV at the m-AgSAE.

Under optimum conditions found, the calibration curves were measured in the concentration range of 0.2–100 $\mu\text{mol L}^{-1}$ (for DCV at the m-AgSAE; $L_Q \approx 0.2 \mu\text{mol L}^{-1}$) and 0.1–100 $\mu\text{mol L}^{-1}$ (for DPV at the m-AgSAE; $L_Q \approx 0.1 \mu\text{mol L}^{-1}$). The lowest L_Q was reached using DPAdSV at the m-AgSAE ($L_Q \approx 0.005 \mu\text{mol L}^{-1}$). Adsorptive accumulation of the analyte on the electrode surface [9] was tested in the optimum medium for the DPAdSV determination of 2-AFN at the m-AgSAE (0.2 mol L^{-1} acetate buffer (AcB) pH 4.0) without presence of methanol which decreased signals of 2-AFN [1]. The optimum potential of accumulation (E_{acc}) 200 mV and optimum time of accumulation (t_{acc}) 3 min were chosen. The calibration curve was constructed for the concentration range from 0.01 to 0.1 $\mu\text{mol L}^{-1}$ of 2-AFN. It is non-linear, therefore, polynomial regression of the second degree was preferably used for this purpose.

The optimum conditions found above for the determination of 2-AFN were used for the direct determination of the substance in model samples of drinking water from the public water line in Prague and river water from the Labe river in Nymburk. The BR buffer pH 4.0 was replaced by the AcB pH 4.0 for simplification. Calibration curves were measured using a mixture of 9.0 mL of a spiked model water sample and 1.0 mL of the AcB pH 4.0 (dilution factor of 0.9). It was found that the calibration curve is linear over the concentration range of 0.1–10 $\mu\text{mol L}^{-1}$ of 2-AFN, with $L_Q \approx 0.2 \mu\text{mol L}^{-1}$ for DCV and $L_Q \approx 0.2 \mu\text{mol L}^{-1}$ for DPV

(for spiked drinking water), and $L_Q \approx 0.4 \mu\text{mol L}^{-1}$ for DCV and $L_Q \approx 0.2 \mu\text{mol L}^{-1}$ for DPV (for spiked river water). DPAdSV at the m-AgSAE was successfully applied not only to the matrix of deionized water, but even also to the matrix of drinking and river water in the concentration range $0.01\text{--}0.1 \mu\text{mol L}^{-1}$ ($L_Q \approx 0.005 \mu\text{mol L}^{-1}$ for drinking water and $L_Q \approx 0.01 \mu\text{mol L}^{-1}$ for river water). There were no interfering substances present in unspiked drinking or river waters giving significant voltammetric response at potentials at which 2-AFN is reduced.

For comparison, the UV spectrophotometric (measured at 274 nm) and fluorimetric (measured at 460 nm, using the excitation wavelength of 314 nm) determination of 2-AFN were carried out in methanol, with L_{QS} of 0.1 and $2 \mu\text{mol L}^{-1}$, respectively. The L_{QS} reached using DCV or DPV at the m-AgSAE are similar to that attained by UV spectrophotometric determination and they are one order of magnitude lower than that attained by fluorimetric determination.

These results confirm that the m-AgSAE is able to efficiently compete with mercury electrodes in the field of ultratrace analysis [3]. Moreover, the sensitivity of the newly developed voltammetric methods can be further increased by a suitable preconcentration technique. Solid phase extraction (SPE) was used for the preconcentration of 2-AFN prior to the voltammetric determination using DPAdSV at the HMDmE [1,2]. Since the results obtained in this work with the m-AgSAE are very similar to those at the HMDmE, it is reasonable to expect that the similar L_Q could be reached when DPAdSV at the m-AgSAE would be preceded by the SPE of 2-AFN.

4.1.2 Miniaturized Electrode System

A newly designed miniaturized electrode system (MES), utilizing a silver solid amalgam electrode which can be either polished or covered by mercury film or mercury meniscus as the working electrode, was constructed for on-site voltammetric determination of electrochemically reducible organic compounds in small sample volumes. The MES is described for the first time in the following scientific paper in the Appendix II [10] and protected by the Czech National Utility Model in the Appendix III [11].

It is a compact three-electrode system consisting of a silver amalgam working electrode, a silver/silver chloride reference electrode, and a platinum auxiliary electrode. The body of the MES was made from the 1 mL micropipette tip. The porous matter (cellulose) ensures electrolytic contact between the inner solution (saturated KCl) and the measured solution. The bottom and upper parts of the body set were sealed by epoxy resin. The MES is designed to

be a stable and mechanically resistant sensor for long-term use. If there are any changes in its electrochemical performance in time (*e.g.*, increasing background current/noise or changing shape/position of the analyte peak), simple regeneration procedures can be performed either with the working or reference electrode, respectively: (i) amalgamation, electrochemical activation, and electrochemical regeneration of the working electrode (the same for m-AgSAE between two regeneration potentials ($E_{\text{reg},1}$ and $E_{\text{reg},2}$)) or (ii) refill of the inner solution (saturated KCl) of the reference electrode.

The main benefits of this prototype are: the possibility of simple field measurements without the need to carry fragile electrodes (*e.g.*, the glass ones), its easy portability when it is connected to a small-size electrochemical analyzer and a notebook or tablet, and the possibility of high-throughput and automatic measurements in microliter sample volumes (using a microtiter plate as a system of electrochemical cells). This MES can be used for the analysis of contaminated waters or soils.

During the development and application of the MES, the problem with oxygen removal during the field measurements has also been solved. The usual way to remove interfering oxygen from the measured solution is bubbling with nitrogen. In the field analyses, the removal of oxygen from the sample solution by bubbling with nitrogen can complicate the measurement. However, this procedure can be replaced by the simple addition of solid Na_2SO_3 to the analyzed sample. Solid Na_2SO_3 is a well-known reducing agent, causing reduction of interfering oxygen to water. A certain disadvantage is that Na_2SO_3 cannot be used in acidic media in which Na_2SO_3 reacts with an acid, liberating gaseous SO_2 , and changing pH. Na_2SO_3 may also react with the analyte, this reaction can cause the decrease of the analyte signal and/or the change in the shape of the voltammetric peak. An advantage is that Na_2SO_3 can be used primarily for field measurements where bubbling with nitrogen is rather difficult. The oxygen removal by bubbling with nitrogen was performed in the case of measurements in the manually adjusted Eppendorf tube, while the addition of solid Na_2SO_3 was used for measurements in the microtiter plate well-based cell (Fig. 4-1) [**10,11**].

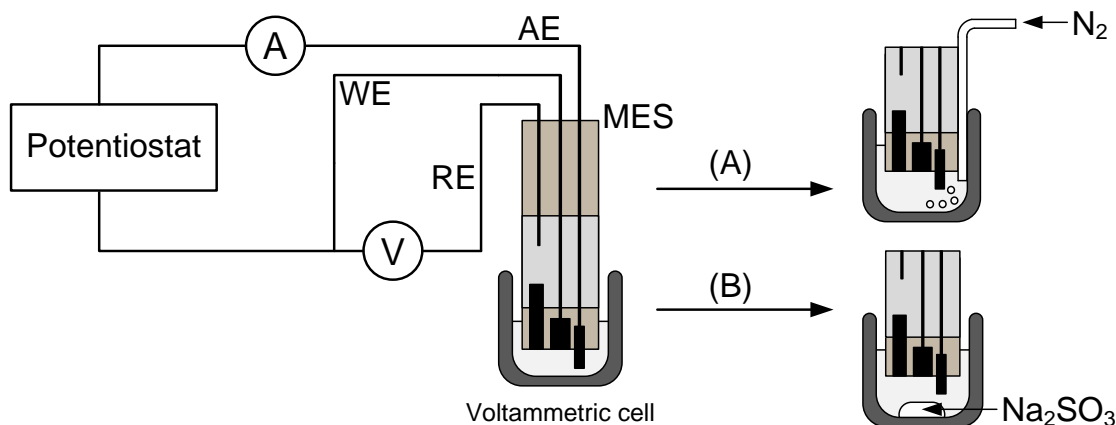


Fig. 4-1 Measurement setup for voltammetric measurements performed using the miniaturized electrode system (MES): (A) in the manually adjusted Eppendorf tube when the oxygen removal by bubbling with nitrogen is applied and (B) in the microtiter plate when the oxygen removal by adding solid Na_2SO_3 is applied. RE – reference silver–silver chloride electrode; WE – working silver solid amalgam electrode; AE – auxiliary platinum electrode.

The determination of 2-AFN was carried out by DPV in 100 μL samples under following conditions, using two different ways of air oxygen removal: (i) a supporting electrolyte methanol–BR buffer pH 4.0 (1:9, v/v), with regeneration potentials $E_{\text{reg},1} = 0$ mV and $E_{\text{reg},2} = -1200$ mV and with oxygen removal by bubbling with nitrogen (the same optimum conditions as in publication dealing with the m-AgSAE (presented in the Appendix I) [3], and (ii) a supporting electrolyte methanol–BR buffer pH 10.0 (1:9, v/v), with regeneration potentials $E_{\text{reg},1} = -200$ mV and $E_{\text{reg},2} = -1600$ mV and with oxygen removal by the addition of solid Na_2SO_3 as a reducing agent. The calibration curves were measured in the concentration range from 1 to 100 $\mu\text{mol L}^{-1}$ of 2-AFN with the $L_Q \approx 0.8$ $\mu\text{mol L}^{-1}$ for oxygen removal by bubbling with nitrogen and $L_Q \approx 1.0$ $\mu\text{mol L}^{-1}$ for oxygen removal by the addition of solid Na_2SO_3 .

The practical applicability of the newly developed DPV using the MES was successfully verified on the direct determination of 2-AFN in model samples of drinking and river water. The concentration range from 1 to 10 $\mu\text{mol L}^{-1}$ was measured in AcB pH 4.0–spiked drinking water (1:9, v/v) ($L_Q \approx 1.0$ $\mu\text{mol L}^{-1}$) and in AcB pH 4–spiked river water (1:9, v/v) ($L_Q \approx 2.0$ $\mu\text{mol L}^{-1}$) for oxygen removal by bubbling with nitrogen. L_Q s obtained in the 0.2 mol L^{-1} borate buffer of pH 10.0 were the same for drinking and river water for oxygen removal by the addition of solid Na_2SO_3 .

An attempt to increase the determination sensitivity using adsorptive accumulation of 2-AFN on the m-AgSAE surface [3] was not successful at the MES [10]. However, the reached L_Q s can be further decreased by the preconcentration of the analyte using the SPE [2].

It was previously verified that the preconcentration of 2-AFN from 100 mL model water samples to 100 μL volumes (with a theoretical preconcentration factor of 1000), which is applicable also for measurements using the MES, allows to reach the L_{QS} of three orders of magnitude lower, *i.e.*, about 1 pmol L^{-1} [10,11].

The MES is predetermined for routine on-site electrochemical analyses. However, it can be used in environmental, clinical, and toxicological laboratories as well, mainly as a substitution of the HMDE. Generally, it can be used for determination of both organic and inorganic electrochemically reducible compounds. To the best of our knowledge, the only one similar concept, which is currently commercially available, can be seen in screen-printed three-electrode assemblies. However, the MES is designed to be a stable and mechanically resistant sensor for long-term use (months or even years). Moreover, silver solid amalgam can be simply replaced within the fabrication of the MES by any solid electrode material (*e.g.*, carbon, platinum, gold, boron-doped diamond, *etc.*) to provide the miniaturized three-electrode system with a tailored working electrode suitable for a specific electroanalytical purpose.

4.2 Sensor Based on Glassy Carbon Electrode

2-AFN contains two electrochemically active functional groups – cathodically reducible carbonyl group and anodically oxidizable amino group. Therefore, it is possible to determine 2-AFN in both the negative and the positive potential regions. A glassy carbon electrode (GCE) has very wide potential window and, therefore, is suitable for investigation of voltammetric behavior of 2-AFN and its determination (presented in the Appendix V) [12].

The electrochemical behavior of 2-AFN was investigated at a bare GCE as a function of pH to provide an overall information regarding electrochemical transformations of 2-AFN in both cathodic and anodic potential regions. In mixtures of methanol and the BR buffer with pH values from 2.0 to 13.0 (1:9, v/v), responses of $100 \text{ }\mu\text{mol L}^{-1}$ 2-AFN were measured using DCV and DPV. In the negative potential region, 2-AFN yielded only one well-developed cathodic voltammetric peak which corresponds to a two-electron transfer (reduction of the carbonyl group to the hydroxyl group) [3,12]. The E_p were shifted towards more negative values with the pH increasing from 2.0 to 7.0. On the other hand, in the pH range from 8.0 to 13.0, the E_p values were practically pH-independent. The highest peaks were obtained for both DCV and DPV in the methanol–BR buffer pH 4.0 (1:9, v/v) medium, which was further [3,10] used for the determination of the analyte in the negative potential. In the positive potential region, 2-AFN gave one well-developed anodic voltammetric peak, corresponding to

the oxidation of the aromatic amino group [13,14], and the E_p were shifted towards more negative values with the increasing pH over the whole pH region. The highest and best-developed DCV and DPV peaks were obtained in the methanol–BR buffer pH 8.0 (1:9, v/v) medium, which was chosen as the optimum one for both DCV and DPV determination of 2-AFN at the GCE in the positive potential region [12].

The GCE surface after repeated measurements is passivated mainly by electrode reaction products. Therefore, the peak current decreased and the peak potentials shifted [15-17]. The repeatability of the cathodic and anodic determination is described in detail in the Appendix V [12]. The results are appropriate for voltammetric determinations at solid electrodes. The best repeatability of the determination was achieved when the GCE was wiped with a microfiber cloth. Thus, this mechanical pretreatment of the GCE surface was further used in the cathodic determination of 2-AFN for both voltammetric techniques after every fifth curve recorded. On the other hand, the repeatability of the anodic determination at the non-pretreated GCE was insufficient because oxidation products strongly passivated the GCE surface. Therefore, the GCE was regenerated mechanically after every curve recorded.

The optimum media for the determination of 2-AFN at the GCE were as follows: methanol–BR buffer pH 4.0 (1:9, v/v) or methanol–BR buffer pH 8.0 (1:9, v/v) for the determination in the negative or positive potential region, respectively. The calibration curves were measured in the concentration ranges of 0.2–100 $\mu\text{mol L}^{-1}$ (for DCV at the GCE; with the $L_Q \approx 0.4 \mu\text{mol L}^{-1}$) and of 0.1–100 $\mu\text{mol L}^{-1}$ (for DPV at the GCE; $L_Q \approx 0.2 \mu\text{mol L}^{-1}$) in the negative potential region. Under the optimal conditions found for positive potential region, the calibration curves were measured for both DCV and DPV in the concentration range from 0.4 to 100 $\mu\text{mol L}^{-1}$, with the L_Q of 0.8 and 0.6 $\mu\text{mol L}^{-1}$, respectively. The determination of 2-AFN using oxidation at the GCE is slightly less sensitive than by reduction [12]. The results of the determination of 2-AFN at the GCE are comparable to those obtained by the same voltammetric methods using the cathodic reduction of the carbonyl group at silver solid amalgam electrodes [3,10,11]. These results for determination of 2-AFN in this Ph.D. Thesis are summarized in Table 1.

The practical applicability of the newly developed voltammetric methods was verified on the direct determination of 2-AFN in model samples of drinking and river water, with the L_Q s in the concentration order of $10^{-7} \text{ mol L}^{-1}$ [12]. They are comparable to those very recently obtained at silver solid amalgam electrodes, too [3,10,11].

Table I

Voltammetric methods for determination of 2-aminofluoren-9-one

Electrode	Technique	Medium Investigated	L_Q ($\mu\text{mol L}^{-1}$)	Ref.
Electrochemical process: reduction				
m-AgSAE	DCV	Methanol–BR buffer pH 4.0 (1:9)	0.2	[4]
m-AgSAE	DPV	Methanol–BR buffer pH 4.0 (1:9)	0.1	[4]
m-AgSAE	DPAdSV	AcB pH 4.0	0.005	[4]
MES	DPV	Methanol–BR buffer pH 4.0 (1:9)	0.8	[10]
MES	DPV	Methanol–BR buffer pH 10.0 (1:9) + Na_2SO_3	1.0	[10]
GCE	DCV	Methanol–BR buffer pH 4.0 (1:9)	0.4	[12]
GCE	DPV	Methanol–BR buffer pH 4.0 (1:9)	0.2	[12]
Electrochemical process: oxidation				
GCE	DCV	Methanol–BR buffer pH 10.0 (1:9)	0.8	[12]
GCE	DPV	Methanol–BR buffer pH 10.0 (1:9)	0.6	[12]

4.3 DNA Biosensor Based on Glassy Carbon Electrode

4.3.1 Preparation of the dsDNA/GCE Biosensor

Electrochemical DNA biosensor on based GCE (dsDNA/GCE) was developed (described in the Appendices IV and VI) [18,19]. This material exhibit several unique properties [20]. The wide electrochemical potential window in the positive direction allows sensitive electrochemical detection of oxidative damage caused to DNA by monitoring the appearance of oxidation peaks of DNA bases [21].

First, a mechanical cleaning and/or an electrochemical activation of the GCE surface were used. Then, adsorptive accumulation was applied for the immobilization of low-molecular-weight double-stranded DNA (dsDNA) from salmon sperm on the surface GCE. The whole preparation of this biosensor (involving removal of the previous dsDNA layer, pretreatment of the regenerated electrode surface, and deposition of the new dsDNA layer) takes no more than 5 min [18,19,22]. This represents a significant shortening of the preparation time in comparison to procedures employing the air-drying of a DNA solution on the electrode surface, which lasts from 6 to 24 hours at room temperature [23,24].

The combination of several electrochemical detection techniques represented a comprehensive electrochemical biosensing approach [25,26]. Direct and indirect electrochemical methods give a description of the preparation dsDNA/GCE biosensor and the detection DNA damage in the positive potential region. Direct electrochemical method based

on the oxidation of dsDNA bases (utilizing the square-wave voltammetry (SWV) technique) and indirect electrochemical methods using the DNA-specific redox active indicator $[\text{Fe}(\text{CN})_6]^{4-/3-}$ (utilizing the cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) techniques) were used [19,27].

For the preparation of the dsDNA/GCE biosensor, the optimum conditions were sought: the dsDNA concentration, the adsorption potential (E_{ads}), and the adsorption time (t_{ads}). The setup of the preparation of the dsDNA/GCE biosensor is described in detail in the Appendix VI [19]. The dsDNA/GCE biosensor was prepared newly after each measurement.

After mechanical cleaning (polishing using the aluminum oxide suspension), it was rinsed thoroughly with deionized water. Then, dsDNA was adsorbed on the GCE – adsorptive accumulation from 0.1 mg mL^{-1} dsDNA in the PB with stirring ($E_{\text{ads}} = 0.5 \text{ V}$, $t_{\text{ads}} = 30 \text{ s}$). The prepared biosensor was washed in the PB for 5 s and SW voltamogram was recorded in the pure PB. There are three oxidation signals of dsDNA bases: p_G – the peak of guanosine, p_A – the peak of adenosine, p_{TC} – the peak of thymidine and cytidine [28]. The relative standard deviations of 15 replicate preparations of the dsDNA/GCE biosensor (measured using guanosine and adenosine SWV peaks) were 5.5% (p_G) and 7.8% (p_A), respectively.

The optimization of the preparation of the dsDNA/GCE biosensor was performed in 0.001 mol L^{-1} hexacyanoferrate/hexacyanoferrite anions ($[\text{Fe}(\text{CN})_6]^{4-/3-}$) in the PB using CV and EIS. The changes in the height of the anodic (I_a) and cathodic (I_c) peaks of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ for CV and the changes in the charge transfer resistance (R_{CT}) for EIS were observed at the bare GCE and at the prepared dsDNA/GCE. Followed by electrochemical activation, the GCE was immersed into $[\text{Fe}(\text{CN})_6]^{4-/3-}$ in the PB and these conditions ($E_{\text{act},1}$ of 1.7 V, $t_{\text{act},1}$ of 60 s, $E_{\text{act},2}$ of -0.5 V , $t_{\text{act},2}$ of 60 s) were applied in stirring solution. Then adsorption of dsDNA on the GCE, adsorptive accumulation from 10 mg mL^{-1} dsDNA in the PB (E_{ads} of DNA of 0.5 V, t_{ads} of DNA of 180 s, stirring), was used. The prepared dsDNA/GCE biosensor was washed in the PB for 5 s and the measurement of CV and EIS were performed in $[\text{Fe}(\text{CN})_6]^{4-/3-}$ in the PB. The relative standard deviations of 10 replicate preparations of the dsDNA/GCE biosensor measured using CV (I_a), CV (I_c), and EIS (R_{CT}) were 4.1%, 5.3%, and 9.8%, respectively [19].

4.3.2 Characterization of the dsDNA/GCE Biosensor

More specific information can then be gained using advanced surface mapping techniques to investigate the biosensor surface. To map the surface of the dsDNA/GCE

biosensor, scanning electrochemical microscopy (SECM), profilometry (laser microscopy), and optical microscopy were used.

Profilometry and optical microscopy monitored roughness and topography of the GCE without and with dsDNA. The layer of dsDNA created spots after drying on the surface of the GCE. Dimensions of spots of dsDNA are from 0.1 to 5 μm . The GCE has a smooth surface, with protrusions to 3 μm and the dsDNA/GCE gives a noisy signal because it contains spots of dsDNA, which get dry on the surface of the GCE [19].

SECM is an attractive technique for investigation of heterogeneity in the electrochemical activity of electrode surfaces [29]. $[\text{Fe}(\text{CN})_6]^{3-}$ as a redox mediator was used for SECM of the bare GCE and the dsDNA/GCE biosensor. The presence of the dsDNA layer on the GCE surface caused a decrease in conductivity of the surface. The GCE surface is blocked by dsDNA, which inhibits the diffusion of $[\text{Fe}(\text{CN})_6]^{3-}$ towards the electrode surface (via repulsions between the negatively charged DNA sugar-phosphate backbone and the negatively charged $[\text{Fe}(\text{CN})_6]^{3-}$ anion) [19]. This research is described in the Appendix VI.

4.3.3 Application of the Prepared dsDNA/GCE Biosensor

4.3.3.1 DNA Damage by Derivatives of Fluorene

DNA-modified electrodes represent suitable tools for the *in vitro* investigation and detection of supramolecular interactions between DNA and studied analytes [30,31].

The prepared dsDNA/GCE biosensor was used for detection of DNA damage by 2-aminofluorene (2-AF), its metabolite 2-acetylaminofluorene (2-AAF), 2,7-diaminofluorene (2,7-DAF), and 2-AFN. All studied derivatives of fluorene (Chapter II) gives oxidation signal or signals because they contain one or two amino groups or an acetyl amino group, which both can be electrochemically oxidized. The application of the prepared dsDNA/GCE biosensor on derivatives of fluorene was performed using SWV (Fig. 4-2) and CV and EIS (Fig. 4-3).

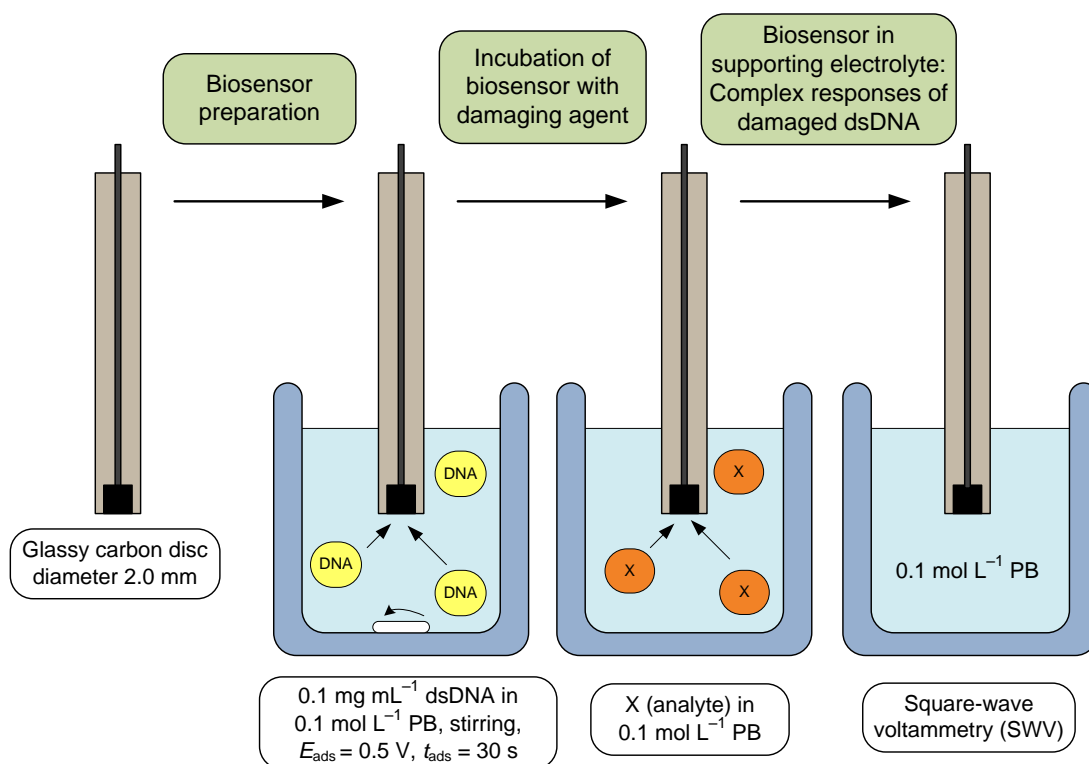


Fig. 4-2 The application of the prepared dsDNA/GCE biosensor on derivatives of fluorene using SWV.

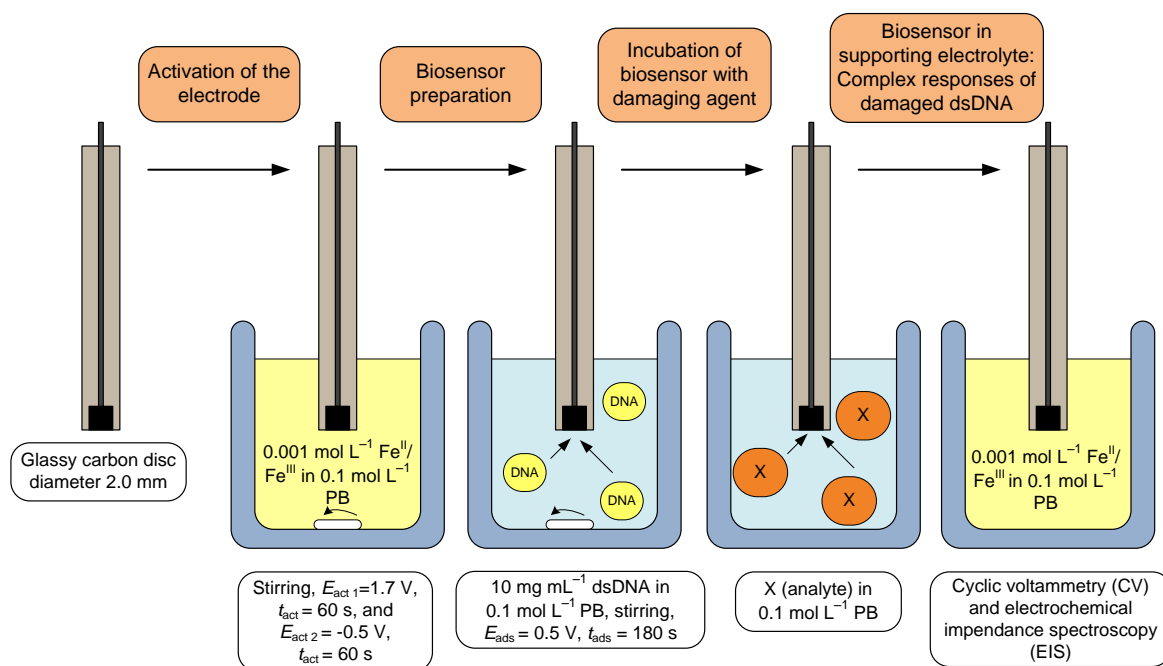


Fig. 4-3 The application of the prepared dsDNA/GCE biosensor on derivatives of fluorene using CV and EIS.

The prepared dsDNA/GCE biosensor was incubated in the solutions of derivatives of fluorene for various times and at various concentrations. SWV was carried out at the

dsDNA/GCE biosensor to monitor the changes in the oxidation signal intensity of dsDNA bases before and after the interaction with derivatives of fluorene. CV and EIS measured the changes in the height of the anodic and cathodic peaks of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ and the changes in the charge transfer resistance.

For SWV, the dsDNA/GCE biosensor was incubated for various times (the incubation lasting for 60, 180, 300, or 600 s) in non-stirred as well as in stirred solution containing $10 \mu\text{mol L}^{-1}$ derivatives of fluorene in the PB. For each incubation time, the dsDNA/GCE biosensor was applied in the pure PB as blank. For 2-AF, the lowest peaks of guanosine and adenosine, representing the highest extent of dsDNA damage, were observed for 300 s in non-stirred incubation solution (35% (p_G), 76% (p_A)). Peaks of guanosine and 2-AAF were not fully separated, therefore, only the peak of adenosine can be used for the research of the dsDNA damage. It decreased to about 52% (for 180 s in non-stirred incubation solution). The peak current of guanosine and adenosine decreased with the increasing incubation time in non-stirred solution for 2,7-DAF (37% (p_G), 63% (p_A)) [19]. For 2-AFN, the incubation time of 180 s was chosen because the peak of p_G was still well separated from the peak of 2-AFN. In non-stirred incubation solution, the decrease was still sufficient 42% (p_G) and 88% (p_A) [12].

The optimum incubation time, which caused the biggest changes in the intensity of the oxidation signals of guanosine and/or adenosine, was used for the experiment investigating the influence of the concentration of derivatives of fluorene (1 to $100 \mu\text{mol L}^{-1}$) in the incubation solution on the extent of dsDNA damage. The higher concentration of the damaging agent caused a deeper degradation of dsDNA.

On the basis of all the results, these selected genotoxic agents interacted with dsDNA and caused damage to the dsDNA structure via formation of double-strand breaks. It can be intercalation of derivatives of fluorene between the dsDNA base pairs. The detailed results of interaction of derivatives of fluorene and dsDNA using dsDNA/GCE biosensor for SWV is described in the Appendices V and VI [12,19].

The anodic (I_a) and cathodic (I_c) peaks of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ were monitored by CV and the charge transfer resistance (R_{CT}) was observed by EIS before and after the interaction the dsDNA/GCE biosensor with one from derivatives of fluorene. The dsDNA/GCE biosensor was incubated for various times (the incubation lasting for 60, 180, 300, or 600 s) in the solution containing $100 \mu\text{mol L}^{-1}$ derivatives of fluorene in the PB in stirred solution. The optimum incubation time was 300 s, which was chosen for all derivatives of fluorene the same for both methods because the higher incubation time did not have better effect to detect

dsDNA damage in any bigger extent. The height of the I_a and I_c peaks (measured using CV) increased up to 25% and 9% for 2-AF, 32% and 21% for 2-AAF, 42% and 10% for 2,7-DAF, and 22% and 12% for 2-AFN. The decrease of the relative biosensor response was 91% for 2-AF, 84% for 2-AAF, 73% for 2,7-DAF, and 78% for 2-AFN (measured using EIS).

For dsDNA damage, the various concentrations of derivatives of fluorene (10, 50, and 100 $\mu\text{mol L}^{-1}$) in the PB were investigated for the incubation time 300 s, with stirring of the incubation solution used. It was found out that derivatives of fluorene exhibited the damaging effect on dsDNA causing the increase of the I_a and I_c peaks of $[\text{Fe}(\text{CN})_6]^{4-/3-}$ because the structure of dsDNA is damaged and nucleic acid fragments probably fall down from the GCE surface. The GCE surface is less blocked and thus inhibits less the $[\text{Fe}(\text{CN})_6]^{4-/3-}$ reduction/oxidation. For EIS, the conductivity of the GCE surface increases with increasing dsDNA damage and the values of R_{CT} decrease. The detailed results obtained within the investigation of the interaction between the derivatives of fluorene and dsDNA (investigated by the dsDNA/GCE biosensor using CV and EIS) are described in the Appendix VI [\[19\]](#).

Measurements at the bare GCE were used to obtain more comprehensive information about dsDNA damage after the interaction with derivatives of fluorene. Firstly, DPV was performed in the solution containing one of the derivatives of fluorene and various concentrations of dsDNA. A given amount of dsDNA (10 μL of 0.1 mg mL^{-1} dsDNA, 10 μL of 10 mg mL^{-1} dsDNA, 500 μL of 10 mg mL^{-1} dsDNA, or 1000 μL of 10 mg mL^{-1} dsDNA) was added to the 10.0 mL solution of 0.1 mmol L^{-1} one of the derivatives of fluorene in the PB. The peak current of the derivatives of fluorene decreased: 2-AF to about 57% and 2-AAF to about 67% (only for small amounts of dsDNA when signal of 2-AAF was separated from the signal of guanosine), the first peak of 2,7-DAF to about 41% , the second peak of 2,7-DAF to about 49%, and 2-AFN to about 73%. Generally, the peak potential of derivatives of fluorene was shifted towards more positive potentials. It can be caused by the formation of a analyte–dsDNA complex which is more difficult to be oxidized than the free form of the analyte. The concentration of the free form is decreasing with the increasing concentration of dsDNA in the measured solution.

Then, DP voltammograms were measured in the solution of dsDNA ($\gamma = 1 \text{ mg mL}^{-1}$) in the PB, with various additions of 1 mmol L^{-1} one of the derivatives of fluorene (10 μL , 100 μL , 500 μL , or 1000 μL) into the 10.0 mL solution of dsDNA at the bare GCE. The peak potentials of guanosine and adenosine units present in the dsDNA structure were shifted towards more positive potentials and their heights decreased with the increasing concentration

of derivatives of fluorene. The detailed results obtained within the investigation of the interaction between the derivatives of fluorene and dsDNA at the bare GCE using DPV is described in the Appendices V and VI [12,19].

The electrochemical behavior of guanosine and adenosine present in dsDNA indicated the formation of the intercalation derivatives of fluorene into dsDNA. The small concentrations of derivatives of fluorene are bounded in the derivatives of fluorene-dsDNA complex and give no DPV peaks at potentials at which free derivatives of fluorene are oxidized, and no significant changes in the original response of dsDNA were visible [12,22].

4.3.3.2 DNA Damage by Hydroxyl Radicals

The prepared dsDNA/GCE biosensor was used for detection of dsDNA damage induced by hydroxyl radicals. After the interaction of the dsDNA/GCE biosensor with hydroxyl radicals, oxidative dsDNA damage was measured using comprehensive electrochemical biosensing approach (detection techniques: SWV, CV, and EIS), too.

Hydroxyl radicals were generated electrochemically on the surface of a boron-doped diamond electrode (BDDE) and chemically (via the Fenton's reaction or the auto-oxidation of Fe(II)). The measurement setup is displayed in Fig. 4-4.

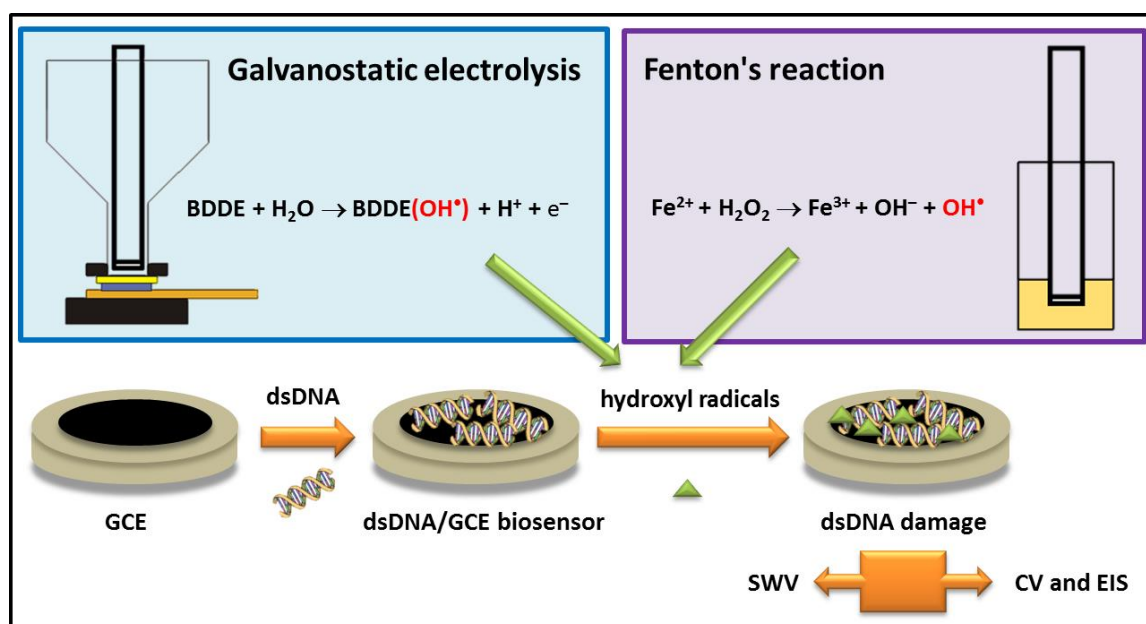


Fig. 4-4 The application of the prepared dsDNA/GCE biosensor on hydroxyl radicals generated on a boron-doped diamond electrode (BDDE) and by the Fenton's reaction.

The BDDE in the form of a plate, which was used for generation of hydroxyl radicals causing oxidative DNA damage, was situated in measurement setup (presented in the Appendix VII) [27]. The prepared dsDNA/GCE biosensor was placed close to the BDDE surface directly against each other (distance of 3 mm). Hydroxyl radicals were generated by the galvanostatic electrolysis on the BDDE [32] in the PB and they migrated in the solution to the dsDNA/GCE biosensor. The current density is an important factor, which corresponds to the ratio between the applied current and the area of the working electrode [33]. Three different current densities ($5\text{--}50\text{ mA cm}^{-2}$) applied for different periods of time were examined.

At SWV, the peak current of guanosine and adenosine decreased with the increasing time of generation of hydroxyl radicals. The higher and more intensive dsDNA damage was observed with increasing current density because more hydroxyl radicals were produced when higher current density was applied. For example, in time 3 min, the peak current of guanosine and adenosine decreased to about 38% (p_G) and 44% (p_A) for the current density of 5 mA cm^{-2} , to about 27% (p_G) and 35% (p_A) for the current density of 10 mA cm^{-2} , and to about 3% (p_G) and 2% (p_A) for the current density of 50 mA cm^{-2} .

At CV, the relative biosensor response (calculated from the values of I_a and I_c) increased with increasing incubation time for the current density 10 mA cm^{-2} . This behavior indicates the formation of a more compact DNA film onto the GCE surface than of the originally adsorbed dsDNA. The electrode surface is more blocked for $[\text{Fe}(\text{CN})_6]^{4-/3-}$. For higher current density of 50 mA cm^{-2} , the situation is dramatically different. First, the relative biosensor response increased during 3 min. For a longer incubation time (30 min), the relative biosensor response decreased to about 60% (I_a) and to about 80% (I_c). Initially, native dsDNA was attacked and oxidized by hydroxyl radicals, which resulted probably in the release of nucleic acid bases and an interruption of the phosphodiester bonds. Damaged dsDNA thus released from the GCE surface.

The relative biosensor response for the current density of 10 mA cm^{-2} (calculated from the R_{CT} value obtained using EIS) increased with the increasing incubation time. It indicates smaller conductivity and higher blocking of the GCE surface. At higher current density (50 mA cm^{-2}), the relative biosensor response increased in time 1 min, but then it decreased with increasing incubation time (to about 30% (R_{CT}) for 30 min). Damaged dsDNA probably fell down from the GCE surface, therefore, the surface had higher conductivity and was accessible for more molecules of $[\text{Fe}(\text{CN})_6]^{4-/3-}$.

In all experiments performed, dsDNA was attacked and oxidized by hydroxyl radicals, which resulted in the release of nucleic acid bases and the interruption of phosphodiester bonds. Consequently, fragments of damaged dsDNA fell down from the GCE surface or they formed a more compact film and thus blocked the GCE surface more intensively. This behavior is described in the Appendix VII [27].

Fenton's reaction is very known source of hydroxyl radicals [34-36]. The optimum molar ratio of Fe(II):EDTA:H₂O₂ for running the Fenton's reaction was found. Hydroxyl radicals were formed by mixing Fe(II):EDTA:H₂O₂ (5:5:50 mmol L⁻¹) in the molar ratio of 1:1:10. The solution with Fenton's reagents was filled up to 10 mL with the 0.1 mol L⁻¹ acetate buffer of pH 4.7. The Fenton's mixture was prepared freshly before each measurement.

The prepared dsDNA/GCE biosensor was immersed into the fresh Fenton's mixture for various incubation times (0.5–30 min) under non-stirred conditions. SW voltammograms were recorded in pure PB. The peak current of guanosine and adenosine decreased with the increasing incubation time. They decreased to about 49% (*p_G*) and to about 28% (*p_A*) after 10 min incubation. This behavior is similar as with hydroxyl radicals generated on the BDDE after application of the small current density.

At CV, the relative biosensor response (calculated from the values of *I_a* and *I_c*) decreased with the increasing incubation time. There was a decrease to about 68% (*I_a*) and to about 74% (*I_c*) for 1 min incubation, to about 32% (*I_a*) and to about 46% (*I_c*) for 5 min incubation, and to about 18% (*I_a*) and to about 35% (*I_c*) for 10 min incubation. On the GCE surface, the more compact DNA film was created. The electrode surface was more blocked for [Fe(CN)₆]⁴⁻³⁻, therefore, *I_a* and *I_c* are lower.

At EIS, the relative biosensor response (calculated from the value of *R_{CT}*) increased with the increasing incubation time. There was an increase to about 139% (*R_{CT}*) for 1 min incubation, to about 299% (*R_{CT}*) for 5 min incubation, and to about 440% (*R_{CT}*) for 10 min incubation. The conductivity of the GCE surface decreased with the increasing extent of dsDNA damage. For longer incubation times, the GCE surface was completely blocked. The compact DNA film was expanded in time and values *R_{CT}* increased.

Moreover, free Fe(II) cations can cause hydroxyl radical formation (via auto-oxidation from Fe(II) to Fe(III)) and thus oxidative dsDNA damage, too. The changes in the intensity of the oxidation signals of dsDNA bases before and after the interaction with solution of Fe(II) in the acetate buffer of pH 4.7 were monitored using SWV. First, the dsDNA/GCE biosensor was immersed into solution containing Fe(II) (*c* = 100 μmol L⁻¹) in incubation time range from 0 to 10 min and SW voltammograms were recorded in pure PB. Then, the dsDNA/GCE

biosensor was incubated in solution of Fe(II) (1 to 1000 $\mu\text{mol L}^{-1}$) for 1 min. After incubation, SWV in pure PB was measured. The peak current of guanosine and adenosine decreased with increasing concentration of Fe(II) in the incubation solution. The detailed results are described in the Appendix VII [27].

This auto-oxidation from Fe(II) to Fe(III) caused oxidative dsDNA damage, but the overall mechanism of iron auto-oxidative DNA cleavage reaction are still not very clear and under debate.

4.4 References

- [1] **A. Hájková**, V. Vyskočil, A. Daňhel, J. Wang, J. Barek, Polarographic and Voltammetric Determination of Genotoxic 2-Aminofluoren-9-one at Mercury Electrodes. *Collect. Czech. Chem. Commun.*, 76, 12 (2011): 1775–1790.
- [2] **A. Hájková**, V. Vyskočil, J. Barek, Voltammetric Determination of Trace Amounts of 2-Aminofluoren-9-one with Preconcentration of the Analyte by Solid-Phase Extraction. *Chem. Listy*, 107, 3, (2013): 234–240.
- [3] **A. Hájková**, J. Hraniček, J. Barek V. Vyskočil, Voltammetric Determination of Trace Amounts of 2-Aminofluoren-9-one at a Mercury Meniscus Modified Silver Solid Amalgam Electrode. *Electroanalysis*, 25, 1 (2013): 295–302.
- [4] J. Grimshaw, in *Organic Electrochemistry*, eds: H. Lund, O. Hammerich (New York: Marcel Dekker, 2001), 411–434.
- [5] M. Ashworth, The Study of a Double-Wave on the Polarographic Curves of Certain Aromatic Ketones and Aldehydes. *Collect. Czech. Chem. Commun.*, 13 (1948): 229–251.
- [6] R. A. Day, J. J. Kirkland, Polarography of Fluorenone, Anthrone and Benzophenone. *J. Am. Chem. Soc.*, 72, 6 (1950): 2766–2767.
- [7] R. A. Day, S. R. Milliken, W. D. Shults, Some Studies of the Polarography of Aromatic Ketones. *J. Am. Chem. Soc.*, 74, 11 (1952): 2741–2744.
- [8] J. T. Gary, R. A. Day, Polarography of Some Aromatic Nitro and Carbonyl Compounds. *J. Electrochem. Soc.*, 107, 7 (1960): 616–618.
- [9] J. Barek, K. Pecková, V. Vyskočil, Adsorptive Stripping Voltammetry of Environmental Carcinogens. *Curr. Anal. Chem.*, 4, 3 (2008): 242–249.
- [10] **A. Hájková**, V. Vyskočil, B. Josypčuk, J. Barek, A Miniaturized Electrode System for Voltammetric Determination of Electrochemically Reducible Environmental Pollutants. *Sensor. Actuat. B: Chem.*, 227 (2016): 263–270.
- [11] **A. Hájková**, V. Vyskočil, J. Barek, Combined Miniature Electrode System with Silver Amalgam Electrode. *Czech National Utility Model* (Number 28234) (2015).

- [12] **A. Hájková**, J. Barek, V. Vyskočil, Voltammetric Determination of 2-Aminofluoren-9-one and Investigation of Its Interaction with DNA on a Glassy Carbon Electrode. *Electroanalysis*, 27, 1 (2015): 101–110.
- [13] V. Vyskočil, J. Barek, Electroanalysis of Nitro and Amino Derivatives of Polycyclic Aromatic Hydrocarbons. *Curr. Org. Chem.*, 15, 17 (2011): 3059–3076.
- [14] E. Steckhan, in *Organic Electrochemistry*, eds: H. Lund, O. Hammerich, (New York: Marcel Dekker, (2001), 545–588.
- [15] M. Pontie, G. Thouand, F. De Nardi, I. Tapsoba, S. Lherbette, Antipassivating Electrochemical Process of Glassy Carbon Electrode (GCE) Dedicated to the Oxidation of Nitrophenol Compound. *Electroanalysis*, 23, 7 (2011): 1579–1584.
- [16] M. Narmadha, M. Noel, V. Suryanarayanan, Relative Deactivation of Boron-Doped Diamond (BDDE) and Glassy Carbon Electrodes in Different Electrolyte Media Containing Substituted Phenols – Voltammetric and Surface Morphologic Studies, *J. Electroanal. Chem.*, 655, 2 (2011): 103–110.
- [17] K. R. Saravanan, S. Sathyamoorthi, D. Velayutham, V. Suryanarayanan, Voltammetric Investigation on the Relative Deactivation of Boron-Doped Diamond, Glassy Carbon and Platinum Electrode During the Anodic Oxidation of Substituted Phenols in Room Temperature Ionic Liquids. *Electrochim. Acta*, 69 (2012): 71–78.
- [18] V. Vyskočil, **A. Hájková**, Novel Electrochemical DNA Biosensors as Tools for Investigation and Detection of DNA Damage, in *Trends in Bioelectroanalysis*, ed. F.-M. Matysik (Cham: Springer International, 2015): published online (doi:10.1007/11663_2015_5002).
- [19] **A. Hájková**, V. Vyskočil, J. Barek, Ch. Iffelsberger, F.-M. Matysik, Electrochemical Investigation of DNA Damage Induced by Genotoxic Derivatives of Fluorene: *prepared for submission*.
- [20] J. Labuda, A. M. Oliveira-Brett, G. Evtugyn, M. Fojta, M. Mascini M, M. Ozsoz, I. Palchetti, E. Paleček, J. Wang, Electrochemical Nucleic Acid-Based Biosensors: Concepts, Terms, and Methodology (IUPAC Technical Report). *Pure Appl. Chem.*, 82, 5 (2010): 1161–1187.
- [21] V. C. Diculescu, A. M. C. Paquim, A. M. Oliveira-Brett, Electrochemical DNA Sensors for Detection of DNA Damage. *Sensors*, 5, 6–10 (2005): 377–393.
- [22] V. Vyskočil, M. Blašková, **A. Hájková**, E. Horáková, Z. Krejčová, K. Stávková, J. Wang, Electrochemical DNA Biosensors – Useful Diagnostic Tools for the Detection of Damage to DNA Caused by Organic Xenobiotics, in *Sensing in Electroanalysis*, eds. K. Vytřas, K. Kalcher, I. Švancara (Pardubice: University Press Centre, 2012), 141–162.
- [23] F. C. Abreu, M. O. F. Goulart, A. M. O. Brett, Detection of the Damage Caused to DNA by Niclosamide Using an Electrochemical DNA-Biosensor. *Biosens. Bioelectron.*, 17, 11–12, (2002): 913–919.
- [24] Y. N. Ni, P. Wang, S. Kokot, Voltammetric Investigation of DNA Damage Induced by Nitrofurazone and Short-Lived Nitro-Radicals with the Use of an Electrochemical DNA Biosensor. *Biosens. Bioelectron.*, 38, 1 (2012): 245–251.
- [25] L. Hlavatá, K. Beníková, V. Vyskočil, J. Labuda, Evaluation of Damage to DNA Induced by UV-C Radiation and Chemical Agents Using Electrochemical Biosensor Based on Low Molecular Weight DNA and Screen-Printed Carbon Electrode. *Electrochim. Acta*, 71 (2012): 134–139.

- [26] G. Ziyatdinova, J. Labuda, Complex Electrochemical and Impedimetric Evaluation of DNA Damage by Using DNA Biosensor Based on a Carbon Screen-Printed Electrode, *Anal. Methods*, 3, 12 (2011): 2777–2782.
- [27] **A. Hájková**, J. Barek, V. Vyskočil, Electrochemical DNA Biosensor for Detection of DNA Damage Induced by Hydroxyl Radicals: *prepared for submission*.
- [28] A. M. Oliveira-Brett, J. A. P. Piedade, L. A. Silva, V. C. Diculescu, Voltammetric Determination of All DNA Nucleotides. *Anal. Biochem.*, 332, 2 (2004): 321–329.
- [29] S. Bergner, P. Vatsyayan, F.-M. Matysik, SECM is an Attractive Technique for Investigation of Heterogeneity in Electrochemical Activity of Electrode Surfaces. *Anal. Chim. Acta*, 775 (2013): 1–13.
- [30] M. Fojta, Detecting DNA Damage with Electrodes, in *Electrochemistry of Nucleic Acids and Proteins*, eds. E. Paleček, F. Scheller, J. Wang (Amsterdam: Elsevier, 2005), 386–431.
- [31] J. Labuda, V. Vyskočil, DNA/Electrode Interface, Detection of Damage to DNA Using DNA-Modified Electrodes, in *Encyclopedia of Applied Electrochemistry*, eds. G. Kreysa, K. Ota, R. Savinell (New York: Springer Science+Business Media, 2014), 346–350.
- [32] S. C. B. Oliveira, A. M. Oliveira-Brett, In Situ DNA Oxidative Damage by Electrochemically Generated Hydroxyl Free Radicals on a Boron-Doped Diamond Electrode, *Langmuir*, 28, 10 (2012): 4896–4901.
- [33] N. Rabaoui, M. E. K. Saad, Y. Moussaoui, M. S. Allagui, A. Bedoui, E. Elaloui, Anodic Oxidation of *o*-Nitrophenol on BDD Electrode: Variable Effects and Mechanisms of Degradation. *J. Hazard. Mater.*, 250 (2013): 447–453.
- [34] M. F. Barroso, C. Delerue-Matos, M. Oliveira, Electrochemical Evaluation of Total Antioxidant Capacity of Beverages Using a Purine-Biosensor, *Food Chem.*, 132, 2 (2012): 1055–1062.
- [35] I. Gualandi, D. Tonelli, A New Electrochemical Sensor for OH Radicals Detection. *Talanta*, 115 (2013) 779–786.
- [36] A. H. Kamel, F. T. C. Moreira, C. Delerue-Matos, M. G. F. Sales, Electrochemical Determination of Antioxidant Capacities in Flavored Waters by Guanine and Adenine Biosensors, *Biosens. Bioelectron.*, 24, 4 (2008): 591–599.

5. CONCLUSION

The presented Ph.D. Thesis represents a contribution to the search for new analytical sensors and electrochemical DNA biosensors. New electrochemical methods for the determination of derivatives of polycyclic aromatic hydrocarbons containing oxo and amino functional groups were developed. They are well-known environmental chemical carcinogens and/or mutagens frequently contaminating water, soil, and sediments. Therefore, the need of continuous monitoring of such environmental pollutants should be raised to the highest priority. They and other xenobiotic compounds cause significant DNA damage and thus induced serious diseases. Therefore, the clarification of damaging mechanisms occurring upon the interaction with DNA is highly demanded, too. This requires development of independent, sensitive, and selective detection techniques and appropriate instrumentation.

2-Aminofluoren-9-one (2-AFN), which is well-known hazardous substance with genotoxic effects and whose occurrence in the environment is associated with the processing and purification of natural gas in gas refineries and with the combustion processes, was selected as a model environmental pollutant. For research on DNA damage, other selected substances were studied: (i) genotoxic derivatives of fluorene – 2-aminofluorene (2-AF), 2-acetylaminofluorene (2-AAF), and 2,7-diaminofluorene (2,7-DAF) and (ii) hydroxyl radicals whose effects in living organisms may range from mild discomfort to serious diseases such as cancer. All the studied xenobiotic compounds are introduced in Chapter 2.

The presented Ph.D. Thesis describes the development and application of a sensor based on silver solid amalgam and its miniaturization and of a sensor and an electrochemical DNA biosensor based on glassy carbon. These newly developed sensors/biosensors are briefly described in Chapter 3.

The obtained results can be summarized as follows:

- Modern voltammetric methods at a mercury meniscus modified silver solid amalgam electrode (m-AgSAE) developed for the determination of trace amounts of 2-AFN in concentration ranges from 0.01 to 100 $\mu\text{mol L}^{-1}$ offer a sensitive, inexpensive, independent, and reliable alternative to more frequently used spectrometric and chromatographic methods.

- The m-AgSAE offers higher stability and easier operation than mercury electrodes. For 2-AFN, the lowest limit of quantification (L_Q) was 0.005 $\mu\text{mol L}^{-1}$, which was reached using differential pulse adsorptive stripping voltammetry (DPAdSV).

- The m-AgSAE represents a versatile sensor offering a variety of applications in voltammetric determination of environmentally significant organic compounds. Therefore, a newly designed miniaturized electrode system (MES) with the m-AgSAE as a working electrode was developed. Its easy portability and possibility of simple field measurements in small sample volumes are big advantages of this system.

- GCE was used for the study of electrochemical behavior and for the sensitive determination of 2-AFN in the negative ($L_Q \approx 0.2 \mu\text{mol L}^{-1}$ for differential pulse voltammetry (DPV)) and in the positive ($L_Q \approx 0.6 \mu\text{mol L}^{-1}$ for DPV) potential range.

- The practical applicability of all the newly developed voltammetric methods (at the m-AgSAE and at the GCE) was verified on the direct determination of 2-AFN in model samples of drinking and river water in submicromolar to micromolar concentrations.

- Electrochemical DNA biosensor based on the GCE (dsDNA/GCE biosensor) was prepared by adsorbing low-molecular-weight double-stranded DNA (dsDNA) from salmon sperm (a biorecognition layer) onto the GCE surface. It was used for the investigation of interactions between dsDNA and genotoxic derivatives of fluorene or hydroxyl radicals.

- DNA damage was observed using a combination of several electrochemical detection techniques (representing a comprehensive electrochemical biosensing approach) at the dsDNA/GCE biosensor: (i) square-wave voltammetry for direct monitoring the oxidation of dsDNA bases, and (ii) cyclic voltammetry and electrochemical impedance spectroscopy as indirect electrochemical methods using the DNA-specific redox active indicator $[\text{Fe}(\text{CN})_6]^{4-/3-}$.

- Damaging effects of derivatives of fluorene on the dsDNA structure were investigated by DPV at a bare GCE, when both dsDNA and one of the fluorene derivatives were present in the measured solution.