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Mechanisms of Analytical Signals Generated by Electrochemical Genosensors - Review

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Abstract. Detection and analysis of specific DNA sequences is an important approach in molecular diagnosis. Avian influenza viruses (AIVs), in particular the highly pathogenic H5 subtype, could cause severe diseases. They are endemic in wild birds and their introduction and conversion to highly pathogenic avian influenza virus in domestic poultry is a cause of serious economic losses as well as a risk for potential transmission to humans. We report a short review of electrochemical genosensors devoted for detection of influenza virus H5N1 gene sequence. We will focus our attention on ion-channel mechanism, E-DNA sensors and genosensors based on redox active layer. A novel a dual DNA electrochemical sensor with "signal-off" and "signal-on" architecture for simultaneous detection of two different sequences of DNA derived from Avian Influenza Virus type H5N1 by means of one electrode is presented.
Key words: electrochemical genosensors; electrode modification; redox active monolayers; mechanisms of analytical signals generation.

Resumen. La detección y análisis de secuencias específicas en el ADN es de relevancia en el diagnóstico molecular. Los virus de influenza aviar, en particular el subtipo altamente patógeno H5, pueden provocar enfermedades graves. Dichos virus son endémicos en aves silvestres y su introducción y conversión en virus de influenza aviar altamente patógenos en aves de corral es una causa de serias pérdidas económicas así como de un riesgo, para su potencial transmisión a humanos. En este trabajo reportamos un revisión breve sobre genosensores electroquímicos útiles para la detección de secuencias genéticas del virus de influenza H5N1. Enfocamos nuestra atención en el estudio del mecanismo de ión-canal, el desarrollo de sensores E-DNA y en genosensores basados en una capa electroactiva. Se presenta un sensor electroquímico dual y novedoso, basado en arquitectura "signal-off"/"signal-on" para la detección simultánea de dos secuencias diferentes en ADN derivadas del virus de la influenza aviar del tipo H5N1.
Palabras clave: genosensores electroquímicos, modificación de electrodos, monocapas electroactivas, mecanismos de generación de señales analíticas.

Introduction

According to the IUPAC definition, a biosensor is a self-contained integrated device which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element, which is in direct spatial contact with a transducer element [1]. Generally, biosensors consist of two main elements:

- A molecular recognition layer that enables the selective recognition of a particular analyte or a group of analytes,
- A signal transducer which converts energetic signal coming from an intermolecular recognition process into another form of energy readable for readout device.

Main parameters describing the quality of biosensors are selectivity, sensitivity (specifically, having a low limit of detection), reproducibility and time of response. Electrochemical biosensors belong to a subclass of biosensors, which contain an electrochemical transductor. Sensors based on a self-assembly layer of DNA strain (24 to 25-mer) as a recognition element at the surface of transducer are called genosensors. The immobilization of DNA probe at the surface of electrode plays a crucial role for future genosensor analytical parameters.

The most popular immobilization methods relay on: biotin-avidin interaction (affinity binding) [2], self-assembly of thiol functionalized DNA probe on the gold electrode surface [3], and carbodiimide covalent binding to an active surface [4].

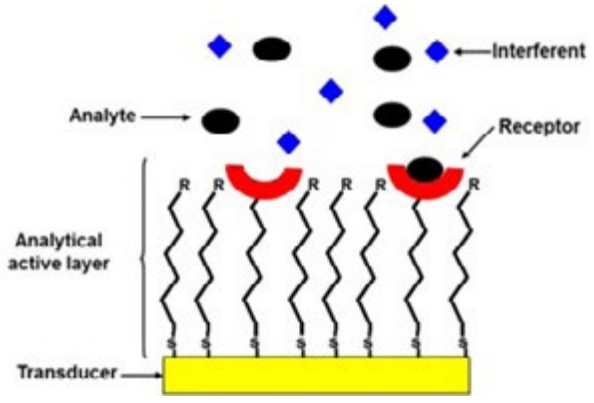

Fig. 1. General scheme of a biosensor.

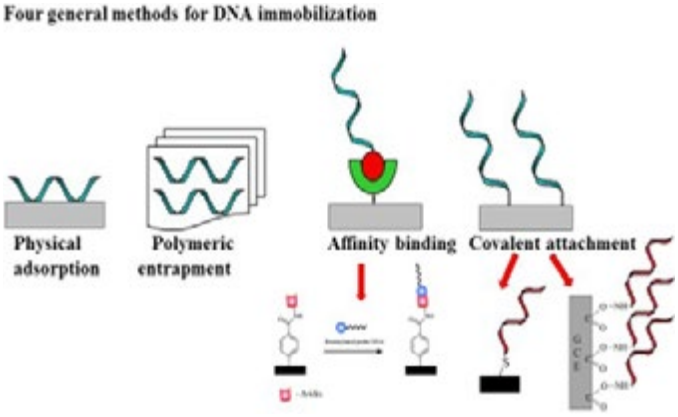

Fig. 2. General methods for ssDNA probe immobilization.

The fundament for the first strategy of electrochemical DNA sensing was given by Paleček in a previous paper concerning the electroactivity of deoxyribonucleic acid [5]. Oxidation of adenine (A) and guanine (G) can be readily observed using carbon electrodes or hanging mercury drop electrodes (HMDEs) which are better for investigation of reduction of nucleic acids. Numerous nucleotide detection methods have been reported based on these reactions. Brabec *et al*. observed oxidation peaks of G and A at about +0.9 V and +1.2 V, respectively, on carbon electrodes (vs. saturated calomel reference electrodes in 0.2 M acetate buffer solution at pH 6.44) [5]. Carbon electrodes are more practical than HMDEs and are suitable for miniaturization based on chip technology since they are solid electrodes, although they have the drawbacks of high background current and low sensitivity. Diamond electrodes are of growing interest and are increasingly being used for electroanalytical applications, including the procedure of DNA sensing based on direct redox reaction of nucleic bases is quite sensitive and selective, but their applicability is rather limited [6]. Their main drawback is background current at the relatively high potentials required for direct oxidation of DNA. In the case of reduction the serious limitation is the necessity to use mercury electrode.

One interesting approach for voltammetric signal amplification was presented by Umezawa and co-workers [7]. In their proposal, the mechanism for generation of an analytical signal was connected with the binding event between electro-inactive analyte and the receptor monolayer located at the electrode surface. This process controlled the redox active marker access to the electrode surface by either electrostatic interaction or by creation of steric hindrance at the surface of electrode. Finally, electron transfer from marker to surface of electrode was affected by analytes binding to the receptor. The heterogeneous rate constant of electron transfer from marker to electrode surface became large or smaller, therefore the redox current increased or decreased. The electrochemical sensors based on this mechanism are called ion-channel mimetic sensors.

Recently in our laboratory we have developed genosensors based on the gold electrode intended for detection of specific DNA sequence of avian influenza virus H5N1 using NH_2-ssDNA or HS-ssDNA probe for modification of a gold electrode [8a, 8b].

Both sensors were used for determination of 20-mer and 180-bp (PCR products) containing the complementary 20-mer sequence in various positions. The localization of complementary fragments is illustrated at the scheme (Fig. 4)

In the case of sensor (A), immobilization of the DNA probe was performed using a thioacid. In the second one (B), the probe was immobilized directly to the gold surface *via* S-Au bonds. As marker ions in both cases, we employed $[Fe(CN)_6]^{3-/4-}$. The sensor based on SH-NC3 probe displayed a detection limit in the 10 pM range, whereas in the case of NH_2-NC3 probe, detection limit was in the fM range. These results showed that the electrode modified with longer spacer molecules show a higher hybridization signal. This is due to improved accessibility of target ssDNA to the probe DNA. But the price for this improvement was a lower selectivity. In the case of using a shorter spacer, the sensor was able to distinguish between the PCR products with different position of complementary parts, whereas the electrode modified with longer spacer molecules was not able to do this. A decrease in the linear current behavior was observed in the case of sensor B, in the presence of all

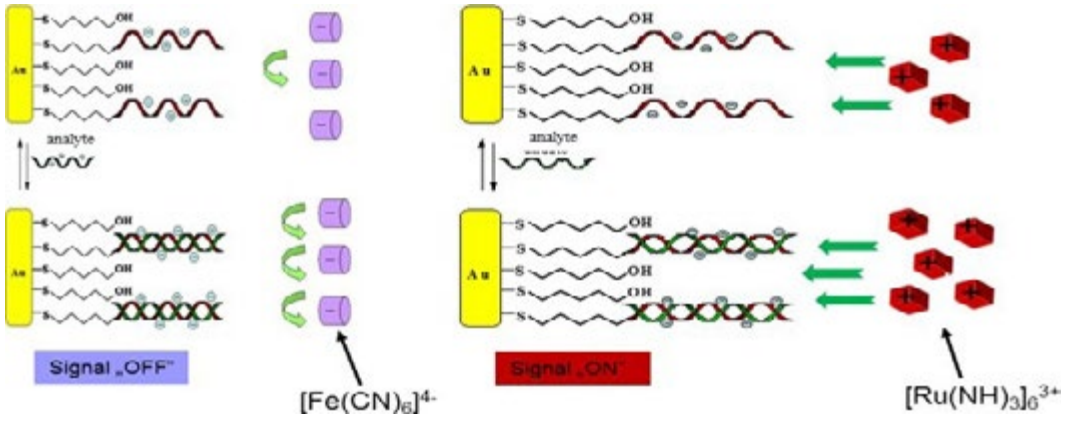

Fig. 3. General scheme of analytical signal generation by ion-channel mimetic sensors.

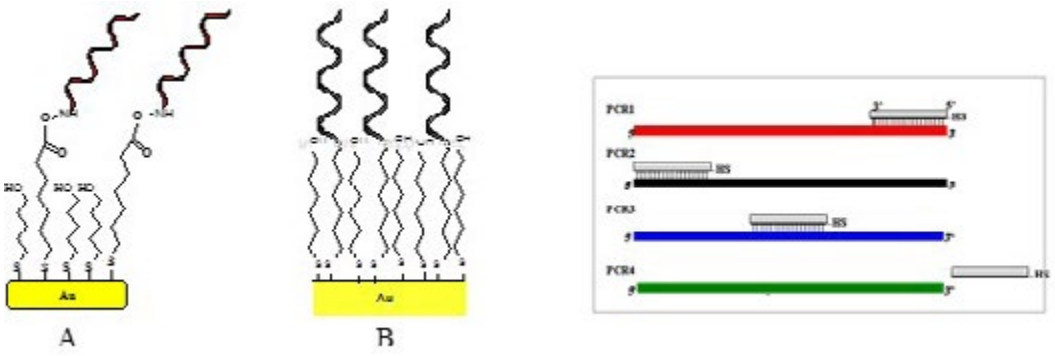

Fig. 4. Scheme of genosoensors: (A) [8a]; (B) [8b] and localization of complementary fragments in PCR products.

target molecules, PCR1, PCR2 and PCR3, in the concentration range from 10 pM to 100 pM. The strongest signal was generated by PCR1 with complementary sequences at the 3'-end. In this case, the hybridization process with oligonucleotide probe takes place at the vicinity of electrode surface, and the part of product which was not involved in the hybridization could be exposed toward the aqueous phase. For PCR2, which has the complementary sequence at opposite side of the strand (at 5'-end), the efficiency of hybridization was very weak. In this case, the accessibility towards oligonucleotide probe immobilized on the surface of electrode was very difficult, because the protruding part (159 nucleotides at 5'-end) was directed toward the electrode surface rather than aqueous phase. In the case of PCR3, where 82 nucleotides at 5'-end precede the sequence complementary to the probe (the region of complementarity to NC3 is in the middle of the PCR3) a "middle" response was generated. The non-complementary product, PCR4, caused only a little increase of current.

Detection of some mutation or damaged DNA bases is very important for early diagnosis of genetic diseases. Application of small DNA–intercalating or groove binding redox active compounds gives the possibility to distinguish between single stranded probe from the double stranded hybrid located at the surface of electrode. Also, application of intercalating molecules as markers of hybridization was based on the fact that DNA base pairs combined to form a continuous π-stacked conduit for charge transfer (CT). The disruption of a single base pair, as in a mismatch, could interrupt the CT pathway. One the most common used intercalator as a redox indicator of DNA duplex formation in DNA sensor is daunomycin [9, 10]. In recent years, a lot of papers describing the electrochemical sensors designated for single base mishmashes determination based on the intercalates as a marker have been published [11-14]. The most important feature of this type of genosensors working with free diffusional redox markers, is the lack of the necessity of labelling of target or DNA probe. Generally, they are very sensitive and selective, in particular those which are based on ion channel mechanism or intercalators as redox markers.

Novel types of hybridization detection techniques being recently developed, exploit the difference in physical flexibility between single-stranded oligonucleotides and double stranded ones. These techniques are based on oligonucleotide probes labeled with electroactive moieties at the end of the probes and tethered to the electrode surface at the other end of the probe. Upon hybridization, the physical changes in the probe structures, including the change in distances of the labeled electroactive moieties to the electrode surface, result in the switching "on/off" of the electrochemical signal. The selectivity and sensitivity of the E-DNA sensors arises from a combination of a conformational change upon hybridisation, together with the redox labels being active at potentials far from those of most electroactive biomolecules typical for clinical and environmental samples, thus being resistant to interfering contaminants. The E-DNA sensors can detect picomoles of ssDNA. Generally the genosensors based on described mechanism belong to very wide "signal-off" mode family. It is worth to note that detection limit of E-DNA working according to "signal-off" mechanism is in the range of 10 pM. Recently, we have observed the dynamic development of E-genosensors based on "signal-off" mechanism [15-17]. The limitation of "signal-off" sensors is that the recognition processes are signalled by the loss of the initial current. So, it is no possible to suppress the original current more than 100%. Thus, it is a strong limitation in sensitivity of such type of sensors.

The first genosensor working according to "signal-on" mechanism was described by Immoos and co-workers [17]. This approach resulted in detection limit of 200 pM and 600 % of current signal increase. Both type of E-DNA sensors, "signal-on" and "signal-off", generated the analytical signal after stimulation with of target DNA without the addition of exogenous reagents. This property is very important from medical diagnosis point of view. This type of sensors have their own strong sides, but also, limitations. They are readily reusable, sequence specific and selective. They could be applied for measurements even in blood serum. The weak side of "signal-off" sensors is suppression of signal generated after stimulation with target. Maximal suppression could be only 100% of the original current. "Signal-on" architecture incorporating redox decorated DNA sequence have a potential for great improving of sensitivity, because the stimulation of these type of sensors with the

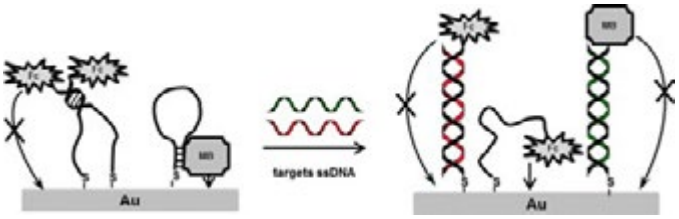

Fig. 5. Schematic representation of working principle of "signal-on" and "signal-off" mode of genosensor consisting of two different ssDNA probe decorated with ferrocene (Fc) and methylene blue (MB) (Reproduced by permission of American Chemical Society [18]).

target does not cause a limited increase of signal. A weak point of these type of E-DNA sensors is the use of rather complicated, not very stable architectures. Most "signal-on" systems contained signal generating strands non-covalent attached to the surface. This approach does not work properly in the complex samples.

Today in medical diagnosis, an ideal biosensor is required to be not only miniaturized and cost effective, but also capable of simultaneous detection of multiple analytes. In our laboratory we have developed a novel dual a E-DNA sensor which generated the analytical signal according to "signal-off" and "signal-on" scheme for simultaneously detection of two different sequences of DNA derived from Avian Influenza Virus (AIV) type H5N1 by means of one electrode [18]. Two sequences of ssDNA characteristic for hemagglutinin decorated with ferrocene (ss-DNA-Fc) and for neuraminidase decorated with methylene blue (ss-DNA-MB) were immobilized covalently together on the surface of gold electrode.

Hybridization process going at the electrode surface was controlled by Osteryoung square-wave voltammetry.

Detection limits determined by graphic method were $4.0x10^{-8}$M and $2.0x10^{-8}$M, for simultaneous analysis of both sequences and for single one, respectively. These values, in particular the detection limits for parallel determinations of two

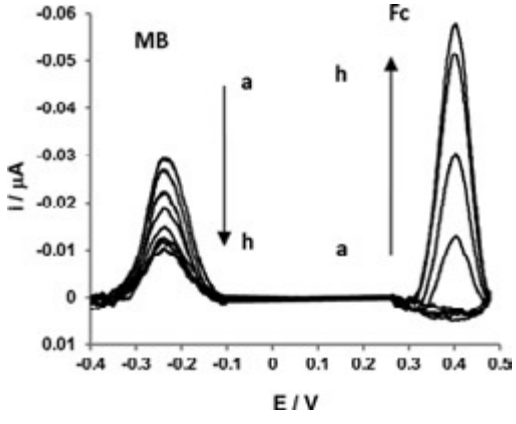

Fig. 6. Representative Osteryoung Square Wave voltammograms recorded with electrodes modified with SH-ssDNA-Fc probe and SH-ssDNA-MB probe. (Reproduced by permission of American Chemical Society [18]).

sequences, is very promising from diagnosing point of view. The duo-genosensor was selective with similar (in the range of 18-21 nM) limits of detection for both targets. The presence of oligonucleotide sequences complementary to SH-ssDNA-Fc probe does not influence the function of the probe decorated with methylene blue and vice versa. The presence of ssDNA complementary to the SH-ssDNA-Fc probe resulted in the increase of ferrocene redox current, which indicates that this probe worked according to the "signal-on" mode. On the other hand, the presence of the ssDNA complementary to the SH-ssDNA-MB probe resulted in a decrease of redox current of methylene blue, which indicates that this probe worked according to the "signal-off" mode. The duo-genosensor is able to detect selectively and with good sensitivity sequences characteristic for genes encoding hemagglutinin (ssDNA-Fc) and neuraminidase (ssDNA-MB) of the influenza H5N1 virus simultaneously by means of single measurement. To our knowledge, this is the first example of a duo-genosensor working in the dual mode: "signal-on" and "signal-off". Detection of influenza virus by means of genosensors may be hampered (put in doubt) by a high mutational rate of the viral genome. With a standard single probe-DNA sensor, especially directed toward a highly variable hemagglutinin gene, this may lead to an increase in false negative results. The duo-sensor would reduce such miss-readings, as the probability of simultaneous appearance of double mutant in two restricted regions of separated targets is rather low. Duo-sensor also should be advantageous by diminishing false positive readings which may appear in the case of non perfect hybridization with component(s) present in non infected host samples. The probability for existence in native samples of components efficiently interacting with two independent DNA probes is limited.

Recently we have developed in our laboratory a new type of genosensors based on gold electrode modified oligonucleotide probe decorated with 3-ironbis(dicarbollide) or cobalt porphyrin complex as a redox label at the "foot" of the oligonucleotide probe, very close to the electrode surface. The proposed genosensors displayed good selectivity and sensitivity towards complementary targets, 20-mer ssDNA and PCR products derived from Avian Influenza Virus, type H5N1. In case of 3-ironbis(dicarbollide) use as a redox label the detection limits recorded for the 20-mer complementary ssDNA and PCR products having complementary sequences at the 3'–end and in the middle of the oligonucleotide were equal to 0.03 and 0.08 fM, respectively, which is superior to many other systems already reported. An additional advantage of this genosensor was its ability to differentiate PCR products containing complementary sequence in different positions [19]. In the case of the genosensor based on the oligonucleotide probe decorated with cobalt(II)- porphyrin complex, the detection limit was 21 fM [20]

The most promising direction of current research in our laboratory is associated with electrochemical biosensors based on redox active layer immobilized on the electrode surface. In such type of analytical active layers, the redox center fulfill double functions: it is both transducer of signal coming from

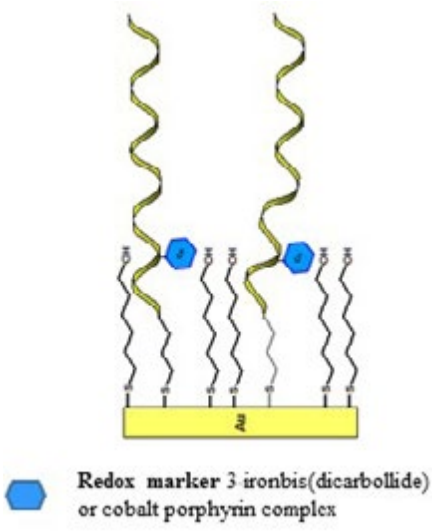

Fig. 7. Scheme of genosensor based on DNA modified with redox label at the "foot" of the oligonucleotide probe.

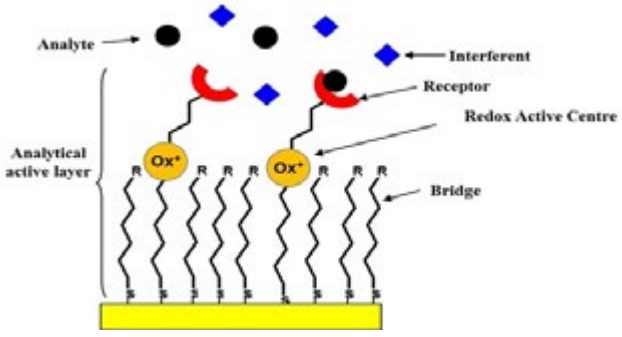

Fig. 8. General scheme of genosensor based on the redox active layer.

recognition into analytical one as well as works as a molecular connector for oriented immobilization of bio-receptors.

We have already developed the new type of sensitive label-free electrochemical genosensors based on $(dipyrromethene)_2Cu(II)$ and $(dipyrromethene)_2Co(II)$ redox-active layers. In the proposed system, the redox centers are bound to the surface of electrode. Thus changes of their distance to the electrode surface are not possible. A novel mechanism of electrochemical signal generation based on changes of the energy of ion-barrier "switch off" system have been proposed. According to this mechanism , the proposed sensors generate an analytical signal because of changes in the environment surrounding the redox center occurring as a result of hybridization processes. The reactions taking place in both redox centers are the following:

(A) $[DPM\text{-}Cu^{+2}\text{-}DPM]^0 \underset{-e^-}{\overset{+e^-}{\rightleftharpoons}} [DPM\text{-}Cu^{+1}\text{-}DPM]^-$ Cations from supporting electrolyte are involved

(B) $[DPM\text{-}Co^{+2}\text{-}DPM]^0 \underset{+e^-}{\overset{-e^-}{\rightleftharpoons}} [DPM\text{-}Co^{+3}\text{-}DPM]^+$ Anions from supporting electrolyte are involved

For the sensor based on the Cu(II) complex, during the redox cycle, Cu(II) is reduced to Cu(I). As a consequence, a single negative charge of the reduced form appears at the surface of the electrode. The precondition of redox reaction run is the compensation of this charge by ions from the supporting electrolyte, involving transport of cations. For the electrode modified with the Co(II) complex, an oxidation process is possible, which generates an extra positive charge of the oxidized form. For its neutralization, anions from the supporting electrolyte will be involved. The mechanism of analytical signal generation by this type of sensors relies on changes in accessibility of ions present in the supporting solution to the redox centers in order to neutralize the charge occurring as a result of oxidation/reduction processes. This phenomenon depends not only on the hydrophobicity/hydrophilicity of ions but on the structure of redox complexes as well. Better analytical parameters have been achieved with DPM-Cu(II)-DPM than with DPM-Co(II)-DPM. The twisted structure of DPM-Cu(II)-DPM created a higher energy barrier for supporting electrolyte ions in comparison to the flat structure of DPM-Co(II)-DPM.

The proposed genosensors have been tested for determination of oligonucleotide sequences specific for avian influenza virus H5N1 with detection limit in the picomolar range. The applied DPM-Me(II)-DPM self-assembled monolayer functionalized with carboxylic groups has universal character and could be used for amide coupling of any NH_2- ssDNA probes for designing of genosensors, which is crucial from a medical diagnosis point of view [21].

Conclusions

By describing the mechanism of analytical signal generation by selected genosensors, we presented a logical trend for their development. Currently, published numerous scientific reports showed extremely strong researchers interest in interdisciplinary fundamental studies on detection methods of hybridization events as well as gene mismatching. On the other hands, the number of genosensors which could be applied for real samples analysing is rather limited. This indicated the strong need of scientific efforts destined for the development of sensitive and selective genosensors, which could be massively produced at reasonable price. Medical diagnosis, food control and environment monitoring are waiting for such analytical devices.

Acknowledgements

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References

1. Thevenot D.R., Toth K., Durst R.A., Wilson G.S., *Pure Appl. Chem*., **1999**, *7*, 2333.
2. Ebersole R.C., Miller J.A., Moran J.R., Ward M.D., *J. Am. Chem. Soc*., **1990**, *112,* 3239.
3. Levicky R., Herne T.M., Tarlov M.J., Satija S.K., *J. Am. Chem. Soc*., **1998**, *120*, 9787.
4. Millan K.M., Spurmanis A.J., Mikkelsen S.R., *Electroanalysis*, **1992**, *4*, 929.
5. Palecek E., *Nature*, **1960**, *188*, 656.
6. (a) Brabec V., Koudelka J., *Bioelectrochem. Bioenerg.*, **1980**, *7*, 793.; (b) Wang J., Cai X., Wang J., Jonsson C., Paleček E., *Anal. Chem.*, **1995**, *67*, 4065.; (c) Cai X., Rivas G., Farias P.A.M., Shiraishi H., Wang J., Fojta M., Paleček E., *Bioelectrochem. Bioenerg*., **1996**, *40*, 41; (d) Fujishima A., Einaga Y., Rao T.N., Tyryk D.A., Elsevier B. V., Amsterdam, The Netherlands, **2005**, pp. 556-574.
7. Aoki H. , Umezawa Y., *Anal. Chem*., 2004, *76*, 320 A.
8. (a) Malecka K., Grabowska I., Radecki J., Stachyra A., Góra-Sochacka A., Sirko A., Radecka H., *Electroanalysis*, **2012**, 24, 439; (b) Malecka K., Grabowska I., Radecki J., Stachyra A., Góra-Sochacka A., Sirko A., Radecka H., *Electroanalysis*, **2013**, *25*, 1871.
9. Blackburn M.G., Gait M.J., *Nucleic Acids in Chemistry and Biology*, IRL Press, New York, **1990**.
10. Mascini M., Palchetti I., Marraza G., *Fresenius' J. Anal. Chem.*, **2001**, *369*, 15.
11. Palanti S., Marrazza G., Mascini M., *Anal. Lett*., **1996**, *29*, 2309.
12. García T., Revenga-Parra M., Abruña H.D., Pariente F., Lorenzo E., *Anal Chem.*, 2008, *80*, 77.
13. Ahangar L.E., Mehrgardi M.A., *Electrochim. Acta*, 2011, *56*, 2725.
14. Mehrgardi M.A., Ahangar L.E., *Biosens. Bioelectron.*, 2011, *26*, 4308.
15. Ricci F., Lai R.Y., Plaxco K.W., *Chem. Commun*., 2007, 3768.
16. Ricci F., Plaxco K.W., *Microchim Acta,* 2008, 163, 149.
17. Immoos C.E., Lee S.J., Grinstaff M.W., *J. Am. Chem. Soc*., 2004, 126, 10814.
18. Grabowska I., Malecka K., Starycha A., Góra-Sochacka A., Sirko A., Zagórski-Ostoja W., Radecka H., Radecki J., *Anal. Chem*., **2013**, *85*, 10167-10173.
19. Grabowska I., Stachyra A., Góra-Sochacka A., Sirko A., Olejniczak A.B., Leśnikowski Z.J., Radecki J., Radecka H., *Biosens. Bioelectron*., **2014**, *51*, 170.
20. Grabowska I, Singleton D, Stachyra. G, A. Góra-Sochacka, Sirko A., Zagórski-Ostoja W., Radecka H., E. Stulz, Radecki J., *Chemical Communications*, **2014**, *50*, 4196-4199.
21. Kurzątkowska K., Sirko A., Zagorski-Ostoja W., Dehaen W., Radecka H., Radecki J., *Anal. Chem*., **2015**, *87*, 9702.