Nanoanalytics

Nanoobjects and Nanotechnologies in Analytical Chemistry

Edited by
er Sergeï Shtykov
Abstract: The development of electrochemical DNA sensors and aptasensors based on nanoparticles different in nature, size, shape, and preparation protocols has been considered with particular emphasis to the mechanism of their influence on signal readout and way of implementation in the biosensor assembly. Most attention is paid to application of Au nanoparticles and carbonaceous nanomaterials though the examples of other applications and hybrid nanomaterials are given. The analytical performance of DNA sensors and aptasensors utilizing nanomaterials is classified in accordance with their targets and role of nanoparticles in sensitivity and selectivity of the response. The trends of future progress in the biochemical applications of nanomaterials are discussed.

8.1 Introduction

There is an urgent need in the development of simple and compact analytical devices providing reliable information on the content of various samples tested. Among many other possible areas of application, chemical sensors are especially demanded in medicine in the framework of the point-of-care diagnostics concept [1, 2]. Being rather simple to operate by unqualified staff and equipped with remote control, personal medical sensors offer new opportunities in the chronic diseases medical treatment and continuous monitoring of the health status. Many medical sensors utilize conventional approaches to increase sensitivity toward particular species, for example, physical accumulation of an analyte followed by its involvement in specific reactions with an ionophore, mediator, or electrode. Nevertheless, further improvement of the biosensor performance as well as extension of the number of the analytes call for the development of new detection principles and broader application of the biochemical recognition. Such approaches assume enzymes, antibodies, or nucleic acids introduced (immobilized) in the assembly of a primary transducer (electrode, optic fiber, cantilever) and involved in an analyte recognition. Appropriate analytical devices based on specific biochemical reactions (enzyme–substrate, antigen–antibody, DNA–intercalator, etc.) are called biosensors (biochemical sensors) [3].

Starting from 1962 with first glucose sensor assembled from the oxygen electrode covered with the permeable membrane containing glucose oxidase [4], biosensors
emerged from the laboratory to a global international market covering more than $14 billion of annual sales [5]. Biosensors mostly used in medicine are intended for the detection of the metabolites (glucose, urea, and lactate analysis), the immuno- [6] and DNA assay [7, 8]. Considering further progress, the DNA sensors demonstrate maximal opportunities among other biochemical receptors due to universal targeting, prospects of miniaturization (lab-on-chip, microfluidic devices [9]), and compatibility with conventional diagnostic systems.

Nanomaterials involve those consisted of the particles sized between 1 and 100 nm in at least one dimension [11]. Their application in electrochemical sensors and biosensors is mostly directed to the increase of the sensitivity of the signal and miniaturization of the measuring devices [7–10, 12–14].

Transduction principle affects the application of the nanomaterials because of the specific requirements existed for all the transducers applied. Thus, most of the nanoparticles used in for electrochemical (bio)sensors show electroconductivity or redox activity. Small size and monolayer deposition make it possible to combine the low- and nonconductive materials with metal and carbonaceous electrodes and avoid at the same time significant worsening in electric wiring of a bioreceptor. Nevertheless, the idea of significant enhancement of the electrode surface and hence of the quantities of recognition elements dominates among other reasons to implement nanoparticles in conventional sensors. Other possible motifs involve simplification of the bioreceptor immobilization protocols, miniaturization of appropriate devices, development of new primary transducers like field effect transistors (FETs) based on a single-walled carbon nanotube (SWCNT) [15].

It should be mentioned that the variety of nanomaterials described in the assembly of electrochemical biosensors remains rather constant during the past decade and does not tend to significant increase because of the limited offers from the manufacturers and rather complex procedures of their synthesis, size-specific separation, and purification. Even for the carbon nanotubes (CNTs) with a rich story of sensor application, few researchers report about the synthesis of some specific sorts of CNTs with advantages over the commercial products, for example, alone standing forest like CNTs, especially for their application in FETs or other types of biosensors [16].

Reduced graphene oxide is mainly synthesized by oxidation of graphite by strong oxidants in acidic media followed by chemical or electrochemical reduction of the products [17, 18]. Multiwalled CNTs (MWCNTs) and SWCNTs, either as-synthesized or treated with oxidants, strong acids, biotin, and other surface modifiers, are available in Sigma-Aldrich catalog together with other carbonaceous nanoparticles [19]. Besides, silicate, ferrite, and noble metal nanoparticles are purchased, both clean and premodified with functional groups requested for the further coupling with receptors [20–22].
Other examples of nanomaterials synthesized for their further application in sensor assembly include metals obtained by controlled electrolysis from metal salts and complexes, metal oxides, and insoluble complexes that are chemically synthesized from appropriate precursors directly on the transducer surface [23–25]. They can be also obtained as suspensions in aqueous and organic media with moderate stability prior to electrode modification and casted on their surface alone or together with other components. Limited reproducibility of size distribution and rather broad range of particles’ shape remain a weak point of such “home-made” protocols of nanomodification.

Owing to the importance of biosensors for medical diagnostics and potential benefits related to the application of nanomaterials in their assemblies, the number of appropriate articles has been enormously increased during the past decade. Some reviews recently published concentrated on specific targets of such biosensors or nature of nanomaterials applied [26–32]. In this chapter, we summarize the progress in the development of electrochemical DNA sensors achieved in the past 5 years with particular emphasis to the role of nanomaterials and mechanisms of their influence on the biosensor signal and its characteristics in real sample assay. The description of the DNA sensors is subdivided in accordance with the nature of recognition element and biological targets.

8.2 DNA sensors: Recognition elements and signal transduction

8.2.1 Biochemical elements applied in the DNA sensors assemblies

The DNA sensors utilize several types of biochemical receptors, either selected from natural nucleotide sequences or synthesized de novo, which are able to highly specific interactions with biological targets. In accordance with their nature and preparation protocol, all the DNA sensors can be classified into several groups.

Genosensors involve rather short single-stranded (ss-) oligonucleotide sequences (also called DNA probes) that interact with complementary strands present in the sample tested with formation of native double-stranded (ds-) DNA molecule fragment [33, 34]. The DNA primers applied in conventional genetic assay and polymerase chain reaction (PCR) are frequently used as the DNA probes in biosensor format. The interaction between the DNA probe and a target sequence called hybridization assumes the formation of stable pairs of the nucleotide bases, that is, adenine–thymine (A–T) and guanine–cytosine (C–T) via hydrogen bonds (Fig. 8.1).

The formation of a hybridization product, that is, ds-DNA piece, confirms existence of an appropriate nucleotide sequence complementary to the probe in the sample. The DNA probes can be related to the variable parts of genes, amplicons, etc.
Genosensors can be used for the direct diagnostics of the pathological microorganisms and the viruses, for the identification of genetically modified organisms (GMOs), selection of the meat origin, etc. Selectivity of agenosensor is estimated by its ability to distinguish between the fully complementary sequence and that differed from it by one, two, etc. nucleotide (so-called single, double, etc. mismatches).

**Aptasensors** include aptamers, ss-DNA or RNA molecules with high specificity toward various analytes, for example, proteins, antibiotics, etc. [35]. They are synthesized *de novo* by selection against targets from the random DNA/RNA libraries of oligonucleotides. SELEX (Systematic Evolution of Ligands by EXponential enrichment) is the most frequently used protocol for aptamer selection [36]. The recognition of aptamer targets is often compared with antigen–antibody interaction. Contrary to the antibodies, aptamers are more stable to hydrolysis and chemical/thermal denaturation. They show in many cases efficiency of analyte binding comparable to that of specific antibodies. For this reason, aptamers are often called as “artificial antibodies.” Once selected, aptamers are easily produced *quantum satis* and seem attractive as biorecognition elements from the point of view of possible commercialization. Many aptamers commercially available contain terminal amino-, carboxylic, or thiol groups or biotin residues necessary for their covalent binding to the careers or the transducers. Although the first SELEX-related patent was filed in 1989, intensive investigations are still performed to select more aptamers against various targets, stabilize their structure, and decrease cross-binding with matrix interferences. Aptamer exist in the structures that might be significantly different from those of native ds-DNA. Thus, guanine-rich areas form flat tetragons stabilized with potassium ions positioned in the center and guanine residues in the vertexes. Such $G_4$-quadruplexes can self-assemble to form stacks or reversibly switch to linear configuration after sharp changes in ionic strength or pH of microenvironment. Partially complementary aptamers form stem-loops that recognize appropriate guest molecules, including short DNA sequences complementary to the main part of the DNA probe. Such an interaction
results in disturbance of the loop and its relative position against transducer interface. The conformational changes described result in serious changes in the permeability of the aptamer layer or the accessibility of the labels implemented in their structure.

**Peptide nucleic acid (PNA) based sensors** utilize synthetic analogs of native DNA that are much more stable to hydrolysis and do not contain charged groups. Due to similar to DNA distance between the nucleic bases, the PNAs are able to hybridization with complementary DNA sequences and form DNA–PNA hybrid structures. PNAs consist of repeating N-(2-aminoethyl)-glycine units linked by amide bonds (Fig. 8.2) [37, 38].

![Chemical structure of a PNA](image)

**Fig. 8.2:** Chemical structure of a PNA. The substituents marked with letters indicate nucleotide base residues.

Among other recognition elements, **native DNA** is rather rarely used in the biosensor assembly due to the large size and problems with reproducible positioning of the biomolecules on the electrode surface. Steric limitations of the DNA deposition onto the electrode surface due to rather rigid helical structure of biomolecule prevent the direct oxidation of the guanine and the adenine residues, so that electrochemical signal is mostly generated by the mediated oxidation of the above nucleotides and by changes in the surface layer permeability for the small redox probes [39, 40]. Even if native DNA is announced as a part of the biosensor assembly, it should be taken into account that manipulations with the DNA solutions (dilution, pipetting, sonication, etc.) result in fragmentation of the DNA molecules. Nevertheless, such DNA sensors have found application for the detection of the oxidative DNA damage [41] or for the determination of low-molecular compounds that are accumulated on the DNA due to intercalation or electrostatic interactions [42]. Autoimmune diseases result in formation of auto-DNA antibodies that specifically interact with ds-DNA molecules and provoke their following cleavage into the pieces. Such interactions are used for the biosensor-based diagnostics of autoimmune character of a disease [43].

**DNA nanostructures.** The progress in the nanotechnologies and investigations directed to the use of nanomaterials in biology and medicine has inspired the synthesis on various three-dimensional (3D) structures using synthetic oligonucleotides
as building blocks [8]. Variety of functional groups and reversible conformational changes controlled by the changes in the microenvironment of these synthetic constructs make them attractive as possible basis of the drug delivery systems, nanomachinery, and nanoscale sensing devices. Thus, the DNA tetrahedrons exert spatial positioning of terminal guest groups on solid support. They are compatible with conventional immobilization protocols based on self-assembling and Au–S binding, whereas the ss-DNA probe opposite to the bottom plane of the tetrahedron selectively binds a biological target [44]. Own size of a tetrahedron limits probe-to-probe spacing of neighboring 3D structures and hence their surface density (about $4.8 \times 10^{12}$ probes/cm$^2$). The DNA origami is another example of 2D and 3D nanostructures that are created using complementary interactions of specific consequences. In mostly used protocol, DNA origami assembling involves folding a long viral ss-DNA by multiple small “staple” strands [45]. Such structures can find application for the detection of the single-nucleotide polymorphism (SNP) [46]. Although the appropriate sensors utilize fluorescence signal and AFM data detection, they promise fast progress in electrochemical devices, too. The 3D structures of some DNA-related receptors applied in biosensor assembly are outlined in Fig. 8.3.

Fig. 8.3: Schematic outlines of DNA-related nanostructures immobilized on the transducer surface. (a) Conventional DNA probe; (b) 3D DNA tetrahedron; (c) stem loop aptamer; (d) aptamer in G4 quadruplex form; and (e) structure of the G4 quadruplex.

8.2.2 Measurement of the signal of electrochemical DNA sensors

Though a variety of the DNA-related species are described in the DNA sensor assembly, a limited number of universal schemes are used for generation of the signal with appropriate transducers. Owing to the domination among other electrochemical sensors, voltammetric (amperometric) and impedimetric sensors will be preferably considered. For them, the protocols of the signal measurement can be subdivided into two groups, that is, label-free and label based methods of signal quantification. The classification principles are presented in Fig. 8.4.
Label-free methods of signal record do not require complex manipulation with the DNA receptors except those necessary for their immobilization. Many of them are conducted in a single step or by consecutive addition of reactants to the same working solution. Taking into account that each additional step is followed by biosensor washing, this significantly reduces total measurement time and possible sources of mistakes related to wrong dilution, incomplete solution mixing, or removal of non-bonded species. On the other hand, the label-based techniques often provide lower limit of detection (LOD) and higher specificity of the response against label-free techniques.

Description of the label-free detection of a recognition event starts with the direct or mediated electrochemical oxidation of guanine residues (1).

\[
\begin{align*}
\text{Guanine /} & \\
\text{8-Oxoguanine} & \\
\text{signals} & \\
\text{Redox active} & \\
\text{intercalators} & \\
\text{Small redox} & \\
\text{probe signals} & \\
\text{EIS} & \\
\text{E-Sensor} & \\
\text{Sandwich mode} & \\
\text{Competitive} & \\
\text{mode} & \\
\end{align*}
\]

The reaction is accelerated in cases of the oxidative DNA damage and mediation with some redox probes. Also, accessibility of guanine is higher in ss-DNA sequences. The latter fact is used for the discrimination of the DNA probe and the product of hybridization with a biological target [47].

Although steric problems prevent the electron transfer from the majority of guanine units, small oxidation current appears on the electrodes covered with the
ds-DNA fragments. It can be recorded in differential pulse (DPV) or square wave voltammetry (SWV) on carbon electrodes. For better discrimination of the signals referred to ss- and ds-DNAs, the guanine residues in the DNA probe can be substituted with inosine fragments [48]. They retain its ability to form pairs with cytosine but are electrochemically inactive, so that the current generated after hybridization fully belongs to the biological target containing guanine. To a lesser extent, oxidation of adenine can be used for the same signal detection [49]. The signals of nucleotides are increased by implementation of the mediators of electron transfer, for example, bipyridine or phenanthroline complexes of Co, Ru, and Rh. The examples of mediators frequently used in the assembly of DNA sensors are presented in Fig. 8.5.

Another label-free technique estimates permeability of the surface layer toward small charge carriers (redox probes). In the simplest way, the charge flux is quantified by appropriate oxidation current measured in the DC mode. Ferricyanide ion $[Fe(CN)_{6}]^{3-}$ is mostly used in such investigations. Hybridization decreases the diffusion rate of ferricyanide and hence the current of appropriate peaks on voltammograms because of implementation of the non-conducting molecules of a biological target in the surface layer and because of the increased negative charge of phosphate groups of oligonucleotides that exert shielding effect on the transfer of negatively charged redox molecules. For aptasensors, similar effect is achieved by the reversible conformation switch of the linear form into $G_4$-quadruplex or steam-loop structure to linear one. The signal of redox probe can be recorded by electrochemical impedance spectroscopy (EIS) as a shift of the charge transfer resistance. Such a phenomenon was observed for ochratoxin A detection with impedimetric aptasensor [50].

Specific interaction of the charge carrier with ss- or ds-DNA also affects the signal. Thus, many planar heteroaromatic molecules penetrate (intercalate) the ds-DNA
helix in between pairs of complementary nucleotides and lose their redox activity [51]. Methylene blue is one of the most frequently used redox active intercalators [52]. Bipyridine and phthalocyanine ligands of metal complexes (see Fig. 8.5) partially intercalate DNA molecules and can either mediate the electron transfer from guanine residues to dissolved oxygen or to the electrode or directly participate in the electron exchange with the electrode. Appropriate changes in the currents indicate conditions of the electron transfer that indicate hybridization, oxidative DNA damage, or DNA–protein interactions. Redox active mediators are often applied as labels or together with labels in appropriate techniques.

**Labels** are rather small molecules, which are covalently bonded to the biochemical reactants and which presence (concentration) is easily quantified by appropriate sensor (transducer). For the electrochemical sensors, carbonaceous and metallic nanoparticles, phenothiazine, phthalocyanine, and ferrocene derivatives can be applied as labels [52] together with enzymes [53] and quantum dots [54].

The operation of label-based DNA sensors is often similar to that in conventional immunochemical assay. Frequently used approaches are illustrated in Fig. 8.6.

**Fig. 8.6:** The operation of label-based DNA sensors. (a) Competitive assay; (b) sandwich assay with capturing and signaling DNA probes; (c) sandwich assay with aptamers and aptamer–protein interactions; and (d) E-sensor with the labeled stem-loop DNA probe.

In **competitive assay**, a sample containing biological target is mixed with the solution of the same sequence bearing a label. Ratio of labeled and non-labeled products of
hybridization on the transducer interface yields from the ratio of their concentrations in the solution: the higher concentration of the target the lower label signal. In **sandwich assay**, two types of DNA probes are utilized. One of them (capturing probe) is attached to the electrode and interacts with a piece of a target containing complementary sequence. In this manner, the hybridization product leaves part of ss-DNA sequence free for the following interaction with a second (signaling) DNA probe that bears label. Its signal increases with the analyte concentration. Similar schemes are used for detection of species specifically interacting with the capturing DNA probe. Thus, DNA–protein interaction can be determined by aptamer specific to the binding site of the protein different from that providing its interaction with capturing aptamer/DNA probe.

Multistep protocol of the sandwich assay is one of the most obvious disadvantages of this measurement protocol because each addition of the reagents assumes time and labor consuming washing steps and increases probability of mechanical distortion of the sensing layer. For this reason, various alternatives have been elaborated to avoid these limitations. In so-called E-sensors, a stem-loop DNA probe is used. It is attached to the electrode by terminal amino or thiol group and labeled with redox active group on the opposite end. Due to partial complementarity of the fragments near both ends of the probe, label is fixed near the electrode surface to establish fast electron exchange and a high amperometric response (see Fig. 8.6d). In the presence of a target sequence, the stem-loop DNA switches to linear form with the same label withdrawn from the electrode [55]. The described mechanism of E-sensor operation is only one example of more general displacement schemes where capturing DNA probe retains their conformation due to partial hybridization with auxiliary sequence that is replaced by target DNA providing either label access or configuration necessary for wiring label to the electrode [56–58]. Thus, the displacement scheme is realized in detection of various analytes. It assumes conformational switches between G₄ quadruplex and linear forms of an aptamer [59]. Displacement-based DNA sensors show unusual calibration curve with a narrow linear range and a sharp rise corresponded to the conformation change initiated by ultra-small quantities of an analyte. For this reason, they are also called as “signal on-off sensors.” Reversibility of the changes observed in E-sensors makes easy their regeneration after use by thermal recovery of initial conformation of the DNA probe. This prolongs the lifetime of the biosensor and decreases the measurement cost.

### 8.3 DNA sensors based on metal nanoparticles

#### 8.3.1 Au nanoparticles

Au nanoparticles are easily produced by electrochemical or chemical reduction performed either in the presence of a sensor transducer or separately to form stable dispersion of nanoparticles, which can be later on casted on the electrode surface [60].
Golden electrodes are quite stable and can be cleaned prior to such modification by treating with nitric acid, hydrogen peroxide, or “piranha solution.” Variety of methods and efficient control of the growth of metal nuclei provide rather narrow size distribution of the particles. Their charge is mainly determined by the synthesis conditions. In most protocols of chemical reduction, the anionic Au complexes (Au(CN)₄⁻, AuCl₄⁻) are used as precursors, so that the surface of the Au nanoparticles becomes negatively charged. This promotes immobilization of the DNA oligonucleotides via terminal thiol groups. Site-specific immobilization is followed by electrostatic orientation of the biomolecules orthogonally to the electrode surface and hence simplifies access of rather bulky target sequences to the probes on the hybridization stage. Together with mild conditions of immobilization and excellent electroconductive properties, this makes Au electrode and Au nanoparticles attractive for the DNA sensor assembling. Besides thiolated biomolecules, dithiols, disulfides, for example, cysteamine, and thiolated carboxylic acids can form surface monolayers modified further by carbodiimide binding (Fig. 8.7).

The surface reaction results in formation of a dense monolayer that prevents nonspecific adsorption of interferences and improves selectivity of the assay. To block undesired reactions, thiolated alcohols and physically adsorbed proteins, for example, bovine serum albumin (BSA) are commonly used for the same purpose. The appropriate treatment is mainly performed after target receptor immobilization or even after analyte binding to reach minimal background signal. The products of modification with organic thiols are called self-assembled monolayers (SAMs). They are used both with bare gold electrodes and Au nanoparticles. In all the protocols described, low thickness and regularity of the composition of the surface layer are achieved.

The Au nanoparticles are compatible with many other modifiers that mediate electron transduction, enhance working area, or promote DNA immobilization. Thus, Au nanoparticles were deposited onto electropolymerized aniline [61] followed by immobilization of thiolated DNA probes and hybridization detection by sandwich assay with alkaline phosphatase and DPV detection of naphthol as a product of enzymatic hydrolysis of α-naphthyl phosphate (2).
Various CNTs are frequently used as supports for the Au nanoparticles, alone or together with the stabilizing layer of chitosan [62]. Other examples of hybrid structures including the Au nanoparticles and performance of the DNA sensors based on such materials are given below in Tab. 8.1.

Reversed approach to the use of Au nanoparticles is described in [65]. Thiolated hairpin DNA probe with free terminal was first immobilized onto golden electrode and then involved in the hybridization reaction. After that, Au nanoparticles were attached to free terminal via thioglycolic acid and changes in the electroconductivity of the surface layer were quantified with EIS. Similarly, to that, Au labeling was used for detection of the homogeneous hybridization [71]. In this work, magnetic nanoparticles were first treated with the cyclodextrin and then dabcyl-modified hairpin DNA probe specific to the hepatitis B virus. The host–guest reaction resulted in liberation of the opposite terminus of the probe containing the Au nanoparticle. The product of the reaction was magnetically separated and dissolved in HCl. Anodic current of Au oxidation made it possible to detect down to 0.993 pM of a target. The steps of labeling with the Au nanoparticles and of hybridization of the complementary parts of the DNA sequences can be repeated for the amplification of the above effect. Stepwise amplification of the signal in the sandwich like assay has been described with the signaling DNA attached to the Au nanoparticles [72] (Fig. 8.8).

In this biosensor, capturing DNA probe 1 was immobilized by the Au–S bond to Au bare electrode. The reaction with a target sequence resulted in the formation of the hybridization product with free part of the sequence ready to the reaction with the signaling DNA probe 2. The amplification was achieved by use of the Au nanoparticles, which surface is modified with two types of oligonucleotides, that is, signaling probe and a shorter sequence, both with methylene blue as label. The ratio of the modifiers is determined on the stage of modification. As a result, direct coupling of a target with such a particle resulted in multiplication of the number of label molecules producing voltammetric signal. Moreover, excessive signaling probes remaining free could be then involved in similar interactions with other Au nanoparticles producing multiplicative number of labels attached.

Dual signal measurement mode has been proposed for detection of the single mutation in apolipoprotein E gene [73]. The Au nanoparticles were first electrochemically deposited onto the transparent indium-tin oxide (ITO) glass electrode.
### Tab. 8.1: The characteristics of electrochemical DNA sensors based on Au nanoparticles

<table>
<thead>
<tr>
<th>Target</th>
<th>Immobilization technique</th>
<th>Signal measurement protocol</th>
<th>Linearity range/LOD</th>
<th>Ref.</th>
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<td></td>
<td>Hybridization detection</td>
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<td>Staphylococcus aureus gene</td>
<td>Au–S coupling on Au nanoparticles deposited onto polyaniline-covered screen-printed carbon electrode</td>
<td>Sandwich assay with alkaline phosphatase – streptavidin, DPV peak current of α-naphthol oxidation</td>
<td>1 fM to 10 nM / 0.33 fM [62]</td>
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<td>Au–S coupling on kappa-carrageenan-polypyrrole-Au nanoparticles composite</td>
<td>DPV signal of diffusively free redox active intercalator, anthraquinone-2-sulfonic acid</td>
<td>5 aM to 1 pM / 5 aM [63]</td>
<td></td>
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<tr>
<td>FLT3 gene</td>
<td>Aminated MWCNTs – Au nanoparticles multilayer obtained by layer-to-layer deposition on Au electrode, thiolated DNA hairpin probe</td>
<td>DPV signal of ferrocene, a terminal label of DNA probe, suppressed due to hybridization of a target DNA and switching conformation of a hairpin probe to linear form</td>
<td>0.1–1000 pM / 0.1 pM [64]</td>
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<td>18-mer sequence</td>
<td>Thiolated hairpin DNA assembled on Au electrode</td>
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<td>Chronocoulometric and EIS measurements</td>
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<td>DPV signal of ferrocene label</td>
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<tr>
<td>18-mer sequence</td>
<td>Thiolated DNA probe attached to Au nanoparticles incorporated into 2D CuS sheet on acetyl black modified glassy carbon electrode</td>
<td>EIS signal with ferricyanide redox robe</td>
<td>0.1 pM to 1 nM / 20 fm [68]</td>
<td></td>
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<tr>
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<td>DPV signal of ferrocene units</td>
<td>350 pM to 25 nM / 275 pM [69]</td>
<td></td>
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<td>Lactococcus lactis genus</td>
<td>Co-deposition of polypyrrole and Au nanoparticles composite followed by covalent linking of p-thiobenzoic acid and aminated DNA probe</td>
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<td>0.1 pM to 2 μM / 0.84 pM [70]</td>
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### Tab. 8.1: (continued)

<table>
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<th>Target</th>
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<th>Signal measurement protocol</th>
<th>Linearity range/LOD</th>
<th>Ref.</th>
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<tr>
<td>Hepatitis B virus</td>
<td>Steam loop DNA probe labeled with Au nanoparticles and dabcy1 interacts with target sequence and receives possibility to be attached to magnetic nanoparticles via dabcy1-cyclodextrin supramolecular binding</td>
<td>After magnetic separation, the complex is dissolved in HCl and oxidized. Then reduction of [AuCl₄]⁻ is recorded by DPV</td>
<td>1.505 pM to 0.301 nM / 0.99 pM</td>
<td>[71]</td>
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<tr>
<td>Specific point mutation in apolipoprotein E gene</td>
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<tr>
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<td>Aminated 27-mer pinhole probe covalently attached to carboxylic groups of mercaptoundecanoic acid by aminated terminus and to Au nanoparticles with thiol group</td>
<td>Changes in electron transduction of the surface layer measured by EIS with ferricyanide redox probe</td>
<td>5 fM to 500 pM / 0.3 fM</td>
<td>[73]</td>
</tr>
<tr>
<td>Codeine</td>
<td>Au–S binding on Au-mesoporous silica nanoparticles</td>
<td>Changes in charge transfer resistance measured by EIS with ferricyanide redox probe</td>
<td>10 pM to 100 nM / 3 pM</td>
<td>[74]</td>
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<tr>
<td>L-Histidine</td>
<td>Au-S binding of a pair of thiolated aptamers bearing ferrocene units on Au nanocrystals with high-index facets</td>
<td>SWV signal of ferrocene after cleavage of the aptamer sequence caused by DNAzyme activity of the analyte and release of the labeled sequence</td>
<td>0.1 pM to 0.1 µM / 0.01 fM</td>
<td>[75]</td>
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<td>ATP</td>
<td>Au electrode covered with monolayer of thiolated capture probe</td>
<td>ATP displacement of DNA probe from aptamer-DNA duplex followed by interaction with capture probe, saturation with thionine and DPV record of thionine signal</td>
<td>0.1–50 nM / 0.05 nM</td>
<td>[76]</td>
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<tr>
<td>K⁺</td>
<td>Au electrode covered with thiolated aptamer bearing ferrocene units</td>
<td>Electron transduction to DNAzyme mimicking HRP activity consisted of G₄ quadruplex stabilized by an analyte and hemin and mediated by ferrocene units</td>
<td>5–200 µM / 1.6 µM</td>
<td>[77]</td>
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<tr>
<td>Protein tyrosine kinase</td>
<td>Au electrode covered with Au nanoparticles with immobilized aptamer–DNA probe complex bearing Au nanoparticles</td>
<td>DPV signal of methylene blue indicator, displacement mechanism</td>
<td>1–100 pM / 372 fM</td>
<td>[78]</td>
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This process was controlled by measurement of the localized surface plasmon resonance and showed dependence of the particle size and dense packing of their layer on the current density and electrolyte concentration. The hybridization event was recorded by EIS with the ferricyanide redox probe and simultaneous monitoring of the surface nucleation by optic part of the system.

Aptamers utilize the Au nanoparticles as carriers or amplifiers of the signal. Contrary to detection of hybridization effect, the relative mass and volume of the target species are often insufficient for significant changes in the permeability or conductivity of the surface layer. In some cases, this is compensated for by changes in the conformation of the aptamer. Au nanoparticles give privilege to the biosensors because of more dense and regular positioning of binding sites on the transducer interface. Thus, codeine determination showed remarkable sensitivity due to immobilization of thiolated aptamers on the nanoparticles consisting of mesoporous silica with implemented Au nanoparticles [74]. The EIS signal indicated increase of the charge transfer resistance starting from 3 pM of the target. It is interesting to note that the authors have optimized the aptamer structure and reported on the dissociation constant $K_d = 0.91 \mu M$. This value contradicts with super-low LOD and can be explained by some other factors like different accessibilities of aptamers in the pores of nanoparticles and in homogeneous conditions of affinity constant determination.

Although the Au nanoparticles obtained in most common methods have rounded shape, scrupulous tuning of the conditions of their synthesis makes it possible to obtain other forms, for example, octahedrons [75]. For this purpose, a mixture of cationic surfactants was used and the periods of seedings and growth were carefully controlled. The formation of nanocrystals with high-index facets was confirmed by electron microscopy. The Au nanocrystals showed some advantages over more common shapes and resulted in ultra-low LODs of L-histidine. The measurement scheme is presented in Fig. 8.9. The analyte promotes cleavage of the aptamer molecule and dissociation of the complex existed. As a result, ferrocene-bearing aptamer attached to the Au nanocrystals becomes flexible and the current related to the ferrocene redox conversion increases with the concentration of the analyte.

**Fig. 8.8:** Application of Au nanoparticles for multiplication of label signal in sandwich-type hybridization assay with appropriate biosensor.
Displacement protocol is another approach to the detection of low-molecular compounds with appropriate aptasensor. In the work [76], aptamer toward ATP was first hybridized with the complementary DNA sequence bearing thiol group. The aptamer–DNA complex was then adsorbed on magnetic particles and incubated in the ATP solution. The reaction with aptamer resulted in release of the thiolated DNA probe, which was then involved in hybridization reaction with capturing probe on the surface of Au electrode. The resulting product was treated with the Au nanoparticles and then thionine, which redox signal was measured by DPV.

Potassium ion K⁺ was determined with aptasensor due to its stabilization of G₄ quadruplex followed by hemin implementation and its redox signal measurement by DPV [77]. The sensitivity of the assay was provided by the Au nanoparticles bearing the reaction components and attaching them to the electrode surface.

In the work [78], the Au nanoparticles played the role of both aptamer carrier and signal enhancer. In this work, the aptamer was first immobilized on the surface of the Au nanoparticles on the electrode and then reacted with complementary sequence attached to other Au nanoparticles present in the solution. The product of interaction was saturated with methylene blue that was accumulated on the negatively charged phosphate residues of the sequences. In the presence of an analyte, tyrosine kinase, the aptamer–DNA probe complex was dissociated due to conversion of stem-loop structure of the aptamer binding the guest. As a result, most part of methylene blue was removed from the electrode surface and its DPV signal decreased.

In some cases, DNA plays auxiliary function in the sensing layer assembly, which is not related to specific interactions but provides the required charge of the surface or connectivity of other components. Au nanoparticles are intended to compensate for losses in electroconductivity of the layer resulted from addition of non-conductive biopolymer molecules. Many of such sensors use layer-by-layer deposition of charged species, including DNA to reach reproducibility of the properties and regularity of the content of the whole surface film. The successful examples of such an approach are given below. Polyelectrolyte layer formed by MWCNTs, poly(ethylene imine),

Fig. 8.9: Determination of histidine based on aptamers with DNAzyme activity bearing ferrocene (Fc) label and Au nanocrystals.
DNA, the Au nanoparticles, and NAD$^+$ showed remarkable electrocatalytic activity in $\text{H}_2\text{O}_2$ reduction [79]. Electropolymerization of $o$-phenylene diamine in the presence of ds-DNA and the Au nanoparticles was used for modification of the pencil graphite electrode and sensitive determination of dopamine (LOD 6 nM) [80] and Sudan II (LOD 0.3 nM) [81]. Polyaniline nanowires decorated with the Au nanoparticles have been proposed to use as universal platform for the detection of various interactions, including the DNA hybridization, glucose sensing with glucose oxidase and Lamina protein detection via its binding to specific antibody [82]. All of the receptors mentioned were just placed on the polymer net and changes in the polyaniline activity followed biochemical interactions on its surface. Additional examples of joint application of the Au nanoparticles and carbonaceous nanomaterials are given below in Section 8.3.2.

### 8.3.2 Other metal nanoparticles

Although many nanoparticles are applied in electrochemical sensors [20], their variety in the assembly of the DNA sensors is not as high as could be expected from the comparison with other electrochemical biosensors. Probably, most of the possible functions of such additives are performed with the Au and carbonaceous nanomaterials better than could be expected from other metals, which are more complicated in preparation, modification, and entrapment in the biosensor assembly. Nevertheless, some examples of the metal nanoparticles successfully applied in DNA sensors are presented below for the period of 2013–17.

In some sensors, the Au-based alloys are used to improve chemical stability of bare gold and possible influence of the Au–S interactions with biogenic thiols and proteins. The additives can also exert their own specificity in the signal generation. Thus, the Au–Pd system was applied in paper-based electrochemiluminescence DNA sensor for microfluidic detection of the cancer cells [83]. The addition of Pd accelerates the reduction of water required in luminescence generation with peroxodisulfate as precursor.

The Ag nanoparticles are mostly used in the optic biosensors based on the fluorescence quenching, but they also can substitute the Au nanoparticles in electrochemical devices though their chemical and electrochemical stabilities are lower than those of gold. Layered nanocomposite films of the polypyrrole–poly(3,4-ethylenedioxythiophene) (PEDOT)–Ag were used for immobilization of the thiolated DNA probe and following hybridization with the complementary sequence [84]. The hybridization resulted in an increase in the charge transfer resistance measured in the presence of ferricyanide as the redox probe. The formation of separate components by chemical oxidation and electrochemical deposition of the Ag nanoparticles was monitored by EIS and constant-current voltammetry. The relative shift of resistance linearly depended on the concentration of the target analyte in semi-logarithmic scale within 10 fM to 10 pM (LOD of 5.4 fM).
The Ag nanoparticles decorated with supramolecular ligands bearing dopamine groups and protecting particles from aggregation were adsorbed together with the neutral red dye on the glassy carbon electrode, pre-oxidized and “activated” by carbodiimide [85]. The ds-DNA was electrostatically accumulated onto the positively charged dye molecules. The biosensor was used for the detection of the oxidative DNA damage. The treatment of DNA with the Fenton reagent or H2O2/CuSO4 mixture resulted in increased charge transfer resistance measured by EIS and higher peak current of neutral red reduction on voltammogram. The Ag nanoparticles amplified the signal due to better electron transduction and possible participation in the DNA damage.

The Ag and Pt nanoparticles prepared separately by chemical reduction of appropriate salts were introduced in the carbon paste to provide electrical wiring, and immobilization protocol for DNA probe related to the β-thalassemia’s gene sequence. The hybridization was monitored by methylene blue current depended on the target concentration within the range 0.6–12.5 ng/µL (LOD 470 pg/µL). Both Ag and Pt showed increase in the cathodic current of the indicator measured by constant-current voltammetry but taken together as they exerted synergic effect. It is interesting to note that in this particular case the DNA probe molecules did not contain thiol groups for covalent attachment to the metals.

The Pt@Pd nanowires were used together with horseradish peroxidase (HRP) for dual amplification of the signal on the DNA sequence related to *Mycoplasma pneumoniae* [87]. The assay is performed in sandwich mode with capturing probe attached to the gold electrode covered with the Au nanoparticles and signaling probe attached together with enzyme and thionine to Pt@Pd nanowires. The latter ones were synthesized by mixing H2PtCl6 and H2PdCl4 in aqueous ethylene glycol and dimethylformamide and heating the mixture to 170°C. The current recorded after H2O2 addition was referred to the oxidized form of thionine, which is enzymatically recovered on the surface of the metal nanoparticles.

Zinc oxide (ZnO) has found increasing interest as biomolecule carrier and component of the optic and electrochemical biosensors due to high biocompatibility, no toxicity, semiconductor and photosensitizer properties [88–90]. The form and size of the ZnO particles depend on thermal conditions of its synthesis. The variety of shape offers additional opportunities to control the performance of the DNA sensors. Thus, flower-like 3D nanostructures have been obtained by mixing Zn acetate and hexamethylenetetramine at 90°C [91]. They were deposited from suspension in chitosan solution on the glassy carbon electrode and then saturated with the Au nanoparticles obtained separately by the citrate/NaBH4 reduction of HAuCl4 solution. Thiolated DNA probes were assembled onto the Au nanoparticles and then used for hybridization detection. Charge transfer resistance increased with the logarithm of the analyte concentration in the range from 0.1 nM to 0.01 pM (LOD 0.002 pM).

Similar flower-like ZnO particles have been obtained by thermal synthesis directly on platinized silicon [92]. The DNA probe related to *N. meningitides* was adsorbed on
the surface. The hybridization was monitored by EIS and DPV. The DNA sensors made it possible to determine from 5 to 240 ng/µL of target sequence with LOD of 5 ng/µL.

### 8.4 DNA sensors based on carbonaceous materials

#### 8.4.1 Carbon nanotubes

SWCNT can be ascribed as a graphene sheet rolled up into a tube normally capped with fullerene semi-spheres. The raw products obtained mostly by chemical vapor deposition (CVD) [93] are contaminated with residues of the catalyst and amorphous carbon, which are removed prior to use by treatment with strong mineral acids and oxidants, respectively. Oxidation of MWCNTs starts from the defects on side-walls and resulted in removal of the fullerene caps. It is often used to simplify functionalization and implementation of MWCNTs in the biosensor assembly. Besides, non-covalent side-wall functionalization due to hydrophobic interactions is described [94].

The diameter of CNTs, their electroconductivity, and number of layers of MWCNTs depend on the synthesis conditions and carbon source. The diameters of SWCNTs and MWCNTs vary from 0.4 to 3 nm and from 2 to 100 nm, respectively [95]. Most of the commercially available CNTs represent a mixture of types different in the convolution of the graphene roll, and in electroconductivity they varied from metallic to dielectric. Semiconducting SWCNTs behave as hole-doped $p$-type FETs [15]. Hole-doping is commonly provided by adsorbed oxygen [96]. Other compounds able to sorption on the side-wall affect the conductivity of such SWCNTs.

There are several approaches to the development of the DNA sensors based on CNTs. First, the transducer should be modified with the CNT layer. This is made by casting the appropriate dispersions, alone or together with other reagents. The aggregation of CNTs in aqueous media prevents the formation of stable dispersion. The addition of cationic surfactants or strong mineral acids stabilizes the dispersion and improves reproducibility of the properties of the surface layer obtained by casting dispersion on the electrode.

The SWCNTs spontaneously form complexes with the partially wrapping DNAs that can be used for dispersing CNTs, their electrophoretic fractioning by size and charge [97] and for detection of the hybridization event [98]. MWCNTs are applied as components of the carbon paste and the surface layer that improve their mechanical durability and electroconductivity and increase the surface area. To some extent, the CNTs exert electrocatalytic properties and can either accelerate oxidation of the DNA or amplify the activity of other mediators implemented into the surface layer and intended for the measurement of a label signal. Contrary to SWCNTs that prefer non-covalent interaction with DNA probes, covalent immobilization of the DNA probes and auxiliary agents is typical for the MWCNTs pre-oxidized almost as often as
they are used. Oxidation provides carboxylic groups for carbodiimide binding of the aminated DNA probes and increases negative charge affecting electrostatic assembling of the biosensing layer.

The adsorption of ds-DNA onto the CNTs accelerates oxidation of nucleotides due to partial wrapping of nucleic acids and due to the electrocatalytic properties of the adduct. The adenine oxidation was observed in the layer of ionic liquid containing MWCNTs and DNA [99]. The spontaneous adsorption of the DNA from aqueous solution on the MWCNT-covered glassy carbon electrode showed independent oxidation of guanine and adenine that gave irreversible peaks at 0.70 and 0.81 V [100].

Besides adsorption, DNA and CNTs can be immobilized on the transducer surface by electrostatic self-assembling with addition of the cationic substances like polyamine, poly(diallyldimethylammonium chloride), alkali earth metal cations, and small charged organic molecules, that is, thionine [101–103].

In Tab. 8.2, examples of the DNA sensors utilizing CNTs are given for a period from 2013 to 2017. Earlier works are summarized in [10, 13, 15, 31]. As could be seen, CNTs are rarely used alone due to obvious problems with reproducibility of the results obtained with commercial preparations. Moreover, electrocatalytic properties of the CNTs applicable for detection of oxygen, hydrogen peroxide, and antioxidants do not contribute to the improvement of the signal related to redox labels of DNA sensors, which are commonly involved in reversible redox reaction at rather low potential. The detection of enzyme activity might be an exception, but the amplification of the signal due to catalytic conversion of the substrate exceeds the positive influence of CNTs.

Besides own electrocatalytic activity, CNTs show other advantages that make them popular in the DNA sensor assembly, that is, applications for site-specific immobilization of the DNA probes and sharp increase of the real electrode surface accessible for electrochemical reactions against bare electrode. The latter parameter allows quantification by electrochemical tools because the estimation of roughness by AMF or SEM does not take into account the difference in electroconductivity of a variety of CNTs commonly present in commercial preparations. The determination of specific surface concentration of the DNA probe immobilized on the CNTs is another problem that needs optimization. It can be determined from relationships of a monolayer adsorption using redox probes or by current of \( \text{Ru(NH}_3\text{)}_6^{3+} \) reduction, which is proportional to the surface concentration of the oligonucleotide [141]. In the following discussions, increase in the number of the DNA probes attached to CNTs is considered as an advantage of the immobilization protocols though steric limitations can compensate for such an improvement.

One could see, most of the CNT-based DNA sensors and aptasensors utilize additional components like metal nanoparticles and polyelectrolytes. The sequence of their deposition on the electrode varies depending on the source and nature. It should be mentioned that AFM and SEM microphotographs presented in many publications show that the size of CNTs and other nanocomponents is incomparable: CNTs are much bigger and loner. For this reason, preliminary deposition
### Tab. 8.2: The characteristics of electrochemical DNA sensors based on CNTs

<table>
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<tr>
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<th>Signal measurement protocol</th>
<th>Linearity range / LOD</th>
<th>Ref.</th>
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<td>Target</td>
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<td>1–400 nM / 210 pM</td>
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<td>Cytochrome c</td>
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<td>0.001–10 ng/mL / 1 pg/mL</td>
<td>[136]</td>
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<td>[137]</td>
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<tr>
<td>Kanamycin</td>
<td>Au electrode consecutively dripped with Au nanoparticles – chitosan, Au nanoparticles – graphene and MWCNTs – Co phthalocyanine dispersions, aminated aptamer covalently bonded by carboxylic groups</td>
<td>Sandwich assay with biotinylated secondary aptamer and HRP–streptavidin conjugate, constant-current signal of hydroquinone as enzyme substrate</td>
<td>10–150 ng/mL / 5.8 nM</td>
<td>[138]</td>
</tr>
<tr>
<td>Target</td>
<td>Immobilization technique</td>
<td>Signal measurement protocol</td>
<td>Linearity range / LOD</td>
<td>Ref.</td>
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<tr>
<td>Kanamycin</td>
<td>Glassy carbon electrode covered with MWCNTs — ionic liquid composite and then amino functionalized graphene. Aminated aptamer immobilized by carbodiimide binding</td>
<td>DPV registration of own activity of the surface layer</td>
<td>1 nM to 100 µM / 0.87 nM</td>
<td>[139]</td>
</tr>
<tr>
<td>PDGF BB</td>
<td>Capture aptamer modified with magnetic beds interacted with an analyte and signaling aptamer bearing silver nanoparticles</td>
<td>Addition of o-nitrophenol and NaBH₄ resulted in formation of o-aminophenol in the presence of Ag nanoparticles, o-aminophenol was electrodeposited with MWCNTs on screen-printed carbon electrode, DPV signal of coating</td>
<td>0.1–100 ng/mL / 60 pg/mL</td>
<td>[140]</td>
</tr>
</tbody>
</table>

PDGF BB    platelet-derived growth factor BB.  
PDMS      polydimethylsiloxane  
PAMAM     polyamidoamine
of CNTs on the electrode results in the formation of rather spongious net. The underlying electrode surface remains accessible for the following casting of other chemicals necessary for film formation and biosensor operation. For this reason, simultaneous casting of all the components directly added to the CNT dispersion has some advantages like one-step preparation of the surface layer, no necessity in time-consuming intermediate washing steps, etc. Layer-by-layer deposition of the components has sense if electrostatic self-assembling takes place or the chemicals are dissolved in inconsistent media. Another argument in favor of step-wise formation of biosensing layer consists of electrochemical synthesis of metal nanoparticles onto CNTs as templates. Negative charge of the CNT's shape caused by carboxylic groups promotes the formation of rather small particles, which are evenly distributed among the DNA surface. To some extent, this can be referred to the electropolymerization of pyrrole [116, 124], aniline [103], and other redox active polymers [109, 111]. Simultaneous influence of electrostatic forces and hydrophobic attraction of neutral forms of the polymers to the side-walls of CNTs results in wrapping of the CNTs with polymer wires and formation of regular structures with extended areas of redox activity [124].

In addition to rather common approaches to the signal measurement, for example, EIS control of surface layer permeability or hybridization detection via redox probe signal (Adriamycin, methylene blue), some unusual solutions can be mentioned. Thus, direct electron transfer to glucose oxidase active site was performed in [108] for hybridization detection. The enzyme was placed together with Au nanoparticles on the surface of MoS₂–MWCNT composite, ss-DNA probe was covalently attached to the carboxylic groups of the CNTs. The formation of ds-DNA in reaction with biological target suppressed the current recorded in the presence of enzyme substrate, glucose. The application of direct electron transfer is not typical for bioanalytical applications because it is often hindered by steric limitations of the electron transfer. In this particular case, its efficiency can be attributed to another component of the surface layer, molybdenum disulfide, which is similar to graphene in electron conductivity and some other properties.

A variety of nanomaterials have been used for the detection of Aeromonas bacteria related to gastroenteritis infections [112]. In sandwich assay, the complex of the capturing DNA probe with the target sequence related to aerolysin gene reacts with the signaling DNA probe labeled with PbS nanocrystals. After separation of the product of reaction, the PbS particles were released and dissolved. The concentration of Pb²⁺ ions was determined with the electrode modified with MWCNTs and Bi. The selectivity of biosensor was confirmed by its use to detect various target DNAs from Aeromonas strains isolated from tap water.

In several works, the PAMAM bearing ferrocene units was used to detect hybridization event [116] and aptamer–analyte interactions (prions [124], thrombin [125]). This hyperbranched polymer has hydrophilic terminal groups suitable for functionalization and is well compatible with biomolecules. Due to the high number of
substituents typical for dendrimers of 4th generation (64 amino groups per PAMAM molecule), it provides multiplication of the signal due to the high number of redox centers participating in the electron exchange and multiple number of target molecules able to bind with specific sites available on the surface of dendrimers.

A very interesting approach has been proposed for amplification of the sensitivity of protein determination (on example of thrombin) [142]. In this work, the analyte first reacted with capturing aptamer and then with signaling aptamer attached together with HRP to the surface of MWCNTs. Sandwich complex was isolated and tetramethylbenzidine added together with \( \text{H}_2\text{O}_2 \). The reaction resulted in formation of insoluble product, which was then electropolymerized to form insulating film on the electrode. This increased the signal measured by EIS. The aptasensor made it possible to detect down to 0.05 pM of thrombin. In a similar manner, electrochemical postreaction with the product formed due to target interaction of the components combined in sandwich-like complex was described in [139] for platelet-derived growth factor BB (PDGF BB) detection. The only difference was that instead of enzyme, silver nanoparticles catalyzed reduction of \( o \)-nitrophenol to \( o \)-aminophenol with \( \text{NaBH}_4 \) followed by electrodeposition of the \( o \)-aminophenol with the MWCNTs on screen-printed electrode.

The development of FETs on the SWCNTs deserves special attention. The device is based on \( p \)-doped Si wafer where two insulated \( n \)-doped areas (drain and source) separated with so-called gate area. The shift of the current between the drain and the source is a measure of the charge in gate area, which is affected by external stimuli. In CNT-based FETs, the drain and the source areas are connected with the semiconducting SWCNT, which resistance depends on the charge distribution altered due to biochemical interactions. The manufacture of the CNT-based FETs employs two approaches (Fig. 8.10). In the first one, the CNTs are synthesized by CVD using metal coating of the gate area as template (catalyst) [143].

![Fig. 8.10: CNT-based FETs. (a) SWCNT is synthesized in gate area by CVD and aptamers are attached to the side-wall due to hydrophobic interactions with pyrene unit. (b) Alone standing CNTs in gate area are modified with DNA probe or aptamer by carbodiimide binding.](image)
The Au contacts of source and drain areas are formed by nanolithography. Second approach assumes deposition of the carboxylated SWCNTs on the iron oxide intermediate layer, so that vertically aligned SWCNTs fill the gap between the source and the drain areas. In both types of the FETs, the DNA probe or aptamer are immobilized onto the CNTs surface via hydrophobic interactions with side-walls or carbodiimide binding of aminated receptors with terminal carboxylic groups of CNTs. Contrary to many other DNA sensors, the generation of the signal does not require any labels or auxiliary reagents, for example, redox indicators.

Similar FETs have been developed for detection of immunochemical interactions and determination of specific antigens [144]. Meanwhile, the use of aptamers instead of antibodies increases the sensitivity of the analyte detection. This is explained by different sizes of the receptors. The sensitivity of the FET signal to the charge distribution changes depends on the distance from the gate to the receptor position where biochemical interaction takes place in comparison with the Debye length. This is the distance that allows screening the surplus charge of the mobile carriers present in a material [145]. If the distance from the surface of a SWCNT to receptor is bigger than Debye length, biochemical reactions do not affect mobile charges of the material. The typical size of the antibodies (~10–15 nm) is similar to the Debye length and the antigen–antibody binding finds response in the double layer near the CNT surface. The aptamer size is commonly smaller and this increases the sensitivity of the aptamer-based assay as was shown on the example of the IgE determination [146, 147].

The immobilization of several aptamers on the SWCNTs makes it possible to determine two analytes with the same FET [148]. To discriminate the signal, different amplification systems based on HRP and glucose oxidase together with appropriate mediators (ferrocene and toluidine blue) have been applied for determination of the thrombin and the PDGF. The sandwich assay was performed with the analytes first attached to capturing aptamers and then treated with labels consisted of graphene flakes bearing enzymes and mediators immobilized on the same carrier.

Similar to SWCNTs, other nanomaterials can be introduced in the FET assembly. Thus, few-layer MoS2 sensing channel material with electronic structure similar to that of graphene was successfully used for this purpose [149]. The material was obtained by chemical reduction of Mo salt in the presence of H2S in Ar atmosphere followed by annealing the material to remove the oxygen residues. The MoS2-based FET was tested on detection of hybridization with 10 µM ss-DNA probe and complementary targets. Other examples of bio-FETs based on graphene are given in Section 8.4.2.

8.4.2 Graphene-based DNA sensors

Graphene is a 2D sheet of carbon atoms linked by sp2 bonds. The electric properties of graphene are related to electron transduction along the basal plane and electron exchange at the edge of the plane [150]. The relative contribution of the above mechanisms depends on the source of a raw material and production protocol.
Graphene-based nanomaterials applied in electrochemical sensors and biosensors are classified in accordance with the number of planes, their defects, production, and pretreatment protocols. Most often, reduced graphene oxide is applied that can be easily obtained by chemical oxidation of graphite followed by separation of graphene oxide and its chemical or electrochemical oxidation either in suspension or directly on the electrode interface. The formation of graphene from its oxide can be monitored by bands referred to >C = O and –C(O)OH groups in Fourier-transform infrared spectra. The surface area of graphene sheets obtained in such a manner (about 2600 m²/g [151]) exceeds that of SWCNTs (~1000 m²/g [152]). The application of the graphene nanomaterials in electrochemical sensors and biosensors was recently summarized in some reviews [153–155].

The ss-DNA probes can be simultaneously adsorbed on graphene and especially graphene oxide sheets by hydrophobic interactions. The hybridization of DNA increases the density of the negative charge and often results in desorption of biopolymers from the graphene surface. If the DNA probe is labeled with fluorescent particle or molecule, such processes result in quenching and re-establishment of the fluorescence, respectively, and can be used for homogeneous DNA assay [156]. In biosensor assembly, the DNA probe should be immobilized to exclude spontaneous leaching. The immobilization protocols are similar to those already described for other carbonaceous materials. They include carbodiimide binding via carboxylic groups of graphene defects bonded to the aminated DNA probe, electrostatic assembling of oppositely charged layers with intermediate cationic polyelectrolytes and hydrophobic binding to polyaromatic molecules, for example, 3,4,9,10-perylene tetracarboxylic acid (PTCA [156]) or 1-aminopyrene [157] covalently attached to the DNA probe or aptamer. In a similar manner, other nanomaterials can be assembled on graphene support for amplification of their electrochemical signal [158]. In comparison with the CNT-based biosensors, graphene exerts less steric hindrance for interaction of bulky molecules and forms denser layers on electrodes. In some cases, graphene layers are used to prevent undesirable sorption of the reaction components on the transducer interface instead of the use of BSA or thiolated alcohols for the glassy carbon and the Au electrodes, respectively.

In some publications summarized in Tab. 8.2, the CNT adducts are applied together with graphene [125, 138, 139]. The authors announce synergic influence of CNTs and graphene brought together in the biosensing layer though there are now direct evidences of such interactions. Moreover, analytical characteristics of such hybrid CNT–graphene biosensors do not differ dramatically from those reported for biosensors with simpler content of the biosensing layer. Most likely, the positive influence of graphene can be attributed to the higher stability of its dispersions and higher electroconductivity against commercial preparations of the CNTs. In many biosensors, graphene is casted onto the glassy carbon as a support for immobilization of the DNA probe that retains electric wiring of oligonucleotides and enhances label signal against bare glassy carbon. As CNTs, graphene is compatible with metal nanoparticles that are easily synthesized on its surface due to the accumulation of ions due to electrostatic interactions. When used in the label assembly, the graphene sheets
show higher stability in dispersion and lower aggregation and sedimentation ability than CNTs due to lesser size and rather high charge density. This simplifies the measurement protocol and improves accuracy of the signal record.

Graphene-based sensors are often used for the detection of the direct redox conversion of the low-molecular compounds like heavy metal cations [158–160], guanine and adenine [161, 162], antioxidants [163, 164], etc. This is related to the electrocatalytic properties of graphene and its charge promoting accumulation of cationic species prior to their reduction or oxidation on the electrode. The DNA can be involved in the assembly of such sensors as additional accumulation component and receptor required for selective response to certain analytes. Thus, Ag⁺ ions form specific complexes with cytosine-rich areas of the DNA and hence can be accumulated on ss-DNA and cytosine-rich oligonucleotides for the following detection by relative increase in charge transfer resistance measured by EIS [165]. Graphene oxide – Fe₃O₄ composite provided platform for immobilization of DNA and site-specific immobilization of DNA sequences on its surface. LOD of 2 pM was reached together with multiple use of the biosensors based on intermediate washing with cysteine solution.

Such biospecific routes of ion recognition are especially popular for Pb²⁺ and Hg²⁺ ions. Thus, graphene oxide was treated with nitrogen plasma and then with a complex combining DNAzyme, substrate DNA, and binding Hg²⁺ and Pb²⁺ DNA [166]. Binding of the target ions results in conformational changes of the DNA and appropriate shift of the charge transfer resistance within the interval 0.01–100 nM (LOD 7.8 and 5.4 pM for Pb²⁺ and Hg²⁺, respectively). Graphene-based FET with aptamer immobilized in the gate area covered with graphene synthesized in situ by CVD showed sensitive response to Hg²⁺ ions (LOD 10 pM) [167].

The Pb²⁺-dependent DNAzyme was immobilized on the surface of the Au electrode [168]. In the presence of Pb²⁺ ions, it was cleaved to leave ss-DNA sequence attached to the electrode and complementary to DNA probe bearing graphene nanosheets containing MnO₂ and hemin that exert peroxidase mimicking activity. The signal recorded in DPV mode and related to the H₂O₂ reduction makes it possible to determine 0.1 pM to 200 nM Pb²⁺ (LOD 0.034 pM).

Another approach to the Pb²⁺ detection was demonstrated in [169]. The Pb²⁺-sensitive aptamer was immobilized on the Au electrode via Au–S binding. Prior to contact with an analyte, it was saturated with graphene nanosheets bearing thionine molecules hydrophobically retained on its surface. The reaction resulted in removal of graphene label together with thionine and entrapment of Pb²⁺ ions into the folded G₄ quadruplex structure of the aptamer. Besides amplification of the signal due to the high capacity of graphene to thionine molecules, the use of this label suppresses interfering influence of K⁺ ions competing with Pb²⁺ ions for binding site of the aptamer. The aptasensor makes it possible to detect 32 pM of target ion using thionine peak current measured with DPV as a signal (concentration range from 0.16 pM to 0.16 nM). In a similar manner, Pb²⁺–aptamer interaction in gate area was recorded with graphene-based FET [170].
Mercury biosensing is mainly based on its ability to form stable triple complex T–Hg$^{2+}$–T (3) with the thymine-rich areas of ss-DNA or poly(T) sequence.

The latter one was immobilized via terminal thiol group on the Au electrode followed by incubation of the biosensor in dispersion of the graphene oxide and then in Hg$^{2+}$ solution. In the absence of the target, the current of graphene oxide reduction can be observed by constant-current voltammetry. The Hg$^{2+}$ ions are incorporated between thymine bases, so that more rigid analog of ds-DNA is formed. This results in increase of the distance between the graphene units and the electrode followed by increase of the charge transfer resistance measured with EIS and decrease in appropriate current. The biosensor makes it possible to determine 1–300 nM Hg$^{2+}$.

Nanorods of polyaniline obtained in the presence of reduced graphene oxide were used as support for immobilization of T-rich DNA and following detection of Hg$^{2+}$ that formed T–Hg$^{2+}$–T complexes on the surface [170, 171]. The EIS signal was observed from 0.01 to 100 nM (LOD 3.5 pM).

Triple-helix DNA consisted of the ss-DNA, and T-rich ds-DNA was used for silver-amplified mercury detection. In this biosensor, the addition of Hg$^{2+}$ ions removed the ds-DNA due to unwinding caused by introduction of the mercury ions and formation of T–Hg$^{2+}$–T fragments [172]. The ss-DNA is then involved in the reaction with C-rich complementary sequence that is immobilized on Au nanoparticle. The reaction results in formation of C–Ag$^+$–C bridges (4) to form dendrite silver nanostructures after cathodic reduction of entrapped silver ions.

The anodic current of silver dissolution in stripping voltammetry mode is proportional to the Hg$^{2+}$ concentration in the range from 0.1 to 130 nM (LOD 0.035 nM).

The examples of application of graphene in the assembly of the DNA sensors for hybridization detection and the aptasensors are presented in Tab. 8.3 covering 2013–17.
## Tab. 8.3. The characteristics of electrochemical DNA sensors based on graphene

<table>
<thead>
<tr>
<th>Target</th>
<th>Immobilization technique</th>
<th>Signal measurement protocol</th>
<th>Linearity range / LOD</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridization detection</td>
<td></td>
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<tr>
<td>Human papillomavirus</td>
<td>Capture probe immobilized on glassy carbon electrode modified with graphene / Au nanorods / polythionine</td>
<td>Sandwich assay with two long-range signaling probe partially complementary each other to increase ds-DNA area, DPV signal of Ru hexamine as hybridization indicator</td>
<td>0.1 pM to 0.1 nM / 40 pM</td>
<td>[174]</td>
</tr>
<tr>
<td>Staphylococcus aureus nuc gene</td>
<td>ZrO₂/graphene nanocomposite electroreduced on carbon ionic liquid electrode, DNA probe immobilized by phosphate residue interacted with ZrO₂</td>
<td>DPV signal of methylene blue as hybridization indicator</td>
<td>0.1 pM to 1 µM / 32.3 fM</td>
<td>[175]</td>
</tr>
<tr>
<td>BCR/ABL fusion gene</td>
<td>Graphene sheets / polyaniline /Au nanoparticles deposited on glassy carbon electrode, thiolated stem-loop is immobilized to Au nanoparticles via Au–S binding</td>
<td>Capture stem-loop probe is involved in catalyzed stem-loop assembly resulted in formation of biotinylated product interacted with streptavidin–alkaline phosphatase conjugate</td>
<td>10 pM to 20 nM / 1.05 pM</td>
<td>[176]</td>
</tr>
<tr>
<td>HIV1 gene</td>
<td>Printed graphene electrode with electrochemically reduced graphene oxide and DNA probe immobilized by hydrophobic interactions</td>
<td>Peak current of graphene oxide reduction</td>
<td>1 pM to 0.1 µM / 0.1 pM</td>
<td>[177]</td>
</tr>
<tr>
<td>Apolipoprotein E gene associated with Alzheimer's disease</td>
<td>Graphene/mesoporous silica hybrid with covalently attached ferrocenocarboxylic acid, adsorbed methylene blue and DNA probe</td>
<td>Methylene blue current as indicator of hybridization indicator</td>
<td>10 nM to 10 fM / 10 fM</td>
<td>[178]</td>
</tr>
<tr>
<td>HIV gene</td>
<td>Fe₃O₄-reduced graphene oxide composite, physically adsorbed DNA probe</td>
<td>Fe(II/III) signal increases in hybridization step</td>
<td>10 aM to 1 nM / 2 aM</td>
<td>[179]</td>
</tr>
<tr>
<td>Fragment of PML/RARA fusion gene sequence</td>
<td>Graphene oxide electrochemically reduced on polyaniline modified glassy carbon electrode, physically adsorbed DNA probe</td>
<td>Charge transfer resistance measured by EIS in the presence of ferrocyanide ions</td>
<td>1 fM to 10 nM / 0.25 fM</td>
<td>[180]</td>
</tr>
<tr>
<td>Target</td>
<td>Immobilization technique</td>
<td>Signal measurement protocol</td>
<td>Linearity range / LOD</td>
<td>Ref.</td>
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<tr>
<td><em>Escherichia coli</em> O157:H7</td>
<td>Graphene oxide–modified iron oxide – chitosan hybrid nanocomposite electrophoretically deposited on ITO glass electrode, physically adsorbed DNA probe</td>
<td>Charge transfer resistance measured by EIS in the presence of ferrocyanide ions</td>
<td>10 fM to 1 µM / 10 fM</td>
<td>[181]</td>
</tr>
<tr>
<td>18-mer DNA probe</td>
<td>Glassy carbon electrode modified with graphene and Cu(phen)$_2$$^{2+}$ complex acting as anchor for DNA probe</td>
<td>SWV signal of Cu(phen)$_2$$^{2+}$ as hybridization indicator</td>
<td>1 pM to 1 µM / 0.199 pM</td>
<td>[182]</td>
</tr>
<tr>
<td>Fragment of <em>E. coli</em> DNA sequence</td>
<td>Glassy carbon electrode modified with graphene decorated with Au nanoparticles with immobilized thiolated capture DNA probe</td>
<td>Sandwich assay with signaling DNA probe modified with methylene blue, DPV signal of methylene blue</td>
<td>0.1 fM to 0.1 µM / 0.035 fM</td>
<td>[183]</td>
</tr>
<tr>
<td>Breast cancer 1 (BRCA1) gene</td>
<td>Glassy carbon electrode modified with reduced graphene oxide – yttria and DNA probe specifically bonded to yttria with phosphate residue</td>
<td>Sandwich assay with signaling DNA probe labeled with Au nanoparticles, DPV signal of Au nanoparticles</td>
<td>10 aM to 1 nM / 5.95 aM</td>
<td>[184]</td>
</tr>
<tr>
<td>HIV gene</td>
<td>Glassy carbon electrode covered with graphene – Nafion composite, DNA probe physically adsorbed</td>
<td>Charge transfer resistance measured by EIS in the presence of ferrocyanide ions</td>
<td>0.1 pM to 0.1 nM / 0.023 pM</td>
<td>[185]</td>
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<tr>
<td><strong>Aptamer-based DNA sensors (aptasensors)</strong></td>
<td><strong>MoSe$_2$–graphene composite obtained by hydrothermal method, aptamer immobilized via carbodiimide binding</strong></td>
<td><strong>Aptamer reacts with complementary DNA, exonuclease digests ds-DNA releasing complementary DNA for reaction with auxiliary biotinylated DNA, the following binding with streptavidin–peroxidase conjugate provides the signal after addition of hydroquinone and H$_2$O$_2$, PDGF stimulates the formation of G$_4$ quadruplex and stops the above reaction</strong></td>
<td><strong>0.1 pM to 1 nM / 20 fM</strong></td>
<td><strong>[186]</strong></td>
</tr>
<tr>
<td>Target</td>
<td>Immobilization technique</td>
<td>Signal measurement protocol</td>
<td>Linearity range / LOD</td>
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<tr>
<td>Dopamine</td>
<td>PEDOT doped with graphene oxide obtained by electropolymerization, aminated aptamer covalently attached via carbodiimide binding</td>
<td>DPV signal of dopamine</td>
<td>1 pM to 160 nM / 78 fM</td>
<td>[187]</td>
</tr>
<tr>
<td>Neuropeptide Y</td>
<td>Aptamer covalently attached to Au nanoparticles and deposited onto the surface of graphene sheets used as electrode in flow lateral microfluidic device</td>
<td>Constant current signal of oxidation of tyramine residues of neuropeptide Y</td>
<td>10–1000 pM / 10 pM</td>
<td>[188]</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>Reduced graphene oxide was deposited on the glassy carbon electrode and Au nanoparticles were electrochemically synthesized onto its surface, aptamers were immobilized with via Au–S binding or specific antibodies</td>
<td>Sandwich assay with signaling aptamer attached to Au nanoparticles bearing peroxidase, constant current measured after addition of hydroquinone and H₂O₂</td>
<td>0.5 ng/L to 2 mg/L / 0.5 ng/L</td>
<td>[189]</td>
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</table>

Tab. 8.3: (continued)
One could see covalent immobilization was applied much less against hydrophobic interactions via special labels introduced in the assembly of an aptamer or by physical adsorption of the aptamer onto the electrode surface. Contrary to graphene, reduced graphene oxide retains boundary polar groups providing the dispersion and following deposition of the suspension on the electrode surface. Electrochemical reduction is less efficient but offers new opportunities of the formation of hybrid structures due to simultaneous formation of Au nanoparticles or polyaniline (polyanthionine). In all such cases the function of graphene was attributed to the enhancement of electroconductivity and electric wiring of labels (redox indicators) used in measurement protocol. The simultaneous deposition of hybrid coatings provides even distribution and accessibility of the components for diffusionally free agents. This was monitored by EIS and SEM and considered as additional advantage of the modification method. The following detection of target interactions is rarely based on the intrinsic redox activity of the surface layer. In case of small species, they can be involved in redox reaction so that aptamers just concentrate the analyte molecules onto the electrode surface. In many other sensors, the activity of enzymes or redox response of labels introduced in the surface confined complex is measured by conventional methods. Contrary to the CNTs, changes in the permeability or charge distribution of the surface layer estimated by EIS are rarely applied in graphene-based biosensors. This might be referred to higher redox activity and lesser size of graphene against those of CNTs and compensation of steric hindrances appeared after binding bulky analytes. The same reason might explain higher attention to specifically biochemical approaches to the sensitivity amplification, for example, additional treatment of the sensors with exonucleases or formation of catalytic complexes for stem-loop aptamers. To some extent, such sophisticated reaction paths realized in biosensor format level their main advantage, that is, simple format and fast response, but the LOD values and dynamic range of concentrations achieved are very low and allow direct monitoring of appropriate processes in vivo.

8.5 Conclusions

The progress in the development and manufacture of novel nanomaterials for the electrochemical DNA sensors offers new opportunities of their further commercialization and application in medical diagnostics and other areas. Meanwhile the current state-of-the-art also showed some problems related to the choice of nanomaterials and their role in biosensor performance.

Recently reported sensors with DNA probes and aptamers as recognition elements mostly use not one but several nanomaterials that play different role – receptor support, surface enhancer, and electric transduction improvement. This is particularly true for the combination of carbonaceous materials and Au nanoparticles that compensate for
negative effect of non-conductive additives on the electric wiring of labels or indicator redox centers. The experience of application of some nanomaterials like quantum nanodots or silver nanoparticles came from optic methods of assay where they are used as fluorophores or fluorescence quenchers. However, it should be mentioned that the size and quantities of such additives in electrochemical sensors can be significantly different from those in optic devices. Magnetic nanoparticles are used in conventional immuno- and DNA assay for separation of appropriate labeled complexes on intermediate stages of the analysis. However, their use in the assembly of biosensing layer of DNA sensors has much less substantiation excepting mechanical durability of the coating. For the same reason, semiconductors applicable in electronic devices have lesser effect in electrochemical sensors operated in aqueous electrolytes. This refers to ZnO and some metal chalcogenides that are used in DNA sensors but do not participate in electron transduction. The application of up to five different nanoparticles in the assembly of single DNA sensors also has some objections regarding the accuracy of assembling and real necessity of so many species for detection of rather simple redox reactions.

In spite of these difficulties, the application of nanomaterials in DNA sensors increases dramatically in the variety of possible analytes and mechanisms of signal transduction. Concerning nearest future, enhanced application of biochemical steps of signal amplification can be expected because many traditional opportunities of electrochemical analysis are near being exhausted. The popularity and accessibility of FET devices depend only on the offers of their manufacturers. Another possible source of the growth includes the capabilities provided by 3D printing using traditional plastics and those mixed with nanomaterials able to recognize analytes and accumulate their molecules for target interactions with DNA.

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Part III: Application in Electroanalysis


