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Novel DNA-Biosensors for Studies of GMO, Pesticides and Herbicides

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Additional information is available at the end of the chapter

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1. Introduction

The modern intensive and highly-efficient agricultural technologies impose strong demands on the crop protection against pests and weeds. Among others, there are two main ways, often controversial, to achieve an efficient crop protection: to use the pesticides and herbicides and/or to modify the crop genome so that it can become immune or less susceptible to the invaders' attacks. This approach is widely applied in industrial countries and is gaining popularity in the rest of the world. Due to the unavoidable toxicity of pesticides and herbicides, including possible genotoxicity and carcinogenicity, there is an urgent need to extend a pollution control and to monitor the levels of pesticides and herbicides in ground water and soil. On the other hand, the genetically modified organisms (GMOs) are the products of a new technology and the effects of genome modification on human health are largely unknown. Hence the GMOs are not permitted or are restricted in some countries. In European Union, the content of GMO's in foodstuff is restricted to 0.9%. Since the Roundup Ready corn GMO, for instance, is cultivated in USA already on more than 80% of the fields, the analysis of food products for GMO becomes necessary. In this Chapter, the progress in the development of new inexpensive analytical sensors for pesticides, herbicides, and GMO is presented and discussed in view of the necessity of environmental pollution control, as concerns to pesticides and herbicides, and in view of the GMO content in foodstuff, as required by the mandated restrictions. The common feature of the sensors discussed is the strong dependence of the analytical signal of the sensors on the interaction of DNA molecules immobilized on the sensor surface with the analyte. In the case of GMOs, this provides a straightforward means for biorecognition and very sensitive identification of the GMO genes. In the case of pesticides and herbicides, it enables testing formulations that include adjuvants in addition to the main pesticides or herbicides, and their effect on DNA, thereby probing the affinity of analytes to DNA and possible DNA damage.

2. Effect of pesticides and herbicides on DNA

The pesticides and herbicides are designed to either kill or disable pests and weeds. They often act directly onto the DNA of pests or weeds and while they do not appear to be immediately harmful to larger animals, the evidence suggest teratogenicity and in some cases they clearly exhibit genotoxic and carcinogenic properties.

The pre-mutagenic DNA modifications leading to DNA damage (strand scission, mutations) often begins with nitrogen base oxidation. It has recently been found [1] that catechol-containing compounds in the presence of copper(II) or iron(II) ions, may induce a Fenton cascade leading to reactive oxygen species (ROS) generation which are potent enough to damage DNA. Currently, more than one half of the world production of catechol is consumed by the pesticide and herbicide industry. The oxidative damage to DNA structure is of critical importance for many biological processes, including aging, mutation, and carcinogenesis [1-4]. A number of pesticides and herbicides interact with DNA and can cause DNA damage [5-12].

In this Chapter, we describe the extent of DNA damage done by herbicide paraquat (PQ) and the effect of another herbicide, atrazine (Atz), on DNA helix using electrochemical DNA-biosensor method [4, 11, 13, 14]. Also we present DNA-based piezoelectric sensors developed for the detection of genetically modified foodstuff [15].

Paraquat is a broadly used herbicide which is highly toxic and acts nonselectively. This herbicide can cause fatal intoxication in humans and animals [16] since it targets the dopaminergic neurons. It has been reported that paraquat may also induce neurodegenerative diseases such as Parkinson's disease [17-22]. There are known extreme cases of PQ causing widespread damage to many organs [23, 24]. We have demonstrated [4] that paraquat can initiate the formation of ROS, such as $\text{HO}^\bullet$, $\text{O}_2^{\bullet-}$, in the presence of H_2O_2 and induce DNA damage. PQ-mediated DNA damage was found also by Yamamoto and Mohanan [25] and Ali et al. [24]. Tokunaga et al. [26] have reported that paraquat caused oxidation of guanine and increased the 8-hydroxy-deoxyguanosine amount in heart, brain, and lung. Schmuck et al. [27] have shown that PQ can induce an oxidative stress in rat cortical neurons and astrocytes in vitro, leading to the dopaminergic cell death in the nigrostriatum.

Atrazine is another commonly applied herbicide. It is known as an inhibitor of photosystem II (PSII) in plants [28]. Several studies indicate atrazine genotoxicity [7, 8, 29-32]. Because of this, its use has been regulated in many countries [9]. Other studies have shown no toxicity of pure atrazine. There is a growing consensus that atrazine alone may act as a sensitizer [11] increasing the DNA susceptibility to a damage inflicted by various adjuvants of herbicide preparations which then become cytotoxic [32].

3. Biosensors for pesticide/herbicide pollution monitoring and screening of their interaction with DNA

The extensive use of highly toxic herbicides and pesticides and serious risk for the environment and human health compel the development of pollution control. The Food and Agri-

culture Organization (FAO) and the World Health Organization (WHO) have established maximum residue limits for pesticides in food [33-36].

The standard laboratory procedures for routine analysis of pesticides and herbicides are based on gas chromatography, mass spectrometry and high-performance liquid chromatography (HPLC). The application of these methods requires sophisticated and costly instrumentation, laborious sample preparation, and highly trained personnel. Therefore, there is a pressing necessity for the advancement of new analytical platforms able to assist in the rapid and inexpensive field-deployable testing. It becomes apparent that biorecognition-inspired biosensors based on DNA, antibody, and whole cells can fill the gap:

- i. *Electrochemical DNA-biosensors.* In Hepel's laboratory, the interactions of herbicides and pesticides with DNA have recently been widely studied [11, 12], including among herbicides: atrazine (Atz), paraquat (PQ), glufosinate ammonium (GA), and 2,4-dichlorophenoxyacetic acid [2,4-D], and among pesticides: diflubenzuron (DFB), carbofuran (CF), paraoxon-ethyl (PE). The DNA electrochemical biosensor was also used by Mascini to the determination of intercalating and groove-binding drugs and pollutants including daunomycin, polychlorinated biphenyls (PCBs), aflatoxin B1, cisplatin, atrazine, and hydrazine [37]. Moreover, an electrochemical DNA-biosensor has been used to investigate the interactions between DNA and derivatives of 1,3,5-triazine herbicides [38].
- ii. *Piezoelectrochemical biosensors.* The next kind of biosensors used for highly-sensitive, quantitative detection of herbicides has been the antibody based quartz crystal nanobalance biosensor. Halamek et al. [39] have developed a piezoelectric immunosensor for the detection of 2,4-dichlorophenoxyacetic acid [2,4-D] and Pribyl et al. [40, 41] for the detection of atrazine and polychlorinated biphenyls (PCB).
- iii. *Cell-based biosensors.* Immensely significant in the determination of environmental pollutants, toxic chemicals, pesticides or water quality assessment has been the development of cell-based biosensors. These excellent analytical tools based mainly on bacteria, algae or yeast have been widely investigated in recent years [42-46]. Li and coworkers have described fluorescence and bioluminescence bacterial biosensors for the determination of petroleum products such as benzene, toluene, ethylbenzene, and xylenes (BTEX) in groundwater and soil samples as an alternative to conventional HPLC and GC-MS methods of BTEX measurement [47]. Naessens et al. [28] have developed a new algal-based fluorescence biosensor for the detection of inhibitor of photosystem II (PSII) herbicides: atrazine, diuron and simazine with the detection limit of 0.1 µg/L.

4. GMO alternative to pesticides and herbicides

Area of the genetically modified cultivations including: soybean, maize, cotton, and rapeseed increases in recent years and more and more of genetically modified plants or their de-

rivatives are involved into the food industry. Mainly, a soybean and a maize are used in the production of food and feed. Many countries, such as Korea, Japan, and Australia, have developed laws controlling the marketing of the GMOs. The European Union (EU) and Polish legislation (EC Directive 18/2001, 1829/2003, 1830/2003, 1946/2003] imposes a duty to control GMO by qualitative and quantitative assays. The food products and ingredients, containing transgenic material in percentage higher than 0.9 %, 3%, and 5%, have to be labeled in EU, Korea, and Japan, respectively. However, the labeling of GM foods is not compulsory in the United States and Canada. The genetic modification confers plants novel characteristics which improve their agronomic properties (eg. response to herbicides), quality (taste, maturation, shelf life, color), and pest resistance, including viral, fungal, insect, and parasite resistance. The most common of the genetically modified plants inoculated against herbicide glyphosate are Roundup Ready soybean, maize, and cotton. The Roundup Ready genetic insert contains a portion of the cauliflower mosaic virus (CaMV) 35 promoter, the *Petunia* hybrid 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), chloroplast transit peptide (CTP), the CP4 EPSPS coding sequence, and a portion of the 3' non-translated region of the nopaline synthase gene terminator (NOS) [48]. The microbial CP4 gene introduces a glyphosate resistance to the plants. Glyphosate is a broad-spectrum herbicide which controls plants by inhibiting enzyme EPSPS, an essential enzyme in the shikimate pathway which plays a role in the biosynthesis of aromatic amino acids, used in the protein synthesis, cell wall formation, and pathogen defense. Glyphosate is toxic to plants because it prevents the production of tryptophan, tyrosine, and phenylalanine.

5. GMO detection technologies

Presently, the main assays used for GMO detection are DNA- and protein-based methods. In these methods, the genetic modification such as the inserted/altered gene is detected or the product resulting from the genetic modification is identified. DNA-based detection method relies on the inherent ability of the complementary strands to form a double-helix of a double-stranded DNA and may utilize either a Southern Blot or a Polymerase Chain Reaction (PCR) technique [49-55]. For GMO quantification, the Real-Time PCR is used [56, 57]. The methods based on liquid chromatography - mass spectrometry (LC-MS) enable precise analytical measurements. The protein-based detection method relies on finding proteins coded by the transgene. The following techniques are used for the determination of proteins from GMOs: one- and two-dimensional gel electrophoresis, Western Blot, Enzyme Linked Immunosorbent Assay (ELISA) and the lateral flow strip [54, 55]. Rogan et al. have employed immunological methods to measure the 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) protein derived from the *Agrobacterium* sp. strain CP4 in the major processed fractions derived from Roundup Ready soybean [58]. All of these methods are very sensitive but costly, requiring very expensive equipment and reagents, and highly experienced personnel. Therefore, the mandated monitoring of GMOs on the market calls for the development of new, fast, and inexpensive analytical biosensing platforms enabling field-testing of crop, foods and feeds.

Recently investigated biosensors based on DNA hybridization [2] may become a viable alternative. The DNA-biosensors are relatively cheap and easy to use. These devices are used in many fields of research including clinical, environmental, and food industry and many reviews evaluating the progress in DNA-biosensors have been published [59-62]. Some of the electrochemical sensors have utilized inherent electroactivity of nucleic acid bases which undergo electrooxidation processes at carbon and mercury electrodes [63-66]. In others, changes in peak current or potential for redox-active probe-molecules, which selectively bind to DNA grooves, electrostatically interact with negative chain, or intercalate into dsDNA helix, are monitored [67-69].

The electrochemical methods of GMO detection based on DNA biosensors have been utilized in the investigations of the Filipiak's group [70-72]. The Authors have been able to detect a specific *bar* gene coding for the resistance to herbicide, phosphinotricin, by using the electrochemical hybridization indicator – [2,2'-bipyridyl)cobalt(III) [68]. This indicator intercalates into the double-stranded DNA after the completion of hybridization of the immobilized 19-mer or 21-mer single-stranded probe DNA (pDNA) with target-gene single-stranded DNA (tDNA). The cathodic signal of Co(bpy)_3^{3+} was significantly higher after the formation of a DNA double-helix from the *bar* gene target and pDNA. Also, the Authors have detected genetically modified plants with a transgenic coding for resistance to kanamycin (*nptII*). In their investigations, they used an organic dye, methylene blue (MB), which shows considerable affinity toward guanine bases in DNA. After the interaction of the probe with complementary target sequence of *nptII* gene, the electrochemical signal of this indicator has decreased. Meric et al. have been able to detect the most common insert in GMOs, nopaline synthase terminator (NOS) using DNA biosensor [67]. The Authors have based their investigations on an MB intercalator probe as the hybridization indicator. They tested their sensor with short synthetic oligonucleotides and DNA fragments obtained by PCR amplification. We have investigated the dye Nile Blue, used in DNA staining in carcinoma-cell tumors [73], to evaluate the effect of herbicides and pesticides on DNA [13], [14]. The next kinds of biosensors which are promising for the determination of GMOs are based on the surface plasmon resonance and piezoelectric sensors. The Mascini's group has investigated the promoter [35S) and the terminator elements (T-NOS), which are widely used for the production of many transgenic commercially available vegetables. They have performed a hybridization study using short-oligonucleotide of DNA samples isolated from certified reference materials (CRM), soybean powder, real samples of different dietetic products, which were amplified by PCR, as well as the genomic and plasmidic DNA samples non-amplified by PCR [74-76]. Stobiecka et al. [15], have designed a DNA hybridization biosensor for the determination of genetically modified soybean Roundup Ready using a chemically-modified gold piezoelectrode with single-stranded probe DNA immobilized in sensory films using avidin-biotin binding system.

6. Materials and methods

6.1. Chemicals

All chemicals used for investigations were of analytical grade purity. Avidin, 6-mercapto-1-hexanol (MCH), N-[2-hydroxyethyl]piperazine – N'-[2-ethanesulfonic acid] (HEPES),

$K_3[Fe(CN)_6]$, paraquat (PQ), redox active dye Nile Blue A (NB), mercaptopropionic acid (MPA), N-[3-dimethylaminopropyl]-N'-ethylcarbodiimide (EDC), and ethanolamine were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A. or Poznań, Poland). Atrazine was purchased from Supelco (Bellefonte, PA, U.S.A.). The short synthetic oligonucleotides used in investigations with atrazine and paraquat were obtained from Eurofins MWG/Operon (Huntsville, AL, U.S.A.). Synthetic biotinylated oligodeoxynucleotides used as a probe to the detection of the genetically modified soybean and unbiotinylated oligodeoxynucleotides complementary or noncomplementary to probes were synthesized in the Laboratory of DNA Sequencing and Oligonucleotides Synthesis, IBB PAS, Poland. Samples of fragments of DNA amplified by PCR and genomic DNA were prepared in the Genetic Modifications Analysis Laboratory, IBB PAS, Poland. 3,3'-dithiodipropionic acid di(N-succinimidyl ester) was from Fluka-Sigma-Aldrich (Poznań, Poland). Aqueous solutions were prepared using Millipore Milli-Q deionized water (conductivity $\sigma = 55$ nS/cm) (Billerica, MA, U.S.A.) or Simplicity® 185 Water System (Molsheim, France).

6.2. Quartz crystal nanobalance measurements

For nanogravimetric measurements, a Model EQCN-700 Electrochemical Quartz Crystal Nanobalance from Elchema (Potsdam, NY, U.S.A.) and a Model CHI-410 Time-Resolved Electrochemical Quartz Crystal Microbalance (CH Instruments, U.S.A.) were used. Quartz crystals coated with gold on both sides with resonant frequency of 9.975 MHz or 7.995 MHz were used as the substrates for working electrodes, and were obtained from Elchema or CH Instruments, respectively. The geometric surface area of the working electrode was 0.1963 cm^2 and the apparent-mass changes Δm were related to the fundamental frequency shift Δf using the equation: $\Delta f = 0.8673 \Delta m$ or $\Delta f = 1.34 \Delta m$ ng, respectively, based on Sauerbrey equation [77-80]:

$$\Delta f = - \frac{2 \Delta m n f_0^2}{A \sqrt{\mu_q d_q}} \quad (1)$$

where, Δf is the change in the resonant oscillation frequency, Δm is the change in the interfacial mass, A is the piezoelectrically active area, n is the overtone number and f_0 is the fundamental frequency which depends on the quartz properties (density, $d_q = 2.648 \text{ g cm}^{-3}$, and shear modulus, $\mu_q = 2.947 \times 10^{11} \text{ g cm}^{-1} \text{ s}^{-2}$) and resonator thickness (here: 0.166 mm). All experimental variables influencing the resonant frequency [77] of the EQCN electrodes such as the temperature, pressure, viscosity and density of the solution, were kept constant in the apparent mass change measurements.

6.3. Electrochemical measurements

Cyclic voltammetric (CV) measurements were performed with a standard electrochemical setup - a Potentiostat/Galvanostat Model PS-205B with a Data Logger and Control System, Model DAQ-716v, operating under Voltscan 5.0 data acquisition and processing software from Elchema (Potsdam, NY, U.S.A.) or with the Time-Resolved Electrochemical Quartz Crystal Microbalance (CH Instruments, U.S.A.). Potentials were measured versus the double-junction saturated Ag/AgCl reference electrode. As the working electrodes, gold disk

electrodes with an area of 1 mm², gold coated quartz crystal piezoresonators with a real surface area of 0.264 cm² ($f_0 = 9.975$ MHz) obtained from Elchema, and quartz crystals coated with gold on both sides ($f_0 = 7.995$ MHz) obtained from CH Instruments, were used. A platinum wire was used as the counter electrode. First, the surfaces of gold electrodes were polished on a flat pad with two kinds of alumina, 0.3 and 0.05 μm dia., in wet alumina slurry (Coating Service Department, Indianapolis, U.S.A.). Next, the electrodes were cleaned electrochemically in deoxygenated solutions of 1M KOH and 0.1 M H₂SO₄ until the cyclic voltammograms showed no further change. The solutions were deoxygenated by purging with argon. Quartz crystal piezoelectrodes were also cleaned electrochemically.

6.4. Molecular dynamic simulations and quantum mechanical calculations

The molecular dynamics (MD) simulations and quantum mechanical calculation (QC) of electronic structure for a model DNA molecule and herbicides for the analysis of interactions of atrazine and paraquat with DNA, were performed using procedures embedded in Wavefunction Spartan 6 (Irvine, CA, U.S.A.).

7. Design of DNA-based biosensors

In Figure 1, a schematic of the biosensor films with DNA immobilized on a gold electrode is presented for:

- a. Au/MPA/dsDNA_{20-bp} film and
- b. Au/DASE/avidin/RR-gene oligonucleotide film,

where the basal self-assembled monolayer (SAM) film is composed of either the mercaptopropionic acid (MPA) or the 3,3'-dithiodipropionic acid di-(N-succinimidyl ester) (DASE).

In the first kind of biosensor (a), a clean gold electrode was modified with 10 mM mercaptopropionic acid for 1 h. After the activation of carboxyl group in MPA with a 0.1 M N-[3-Dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) solution, the sensor was incubated for 1h with a NH₂-modified oligonucleotide probe 5'NH₂C₆H₁₂-ATTTCGACAGGGATAGTTC-GA3' with final concentration of 1 μM , to attach it to the thiol film. The hybridization process was performed by injecting 1 μM (final concentration) solution of complementary oligonucleotide 5'TCGAACTATCCCTGTCTGAAT3'to PBS solution (pH = 7.4) for 1h. The DNA biosensor prepared in this way was rinsed with 0.02 M PBS buffer (pH = 7.4) and used for testing of DNA damage caused by two herbicides: paraquat (PQ) and atrazine (Atz) using a redox dye Nile Blue (NB) as the probe intercalator and marker of DNA damage. After the interactions of ds DNA helix with herbicides, the DNA based biosensors were discarded and a new modification of gold piezoelectrodes was prepared for next experiments due to the expected DNA damage by atrazine and paraquat. The reproducibility of subsequent modifications was very good (ca. 5 %).

In the second kind of a DNA biosensor (b), a clean gold piezoelectrode was modified with 5 mM solution of 3,3'-dithiodipropionic acid di-(N-succinimidyl ester) (DASE) in chloroform

for 2 h. Then, 1 mM solution of 6-mercapto-1-hexanol (MCH) in ethanol was used to block the remaining free surface of the gold electrode for 1 h. Next, the electrodes were modified with 0.2 mg/mL aqueous solution of avidin for 1 h and additionally for 1 h with 1 mM aqueous solution of 2-aminoethanol (pH 8.00). The biotinylated probes 5'-biotin-ATC CCA CTA TCC TTC GCA AGA-3' were injected with final concentration 300 nM. The DNA biosensor prepared in this way was then tested with short complementary and non-complementary synthetic oligonucleotides and PCR products. Following the tests, the sensor was used for the detection of genetically modified soybean Roundup Ready. Sensors were regenerated by immersing the quartz crystal electrode into the HEPES denaturation buffer pH 8.00, for 10 min at 95 °C, and then cooled down on ice or washing thrice with 10 mM NaOH for 2 min. The reproducibility of the hybridization process in the samples non-amplified by PCR, expressed by the average coefficient of variation, was 20%.

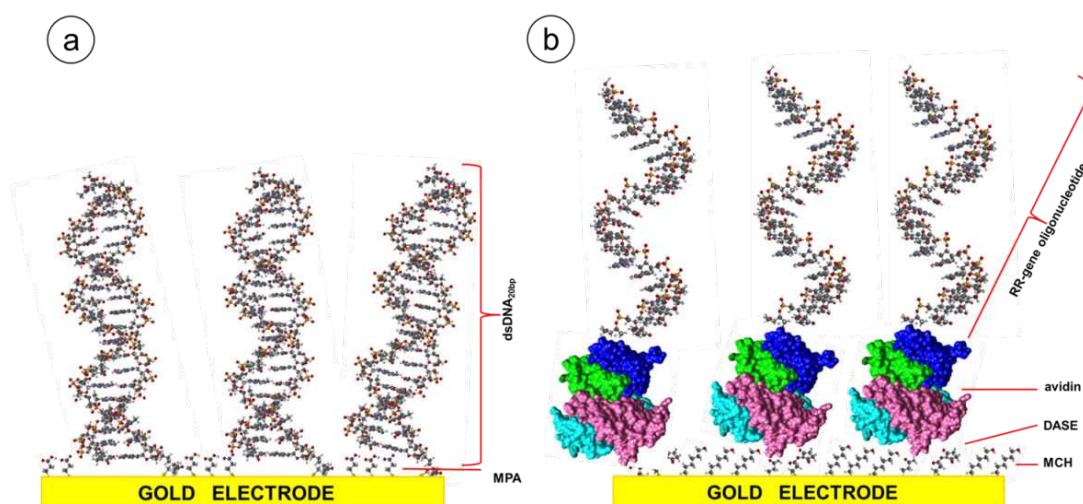


Figure 1. Design of functionalized DNA biosensors for (a) the determination of DNA damage by herbicides (Au/MPA/dsDNA_{20-bp} film) and (b) the detection of the genetically modified soybean Roundup Ready (Au/DASE/avidin/RR-gene oligonucleotide film).

7.1. Studies of DNA damage by atrazine and paraquat using intercalation redox probe

In Figures 2 and 3, cyclic voltammetric response of the Nile Blue redox dye probe incorporated in sensory films of the DNA-biosensors is presented. This electroactive probe was used for the determination of DNA damage by herbicides atrazine and paraquat (in Figure 2 and 3, respectively). Voltammograms were obtained in pure PBS buffer without NB dye. The sensor was incubated first in a 100 μ M solution of NB dye for 10 min, carefully washed in PBS buffer, and tested in the same buffer (curve 2). The NB molecules intercalate into the double-helix structure of DNA. A couple of well resolved redox peaks with cathodic and anodic peak potentials at $E_{pc} = -0.41$ V and $E_{pa} = -0.38$ V vs. Ag/AgCl is observed. The linear dependence of the peak currents, I_{pc} and I_{pa} , on the potential scan rate v indicates that the redox peaks correspond to the surface bound NB species [14]. Then, the sensor was incubated in 100 μ M solution of herbicide (atrazine or paraquat, respectively) and characterized in a

PBS solution without intercalative dye (curve 3). Next, the sensor was soaked once again in the solution of NB, washed in PBS, and characterized in pure PBS buffer (curve 4). The changes in cathodic peak current (I_{pc}) of a Nile Blue probe before and after the interactions of herbicides with DNA were quantified. It is seen that after the interaction of Atz or PQ with DNA, the I_{pc} of a NB bound to DNA has changed considerably. The DNA damage φ was measured as the relative current increase/decrease in the NB uptake after interaction of DNA with herbicide:

$$\varphi = 100 \left(\frac{I_{pc,herbicide} - I_{b1}}{I_{pc,0} - I_{b0}} \right) \quad (2)$$

where, $I_{pc,0}$ and $I_{pc,herbicide}$ are the cathodic peak currents for the reduction of NB intercalated in dsDNA helix on electrode before and after interactions of DNA with herbicides, respectively; and I_{b0} and I_{b1} are the background currents.

7.2. Interaction of atrazine with DNA

After the incubation of a dsDNA sensor in atrazine solution, the cyclic voltammetric characteristic of NB, recorded in pure PBS buffer solution without redox dye, shows a considerable increase of the cathodic current of NB (Figure 2, curve 4) in comparison to the cathodic current of NB before interaction of dsDNA with the herbicide (Figure 2, curve 2) [13],[11].

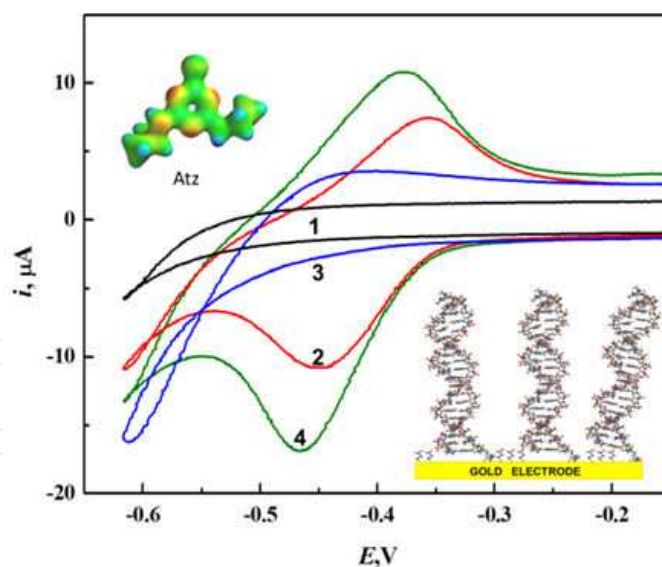


Figure 2. Cyclic voltammograms for an Au/MPA/dsDNA_{20-bp} film after subsequent treatments: [1] PBS only, [2] after 10 min soaking in 100 μ M NB solution; [3] after 40 min soaking in 100 μ M atrazine solution; [4] after 10 min soaking in 100 μ M NB solution; scan rate $v = 400$ mV/s, solution: 0.02 M PBS, pH = 7.4. Modified from reference [13] with permission. Copyright 2010 The Electrochemical Society.

It indicates that more molecules of the electroactive dye were intercalated into the DNA helix. It is reasonable to conclude that the higher capacity of dsDNA towards the Nile Blue

probe is associated with *B*-DNA structure altering caused by the herbicide. It is interesting that the second incubation of DNA sensor with NB dyes, after the interaction of dsDNA sensor with atrazine, resulted in so large an increase in the NB uptake ($\varphi = 65\%$). Separate experiments performed with not fully matched complementary oligonucleotides (C-A mismatched oligonucleotides) have also led to the increased NB uptake but only by $\varphi = 17\%$ [14]. This indicates a high sensitivity of the DNA biosensor proposed. Molecular dynamic simulations have confirmed that atrazine molecules cause underwinding of double-stranded helix of DNA and increase the uptake of Nile Blue redox probe due to the increase in the inter-base spacing in the base stacks.

7.3. Effect of paraquat on DNA biosensor responses

The interactions of paraquat with dsDNA, immobilized on a sensor surface, have been investigated [4]. Changes in cyclic voltammograms of a Nile Blue intercalative redox probe have been observed after the incubation of a DNA-biosensor in a paraquat solutions. For the incubation times of a DNA-biosensor in a paraquat solution for up to 55 min and soaking the biosensors for 10 min in solution of a Nile Blue (100 μM), the peak current of NB reduction was observed to increase in comparison to the peak current of NB before the interactions of DNA with paraquat. The uptake of NB intercalated into the dsDNA helix after the interactions with PQ increased on average by $\varphi = 13.9\%$ (for interaction times of paraquat with dsDNA of 10, 35, and 55 min). This indicates the unwinding of the dsDNA helix, similar to the effect of atrazine on dsDNA, resulting in the increased uptake of NB molecules into the DNA duplex. However, after longer interaction time of paraquat with dsDNA ($t = 80$ min), the peak current of NB reduction has been found to decrease (Figure 3, curve 4) in comparison to the peak current of the probe in an undamaged dsDNA (Figure 3, curve 2). It clearly indicates on a break and fragmentation of dsDNA caused by long-lasting action of paraquat which leads to the observed diminished uptake of NB probe into the DNA helix, with $\varphi = -32.1\%$.

7.4. Characterization of the biofilm and testing of EQCN-based DNA biosensor

Each modification step during the construction of a DNA biosensor used for probing the affinity of atrazine to DNA and possible DNA damage was monitored by quartz crystal nanobalance technique (Figure 4). For instance, the immobilization of the mercaptopropionic acid (MPA) on a gold surface of a piezoelectrode has led to a total resonant frequency shift of $\Delta f = 24.3$ Hz, corresponding to the apparent mass change of $\Delta m = 21.1$ ng and surface coverage $\gamma_{\text{MPA}} = 0.76$ nmol/cm² ($A_{\text{geo}} = 0.22$ cm², roughness factor $R = 1.2$, and real surface area: $A = 0.264$ cm²). Next, after activation of the carboxylic groups of the thiol film with EDC, the NH₂-modified oligonucleotide probes (pDNA) and complementary to them target oligonucleotides (tDNA) were attached. After the injection of oligonucleotides, the frequency shifts $\Delta f = 73.8$ Hz and $\Delta f = 72.3$ Hz were observed, for pDNA and tDNA, respectively. The main mass increase was observed within 45 min which was considered as the time necessary to attain stable and full-monolayer coverage of dsDNA biofilm. The dsDNA surface coverage $\gamma_{\text{DNA}} = 34$ pmol/cm² determined from the experimental mass of DNA film was close to the theoretical value for hexagonal close packing and corresponds to the loading of 0.68

nmolbp/cm² [13],[11]. The DNA-biosensor prepared in this way was subsequently used for the investigations of the behavior of atrazine and possible DNA damage detection.

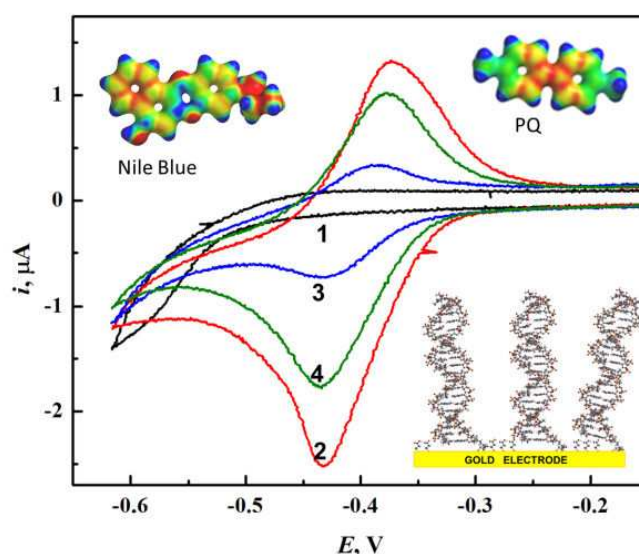


Figure 3. Cyclic voltammograms for an Au/MPA/ dsDNA_{20-bp} film after subsequent treatments: [1] PBS only, [2] 10 min soaking in NB solution (100 μM); [3] after 80 min soaking in PQ solution (100 μM); [4] after soaking in PQ and 10 min soaking in NB solution (100 μM); scan rate $v = 100$ mV/s, solution: 0.02 M PBS, pH = 7.4. Modified from reference [4]. Adapted with permission from Antioxidant Effectiveness in Preventing Paraquat-Mediated Oxidative DNA Damage in the Presence of H₂O₂, M. Stobiecka, A. Prance, K. Coopersmith, M. Hepel, 2011, 211-233, in: S. Andreescu, M. Hepel (Ed.) Oxidative Stress: Diagnostics, Prevention, and Therapy. Copyright 2011 American Chemical Society.

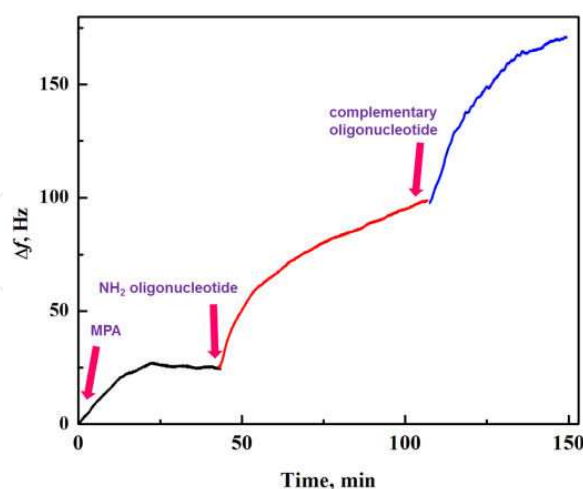


Figure 4. Resonant frequency shift recorded for a gold piezosensor after injection of mercaptopropionic acid (MPA), NH₂-modified oligonucleotide (5'-NH₂C₆H₁₂-ATTCGACAGGGATAGTTCGA3') (pDNA) and a complementary oligonucleotide (5'-TCGAACATATCCCTGTC GAAT3') (tDNA); solution: 0.02 M PBS, pH = 7.4; $C_{\text{MPA}} = 10$ mM, $C_{\text{pDNA}} = 1$ μM, $C_{\text{tDNA}} = 1$ μM, all concentrations are the final concentrations. Modified from reference [13] with permission. Copyright 2010 The Electrochemical Society.

8. Detection of genetically modified soybean Roundup Ready by quartz crystal nanogravimetric technique

A DNA biosensor for the determination of genetically modified soybean Roundup Ready was examined using quartz crystal nanobalance technique [15], [69]. In Figure 5, cyclic voltammograms for ferricyanide redox probe obtained after each step of the modification of a gold piezoelectrode are presented. The voltammogram obtained for a bare gold electrode (Figure 5, curve 1) shows a couple of well-developed redox peaks for the marker ion with peak separation, $\Delta E_p = 111$ mV. After forming the 3,3'-dithiodipropionic acid di-(N-succinimidyl ester) (DASE) self-assembling monolayer on a gold surface, a repulsion of the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ ions from the film was observed and the peak separation of the redox probe couple in voltammetric characteristics has increased to $\Delta E_p = 135$ mV for the ester-modified gold piezoelectrode (Figure 5, curve 2).

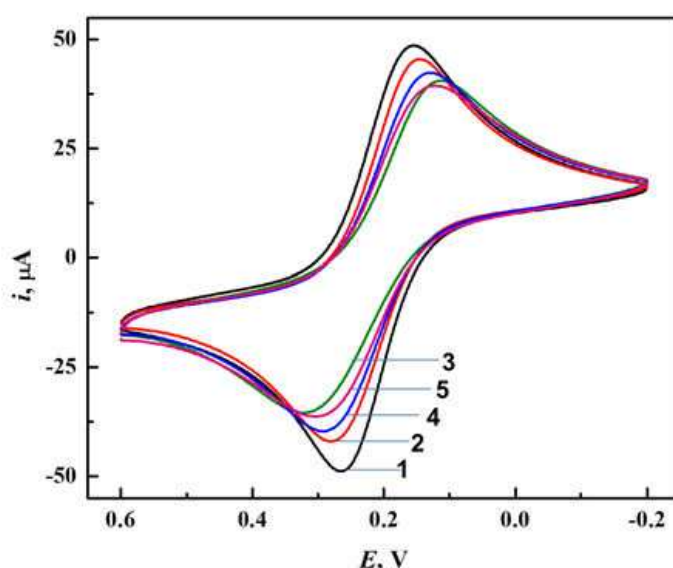


Figure 5. Cyclic voltammograms for a $\text{K}_3\text{Fe}(\text{CN})_6$ test solution recorded after subsequent steps of the DNA biosensor construction: [1] bare gold piezoelectrode, [2-5] gold piezoelectrode after immobilization of successive layers of [2] 3,3'-dithiodipropionic acid di-(N-succinimidyl ester) (DASE) (5 mM), [3] avidin (0.2 mg/mL), [4] aminoethanol (AET) (1 mM), [5] biotin-oligonucleotide (300 nM), $v = 100$ mV/s, $C_{\text{compl}} = 80$ nM, $C_{\text{non-compl}} = 72$ nM; the solution composition: 0.1 M phosphate buffer pH = 7.2, 0.1 M KCl and 0.001 M $\text{K}_3\text{Fe}(\text{CN})_6$, scan rate 0.1V/s. Modified from reference [69], unpublished data

The addition of an avidin solution, has led to further decrease in the marker signal and an increase in the peak separation for ferricyanide ions to $\Delta E = 208$ mV (Figure 5, curve 3). After the immobilization of the protein, the electron transfer of the $\text{Fe}(\text{CN})_6^{3-/4-}$ couple has decreased due to the formation of a blocking avidin layer, reduced surface accessibility, and steric hindrance. The immobilization of aminoethanol (AET) molecules promoted an electron transfer between the redox molecules and the electrode surface. Hence, the immobilization of AET on a gold electrode surface resulted in the increase of the redox marker reaction reversibility and a dramatic decrease of the peak separation of the redox probe to $\Delta E_p = 163$

mV (Figure 5, curve 4). The attachment of the biotinylated oligonucleotides resulted in a decrease of the redox response of the electroactive marker and an increase in the peak separation for ferricyanide ions to $\Delta E = 182$ mV. The decrease of the ferricyanide probe signal after the immobilization of oligonucleotides was expected due to repulsions between negatively charged DNA chains and negative ferricyanide ions.

A freshly prepared DNA-biosensor was first functionalized with short synthetic oligonucleotides using quartz crystal nanobalance technique [15] for film formation monitoring. In Figure 6, the hybridization process of short synthetic oligonucleotides, complementary and non-complementary, to the oligonucleotide probe immobilized on the sensor surface is presented. The probe was related to the 5-enolpyruvylshikimate – phosphate synthase (*EPSPS*) gene, which is an active component of an insert integrated into a Roundup Ready soybean. Next, the DNA biosensor was used for testing with a 169 base-pair fragment of *EPSPS* gene extracted from Roundup Ready soybean genome and amplified by PCR, and with a non-complementary 138 base-pair fragment amplified by PCR on maize alcohol dehydrogenase gene template. Finally, the DNA biosensor was employed for the detection of *EPSPS* sequence in PCR non-amplified DNA samples extracted from animal feed containing 30% of the genetically modified soybean RR. The sensor was able to distinguish between a transgene sequence of the modified and unmodified soybean DNA at the genomic DNA quantities used in the analysis: 3.6, 4.6 and 5.4 μg . The detection limit was in the range of 4.7×10^5 genome copies in 200 μL of a QCN cell [15].

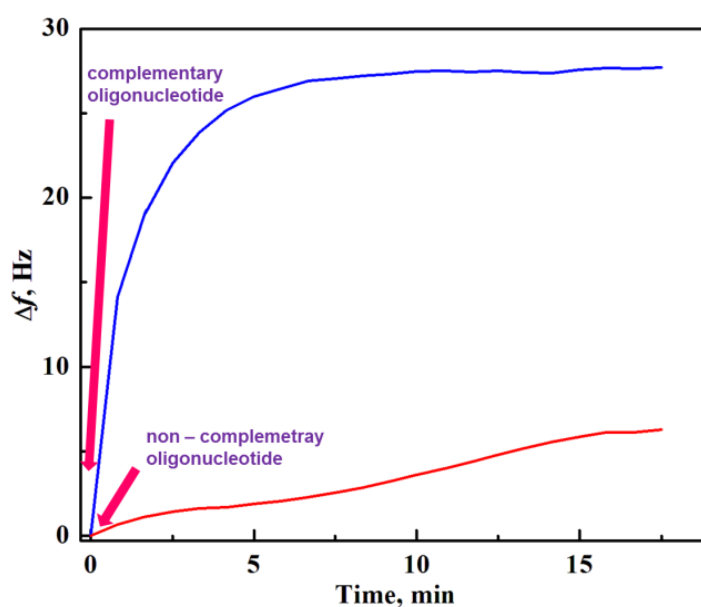


Figure 6. Resonant frequency shift recorded for an Au/DASE/avidin film after injection of a complementary and non-complementary oligonucleotides, $C_{\text{compl}} = 80$ nM, $C_{\text{non-compl}} = 72$ nM; the solution composition: 27 mM HEPES, 55 mM NaCl, pH 7.5; total volume in the QCM cell: 200 μL . Modified from reference [15] Copyright 2007 M. Stobiecka, J.M. Cieřla, B. Janowska, B. Tudek, H. Radecka; licensee MDPI, Basel, Switzerland.

9. Molecular dynamics

To better understand the interactions of herbicides with dsDNA, further investigations were carried out using molecular dynamics (MD) simulation and quantum mechanical calculation (QC) of electronic structures. In MD simulations, atrazine and Nile Blue molecules were: [1] docked on the sugar-phosphate chain and at minor and major grooves of DNA to evaluate hydrogen bonding and electrostatic interactions and [2] were inserted into a gap between two stacked bases in model structures of ssDNA and dsDNA [11],[14],[13]. In investigations carried out for the herbicide paraquat, three molecules of the herbicide were placed between the bases in a model dsDNA consisting of 11 nucleic base-pairs [4]. In these investigations, we have found that the intercalation of Atz and PQ in dsDNA helix leads to a conformational alterations of the DNA structure. The herbicides caused an increase in the interbase distance, longitudinal helix expansion and unwinding of the dsDNA confirmed by the higher binding capacity of DNA toward the Nile Blue intercalative redox probe and a higher uptake of the probe after incubation of DNA with herbicides. Long time interactions of paraquat with model dsDNA resulted in the denaturation of the double-stranded helix to single strands decreasing the NB uptake. The molecular dynamics simulations strongly corroborate cyclic voltammetry and fluorimetric measurements carried out by our groups. In Figure 7, an example of a simulation of the unwinding process of a dsDNA during its interactions with atrazine is presented. A clear elongation of the DNA duplex is readily discernible.

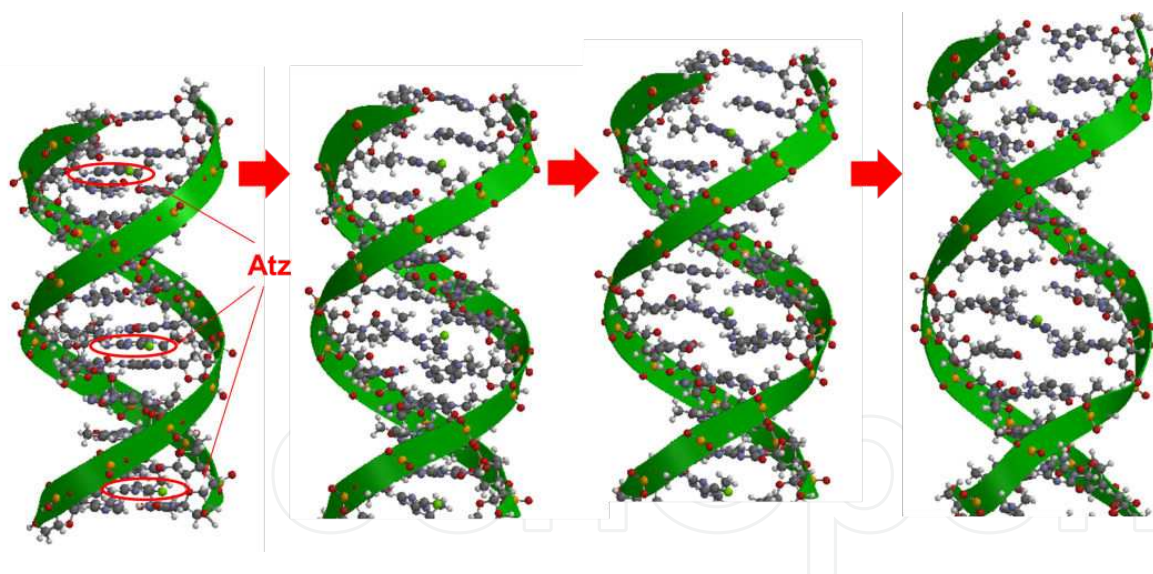


Figure 7. Molecular dynamics simulation of the interactions of atrazine (Atz) with ds DNA helix.

10. Conclusions

In this Chapter, the principles of sensory film designs for DNA-biosensors using nanogravimetric and voltammetric signal transduction techniques were presented. The biosensing platforms we have studied include the electrochemical biosensing with intercalating redox

probe (NB) and travelling redox probe (ferricyanide ions), as well as the EQCN monitoring of conformational film changes correlated with herbicide-induced DNA alterations. Applications of DNA-biosensors for probing the affinity of atrazine and paraquat to DNA, the interactions of herbicides with DNA, assessment of DNA damage, and detection of the genetically modified soybean RR, have been reviewed. We have shown that the interactions of herbicides with DNA caused an evident alteration of the *B*-DNA conformation including unwinding of the helix and an increase in the interbase distance. The unwinding results in an increase in the capacity of dsDNA to bind an intercalative electrochemical probe and higher uptake of Nile Blue molecules into the DNA duplex. At longer interaction times of DNA with paraquat, strand breaks and DNA fragmentation has been observed as evidenced by the lower Nile Blue uptake into the DNA helix. The piezoelectric DNA-biosensor based assay developed was utilized as an alternative, highly sensitive, inexpensive, and simple method for the detection of genetically modified soybean in samples that do not require PCR amplification.

List of abbreviations

AET - aminoethanol

Atz- atrazine

CaMV - cauliflower mosaic virus 35 promoter

CRM - certified reference materials

CV - cyclic voltammetric measurements

DASE - 3,3'-dithiodipropionic acid di-(N-succinimidyl ester)

EDC - N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide

ELISA - Enzyme Linked Immunosorbent Assay

EPSPS-5-enolpyruvylshikimate-3-phosphate synthase gene

EQCN - Electrochemical Quartz Crystal Nanobalance

GMOs -genetically modified organisms

HEPES -N-[2-hydroxyethyl]piperazine – N'-[2-ethanesulfonic acid]

HPLC - high-performance liquid chromatography

I_{pc} -cathodic peak current

LC-MS -liquid chromatography - mass spectrometry

MCH - 6-mercapto-1-hexanol

MD - molecular dynamics

MPA -mercaptopropionic acid

NB - redox active dye Nile Blue A

NOS - nopaline synthase terminator

PCR- Polymerase Chain Reaction technique

pDNA - single-stranded probe DNA

PQ - paraquat

QC -quantum mechanical calculation

ROS -reactive oxygen species

RR soybean - genetically modified soybean Roundup Ready

tDNA - target-gene single-stranded DNA

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