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SUMMARY OF PROFESSIONAL ACCOMPLISHMENTS

Warsaw, December 2015

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I | *personal data*

Agnieszka Więckowska

II | *scientific degrees*

1. **Ph.D. in chemistry –** cum laude 2003r., University of Warsaw, Faculty of Chemistry
   thesis entitled: "Electrochemical studies of molecular interactions in multicentre complexes of selected transition metals"
   supervisor: Prof. Renata Bilewicz, Ph.D.
2. **M.Sc. –** 1998, University of Warsaw, Faculty of Chemistry
   thesis entitled: „ Studies of tetraazamacrocyclic complexes of Ni(II) and Cu(II) as potential donors in the donor-acceptor systems"
   supervisor: Prof. Renata Bilewicz, Ph.D.

III | *employment*

1.10.2015 –up to now	Faculty of Chemistry UW, lecturer
1.10.2003 – 20.09.2015	Faculty of Chemistry UW, adjunct
18.03.2006 – 1.04.2006	VTT Technical Research Centre of Finland Tampere, Finland research fellow
1.02.2007 – 31.01.2008	Institute of Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel post-doctoral fellow

IV | *scientific achievement*

A | *type of scientific achievement:*

monothematic series of publications entitled:
„Self-assembled monolayers attached to the gold nanoparticles and solid substrate."

B | *list of scientific publications*

[H1] **A. Wieckowska**, M. Wiśniewska, M. Chrzanowski, J. Kowalski, B. Korybut-Daszkiewicz, R. Bilewicz;
"Self-assembly of nickel(II) pseudorotaxane nanostructure on a gold surface"
Pure and Applied Chemistry, 2007, 79, 1077-1085.

[H2] **A. Wieckowska**, A.B. Braunschweig, I. Willner;
"Electrochemical control of surface properties using a quinone-functionalized monolayer: effects of donor–acceptor complexes"
Chem. Commun., 2007, 3918-3920.

[H3] **A. Wieckowska**, D. Li, R. Gill, I Willner;
"Following Protein Kinase Activity by Electrochemical Means and Contact Angle Measurements"
Chem. Commun., 2008, 2376-2378.

[H4] O.I. Wilner, C. Guidotti, **A. Wieckowska**, R. Gill, I. Willner;
"Probing Kinase Activities by Electrochemistry, Contact Angle and Molecular Force Interactions"
Chem. A Eur. J, 2008, 14, 7774-7781.

[H5] **A. Wieckowska**, E. Jabłonowska, E. Rogalska, R. Bilewicz;
„Structuring of supported hybrid phospholipid bilayers on electrodes with phospholipase A2"
Phys. Chem. Chem. Phys. 2011, 13, 9716-9724.

[H6] E. Jabłonowska, **A. Wieckowska**, E. Rogalska, R. Bilewicz;
"Phospholipase A(2) activity on supported thiolipid monolayers monitored by electrochemical and SPR methods"
J. Electroanal. Chem. 2011, 660, 360–366.

[H7] M. Karaskiewicz, D. Majdecka, **A. Wieckowska**, J.F. Biernat, J. Rogalski, R. Bilewicz;
„Induced-fit binding of laccase to gold and carbon electrodes for the biological fuel cell applications";
Electrochimica Acta 2014, 126,132-138.

[H8] **A. Wieckowska,** M.Dzwonek;
„Ultrasmall Au nanoparticles coated with hexanethiol and anthraquinone/hexanethiol for enzyme-catalyzed oxygen reduction"
Sensors and Actuators B 2016, 224, 514-520.

[H9] D. Li, **A. Wieckowska**, I. Willner;
"Optical analysis of Hg^{2+} ions by oligonucleotide-Au nanoparticles hybrids and DNA-based machines"
Angew. Chemie Int. Ed., 2008, 47, 3927-3931.

C | *the discussion of scientific aim of the above-mentioned scientific publications and the results achieved, together with a discussion of their possible application*

1. | *scientific aim*

Designing and developing methods of preparation and application of layers immobilized on the electrode or on the nanoparticle surface in order to use the proposed arrangements for specific research purposes, such as changing the surface properties of the electrodes, the detection of the substance, or the study of intermolecular interactions or mechanisms of processes taking place in monomolecular layers.

2. introduction

Nanotechnology is currently one of the fastest growing branches of science. Chemical, optical, mechanical and electrical properties of nanosystems are of special interest because they differ significantly from those measured for macroscopic structures [1]. There are already commercially available compounds that act as transistors, diodes, memory cells, wires and other components of the microcircuits. However, miniaturization achieved by mechanical methods i.e. route "top-down" has already reached its theoretical limit of 50 nm [2]. Accordingly increasing interest in the "bottom-up" method has been awakened, where the starting point is individual atoms and molecules, and larger systems are built from them. A direct consequence of this approach is the design of systems anchored on solid surfaces and studies of their properties.

A scientific area related to nanotechnology is supramolecular chemistry, the science of compounds existing due to weak intermolecular interactions. Non-covalent binding methods include: guest-host interactions, hydrogen bonding, donor-acceptor interactions, π-π interactions, or Van der Waals forces. Thus, when covalent bonds between the structural elements are not present, we are talking about the mechanical bonds, such as observed e.g. in catenanes or rotaxanes [3, 4].

My scientific activity has been focused on basic research of systems immobilized on a solid support. Monolayers may alter the functionality of the substrate on which they are immobilized and can therefore be used to passivate the substrate and to introduce new features on the solid surface. Application of monolayers is connected with the formation of biosensors, corrosion resistant coatings or new electronic devices. An understanding of the forces that are responsible for self-assembly of monolayer process on a substrate is important in designing systems with specific surface properties.

The first technique for forming structured thin films (from angstroms to micrometers) was the Langmuir-Blodgett technique [6] based on the formation of amphiphilic monolayer at the water-air interface. In order to transfer the layer on the solid substrate the substrate is resurfaced or dipped into subphase coated with monolayer, depending on the nature of the solid surface [7, 8].

In the 40s of the last century a phenomenon of spontaneous organization of oleic acid to form a stable monolayer on clean platinum was observed for the first time [9]. Monolayers form spontaneously by immobilizing the substrate in a solution of a compound having affinity

for the substrate. One possibility of anchoring layer on a solid substrate (SAM-self assembled monolayers) is a method involving the formation of a covalent bond between a gold substrate and a sulphur atom of the compounds of the type RSH RSSR [10, 11]. Gold is the most commonly used substrate due to the absence of interfering surface oxide and the binding energy of Au-S [12]. Stability of thiol monolayers on gold surfaces is based on several factors: the interaction of sulphur bonding group with the substrate, interaction between the backbones of molecules (depending on the chemical nature these may be Van der Waals forces, π–π interactions, electrostatic interactions, including dipole- dipole interactions) [13] or the effects of end groups [14]. The molecules of thiols on gold surface of the substrate have a hexagonal packing, and the distance between the chains of alkanethiols is about 5 Å. The alkyl chains are bent relative to the surface, and this angle is dependent mainly on the nature of the chain [12]. Thus, the thickness of the monolayer is smaller than the length of the molecules forming the monolayer.

Part of the research in which I participated in recent years, which is the basis for the application for habilitation procedure, concerned with designing, preparing and studying of systems organized at the electrode surface, and the application of produced system for cognitive and analytical purposes.

3. discussion of publications

One of the examples of applying the method of thiol derivative self-assembly on a gold electrode is publication [H1] on the construction of nanomachines (molecular machines) using poliazamacrocyclic transition metal complexes. The aim was to create a rotaxane by interaction between the axis-molecule that forms a bond with the gold substrate and the ring-molecules, and the observation of this process by electrochemical means.

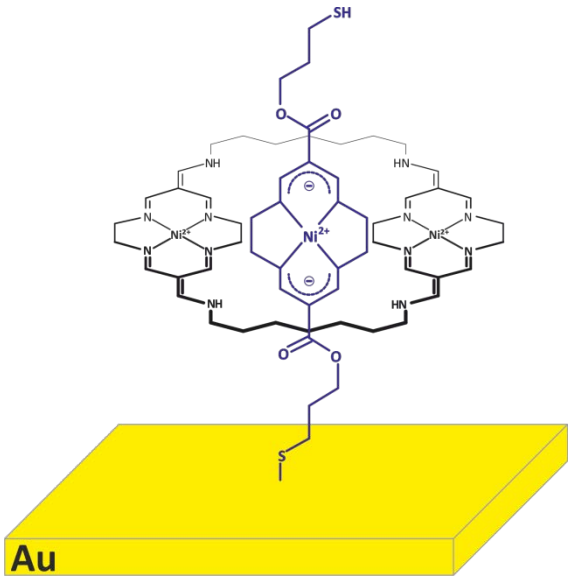

Fig. 1. Scheme of rotaxane on electrode surface [H1].

Tetraazamacrocyclic complexes of nickel and copper are compounds with many interesting features. The metal ion can undergo reversible oxidation process to oxidation state +3. The oxidation process of metal ion can occur at different potential values, depending on the substituents in the ring, the ring size or the presence of other

molecules in its immediate vicinity. Therefore, such compounds may be used as ingredients of catenanes or rotaxanes showing the phenomenon of an electrochemically-triggered intramolecular motion (rotation) [15, 16]. The monomeric nickel complex has been modified at the opposite sides of the molecule with two -SH free groups, allowing anchoring the gold surface. Such a molecule can adopt two orientations on the surface of the electrode: if only one -SH group is attached to the surface and the compound assumes an orientation parallel to the normal, or two -SH groups reacted with the gold surface and the molecule is placed flat on the electrode surface. Only the first orientation allows creating rotaxane by threading the molecule ring. The area occupied by one molecule (43 ± 1.5 Å), compared to that observed for alkanethiol monolayers (≈20 Å) leads to the conclusion that electrostatic interactions are present between charged nickel centres, most of which have the same orientation in relation to the substrate surface. After optimization of the adsorption process and determination of the type and ratio of thiol dilution, the resulting layer was examined by scanning tunnelling microscopy using gold nanoparticles as markers for free thiol groups. The process of oxidation of Ni(II) centre to Ni(III) takes place at a potential 0.85 V vs. Ag/AgCl. Creation of the rotaxane at the electrode surface is based on the use of bismacrocyclic complex of nickel - acceptor, as the ring, which is also electroactive; however, due to the different nature of tetraazamacrocyclic rings complexing metal centres, oxidation of metal ions occurs at a potential of 1.4 V vs. Ag/AgCl. This process is too positively placed to observe the changes during creating the rotaxane process without destroying the layer. Therefore, I observed the creation of rotaxane by the changes of the electrochemical behaviour of axis-molecules immobilized on the electrode surface. The shift of oxidation potential of Ni^{2+}/Ni^{3+} towards more positive values while creating an intertwining structure proves the changes in the environment of redox centre. Rotaxane is positively charged, while the net charge of the axis molecule is zero, and therefore the formation of rotaxane hinders oxidation of the axis molecule (more positive potential values). Formation of such intertwined structure is kinetically relatively slow process, but it can be enforced by applying a potential corresponding to the form of Ni^{2+} complex which is the rotaxane axis.

The application of electrochemical methods to study the processes of formation of intertwined systems is possible only if the components are electroactive, but the interpretation of the results obtained is sometimes complicated. The most important achievement presented in [H1] is the development of an effective method of adsorption of axis molecules, and electrochemical observation of the process of creating a new individual on

the electrode surface. In contrast to the work of Stoddart [17, 18], where compounds without metal ions were used or Sauvage [19, 20] where the process of creating of a intertwined structure required templating effect around the metal ion, presented rotaxane contains metal centres, and the change of oxidation state of metal centres facilitates creation of rotaxane and observation of this process is based on electrochemical processes of metal centres.

The donor-acceptor interactions may enable the preparation of surfaces with controlled wettability. The surfaces for which the hydrophobic-hydrophilic are changed under the influence of external stimuli are of interest because of the possibility of their use as self-cleaning surfaces [21]. The control of surface wettability can be achieved, inter alia, through self-organization of long-chain alkanes with charged head groups [22] or by using electroactive thiolated derivatives that response to changes in the applied potential by changing the degree of wettability of the surface [23]. The publication [H2] presents the design of surface with controlled hydrophilic-hydrophobic properties through the use of thiol modified derivatives. Factors affecting the control characteristics of the surface can be of chemical and electrochemical nature.

In order to prepare surfaces with various wettability, a monolayer of cysteine was immobilized on a gold electrode and 1, 4-benzoquinone moieties were attached. By electrochemical means I determined a surface concentration of quinone derivatives as $1.1 \cdot 10^{-11}$ mol·cm^{-2}. The value of thiol surface concentration indicates the incomplete coverage of the electrode surface. After sealing the monolayer with additional butanethiol molecules, surface concentration of quinone derivatives has decreased to $7.9 \cdot 10^{-12}$ mol·cm^{-2}, suggesting a partial replacement of electroactive molecules by butanethiol molecules during the sealing procedure. The quinone form of the monolayer can be reversibly reduced to the hydroquinone form after application of a suitable potential. The mixed layer modified electrode comprising in the outer plane the quinone group was reacted with π-acceptor which is methylviologen. The formation of a donor-acceptor complex is possible only after the reduction of quinone to hydroquinone, which indicates good π-donor properties.

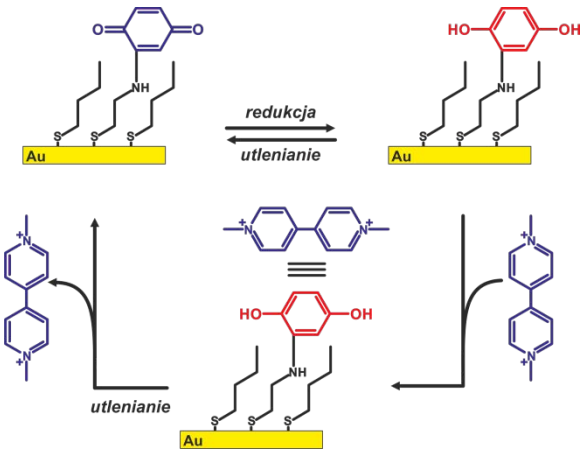

Fig. 2. Scheme of the system [H2].

Changes in hydrophobic-hydrophilic properties of the donor-acceptor complex modifying surface are induced by external factors such as applied potential, or the presence of the reducer on the surface and investigated by the changes of the contact angle or atomic force spectroscopy (AFM). The AFM probe modified with thiol derivative of viologen interacts with the substrate covered with a monolayer of hydroquinone, but after the chemical or electrochemical oxidation of the monolayer-modified surface to quinone, this effect disappears. Similarly, changes in the contact angle measurement suggest that the reduction of modified surface of the gold increases the hydrophilic properties of the layer. A change in the contact angle from 66°±3° to 48°±6° proceeds after applying the potential of -0.55 V vs. silver wire. However, after the return to the potential of 0 V vs. Ag wire, the value of the contact angle has increased to 54°±4°. The formation of a donor-acceptor complex between the reduced form of a quinone on the surface and viologen in the solution leads to decrease in the contact angle to 36°±4°.

This behaviour reflects the formation of donor-acceptor complex between the π-acceptor - viologen and rich in electrons hydroquinone, which is anchored on the electrode surface. Such research might in the future be applied in microfluidics area for creating a self-cleaning surfaces or surfaces that detect biological analytes. Electrochemical and microscopic methods can be complementary to describe controllable wettability surface.

Another example of studies employing substrate anchored on the surface is the study of enzyme activity. Changes in the activity of enzymes are important diagnostic parameters and may reflect lesions in organs. Defective functioning of organs is associated with changes in the permeability of cell membranes of organs or the damage in structures, responsible for flow of the enzymes and alters their activity in body fluids.

Casein kinase, which is an enzyme responsible for phosphorylation of proteins, plays crucial role in functioning of cells, their life cycle and fission [24]. Monitoring the activity of this enzyme is especially important in the diagnosis of many neurodegenerative diseases and cancer.

The most common methods to determine the kinase activity include the use of fluorescently or isotopically labelled antibody [25, 29], spectroscopy of surface plasmon resonance [26], systems based on FRET (energy transfer between the two fluorophores) [27], as well as recently popular methods using gold nanoparticles. Methods based on interaction with the gold nanoparticles also require labelling of, for example, the use of biotin-modified ATP. After phosphorylation of the biotinyl-containing peptide, capable of reacting with avidin-coated nanoparticles [28, 30]. The presence of gold nanoparticles can be detected electrochemically, while silver-plated gold nanoparticles will enhance the signal in resonance light scattering (RLS).

Determination of casein kinase was the objective of research described in publication [H3]. I used electrochemical methods and electrode with surface modified with specific protein. The unique feature of this research is that it was based on simple idea and the product labelling is cost-effective.

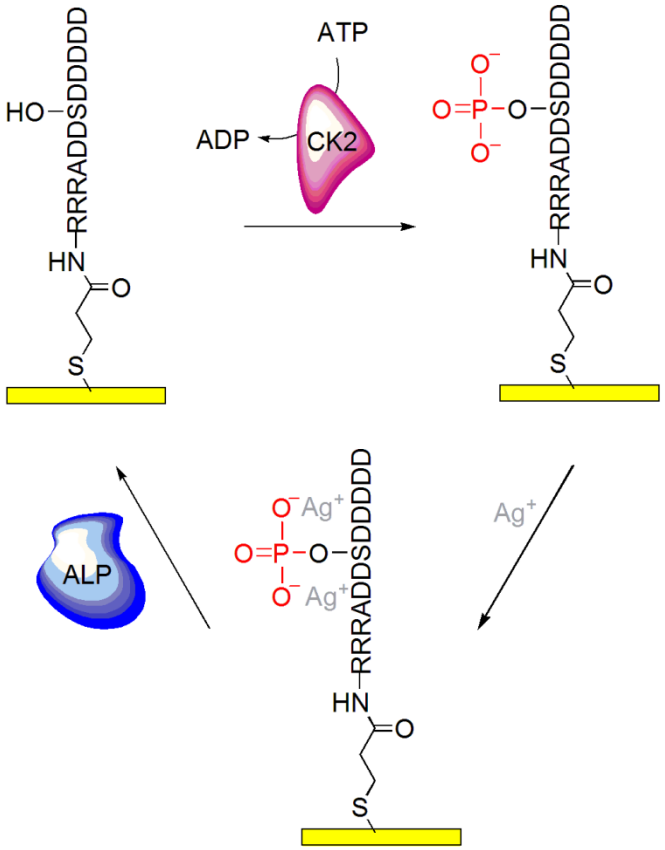

Fig. 3 Scheme of the system for determination of kinase activity [H3].
where: R - arginine, A - alanine, D – aspartic acid, S - serine

Determination of casein kinase is based on the electrochemical reduction of silver ions associated with the phosphorylated centres of proteins immobilized on a gold electrode [H3]. The process was monitored electrochemically and with microscopy of contact angle. In order to anchor the substrate (protein) of enzyme reaction on a gold electrode, thiolated derivative of the active N-hydroxysuccinimide ester was adsorbed. Then the reaction of coupling of the peptide to the gold surface was conducted. Prepared electrode modified with substrate was treated by enzyme of constant activity in the presence of ATP molecules, which resulted in phosphorylation of the serine in the peptide. The extent of phosphorylation

was modified by increasing the time of enzymatic reaction until the plateau was reached (20 minutes). Then, the electrode was placed in a solution of silver ions that bounded to the phosphate groups that has been generated by the casein kinase. The electrochemical study of the reduction of Ag^+ ions provides the quantitative information about the number of generated phosphate groups, which corresponds to the activity of casein kinase. Analogous measurements were performed in the system with modified electrode after immersion in the solutions with kinase of different activity. Based on previous measurements the reaction time was set as 20 minutes. The plot of the reduction of silver ions signal versus activity of the enzyme is the basis of quantitation of CK2.

The surface of the electrode can be regenerated by the use of alkaline phosphatase that removes phosphate groups from the substrate immobilized on the surface. The surface concentration of immobilized peptide derived from the quartz microbalance experiments (QCM) reached: $3 \cdot 10^{-10}$ mol cm^{-2}.

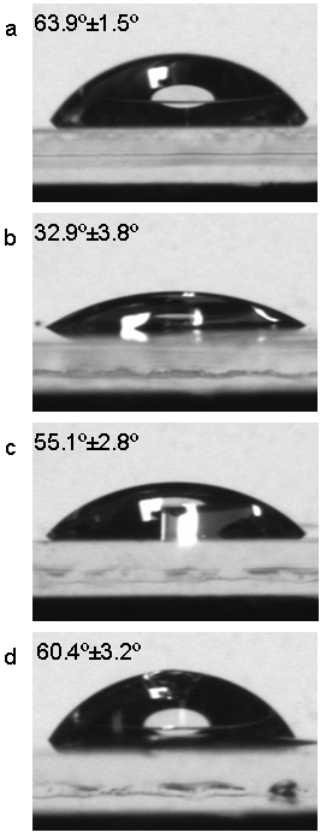

Fig.4. Images of HEPES droplet (20 μl) on the surface of electrode modified with peptide (a), after phosphorylation (b), after adsorption of silver ions Ag^+ (c), after reconstruction of the surface using alkaline phosphatase (d).

In order to confirm the specificity of the reaction I used electrode modified with foreign peptide, which is not a substrate for the enzymatic reaction. The experiments with foreign peptide resulted in very low response current.

Besides of electrochemical techniques I used microscopy contact angle for determining the changes in hydrophilic-hydrophobic properties of peptide monolayer during the reaction with enzymes. Phosphorylation of a substrate which is immobilized as a monolayer on the electrode surface significantly increased the hydrophilicity of the monolayer, but the formation of a complex between phosphate groups and the silver ions alters the hydrophilic nature of the film towards more hydrophobic. The enzymatic reaction with alkaline phosphatase completely removes the phosphate group from the substrate and renews the hydrophobic layer of unphosphorylated protein. To verify these results as I used photoelectron spectroscopy (XPS) and I confirmed the formation of a complex between silver ions and phosphate groups of protein, produced by casein kinase action.

In summary, in [H3] I developed electrochemical sensor of kinase activity based on the reduction of Ag^+ ions associated with

the enzymatically generated phosphate groups. The observations of XPS and contact angle microscopy answers resulting from changes in the hydrophobic/hydrophilic properties, as the effect of the enzymatic reaction support the measurements by Osteryoung square wave voltammetry for characterizing the process of phosphorylation and dephosphorylation of the enzymatic reaction substrate immobilized on the electrode surface.

Another approach to determination of casein kinase is based on the interaction of phosphorylated substrate of casein kinase with an appropriate antibody and using impedance spectroscopy EIS, contact angle measurements CAM and atomic force microscopy AFM [H4]. Impedance spectroscopy is a technique involving the application of the AC voltage signal of small amplitude to the system and analysis of the AC response. The advantage of this technique is the possibility of separation processes with different time constants, including diffusion, charge transfer, the resistance of the electrolyte, the resistance of layer immobilized on the electrode and capacitance of the double layer. Separation of such processes is possible because response of the system depends on the frequency of the signal [31, 32].

The most important assumption of impedance measurements is linearity, which means that the system cannot be changed during the measurement. Electrochemical or physical processes are considered parts of the electrical equivalent circuits characterized by the relevant time constants. In particular, a very useful model to interpret the phenomena occurring at modified electrodes is Randles - Ershler model $R_s(R_{ct}(C_{dl}W))$ wherein R_s is the resistance of the electrolyte solution, R_{ct} is the resistance of the charge transfer, C_{dl} corresponds to the capacity of a double layer, and W is the Warburg impedance resulting from ion diffusion to the electrode surface. In this publication [H4] I conducted kinase activity assay based on interaction between protein - substrate with an appropriate antibody, which also is a protein. Proteins are characterized by weak conductivity and hinder electron transfer due to blocking of the electrode surface. The increase in thickness of the layer on the working electrode surface, and the changes of the blocking layer on the electrode was monitored by Faradaic impedance.

The protein-modified electrode, which is a substrate for casein kinase has been treated with the enzyme in the presence of ATP. The enzymatic reaction leads to the phosphorylation of serine in the substrate and the appearance of a negative charge that repels the negative redox probe $[Fe(CN)_6]^{3-/4-}$ present in the solution. On impedance spectrum this phenomenon manifests as an hindrance of electron transfer through a layer, which means increase of the electron transfer resistance R_{ct}. $[Fe(CN)_6]^{3-/4-}$ ions cannot penetrate to the

surface of electrode via a layer with good blocking properties and therefore the electron must tunnel from the probe in the solution, through layer, up to the surface of the electrode. A direct transfer of probe may occur if there are defects in the monolayer on the electrode surface.

Using quartz microbalance I determined the surface concentration of substrate to be equal to: $3.3 \cdot 10^{-11}$ mol cm^{-2}.

With the prolongation of the enzymatic reaction the resistance of charge transfer increases, reaching a plateau in approximately 20 minutes, suggesting complete phosphorylation of the monolayers, or repelling of ATP molecules from more negatively charged layer.

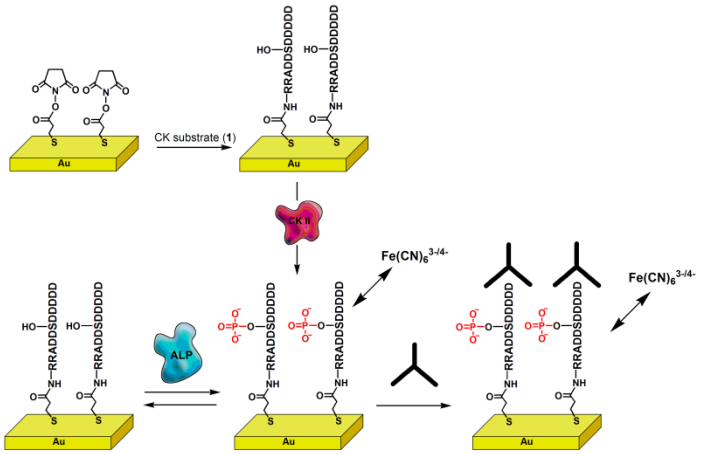

Fig. 5 Scheme of the system for determining of kinase activity [H4].

Control experiments carried out only in the presence of the enzyme, or only in the presence of the molecules of ATP did not show an increase in R_{ct} value. Treatment of the phosphorylated surface with a solution of alkaline phosphatase leads to renewal of the substrate layer, which is restores the initial values (before the reaction with the casein kinase) of the equivalent circuit. I confirmed the linearity of the system using increasing kinase activity. The extent of phosphorylation of the peptide substrate is controlled by the concentration of casein kinase CK2, therefore the increase in the charge transfer resistance results from the increased concentration (activity) of the enzyme - kinase. These changes are less accurate at lower enzyme concentrations, so in order to enhance the effect I used antibodies specific for the phosphorylated substrate. After being attached to the phosphorylated protein in a monolayer, antibodies further block the electron transfer between the redox probe and the electrode surface. AB antibodies are protein molecules, thus attaching them to the surface of the electrode greatly increases the blocking properties toward the redox probe. Impedance signals, i.e. the charge transfer resistance increases linearly with increasing enzyme activity, which enables quantitative determination of the enzyme activity, even in the case of enzyme

activity of 10 U. I performed control experiments using the foreign enzyme - tyrosine kinase and foreign anti-BSA antibody. Responses of the system were minimal, as when the specificity of casein kinase was investigated using foreign substrate (substrate-specific tyrosine kinase). I confirmed these experiments by the contact angle means. Similar results were obtained using the AFM technique. The surface of a gold electrode was covered with the substrate of the enzymatic reaction while the AFM probe has been modified with antibody anti-phosphorylated protein. When the surface of modified gold electrode was reacted with casein kinase in the presence of ATP, atomic force microscopy allowed to observe the interaction between the surface and the probe, while after application of alkaline phosphatase that removes phosphate groups from the gold surface or after application of a foreign enzyme or the substrate not specific for the kinase such effects were not observed.

The employment of a specific substrate of the enzymatic reaction, the specific antibodies and impedance spectroscopy allowed to enhance the signal that corresponds to the enzyme and to determine its activity.

Enzymes can be used as a tool for engineering of nanostructured surfaces for bioelectronics and sensor applications. The enzymes from the group of lipases or phospholipases can be used to modify architecture of the surface coated with lipids. The lipid bilayers are used not only as models of biological membranes, but also as matrix in the sensors. Lipid membrane permeability to small ions is limited [33] and therefore there are many substances that allow ions to pass through the membrane. The first group includes nonactin, monensin or valine, which form complexes with ions, and can diffuse through the hydrophobic environment of the membrane [34]. The second group of compounds acts by a different mechanism, namely, forms a membrane ion channels to allow transport through the membrane. An example of second group can be gramicidin A [35].

Lipases and phospholipases are enzymes catalysing the hydrolysis of ester bonds in glycerides and phosphoglicerides respectively [36]. Hydrolysis or esterification reactions of (phospho)lipids catalysed by these enzymes were used as a new method for the controlled formation of channels in lipid membranes [H5]. Selectivity of diffusion through the pores to the electrode on which membrane is mounted was studied by electrochemical methods using electroactive probes, such as doxorubicin or ferrocyanide ions. Development of method for obtaining nanostructured lipid membranes of selective permeability for construction of conductive surfaces with the scheduled architecture and functionality was the purpose of the work in which I used an enzyme - phospholipase A_2.

The model membrane I used is a lipid bilayer, but in order to immobilize layer on the electrode surface I used thiol derivative. Hybrid lipid layers were prepared by adsorption of thiol derivative on gold electrode surface and transfer of the second- lipid layer by Langmuir-Schaefer technique, or by spreading prepared liposomes. In order to investigate the properties of the obtained hybrid layers I applied impedance spectroscopy in the presence of the redox probe: $[Fe(CN)_6]^{3-}/^{4-}$. Observation of the process catalysed by a phospholipase A_2 was also performed using faradic impedance. During catalytic reaction of the phospholipase on hybrid lipid layers I observed two contrary effects. The first is the adsorption of the enzyme on the hybrid bilayer, resulting in increased thickness of the film on the electrode and thus hindering the electrons transfer between the redox probe in the solution and the electrode. The second effect can be observed after a certain period of time and is related to the enzymatic reaction, which is the hydrolysis of the ester bond sn-2 at the molecules of phospholipids and the release of the product molecules of free fatty acids.

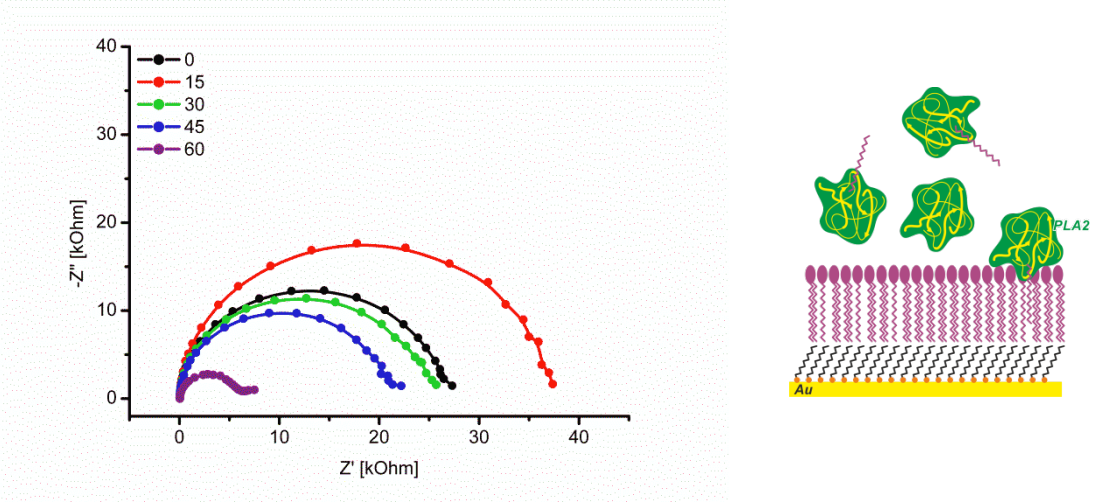

Fig. 6. Nyquist plots for hybrid bilayer dodecanethiol–DPPC + cholesterol (7 : 3) attached to gold electrode after incubation in PLA_2 solution (0.01 $U \cdot ml^{-1}$) in solution containing $K_3[Fe(CN)_6]$ (5mM), $K_4[Fe(CN)_6]$ (5 mM) and electolyte: TRIS (10 mM), NaCl (150 mM), $CaCl_2$ (5 mM), pH=8.9, E= 0.26 V, Amp.: 4 mV. Range: 100 mHz–10 kHz. [H5]

The release of acid molecules from the layer causes the formation of defects in the film and facilitates the electrons transport between the redox probe and the electrode surface. The first stage i.e. adsorption of the enzyme manifests on the Nyquist plot as a decrease in of double layer capacity C_{dl} and an increase in the charge transfer resistance R_{ct}. This phase is called the delay phase ("lag phase") and lasts from the time of enzyme injection until the pulse of enzyme activity [37]. The greater increase in blocking properties upon addition of enzyme, the longer delay phase will be. For the hybrid system dodecanethiol - DPPC

(dipalmitoylphosphatidylcholine) delay phase was not observed at all, suggesting a large number of defects arising during the transfer of the second layer by Langmuir-Blodgett method. In the case of rigid and compact layer comprising also cholesterol in addition to the DPPC, the phase delay lasted approx. 30 minutes. If the second layer was formed by spreading of liposomes, there are domains with a greater number of layers than expected on the surface, and the delay time in this case was even longer (approx. 40 minutes), due to the greater thickness of the film on the electrode.

The effect of the enzymatic reaction and channels created in the bilayer can be observed in the impedance plot as decreasing R_{ct} value with increase of the enzymatic reaction time and finally the appearance of Warburg impedance, proving the diffusion as way of transport of redox probe, after 60 minutes for dodecanethiol - DPPC + cholesterol layers. I performed control experiments with mercaptoundecanoic acid as a second layer of hybrid bilayer and no effects of the enzyme PLA_2 were observed.

The enzymatic reaction can also be observed by examining hydrophilic-hydrophobic properties of the surface. The hybrid bilayer is hydrophilic due to the presence of polar head groups of lipids in the outer plane of the bilayer on the electrode. The lipase partially removes fatty acid chains, whereby the hydrophobic part of the bilayer is exposed to the outside, which increases the contact angle to a value above 100°, characteristic for hydrophobic systems.

In order to explain the mechanism of action of the lipase A_2 on hybrid lipid layers immobilized on gold, I employed the technique of surface plasmon resonance (SPR). The phenomenon of surface plasmon resonance occurs when light is reflected off thin metal layer (gold). This technique allows observation of the interaction between the immobilized layer on the sensor and a second individual in solution, in the flow system.

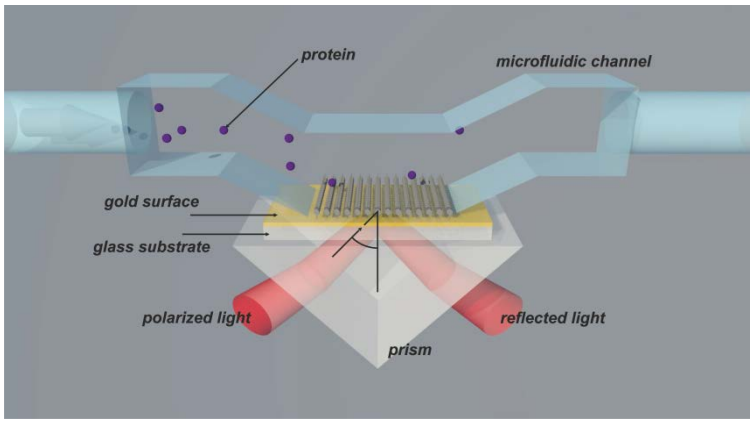

Fig. 7. Scheme of the surface plasmon resonance principles.

When the light beam is passing through a medium with a higher refractive index, and encounters the interface with a medium having a lower refractive index at an angle greater than the critical angle, it is totally internally reflected at the interface and returns to a medium with a higher refractive index [38].

However, during total internal reflection very small part of the energy of the beam penetrates the medium of a lower refractive index as evanescent wave. The amplitude of the wave decreases exponentially with the distance from the interface and disappear at a distance equal to the wavelength from the surface. If, in the conditions of total internal reflection, thin (nanometer) layer of metal is placed at the interface, the evanescent wave will penetrate the metal layer and induce the surface plasmon wave propagating on the surface in contact with a medium having lower refractive index. In the case of metals such as gold, plasmon wave will also generate an enhanced evanescent wave. One of the components of the electric field of the wave is directed to the inside of a medium with a lower refractive index. For this reason, the conditions of the SPR are very sensitive to changes in refractive index of light on a gold surface. During the plasmon excitation by photons there must be conserved energy and momentum. This requirement is met when the wave vectors of the photon and plasmon are equal in magnitude and direction for the same frequency. The wave vector for the plasmon wave depends on the refractive index of gold and a medium with lower refractive index [39]. Adjusting the wave vector and energy allows plasmonic excitation (SPR), causing a characteristic decrease in the intensity of the reflected light. For a given wavelength of the incident light, the SPR is seen as a reduction (dip) in intensity of light reflected at a specific angle of incidence. Medium with low refractive index is a layer covering the surface of the sensor and the solution surrounding the sample. Interactions on the surface change in the concentration of the solution and thus the refractive index within the area of presence of the evanescent wave. Therefore, the angle of incidence required for an SPR phenomenon is changing and this change is measured as a response signal. Sensograms is the curve of changes in SPR angle over time, where one can observe kinetic events on the sensor surface.

In publication [H5] I used surface plasmon resonance technique for observation of the spreading of liposomes as a second layer of hybrid membranes and to examine the impact of lipase A_2 on membrane formed in this way. While conducting an SPR experiment, second layer (lipid) was formed *in situ* on the surface of gold previously modified with thiol derivative. From the sensogram recorded during the spreading of liposomes composed of DPPC I determined a surface concentration of DPPC and it equals $1.02 \pm 0.07 \cdot 10^{-9}$ mol·cm^{-2}. Then, the reaction of hydrolysis of lipid layer with phospholipase A_2 was observed in real time with SPR. The reaction product is free palmitic acid. During the reaction between lipase A_2 and lipid layer obtained by liposomes spreading approx. $6.48 \pm 0.42 \cdot 10^{-10}$ mol·cm^{-2} of palmitic acid was removed. For hybrid membrane obtained using the Langmuir-Schaefer technique the hydrolysis removed

palmitic acid in amount of $6.60 \pm 0.50 \cdot 10^{-10}$ mol·cm^{-2}. The amount of product in both cases is similar, but the reaction time was shorter in the case of the membrane formed by a spreading of liposomes, suggesting more disordered layer structure and easier access of the enzyme to the reaction sites.

Doxorubicin has been used as a marker for the blocking properties of the hybrid membrane. Well-ordered bilayer with hydrophilic properties is blocking toward doxorubicin as a redox probe. However, after the formation of defects in the membrane by the action of phospholipase A_2, doxorubicin can diffuse to the electrode surface and undergo there a reduction, which is observed at voltammograms as the appearance of signals at potential values which corresponds to the doxorubicin reduction on an uncoated electrode. However, when the formed channels are not sufficiently deep, and doxorubicin molecule cannot diffuse to the gold surface, the observed signals of doxorubicin reduction are located at a more negative potential values and correspond to the reduction process of probe located in the outer layer of the membrane. Based on the analysis of reduction signals of doxorubicin, the degree of occurrence of catalytic hydrolysis of lipids in the membrane can be concluded.

Modification of lipid side chains with thiol groups may increase the stability of obtained films, and the lipid environment provides greater freedom to communicate and a convenient orientation with respect to the electrode. One of the examples used for anchoring the lipid bilayers model on the surface of electrode is 1,2-dipalmitoyl-sn-glycero-3-phosphothioethanol (DPPTE) [40]. DPPTE monolayer on the surface of the electrode was examined in the presence of the redox probe during a reaction catalysed by phospholipase A_2. In previous work I showed, that the first stage of the enzymatic reaction is adsorption, which is also known from the literature [41]. The products of the enzyme reaction are fatty acid, and lysolipid. Defects formed during the catalytic reaction allow the penetration of the redox probe into the interior layer and the charge transfer resistance value is reduced. With increasing reaction time, blocking properties of the lipid layer become weaker.

In publication [H6] surface plasmon resonance was used to observe PLA_2 enzyme adsorption on thiolated lipid layer - DPPTE immobilized on a gold electrode. The next step is the hydrolysis reaction and attempt to remove the products from the layer. Since the use of a buffer or water did not produce expected result I used β-cyclodextrin solution to remove the enzymatic reaction products from layer. However, this procedure has proven to be ineffective, in contrast to the observation of Mirsky et al [42]. Then, I used a surfactant i.e. Triton X-100

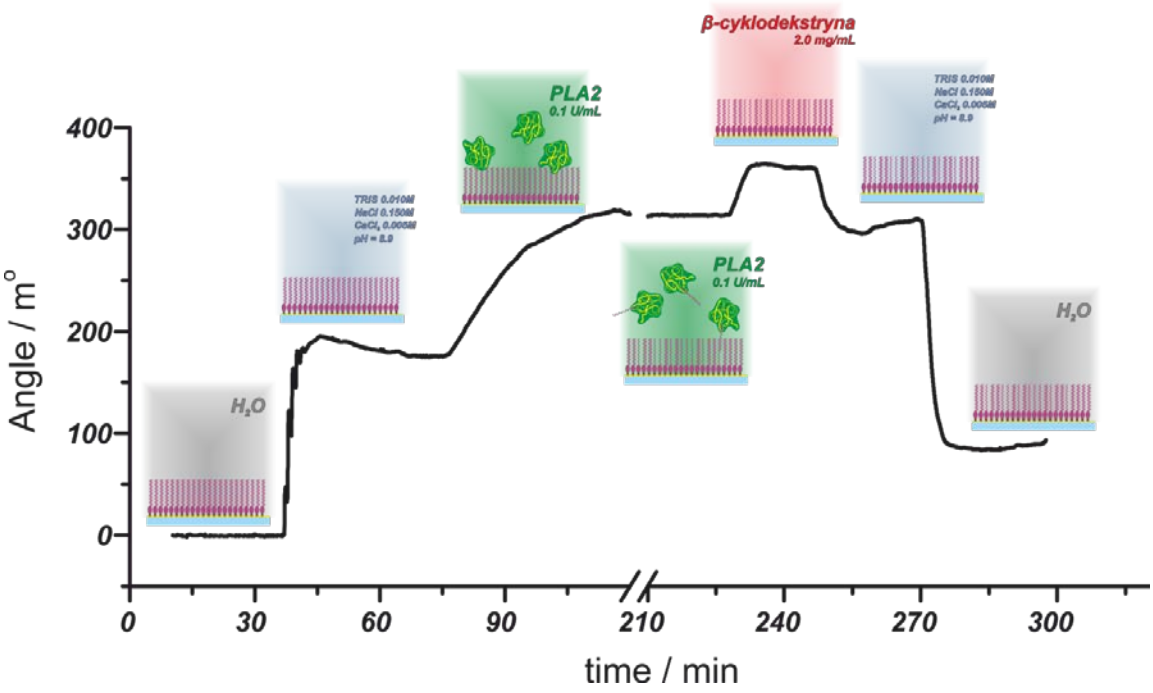

Fig. 8. Sensogram recorded for gold disc modified with thiolipid DPPTE:
(1) TRIS (0.010 M), NaCl (0.150 M), $CaCl_2$ (0.005 M), pH = 8.9 (2) PLA_2 0.1 U/mL, (3) β-cyclodextrin 2 mg/mL, (4) buffer, (5) water.

(polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether) to remove products, which effectively removed fatty acids molecules present after reaction in the lipid layer on the electrode. Triton X-100 was not able to remove the thiolated lipid molecules from the surface of the electrode, meaning that bound between a sulphur atom of the lipid and a gold surface is not broken. Thus, the surfactant has not degraded lipid layer but only allowed rinsing of enzymatic reaction products. The surface concentration corresponding to the elution of the product from the layer reached 1.60 10^{-10} mol·cm^{-2}.

In this publication [H6] I used doxorubicin as a redox probe of the properties of the lipid monolayer anchored on the electrode. In solution at pH = 4 doxorubicin is reduced on the glassy carbon electrode at a potential -0.46 V vs. Ag/AgCl and oxidation process takes place at a potential of 0.70 V vs. Ag/AgCl. None of these signals is observed in the solution of doxorubicin on a gold electrode modified with a layer of DPPTE lipid. However, after exposure of the lipid layer to the lipase A_2 action, two different electrochemical behaviours of doxorubicin can be observed, depending on the type of the defect. The first is the simultaneous adsorption of doxorubicin with the enzyme on the surface of the lipid layer, with reduction peak of the drug appearing at a negative potential values (-0.60 V vs. Ag/AgCl). The second type of behaviour can be observed only after a certain time from starting the

hydrolysis reaction and it is associated with the reduction of doxorubicin in the pores formed as a result of hydrolysis of the ester bonds of lipid layer.

The enzymatic reaction leads to changes in the electrical properties of the lipid monolayer on the electrode. Decreasing the barrier properties of the layer can be observed in both faradaic impedance and electrochemical methods, using a drug (doxorubicin), which may penetrate into pores and defects in the lipid layer. Therefore the use of electrochemical techniques in such studies appears to be very helpful.

The use of enzymes with specific substrates and harmless products enable the design of biofuel cell, which operate at room temperature and neutral pH. However, one of the most important issues associated with the design of bioelectrodes is direct electron transfer (DET) from redox protein to the surface of the electrode. Extremely important challenge is to ensure good contact between the active centre of the enzyme and the electrode [43]. For most of the oxidoreductases, redox centre of the enzyme is electrically isolated by the protein shell. Due to the presence of this shell, the protein cannot be either reduced or oxidised at the electrode surface. For this reason mediators have to be used. Mediators are redox molecules that enable electrical contact between the enzyme and the electrode. Direct transport of electrons can occur for certain redox proteins using gold nanoparticles.

In publication [H7] I used laccase as the enzyme for the construction of biocathode; laccase is four-copper protein that catalyses four-electron oxygen reduction to water, without the intermediate step of generating hydrogen peroxide. The active centre of the enzyme contains four copper ions divided into three groups. T1 centre is responsible for the catalytic oxidation of substrates. The electrons released in this process are transferred to centres of T2 and T3 (1.2 nm from the T1center), where the process of oxygen reduction takes place [44]. T1 centre located in the hydrophobic cavity can be reached by both electrons from the electrodes and organic mediators. Furthermore, the hydrophobic pocket of the enzyme has affinity to molecules containing aryl moieties, which immobilized at the electrode surface can enforce the appropriate orientation of the enzyme relative to the electrode and thereby enable efficient electron transfer [45].

The immobilisation of the enzyme directly on the electrode results in low density of catalytic current because of the low degree of enzyme surface coverage. Conductive nanomaterials such as carbon nanotubes [46,47], acetylene black, graphene [48] or metallic nanoparticles are frequently used to increase the real working surface of the electrode, and ensure good electrical contact. The great advantage of carbon nanotubes as substrates for

enzyme immobilization is the possibility of modification of the ends of nanotubes or defects, so one can control the dispersion and binding enzymes [46, 47]. Compared to the carbon nanostructures there are fewer examples of metallic nanoparticles used to immobilize the enzymes [49].

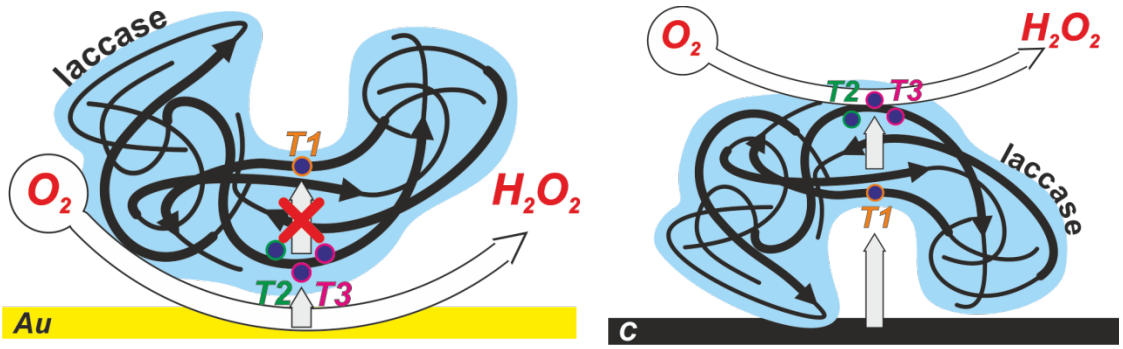

Fig 9. Scheme of laccase orientation on different type of electrodes

In the case of gold this may be related to the fact that oxidoreductases on the surface of this material have unfavourable orientation. Because it was demonstrated that T1 centre of laccase on a carbon electrode is placed toward the surface of the electrode, whereas on the gold electrode the orientation of the enzyme molecules is reversed, the T2 centre is the closest to the surface of the electrode [50]. However, one can also find reports in which authors could see DET of laccase on the surface of carbon electrode with immobilized gold nanoparticles [49].

An interesting solution of the problem of the enzyme orientation toward the electrode surface is the employment of groups having a strong affinity to the hydrophobic pocket of laccase. The most suitable for this purpose tend to be molecules derived from natural substrates of the enzyme. In publication [H7] I examined the effect of the presence of "addressing" groups on the process of enzyme adsorption and subsequent catalytic reduction of oxygen on prepared surfaces. Surface plasmon resonance allowes to study the interactions between laccase and the surface modified with veratric acid derivative. Shape of sensogram reflects adsorption of laccase on a gold surface modified with derivative. The change of the resonance angle allows to determine surface concentration of laccase as 286.4 ± 5.4 ng·cm^{-2}.

Both derivatives of veratric acid fit well to the hydrophobic pocket of the enzyme. Another factor supporting the permanent binding of the enzyme to the electrode surface is the inability of the enzyme to be washed out with water or buffer. This behaviour indicates a high affinity of the enzyme to the surface. The usefulness of gold electrode covered with a

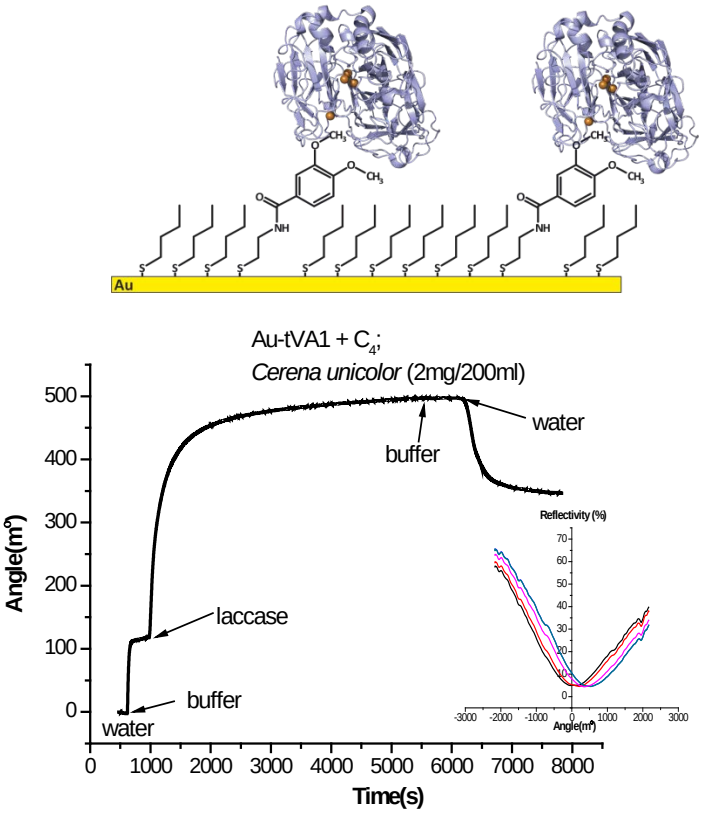

Fig.10. Scheme of the system [H7].

Sensogram recorded on gold sensor modified with: thiolated veratric acid and butanethiol (1:9) during experiment

monolayer of thiolated compound able to bind to laccase is limited by small amount of enzyme molecules on the surface. To achieve high oxygen reduction current density at the lowest possible overpotential one should focus on the formation of three-dimensional carbon nanotube hybrid system, capable of accumulating a much larger amount of enzyme [47]. Therefore, for the construction of biocathode I used carbon nanotubes modified with similar groups, which are derived from natural substrates of laccase. In this publication there is a comparison of several types of modified carbon nanotubes with the substrate analogues of laccase. Oxygen reduction catalytic current on the electrodes modified with analogues of laccase substrates is much greater than in the case of an unmodified nanotubes, or nanotubes with benzoyl substituents. The structure of these compounds makes them geometrically better fitted to the hydrophobic cavity of laccase. Due to the application of such system, the enzyme may be arranged in a preferred orientation and closer to the surface of the electrode, facilitating direct electron transfer. The process of oxygen catalytic reduction is observed at a potential 0.55 V vs. Ag/AgCl, corresponding to the potential of copper T1 centre in laccase. Michaelis-Menten constant measured in solution experiments suggested that carbon nanotubes modified with syringic or veratric acid should have the highest affinity toward laccase, and thus so-modified electrodes should show the highest current density. However, we found that the highest value ratio of the catalytic current to background current was observed at electrodes covered by carbon nanotubes modified with ferulic acid residues.

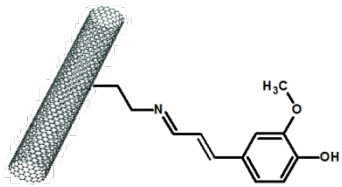

Fig. 11. Scheme of carbon nanotube modified with ferulic acid [H7].

The affinity of the laccase depends strongly on the origin of the enzyme, its purity and the nature of the substrate. Combining of biocathode covered by carbon nanotubes with ferulic acid residues and adsorbed laccase in the hybrid cell with zinc wire as the anode leads to the circuit in which the open circuit potential is 1.71 V and the maximum power density is 5.1 $mW \cdot cm^{-2}$ at a potential of 0.6 V vs. Ag/AgCl. The value is much higher than for the analogous system without adsorbed laccase.

The continuation of promising research on the use of laccase for the biocathode construction was the concept of enzyme immobilization on three-dimensional network formed by gold nanoparticles. I decided to use gold nanoparticles with sizes below 2 nm with completely different properties than larger nanoparticles. Size of nanoparticles affects their optical and electrical properties. For example, surface plasmon band (SPB) is observed for gold nanoparticles of a size greater than 2 nm [51], and is not present when the size of the nanoparticles is below 2 nm and in the spectrum new different bands appear at 680, 450 and 400 nm [52].

Electrochemical properties of nanoparticles also depend on the size of the core [53]. There are three types of electrochemical behaviour of nanoparticles in solution: "bulk-continuum" behaviour, quantum double layer charging and behaviour similar to the behaviour of molecules ("molecule-like"). In the literature defined boundaries of the nanoparticles core size for which the specific type of behaviour can be observed are described. Thus, below 1.4 nm nanoparticles called clusters behave as redox-active molecules, which means a single electron transfer process on the voltammetric curve is observed. The energy gap between the process of adoption of the first electron and the process of releasing the first electron is described as "the molecule-like HOMO-LUMO band gap" [54]. With the increase of the core a plurality of signals derived from the charging nanoparticles size can be observed (quantum double layer charging behaviour). In contrast, when the size of the core of the nanoparticles exceeds 2.2 nm no signals are seen on the voltammetric curve.

In the 90’ of the last century Nathan et al. showed that the layer of gold nanoparticles immobilized on the electrode surface coated with alkanethiol monolayer behaves like a gold electrode [55]. Perhaps this is the reason why in a number of papers about immobilization of laccase on the surface-modified gold nanoparticles authors claimed lack of enzyme activity. Later experiments showed that layer of gold nanoparticles adsorbed onto the thiol blocking

layer on the electrode may "turn on" faradaic behaviour of redox probe in solution [56, 57]. This means that the electron transfer rate constant between the layer of gold nanoparticles and the surface electrode, despite the presence of alkanethiol layer between them, does not depend on the thickness of the layer (approx. 20 Å). Due to the low capacitance resulting from the presence of the alkanethiol layer such systems, constructed using the electrode coated with thiol layer and gold nanoparticles, provide a good matrix for the immobilization of enzymes and facilitate the coupling between the enzyme active centre and the surface of the electrode [58]

The publication [H8] relates to the use of gold nanoparticles with small sizes to facilitate the immobilization of laccase on the electrode surface, and examine the activity of the catalytic system. I developed a method for the preparation of gold nanoparticles with a size approx. 2 nm modified with two different thiols. Thus the step of ligand exchange on gold nanoparticles after the synthesis was eliminated. A key issue of the process is the step of reducing of gold ions (III), and forming polymer between the gold ions (I) and thiol derivatives. In the next step mixtures of more or less monodisperse gold nanoparticles are obtained, depending on the size of the resulting polymers. Also, the step of aging the nanoparticles affects the polydispersity of the samples, because according to the literature, gold nanoparticles consisted of favoured number of gold atoms require tens of hours of aging at low temperature [52].

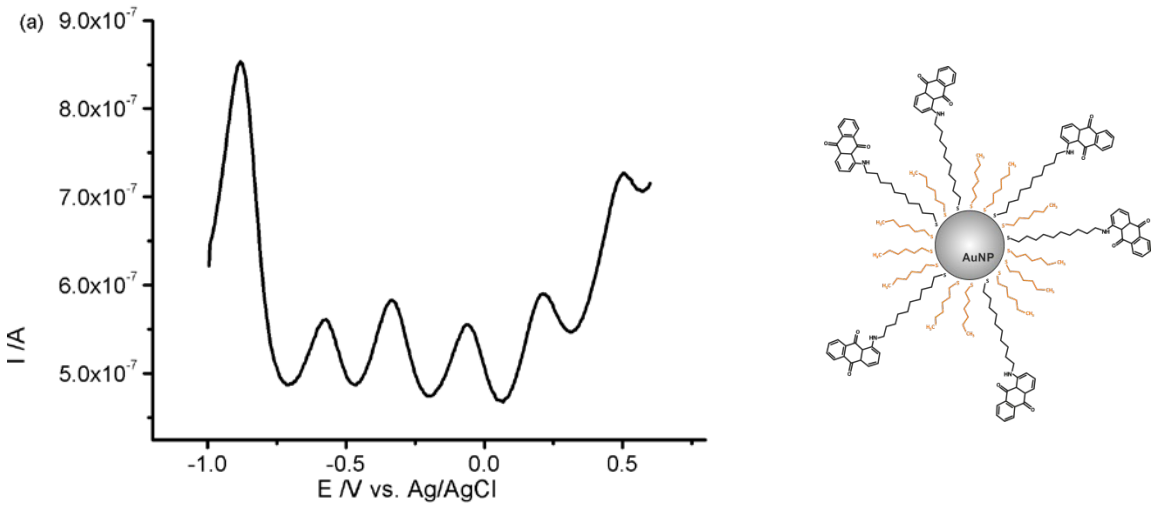

Fig.12. OSWV recorded in 0.1M TBAHFP/ CH_2Cl_2 containing AuNPs modified with hexanethiol and anthraquinone derivative [H8].

The size of obtained nanoparticles was confirmed using dynamic light scattering (DLS) method and transmission electron microscopy (TEM). The hydrodynamic radius of the

nanoparticles modified with hexanethiol was 1.55±0.10 nm, and the nanoparticles modified with both hexanethiol and anthraquinone derivative was 3.64±0.12 nm. However, TEM measurements enable to determine core size of the obtained gold nanoparticles and it was: 1.25±0.29 nm and 1.94±0.74 nm for AuNPs modified with hexanethiol and AuNPs modified with hexanethiol and anthraquinone derivative, respectively. Also, the optical properties of the gold nanoparticles were confirmed by UV/Vis spectroscopy. The size of the core of gold nanoparticles modified hexanethiol and anthraquinone derivative suggests that the electrochemical behaviour of such nanoparticles should remind the quantum double layer charging.

And indeed, number of core loading signal and ligand anthraquinone reduction signal at a potential value of about -0.9 V vs. Ag/AgCl can be observed on the voltammetric curve.

The aim of the project was to immobilize the enzyme - laccase on the gold nanoparticles. The reason why I used anthraquinone derivatives to modify the AuNPs surface was their affinity toward hydrophobic cavity of enzyme.

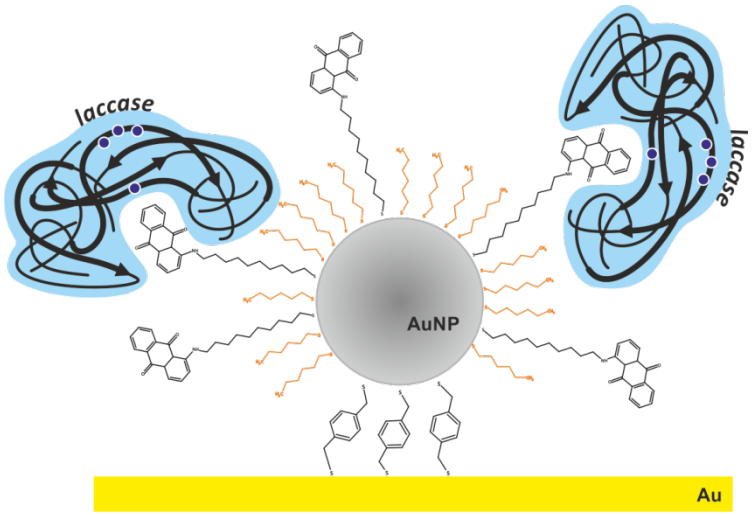

Fig.13. Scheme of the system for catalytic reduction of oxygen [H8].

The process of formation of successive layers on the electrode was monitored using impedance spectroscopy and surface plasmon spectroscopy. I conducted faradaic impedance experiments in the presence of redox probe $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$, and after the recording and verification of spectra I developed an equivalent circuit model, and on that basis I set the parameters describing the properties of the modified electrode. The first layer, 1,4-benzenedimethanethiol layer, has blocking properties and thus leads to the formation of half-circle on the Nyquist plot corresponding to the charge transfer resistance. The adsorption of gold nanoparticles causes a reduction in the charge transfer resistance, which is consistent with the literature [56, 57, 58]. The adsorption of the enzyme on the layer of nanoparticles

results in increase of the R_{ct} value, and the effect is the highest in the case of small nanoparticles modified with hexanethiol and groups with high affinity for laccase and the smallest for 10-nm AuNPs. The results were confirmed by SPR experiments. The biggest weight gain of the enzyme on the SPR sensor was observed if the binding layer consists of gold nanoparticles modified with anthraquinone derivative and hexanethiol. This value is: 469.7±7.2 ng·cm^{-2}. Such conclusion is expected because based on the research paper [H7] we knew that groups with a high affinity for laccase would yield an increased adsorption of the enzyme on a flat surface. In the case of gold nanoparticles modified with anthraquinone moieties, there is a three-dimensional network with more anthraquinone groups than on a flat surface, so the degree of enzyme adsorption is much larger.

The enzyme surface coverage of the electrode depends on both the presence of "addressing" groups as well as the size of the gold nanoparticles. For the biggest nanoparticles layer the least enzyme coverage was calculated based on SPR experiments.

For applications in biofuel cell the most important is not the enzyme coverage of the electrode but the enzyme activity. Experiments performed in Mc'Ilvaine buffer demonstrated that in the presence of oxygen laccases immobilized on the AuNPs modified with hexanethiol and anthraquinone derivative layer catalysed reduction of oxygen. The potential observed for oxygen reduction catalysis is 0.6 V vs. Ag/AgCl and corresponds to the thermodynamic potential of T1 centre in laccase, which proves the maintaining of laccase activity after immobilization in a hybrid system on the electrode.

In conclusion, I proposed a method of synthesis of a suitably modified gold nanoparticles with a size of less than 2 nm, designed a three-dimensional arrangement of modified gold nanoparticles for enzyme immobilization, I have examined the process of adsorption of the enzyme and have proved laccase activity in the proposed system comprising of small gold nanoparticles modified with anthraquinone moieties, dithiol layer, and a gold electrode.

Larger gold nanoparticles were used for the determination of heavy metal ions. The studies described in the paper [H9] concern determination of mercury (II) ions by utilizing the phenomenon of aggregation of gold nanoparticles. The aggregation of gold nanoparticles into larger groups is due to the light, protective coating loss, a sudden increase in the ionic strength of gold nanoparticles solution. 10-20 nm AuNPs are usually obtained by Turkevich method [59] and stabilized with citrate ions, electrostatically associated with a nanoparticle core, which means that they can easily aggregate. When in a solution of citrate-stabilized gold

nanoparticles there are other molecules with a higher affinity for the positively charged core of nanoparticles, citrate ions are removed from the surface and replaced by new ligands. In this way, the negatively charged DNA strands interact with the gold nanoparticles and adsorb onto their surface, which prevents aggregation. Applied in publication [H9] DNA sequence (5'-TTCTTTCTTCCCTTGTTTGTT-3') was designed in order to form a hairpin structure in the presence of mercury ions, which form a stable complex with thymine residues of nucleic acid. Therefore, the addition of Hg^{2+} ions to a solution of gold clusters covered by designed DNA results in the formation of the hairpin structure between DNA strands an mercury ions.

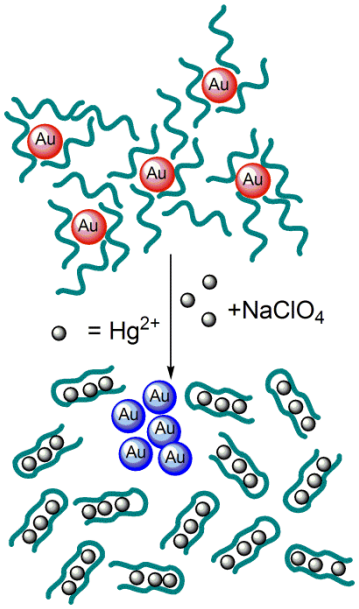

Fig 14. Scheme of mercury ions detection using AuNPs modified with DNA [H9].

The gold nanoparticles, which are not stabilized by DNA molecules, in the presence of a large excess salts, aggregate, which is reflected in a solution colour change from pink-red to violet. The salt used to increase the ionic strength of the solution is sodium perchlorate, which does not form difficult soluble salts with mercury ions. The colour of gold nanoparticle solution may be followed by UV /Vis spectroscopy. Synthesized clusters have a diameter of approx. 12-14 nm, so that the electronic spectrum exhibits an absorption band of approx. 520 nm, whereas the process of aggregation changes the position of the band. The detection limit of this method is 2ppb of Hg^{2+} ions. In order to confirm the aggregation of gold nanoparticles in the presence of mercury ions I used transmission electron microscopy. In contrast, gold nanoparticles stabilized by foreign DNA sequence (5'-CCAACCCCCCAGAAAGAA-3') does not undergo a process of aggregation in the presence of ions Hg^{2+}. There is not complex formation between the mercury ions of and the DNA strand due to the lack of thymine residues in the strands. Therefore, the gold nanoparticles in the presence of excess salt in the solution remains protected by DNA molecules and do not undergo aggregation.

The second presented method for determining mercury ions is based on DNA machine with a signal amplification system. DNA machine in the presence of the analyte and the corresponding substrates, and the enzyme begins to operate. Processes that can be perform by DNA machine include processes: scission, replication, displacement, etc. [60]. Designed strand (5'-CCCAACCCGCCCTACCCGCTGAGGTTCCCCAGATTCTTTCTTCCCTTGTTTGTTTCTGGGG-3')

called the „track" contains three domains. The first domain is designed to recognize and bind the mercury ions and comprises a plurality of thymine residues. The second one is a sequence complementary to a sequence which, after replication will be recognized by a cleaving enzyme Nt BbvC 1A. And the third domain contains a sequence complementary to the DNA-zyme, which mimics the action of horseradish peroxidase. DNA-zyme is a short DNA strand having a catalytic activity. In this work I used DNA-zyme, which is G-quadruplex with hemin complex. Formed complex can catalyse the reaction between the substrate (eg. ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt) and hydrogen peroxide.

In the presence of mercury ions the first domain folds into a hairpin structure, wherein the 3'-end hybridizes to a corresponding part of "DNA track". The formation of double-stranded region starts replication process and construction of the complementary strand in the presence of the free bases and polymerase.

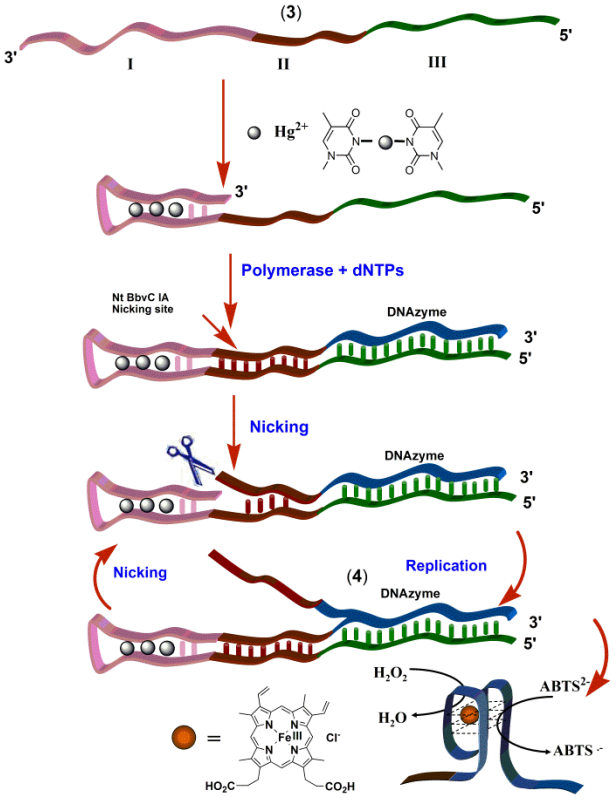

Fig. 15. Principles of determination of Hg^{2+} ions by operation of DNA machine [H9].

In the replicated second there is a specific sequence for the enzyme cutting and the presence of this enzyme product comprising the DNA-zyme is cut-off. Replication starts again and the resulting DNA-zyme in the presence of hemin folds into G-quadruplex, which mimics the horseradish peroxidase and catalyses the oxidation of $ABTS^{2-}$ by H_2O_2. The formation the coloured product $ABTS^{\cdot-}$ is observed as a change in absorbance at a particular wavelength and is proportional to the concentration of Hg^{2+} ions. Detection limit of this method is even lower than the previous one and is 200ppt of mercury ions.In the absence of mercury ions in the solution there appears residual response resulting from spontaneous folding of a DNA strand without the mercury ion. Also an attempt to start the DNA machine by other ions causes residual response. In addition, we preformed gel

electrophoresis experiments to confirm the previous results. In the presence of Hg^{2+} ions a new band corresponding to the mass of resulting DNA-zyme appeared.

In the presented work I used two approaches for determining mercury ions based on the interaction of a specially designed DNA strand with ions of mercury. The first, using the process of aggregation of gold nanoparticles has a high simplicity and the second, using the operation of the DNA machine enables double signal amplification. The presence of Hg^{2+} ions enables autonomous operation of the machine, which synthesizes DNA-zyme molecules. Repeated synthesis of DNA-zyme and its catalytic activity are responsible for enhancement of the signal corresponding to the concentration of mercury ions.

4. summary

My most important achievements:

- construction of a multimetallic rotaxane on the surface of gold electrodes and a description of its formation by an electrochemical means (preparation of functional nanomachines),
- application of donor-acceptor interactions to create a surface with controlled wettability; a description of the phenomenon by using electrochemical techniques,
- determination of enzyme activity (from the group of kinases) by designing the sensor layer using electrochemical techniques, including faradaic impedance,
- the development of model membranes on the surface and modification of their structure with defects using hydrolysing enzymes and the application of impedance spectroscopy and surface plasmon resonance to describe the phenomenon,
- design of biocathode based on the laccase immobilized on modified surface, investigation of the binding effects using surface plasmon resonance,
- development of method for the synthesis of gold nanoparticles with a size less than 2 nm modified with two different thiol derivatives and the use of the nanoparticles network for the immobilization of the enzyme - laccase maintaining its catalytic activity towards oxygen reduction.
- development of methods for the determination of mercury ions in the water by the interactions between ions Hg^{2+} and thymine residues of DNA using strands of DNA and gold nanoparticles.

In my research I use modern chemical methods, but also techniques associated with molecular biology, experimental physics, and other fields, including electrochemical techniques, impedance spectroscopy, UV/Vis spectroscopy, surface plasmon resonance spectroscopy, atomic force microscopy, contact angle measurements, Langmuir -Blodgett technique, a quartz microbalance technique, gel electrophoresis and others.

I investigated mainly systems immobilized on the electrode surface using voltammetric method for describing the layers properties and their interactions with specific individuals. With electrochemical methods I have observed the formation of the intertwined structure - rotaxane built of macrocyclic complexes that force defined architecture and allow the electrochemical observation.

I also designed a surface of chemically and electrochemically changeable hydrophilic/hydrophobic properties, by the existence of donor-acceptor interactions. I also designed surfaces of the sensor for determining the activity of enzymes from the class of kinases using specific reactions and specific antibodies.

I applied the enzymes from the phospholipases group as the modifying agent for the lipid layer immobilized on the electrode surface. The use of enzymes allows the formation of intermembrane channels, and the process was examined both by spectroscopic and electrochemical means. The result of the research is the development of technology of obtaining nanostructured lipid membranes of selective permeability for construction of conductive surfaces with the designed architecture and functionality for bioelectronics and miniature electrochemical sensors.

I developed the biocathode for biofuel cell using laccase that catalyses the 4-electron reduction of oxygen to water, without the intermediate step of generating hydrogen peroxide. To optimize the performance of biocathode I tested different procedures of enzymes immobilization in order to ensure effective contact between enzyme and the electrode.

I proposed the method for the synthesis of small gold nanoparticles modified with groups having an affinity for the laccase. I designed a three-dimensional network comprising modified gold nanoparticles for enzyme immobilization. I employed impedance spectroscopy and surface plasmon resonance for description of enzyme adsorption on the nanoparticles layer. I proved that despite of the presence of groups with high affinity to the enzyme, the size of gold nanoparticles is also important factor affecting the adsorption process. Laccase adsorbed on the proposed system comprising a layer of small gold nanoparticles modified with anthraquinone moieties, dithiol layer, and a gold electrode remains active.

I developed methods for determination of mercury ions through the interactions of these ions with thymine residues of specially designed DNA sequences. The first of these bio-analytical method is based on the process of aggregation of DNA protected gold nanoparticles after deprotection by formation of a complex between DNA and Hg^{2+} ions. The second method that I developed is based on an autonomous operation of the DNA machines operated by the presence of mercury ions. The machine generates multiple DNA-zyme molecules, which catalyse a colour reaction, thus enhancing the signal.

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