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Chiral Biosensors and Immunosensors

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1. Introduction

Investigation of compounds having the asymmetric carbon in their structure has a fundamental significance for understanding all processes that occur in living organisms. Biologically active compounds as amino acids, sugars, peptides, proteins and polysaccharides posses a different stereochemistry. All of these compounds are involve in chiral interactions in biochemical systems functioning in living organisms. Animal and human peptides consist of almost only left-handed (L) amino acids as building blocks for peptides. Right-handed (D) amino acids occur in unicellular lower organisms. A huge interest in chirality results also from the fact that present pharmaceutical and chemical industry to large extent is based on the synthesis of compounds that may have a different stereochemistry. Enantiomeric pharmaceuticals, pesticides or food additives can have a different influence on living organisms. The first observations on the pharmacological role of enantiomers are assigned to Abderhalde and Müller, who described in 1908 a difference in raising blood pressure by enantiomers of epinephrine. These observation started research on the effects of the enantiomers in the context of the pharmacological effect.
Clinical tests indicate that the replacement of racemate by a single active enantiomer of pharmaceutical allows to use of lower doses of drugs, increasing the therapeutic efficacy of individual doses. It helps to avoid possible harmful interactions with other drugs, to minimize the differences in drug metabolism between species and reduce the toxicity caused by supplementation of inactive isomers (Agranat et al., 2002; Baumann et al., 2002). There has been a trend towards to ensure that pharmaceuticals that were invented, approved and marketed as a racemate or a mixture of diastereomers, have been re-marketed as single enantiomers. Such compounds are called "chiral switches" (Caner et al., 2004; Hutt & Valentová, 2003)
The enantiomers of the same compound, indistinguishable by physical and chemical properties show sometimes different physiological effects. There are a number of examples demonstrated the need to test the impact of individual enantiomers, and in some cases, the chiral purity control of the compound. Enantiomers may differ in smell which can be exemplified by carvone, S and R-enantiomer have the scent of caraway and mint respectively (Laska et al., 1999). They may have a different taste - for example, isoleucine and asparagine, have a bitter taste as a form of L and sweet in the form of D (Zawirska-Wojtasiak, 2006) and activity effects on organisms, such as ephedrine and adrenaline. The stimulating effect of (+) ephedrine is 80% of the effect caused by (-) ephedrine (Herráez-Hernández & Campíns-Falco, 2001). There have also been known cases in which one of the enantiomers of a compound have a beneficial influence for the body and the other caused

serious problems. The most famous example was the drug with analgesic and calming effect - thalidomide administered to pregnant women in the racemic form. It was found that the R enantiomer has a therapeutic effect, while the other enantiomer was strongly teratogenic. It elicited phocomelia, disease involving the disappearance of the long bones of limbs (Nakanishi et al., 2004; Lenz, 1988). Currently, researches on the use of this drug for cancer are conducted. It is used to treat multiple myeloma, especially in the elderly. Its inhibiting effect on the formation of blood vessels around tumours was also noted. Also, the enantiomers of many other drugs have different effects. L-DOPA is used to treat Parkinson's disease, while the D-enantiomer exhibits strong toxicity. Similarly, in the case of penicillamine, D-enantiomer is used as an antiarthritic drug, and the L-enantiomer is highly toxic (Eichelbaum & Gross, 1996). Also, a number of drugs belonging to the group of antidepressants and psychotropic drugs have a different effect of both enantiomers (Lane & Baker, 1999).

Literature presents many examples showing that enantiomers differ in activity, rate of reaction or time of dissolution. The example of difference in time of dissolution can be fungicide metalaxyl. In a neutral pH the R enantiomer in its active form shows over four times higher dissolution constant than S enantiomer. (R)-(+)-isomer of organophosphorus pesticide methamidophos reveals the higher insecticidal activity against flies than other enantiomer and racemate (Miyazaki et al.,1988). On the other hand (S)-(-)-methamidophos appear more toxic after application against German cockroaches in a short time. During first 5 hours the same dose of S enantiomer caused death of 75% of insects whereas R enantiomer caused death of only 20%.

Research of the individual enantiomers can be a valuable indicator of the quality of different food products. D amino acid content in natural samples of milk, juice and honey is an indication of bacterial contamination, prolonged storage time of products or poor quality of fruit used in the production of juice. The content of D or L enantiomers of lactic acid in fermented products provides the type of bacteria responsible for the process. Similarly the presence of D-asparagine in yogurt samples indicates the presence of specific bacteria. Also, chiral alcohols can be used to control product quality. For determination of 2-butanol in distilled spirits, R-enantiomer derived from bacteria that may be present in the mash, and S enantiomer is produced only by yeast. The content of R-2-butanol is a marker of bacterial contamination. Also from chiral linalool (affecting the taste of oranges), the S form is present in sweet oranges, and R is the typical to bitter. It can be an indicator of adulteration of the concentrate's composition (Marchelli et al., 1996).

Enantiomers of some compounds can be used also to trace sources of water contamination. A non-selective beta blocker mainly used in the treatment of hypertension-propranolol exists in untreated sewage as a racemate. During successive steps of sewage treatment the amount of R enantiomer in relation to both enantiomers decreased to even less then 40% regardless the concentration of compound. Determination of enantiomeric fraction (the ratio of the concentration of one of the isomers to the total concentration) can be useful indicator to evaluate if examined water is significantly affected by untreated sewage, for example as a result of leaking sewers and to apportion the contribution of treated and untreated sewage into surface waters (Fono & Sedlak, 2005).

In a similar manner the source of groundwater contamination in Switzerland by specifying the content of enantiomers of the herbicide Mecoprop was determined. In order to protect crops in agriculture, it is sold as a pure enantiomer, while the same compound was also used to protect roofs against fouling by plants. In latter case a racemate was used.

Enantiomer ratio of 0.5 was an indication that about 50% of the herbicide in surface water origin from roofs security system, and not - as expected - mostly from agriculture (Bucheli et al., 1998).

There are also works in which attempts to use information about the racemization rate in the study to determine the age of archaeological finds on the basis of the ratio of amino acids enantiomers contained in samples of bone, shell or teeth. In living organisms, the ratio of amino acid D / L is zero. After the death of the body's proteins break down and the process of racemization begins. It leads to increase the ratio of amino acids D / L up to one. This process is lengthy and depends on many factors such as the structure of the amino acid sequence of amino acids in the protein, pH, buffering effect, humidity, temperature, as well as the presence of catalysts. Due to the large number of parameters that must be controlled, this technique is challenged by many researchers, even though it is used for 30 years and has resulted in many interesting publications (Robins et al. 2001).

Optically pure compounds are also used in chiral synthesis. In 2001, Sharpless, Knowles and Noyori received the Nobel Prize for research on the oxidation and hydrogenation reactions using optically active compounds which have found application in the production of many antibiotics and anti-inflammatory and cardiac drugs (Kaniewska 2009).

The enantiomers of the same compound are characterized by almost identical physical properties. For this reason they can not be separated by widely used methods such as fractional distillation or fractional crystallization except that the solvent is optically active. Methods used for separation and determination of individual isomers are based on interaction with substances exhibiting optical activity. Currently used methods for the analysis of optically active compounds are mainly separation methods such as gas chromatography (GC), liquid chromatography (LC) and high performance liquid chromatography (HPLC) using chiral stationary phases, chiral selectors in the mobile phase or flow reactors for derivatization and highly efficient electromigration techniques as capillary electrophoresis (CE) using chiral selectors. Other methods are mass spectroscopy, NMR to the study of molecular recognition, as well as some spectroscopic techniques. These techniques require expensive equipment and the analysis is in most cases time-consuming.

Biosensors are widely used for analytical application for example in clinical of food analysis, environmental monitoring or chemical processes. They are characterized by good precision and sensitivity. The measurement is fast and stable. Biosensors can be miniaturized and used in portable analysers. They contain the biological material which often has the stereoselective or stereospecific properties. For this reason, biosensors may be competitive to the separation methods for the analysis of optically active compounds.

2. Enantioselective enzymatic biosensors

2.1 Electrochemical biosensors

Among natural receptors in construction of biosensors most often enzymes are employed. The reasons are the wide range of measurable parameters that can be utilized as result of biocatalytic process (chemical products, ions, protons, light, electrons) and a large number of available, isolated enzymes (Subrahmanyam et al. 2002). Numerous number of enzymes employed for long years in design of biosensors catalyses enantioselectively reactions of particular isomers of substrates, but very few reports can be found on their enantioselectivity and potential applications. The most examples can be find in reviews (Schlügerl et al. 1996; Stefan et al. 1999)).

Enzymes as compounds involved in life processes catalyze the reactions in which the substrate is a compound in the form of pure enantiomer. Enzymes may exhibit, depending on the method and mechanism of interaction with substrates, the absolute, or stereochemical specificity. The specificity may relate the D and L forms, geometric isomers, the position of binding, the coenzyme spatial settings, and the asymmetry of the complex enzyme-substrate. Stereochemical specificity is the perfect matching of the substrate configuration of the spatial points of interaction in the active centre. Active complex is generated only in the case of appropriate size. Enzymes catalyse reactions by creating new reaction pathways of lower energy transition state. The first step is usually to produce an enzyme-substrate complex. Substrate binds to the enzyme active site, which is a small recess or slot for the characteristic structure of the enzyme. Binding specificity depends on the specific arrangement of atoms in the active site. The fit is possible only if the substrate have an appropriate shape. In the case of chiral compounds the velocity of the reaction of substrate molecule with the enzyme is usually different for both enantiomers. Enantioselectivity factor value (E) can be determined from the equation (1) (Chen C. S. et al. 1982)

$$E = \frac{V_{\max 1} / K_{M1}}{V_{\max 2} / K_{M2}} \tag{1}$$

The difference of reaction rates of competing substrates due to the difference in Gibbs free energy of the subsequent stages of the reaction (Fig. 1) (Overbeeke et al., 1998).

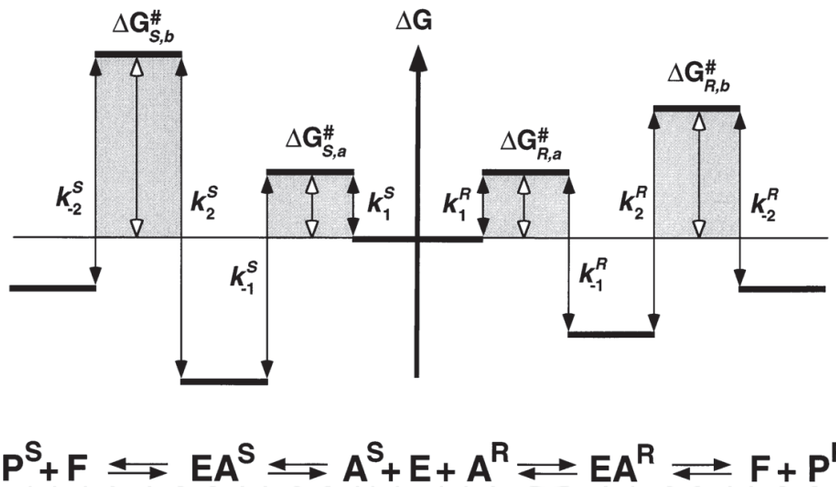


Fig. 1. Gibbs free energy change in subsequent reactions catalysed by the enantioselective enzyme

Then the enantioselectivity is determined from the expression (2):

$$-RT \ln E = \Delta\Delta G^{\neq}_{R,S} \cong \Delta G^{\neq}_{Rb} - \Delta G^{\neq}_{Sb} \tag{2}$$

2.1.1 Chiral catalysis

The most often developed, so far, are systems with pair of enzymes specific for each enantiomer. One of the mostly used examples are amino acid oxidases. D-amino acid oxidase, by the presence of adenine flavin dinucleotide (FAD) as cofactor, catalyses the

oxidation of D amino acids to imino acids which are immediately hydrolyzed to the corresponding α-keto acids and ammonium ions (Moreno et al. 1996). D-AAOx does not catalyze the oxidation of the opposite enantiomer and it is not inhibited by L amino acids. The properties of D-amino acid oxidase differ depending on the species. For example, the structure of the active centre of enzyme derived from mould is more open, which is the cause of a much broader range of catalytic activity (Pollegioni et al. 2002).

Enantiomers of amino acids were determined electrochemically with the biosensors that differ by way of immobilization, enzyme systems, or a kind of mediator. D-AAOx or L-AAOx were involved to the construction of biosensor sensitive to D or L amino acids. Value of the signal obtained for over 20 common L-amino acids and six important D-amino acids were presented in (Sarkar et al. 1999) and compared with the results obtained in (Kacaniklic et al. 1994). In the first case an enzyme was immobilized on the screen-printed electrode with the addition of polyethylamine (PEI) to the working electrode paste. The working electrode incorporated rhodinized carbon to facilitate hydrogen peroxide oxidation. Sensor showed response to six of the seven tested D amino acids, except D-proline. Only the average response of the biosensor for the relevant amino acid at a concentration of 0.1 mol L-1 was presented. In the latter case the enantioselective enzyme was coimmobilized with the horseradish peroxidase in the graphite paste with the addition of polyethylamine. In another example D-or L-AAOx were immobilized together with horseradish peroxidase and ferrocene as a mediator in the graphite-Teflon paste. The sensor consists of two electrodes – each with a different amino acid oxidase, L or D was used to determine amino acids in the racemic samples, or to determine the L amino acid in samples of grapes. Measurements were carried out in batch and flow (Dominguez et al. 2001). For the rapid determination of D and L amino acids the system of D-or L-AAOx biosensors immobilized in a membrane protein with glutaraldehyde was used. The chiral selectivity of the reactor was checked. The system was used for determination of amino acids in samples of two beers during the process of fermentation (Varadi et al. 1999). For determination of D-alanine a system of two sensors was used. System contained a reactor with DAAOx immobilized by glutaraldehyde on glass porous CPG (long chain alkyl amino-controlled pore glass) coupled with a sensor with the pyruvate oxidase (PyOx). D-amino acid oxidase catalysed the oxidation of D-amino acids, including the formation of pyruvate from D-alanine. This compound was then oxidized in a reaction catalysed by the pyruvate oxidase and the amount of oxygen consumed in this reaction corresponded to the contents of D-alanine in the sample (Inaba et al. 2003a). A different design of a sensor based on the same reactions was also used. Pyruvate oxidase was immobilized in the membrane on the surface of the Clark oxygen electrode. Suitable amino acid oxidase D-or L- in the buffer was added to a sample of amino acids and oxygen-saturated solution was incubated with catalase. After a specified time, the concentration of pyruvic acid formed was measured using electrode with PyOx (Inaba et al. 2003a). DAAOx was also immobilized on Prussian blue film by electrochemical codeposition. The electroactive layer was covered with Nafion. Biosensor was calibrated with D-alanine, but had no specific answer to this amino acid (Shi & Dong, 1995)

The screen-printed electrodes with the Prussian Blue as a mediator were used to prepare the biosensor for D-amino acids (Wcislo et al. 2007). The surface of the working electrode covered with the mediator has been additionally protected by the Nafion layer and then modified by evaporation of mixture containing DAAOx, bovine serum albumin and glutaraldehyde. The short response time and good reproducibility was obtained in

measurements of D-alanine and some chosen D-amino acids. Biosensor showed the linear response to D-alanine in the phosphate buffer with addition of FAD in the concentration range 10-300 μM. L-alanine did not give any response (Fig. 2). The biosensor was examined towards some other D amino acids and all L amino acids. The total amount of D-amino acids was estimated for the samples of milk and juices.

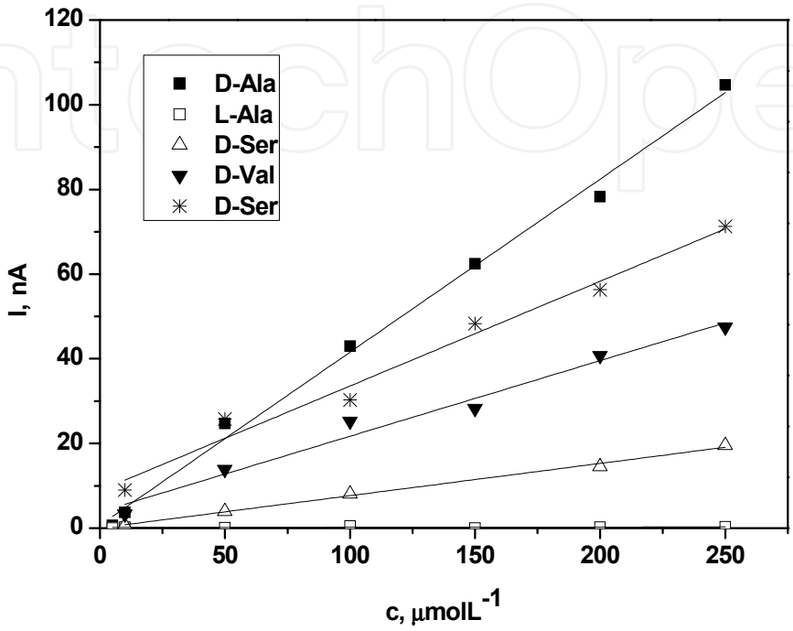


Fig. 2. The amperometric response of screen-printed enzymatic biosensor to enantiomers of alanine. Measurements made by placing a drop of measuring solution on the SPE sensor at –0.05 V polarizing potential. Solution of alanine made in 50 mM phosphate buffer of pH 7.4 containing 0.1 M KCl and 10 μM FAD (Wcislo et al. 2007) .

The graphite paste biosensors with immobilized L-AAOx were employed for enantioselective detection of S-capropril (Stefan et al. 2003a). With amino acid oxidases also multi-enzyme biosensors were developed for determination of various drugs from the group of angiotensin-converting enzyme inhibitors such as cilizapril, pentopril (Aboul-Enein et al., 1999a), enalapril, ramipril, trandolapril (Stefan et al., 1998) and perindopril (Aboul-Enein et al., 1999b). For determination of D- or L-methotrexate (substituted glutamic acid) D-AAOx or L-AAOx were immobilized together with glutamate oxidase and horseradish peroxidase in the graphite paste. Different combinations of enzymes were examined. Mono-, bi- and trienzyme electrodes were constructed. The best enantioselectivity was obtained for three enzyme system with L-AAOx (selectivity coefficient $pK^{amp} = 3.09$). The selectivity of all biosensors was checked by both separate and mixed solution method. In mixed solution method the ratio between the concentration of the main and the interfering enantiomer was 1:10 (Stefan et al. 2003b). For the same multi-enzymatic system also a covalent immobilization with glutaraldehyde on the graphite support was examined with carbodiimide and thermal hardening, but immobilization in graphite paste was found simpler and providing better results in terms of enantioselectivity. Similar methods of enzyme immobilization have been used for detection of pipecolic acid.

The graphite paste biosensor consist D or L amino acid oxidase or the one of the amino acid oxidase combined with horseradish peroxidase in order to improve detection of hydrogen peroxide. The best result was obtained for bienzyme biosensor containing L-AAOx with HRP (selectivity coefficient $pK^{amp} = 3.82$). The values of amperometric selectivity coefficients for all the biosensors designed for L and D pipecolic acid were higher than 2. Proposed biosensors were used in clinical analysis to detect L or D-pipecolic acid in serum samples (Stefan et al. 2003c).

Another similar pair of enzymes sensitive to enantiomers is D- and L-lactate dehydrogenases (D-, L-LDH). Biosensors with these enzymes immobilized on porous carbon electrodes with the use of osmium complex $(Os(bpy)_3)(PF_6)_2$ as mediator have been used for determination of D and L-lactic acids. Changes in the concentration of lactic acid enantiomers measured by cyclic voltammetry were linear for L-enantiomer in the range of 0.1 -10 mmol L-1 and D-enantiomer of 1-20 mmol L-1. The enantioselectivity was determined by marking of error of one of the enantiomers determination at 10 fold excess of another. In the case of biosensor with L-lactate dehydrogenase, L-lactic acid was determined with an error of 4.9% with 10 fold excess of D-lactic acid. In the opposite situation the error was 5.4% (Motonaka et al. 1998). In the design of enzyme field effect transistor with D-LDH, the enzyme was immobilized on pH sensitive gate. The enantioselectivity of the biosensor is presented in Fig. 3. The authors admited, however, that the equilibrium of these reactions was shifted to the left and therefore the reaction product had to be removed. The signals were also influenced by pH, buffer capacity, temperature and flow-rate (Kullick et al. 1994). In a similar way biosensors with D- and L-malate dehydrogenases have been prepared (Schlügerl et al 1996).

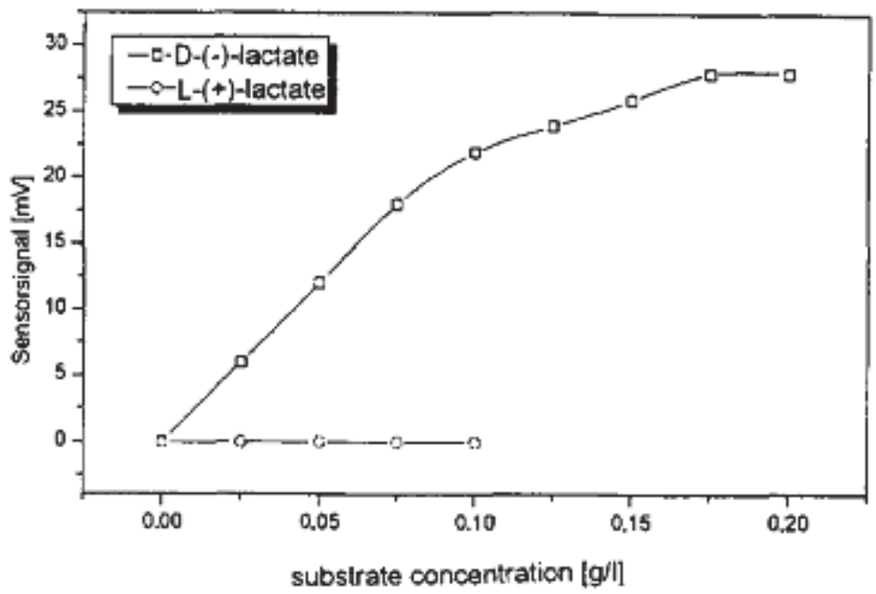


Fig. 3. The potentiometric response of D-LDH FET biosensor to enantiomers of lactate (Kullick et al. 1994).

In cases where there are no specific enzymes for both enantiomers another method of determination can be used. Two biosensors can be simultaneously employed – one sensing

with similar sensitivity both D and L species, and another one, sensitive to particular enantiomer. A multichannel system with eight pH field effect transistors has been developed for determination of hydrophobic esters of amino acids. Esterase EC 3.1.1.1 was used as a non-enantioselective enzyme while α-chymotrypsin only catalysed the reaction of L-amino acid esters. The first biosensor could detect the total amount of amino acid esters and the second gave the information about the enantiomeric ratio in the sample. The same authors present the example of the sensor containing the esterase and lipase for the enantioselective determination of β-hydroxy acid esters, where lipase catalysed the reaction of D-β-hydroxy esters (Kullick et al. 1994). The authors also mention the possibility of construction of system of two enzymes – amidase and aminoacylaze - which catalyze the reactions of deacylation. Amidase catalyzes the non-selective reaction of N-acetyl amino acids and aminoacylaze selectively catalyses only N-acetyl-L-amino acids (Schlügerl et al. 1996)

Also a biosensor design has been reported, where with the use of non-enantioselective enzyme, the enantioselectivity has been gained by the modification of sensing electrode surface with appropriate conducting polymer, which additionally serves as support for immobilization of enzyme. The surface of glassy carbon electrode was modified by electropolymerization of the chiral dicarbazole-biotin. The modification provided differentiation of current magnitude for anodic oxidation of L- and D-norepinephrine of about 50% at 0.5 V *vs.* SCE. Electrode was modified by six enzyme layers constructed by six cycles of sussesive deposition of avidine and biotinylated polyphenol oxidase (PPO). The immobilization resulted in obtaining of biosensor for measurements of D-norepinephrine with 4.8 times larger sensitivity than for L isomer at 0.2 V *vs.* SCE (Cosnier et al. 2003).

Finely there are enzymes with well defined enantioselectivity still not involved to biosensor design. One of the examples is Quinohaemoprotein alcohol dehydrogenase (Jongejan et al., 2000). The enzyme is enantioselective in the oxidation of secondary alcohols. A strong preference is observed for the S-2-alcohols. The enantioselectivity increases with increasing chain length. The same enzyme was immobilized on the surface of electrode and used in that form for preparative purposes (Somers et al., 1998). Although another type of alcohol dehydrogenases were used for biosensor design (Jiang et al. 2009) Quinohaemoprotein alcohol dehydrogenase as yet didn't find any analytical application.

2.1.2 Chiral inhibition

There is a large representation of chiral compounds among pesticides. 25% of the active pesticide ingredients appear in the form of enantiomers (Garrison, 2006). A vast majority of these compounds is produced and sold as a racemate. The operating principle for a large number of phosphoogranic pesticides is precisely acetylcholinesterase inhibition. Unimmobilized enzyme inhibition studies using phosphoogranic pesticides in solution and in vivo in water microorganisms, indicate differences in the inhibition rate and toxicity against selected organisms for the enantiomers of the compounds used for tests. These differences may depend on the origin of the enzyme. Depending on whether it was tested in vitro or in living organisms opposite inhibition was observed. (-)-Profenofos has more than 8-fold greater toxicity on Daphnia manga in vivo, while (+)-Profenofos in vitro shows a rate of inhibition over 71 times stronger towards HR-AChE (Nillos et al., 2007). In the case of fenoxon sulfoxide, IC50 (half maximal inhibitor concentration) has been presented. This factor was estimated for two enzymes: HR-AChE and EE AChE with the average figures at

6.9 μM and 6.5 μM for R (+) fenoxon sulfoxide and 230 μM and 111 μM for S (-) fenoxon sulfoxide respectively (Gadepalli et al., 2007). Another example of chiral phosphoogranic pesticide is the nematicide fosthiazate. The examination of individual enantiomers' reactivity showed a 1.4 difference in the inhibition of EE AChE in vitro and a 3.1 fold difference in toxicity against Daphnia manga (Lin K. D. Linet al., 2007). Significant differences of AChE inhibition can also be observed for the insecticide chloramidofos, which has 2 pairs of diastereoisomers; depending on the chosen pair of enantiomers the differences range from 1.1 to 18.1 (for measurements in vitro) and from 1.2 to 13 (in vivo) (Zhou et al., 2007).
The operating principle of malathion and malaoxon also is based on the inhibition of acetylcholinesterase. The differences in the inhibition rate dependent on the enantiomer applied, as well as on the origin of the enzyme have been observed. R malaoxon inhibited RB AChE (rat brain) 8.6 times faster than S malaoxon (Berkman et al., 1993a). In turn, BE AChE (bovine erythrocytes) difference in the rate of inhibition of R / S is 22.5 (Rodriguez et al., 1997).
Below we present for the first time in literature a screen printed biosensor which can be used to measure the inhibition of the immobilized enzyme in both batch and flow analysis.
Screen printed electrode mediated with Prussian Blue and protected by Nafion layer was used to prepare the bienzymatic biosensor. The membrane was obtained by mixing acetylcholinesterase, choline oxidase and BSA solution phosphate buffer with the addition of KCl, and then with glutaraldehyde solution in water. The scope of straightness for the signal received in batch measurements was 10-500 μM for acetylcholine chloride. Static measurements indicated that the enzyme immobilized by the R-enantiomer is inhibited stronger than the one immobilized with the S-enantiomer by approximately 1.25 times. For malathion the inhibition ratio for the enantiomers R / S was 1.3 (Fig 4A).
Batch measurements were also carried out for B394-strain acetylcholinesterase – a mutant strain of the enzyme isolated from the fruit fly. The biosensor with immobilized B394 strain acetylcholinesterase showed practically no difference in inhibition by the two pesticides (malaoxon and malathion) (Fig 4B)
For the flow measurements the biosensor with EEAChE showed linear response to the analyte in the range of concentration from 50 to 800 μM. The ratio of the original signal of the biosensor inhibited by R and S enantiomers of malaoxon in flow analysis after the first injection was 2.7. Then, after the second injection of the inhibitor solution, it was as high as 22 and the subsequent injections caused practically no signal from the enzyme immobilized on the surface of the biosensor by R-enantiomer (Fig 4C). The enzyme inhibited by S-enantiomer, on the other hand, still showed some activity. The presented study also confirms that the differences in inhibition depend on the chiral origin of the enzyme (Kaniewska, 2009).

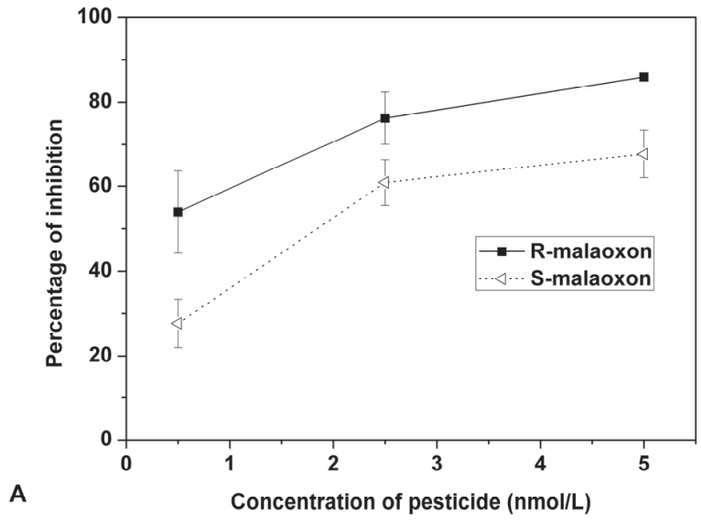
3. Enantioselective immunosensors

It is well known that antibodies can differentiate enantiomers of antigens (Landsteiner & van der Scheer, 1928). Bedsides numerous other selectors as cyclodextrins, crown ethers, macrocyclic antibiotics, Pirkles, proteins or cellulose commonly used for obtaining a chiral stationary phases for HPLC, chiral separation can be obtained by immobilizing suitably raised stereoselective antibodies onto a stationary phase (Hofstetter et al., 2002; Kim H. et al., 2004).
The development of the affinity-based biosensors is on of the fastest growing area in the biosensor field (Rogers 2000). Various strategies have been developed for design of separation-free electrochemical immunosensors, based mostly on heterogeneous immunoassay procedures (Killard & Smyth, 2000).

3.1 Electrochemical immunosensors

The possibility of the construction of enantioselective immunosensors was indicated by the use a stereoselective antibody sensitive to the chiral centre of α-amino acids (Hofstetter et al., 1998). The interactions of rabbit antibodies was detected for amino acids in an enzyme-linked immunosorbent assay (ELISA). The enantiospecificity was observed for free amino acids p-aminophenylalanine and phenylalanine, whose structures overlap with the hapten, but also it was exhibited for other amino acids, aromatic and aliphatic. The same antibodies were employed for the development of a highly enantioselective electrochemical immunosensor. Stereoselective binding of an anti-D-amino acid antibody to the hapten-modified sensor surface resulted in capacitance changes that were detected with high sensitivity by a potentiostatic step method enabled to detect impurities of D-phenylalanine as low as 0.001%. The L-enantiomer was not bound by the antibody (Zhang S. et al. 2006).

Another example of electrochemical sensor is based on the stereoselective interaction of proline with carcinoembrionic antibody (anti-CEA). The selected pure enantiomer of proline was assembled on the glassy carbon electrode surface. Then the anti-CEA was loaded and



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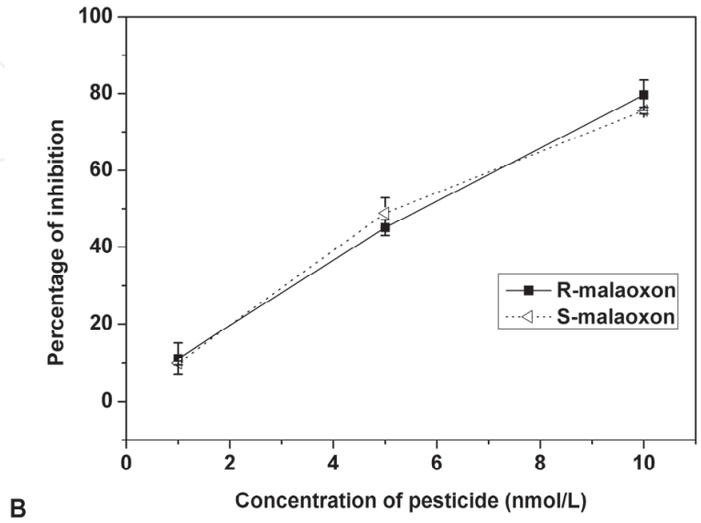

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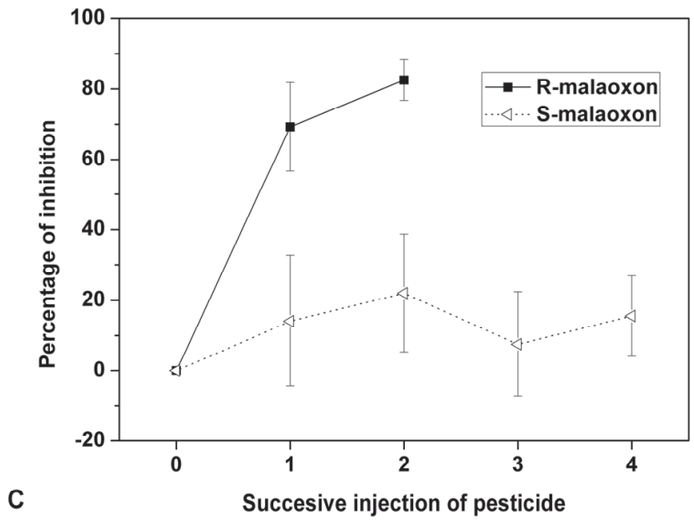

Fig. 4. The percentage of inhibition of AChE immobilized on the surface of the biosensors by malaoxon enantiomers. (A) Inhibition of EE AChE in batch measurement. A signal measurement was performed for three samples of the substrate (concentration 100 µM), preceded by baseline measurements. Then the electrode was incubated for 20 minutes in a solution of a given concentration of the pesticide. (B) Inhibition of B394 strain AChE in batch measurements. Signal measurement was performed for three samples in the substrate concentration of 1mmol L^{-1} preceded by baseline measurements. (C) Inhibition of EE AChE immobilized on the surface of biosensors inhibited by malaoxon enantiomers (40 nM concentration) in the flow system. 100 µL of solution of the substrate at a concentration of 400 µM and 100 µL of inhibitor solution were injected. Subsequent injections were preceded by rinsing the biosensor phosphate buffer (Kaniewska, 2009).

the ready immunosensors were tested over different concentration of carcinoembryonic antigen (CEA) (Fig 5.). The experimental results demonstrated that electrodes modified with D-proline had a better recognition function to anti-CEA, which is in good correlation with images of electrode surface obtained by atomic force microscopy. Authors suggested that designing chiral surfaces of amino acids may bring a new direction for biomaterials and help to understand the origin of stereoselectivity in pharmaceutical systems and clinic diagnoses (Chen M. et al.2009).

An amperometric immunosensor based on a graphite paste was presented in (Stefan & Aboul-Enein 2002). Mouse monoclonal anti-(+)-3,3′,5,5′-tetraiodo-L-thyronine (anti-L-T4) was used to the construction of immunosensor sensitive to thyroid hormone L-T4 known also as L-thyroxine. The selectivity coefficient obtained over D-T4 was $1.7 \cdot 10^{-4}$.

3.2 Other type of detection

Anti-D-AA antibodies have been employed for the determination of trace amounts of enantiomeric impurities of amino acids in the design of immunosensor based on different detection methods. The binding of stereoselective antibodies sensitive to the chiral centre of D-phenylalanine tested by a competitive enzyme-linked immunosorbent assay enabled to detect D- phenylalanine concentrations as low as 0.1 mM in the presence of 10 mM L-phenylalanine which corresponds to an enantiomer excess of 99.998% (Hofstetter et al., 2000).

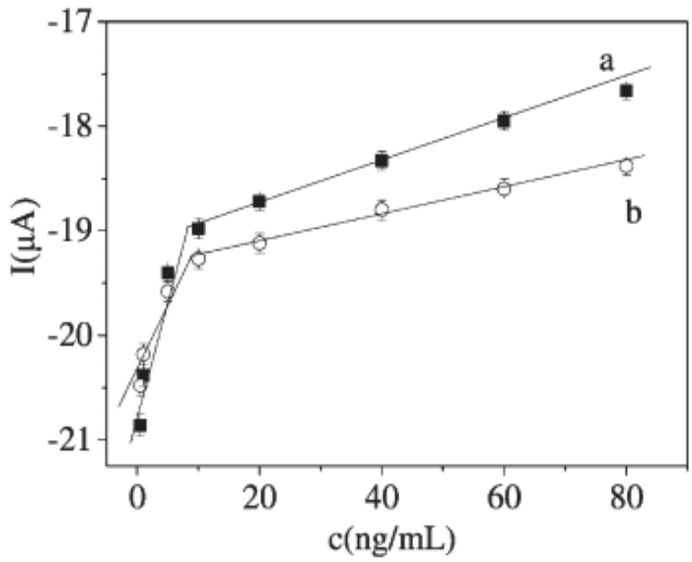

Fig. 5. Calibration plots of the cathodic peak current response vs. concentration of CEA (0.5-80 ng/mL) with D (closed squares) and L (open circles) modified immunoelectrodes under optimal conditions (Chen M. et al.2009).

The same interactions were used in construction of immunosensors based on surface plasmon resonance detection. Applications of immunoglobulins in a sensor design allowed to detect a low-molecular-weight analytes as amino acids by the SPR method. Compounds called D1 (p-amino-D-phenylalanine) and L1 (p-amino-L-phenylalanine) were immobilized onto separate channels of the sensor coated by the streptavidin covalently linked to carboxymethyl-dextran. Polyclonal rabbit antibody sensitive to D-amino acids interacted with the sensor while no increase of the SPR signal was observed for antibody sensitive to L-amino acids. Chiral discrimination was shown for the enantiomers of tyrosine, DOPA (3,4-dihydroxyphenylalanine), norleucine (Fig 6.) and tryptophan. The immunosensor allowed to detect 0.01% of the minor enantiomer present in the major enantiomer sample (Hofstetter et al., 1999).

Also a magnetic relaxation switching appears can be applied as a detection method for the construction of chiral immunosensors. This method allows for a rapid determination of enantiomeric excess in a high-throughput format. The MRS immunosensor was based on magnetic nanoparticles consisted of superparamagnetic iron oxide core with an aminated cross-linked dextran coating (CLIO) labelled with a derivative of D-phenylalanine. A decrease of more than 100 ms in the relaxation time was obtained by self-assembly of antibodies specific to D-amino acids (anti-D-AA) added to the CLIO-D-Phe. Upon addition of mixtures of the phenylalanine enantiomers to the CLIO-D-Phe/anti-D-AA self assembled structures, the presence of D-Phe impurities resulted in the dispersion of the nanoparticles by competing with the CLIO-D-Phe conjugates for antibody binding sites. The immunosensor allowed to detect 0.1 μM D-Phe in the presence of 10 mM L-Phe, which is an equivalent to 99,998% enantiomer excess (Fig 7.) (Tsourkas et al., 2004).

For the less sensitive determination of enantiomeric impurities a simple and inexpensive membrane-based optical immunosensor was invented. The immunosensor was based on a competitive reaction between an analyte and a biotin-derivatized analogue for the binding

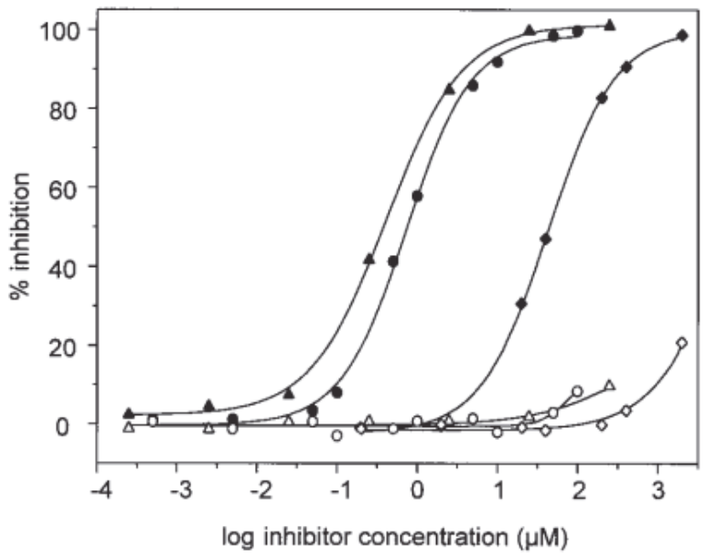

Fig. 6. Stereoselective binding of antibody to various amino acids: D-tyrosine (closed triangles), L-tyrosine (open triangles), D-DOPA (open circles), L-DOPA (closed circles), D-norleucine (closed diamonds), L-norleucine (open diamonds). The SPR values were converted into percentage of inhibition.

sites of a stereoselective membrane immobilized antibody. The antibody-bound was detected with peroxidase-conjugated avidin that converted a colourless substrate into an insoluble dye. The colour intensity was inversely related to the concentration of an analyte. The immunosensor allowed for quantitative determination of chiral phenylalanine up to an enantiomer excess 99.9% (Hofsetter et al. 2005)

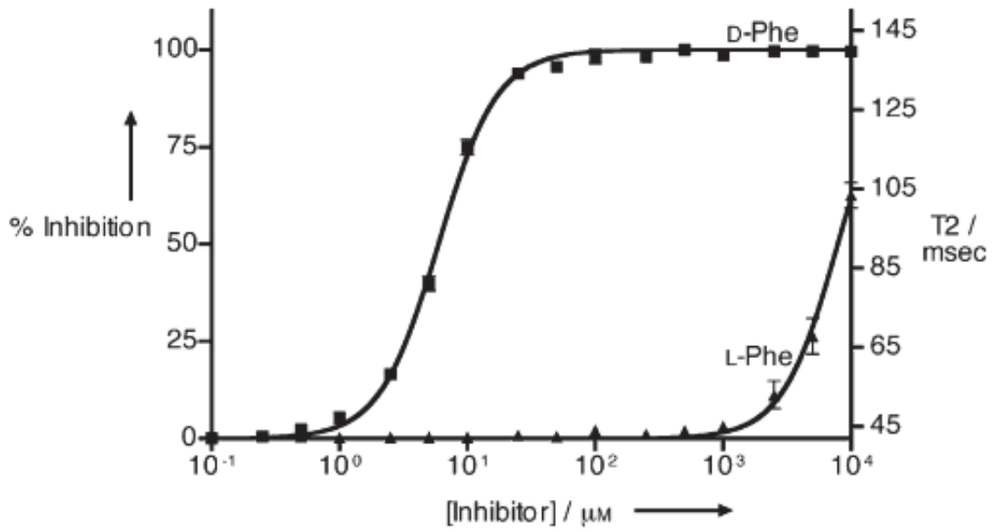

Fig. 7. Inhibition of the CLIO-D-Phe/anti-D-AA self assembly in the presence of increasing concentrations of L- or D-Phe as detected by changes in the T2 relaxation time (Tsourkas A. et al 2004)

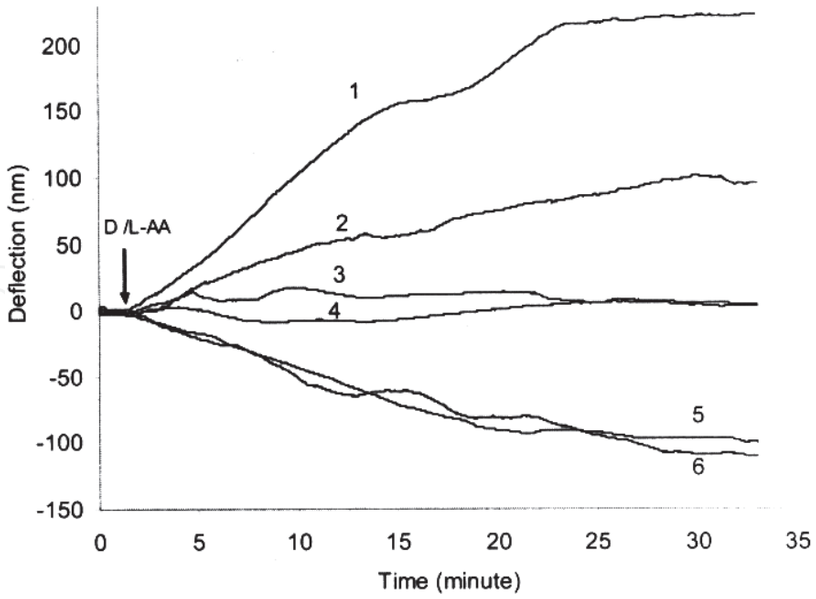

Fig. 8. Time trace of cantilever defections resulting from the binding of enantiomers of amino acids to micro cantilevers modified with covalently anti-L-amino acid antibody (1-4) or human immunoglobulin G (5,6) 1-50 mg/L L-tryptophan, 2,5- 50mg/L L-phenylalanine, 3- 50 mg/L D-tryptophan, 4,6- 50 mg/L D-phenylalanine. (Dutta et al. 2003).

These antibodies have been also employed for enantioselective sequential-injection chemiluminescence immunoassay of triiodothhyronine and tetraiodothyronine with immunoreactor with immobilized haptens. It has been shown that the detection of <0.01% of the L enantiomers in samples of D enantiomers is possible in less than 5 minutes including regeneration of immunoreactor (Silvaieh et al. 2002). Anti-D-AA was used in microfabricated cantilevers for enantioselective detection of amino acids based on inducing surface stress by intermolecular forces arising from analyte adsorption on surface-immobilized antibodies (Dutta et al. 2003). The temporal response of the cantilever allowed the quantitative determination of enantiomeric purity up to an enantiomeric excess of 99.8%. Based on the slope of response curves or anti-D-amino acid antibody, the selectivity coefficients for D- enantiomer towards L-isomer were 6.5, 7.7, and 37.5 for D-phenylalanine, D-tryptophan (Fig 8.) and D-methionine respectively. The largest enantioselectivity has been observed for D-valine (104).

4. Enantioselective bioreceptors

4.1 Mass-based biosensors

There are many examples of sensors exhibiting the enantioselective properties based on quartz crystal microbalance technique for example sensor for L-histidine (Zhang Z. et al. 2005), (+) methyl lactate (Ng et al. 2002), L-cysteine (Chen Z. et al. 2000), L-phenylalanine (Huang et al. 2003) or (-) menthol (Tanese et al. 2004). However the combination of biological macromolecules and QCM technique has been rarely reported for the studies of chiral discrimination.

Two sensors were developed by immobilization of human serum albumin (HAS) and bovine serum albumin (BSA) onto gold electrode combined with quartz plate by self-

assembled monolayer technique. The decreased frequency demonstrated interactions between albumines and enantiomers of R,S-1-(3-Metoxyphenyl)ethylamine (R,S-3-MPEA), R,S-1-(4-Metoxyphenyl)ethylamine (R,S-4-MPEA), R,S-tetrahydronaphthylamine (R,S-TNA), R,S-2-octanol (R,S-2-OT) and R,S-methyl lactate (R,S-MEL). The binding affinity of BSA and HSA for all five pairs of enantiomers was stereodependent. The effectiveness of the QCM sensor was described by the chiral discrimination factor $\alpha_{QCM,}$ defined as a quotient of the frequency decrease for enantiomer R and S respectively. For both sensors the highest discrimination factor were obtained for R,S-TNA. The value were for BSA sensor α_{QCM} =1.34 while for HSA sensor α_{QCM} =1.57 (Su et al. 2009).

4.2 Optical biosensors

The Surface Plasmon Resonance method was used for monitoring real time interactions of enantiomeric drug compounds to biomolecules immobilized on the surface of the sensor chip. The example of such biosensor for the first time was used to check the binding of the unnamed chiral drugs to human and rat albumins. However the enantiomers showed slight differences in their affinities towards the immobilized albumins, authors admitted that they were not able to detect whatever subtle differences could be due to differences in the enantiomers or it could be due to experimental errors (Ahmad et al. 2003). The next SPR biosensors were used to a detailed investigation of enantioselective interactions between protein and chiral small drugs. The binding of β-blockers alprenolol and propranolol to Cel7a cellulase was used as a model system. Cel7a was immobilized onto the sensor chip by PDEA-mediated thiol coupling. The single enantiomers of β-blockers were injected in a series with broad concentration range and a different pH of the solution was examined. The results were compared with the previously validated HPLC perturbation method. (Arnell et al., 2006). Similar interactions of drugs were examined for the SPR biosensors with two types of proteins-transport and target, immobilized onto the sensor chip. Different type of strong, intermediated and week interactions were exhibited by the models of binding of propranolol enantiomers to α_1-acid glycoprotein (AGP), R- and S-warfarin to human serum albumin (HSA) and RS and SR-melagratan to thrombin, AGP and HSA. Strong binding occurred in the case of RS-melagratan-trombin interaction. The other enantiomer did not interact at all with the protein (Sandblad et al., 2009)

4.3 Ion channel biosensors

The enantioselectivity was also reported for coulometric ion channel sensor for glutamic acid. The sensor was based on the use of glutamate receptor ion channel protein. The glutamate receptor was immobilized within an artificial bilayer lipid membrane formed by applying the folding method across a small circular aperture bored through a thin polyimide-film. The detection of L-glutamic acid was performed at a concentration as low as 10^{-8} M. The observed enantioselectivity for the channel activation was attributed to a combined effect of both the relative strength of binding isomers to the receptor protein and the relative potency of bound isomers to induce the ion channel current (Minami et al., 1991).

5. Enantioselective aptamers

DNA aptamers are a new group of chiral selectors. They are a single-stranded oligonucleotide sequences that can fold into a 3D shape with binding pocket and clefts that

allow them to bind many molecular targets as proteins, amino acids, peptides, cells and viruses with specificity that allows them to distinguish even strictly structurally related molecules. Aptamers are able to bind the target molecules with a very high affinity, equal or sometimes even superior to those of antibodies. Comparing to antibodies they present also some important advantages as well defined sequences produced by reproducible solid phase synthesis which allows an accurate modulation of their selectivity and binding parameters. Aptamers are much smaller than antibodies, permitting a higher density of molecules to be attached to surfaces. Their production does not require animal's immunization. It's also possible to obtain aptamers towards molecules that do not stimulate immunoresponce or that are toxic. Selections are not limited by physiological constraints allowing aptamers that bind their targets in extreme conditions to be isolated. Aptamers will refold to regain functionality after exposure to denaturing conditions (Mosing & Bowser, 2007). They are attractive host molecules, because they can be tailored to a variety of guest targets by the method of systematic evolution of ligands by exponential enrichment (SELEX) (Giovannoli et al., 2008).

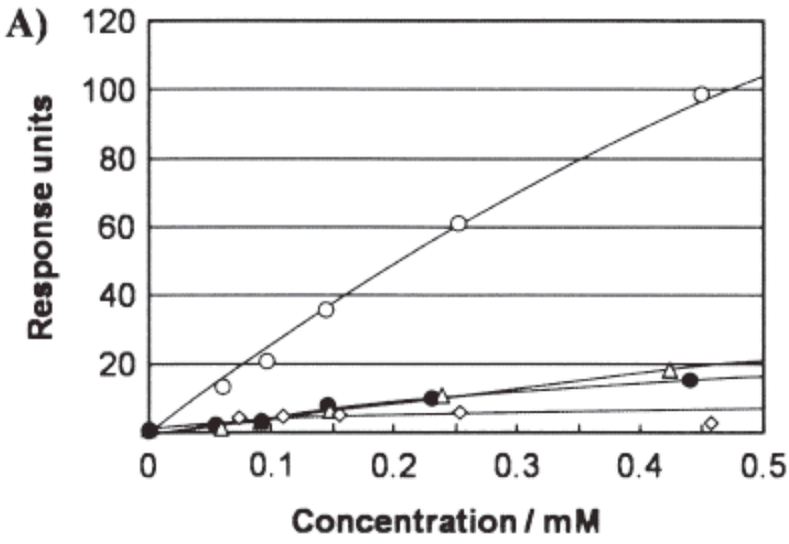

Fig. 9. SPR analyses of enantioselective binding interactions of selected aptamer with complex of avidine and biotinylated L-glutamic acid- α,γ-di-t-butylester (closed circles), D-glutamic acid- α,γ-di-t-butylester (open circles), glycine t-butyl ester (open triangles) and aptamers complex with avidine and biotin (open diamonds) (Ohsawa et al., 2008)

Aptamers can be successfully used to the biosensor design. As a biocomponents in biosensors they offers a multitude of advantages, such as the possibility of easily regenerate the function of immobilized aptamers, their homogeneous preparation and the possibility of using different detection methods due to easy labeling (Tombelli et al., 2005). A different detection techniques can be use for the aptasensor design as for example electrochemical (Liu et al., 2010), optical (Lee & Walt, 2000) or mass-based (Minunni et al., 2004). Although many examples of aptamer biosensor are presented in the literature only few of them considers the enantioselective properties.

The enzymatically prepared the biotinylated aptamers were immobilized on the sensor chip attached with streptavidin. Two of three selected amptamers showed enantioselective

recognition of the dicarboxylic acid moiety of glutamic acid. The binding affinity and enantioselectivity were successfully evaluated by SPR measurements, and the binding ability of these aptamers was eliminated by the absence of arginyl groups, indicating that modified groups are indispensable due to their binding affinity and enantioselectivity. The enantioselective response of selected aptamer is presented in Fig 9. (Ohsawa et al., 2008).
Another example presented in (Perrier et al., 2010) is based on the induced-fit binding mechanism of end-labelled nucleic acid aptamers to the small molecule. The anti-adenosine DNA aptamer, labelled by a single fluorescein dye was employed as a model functional nucleic acid probe. Target binding is converted into a significant increase of the fluorescence anisotropy signal presumably produced by the reduction of the local motional freedom of the dye and detected by fluorescence polarization sensor. In case of target molecule the difference in the anisotropy fluorescence signal generated by D and L enantiomers was not enough to allow the enantioselective detection of adenosine. The presented DNA aptamer was also able to bind the adenine nucleotides such as adenosine monophosphate AMP. In latter case aptasensor exhibited important enantioselective properties. Titration curves obtained by the addition of D-AMP show an FP response while for L-AMP does not cause any significant response Fig 10.

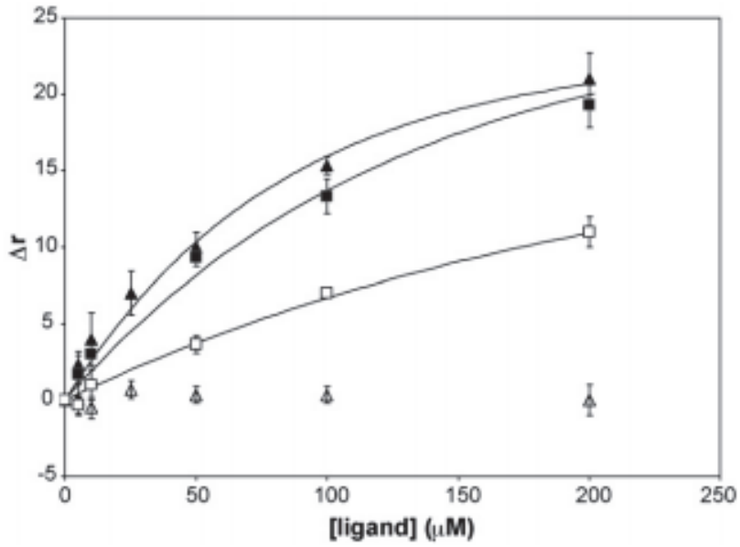

Fig. 10. Titration curves of the 3'-F-21-Apt probe with increasing concentration of enantiomers D-Ade (closed squares), L-Ade (open squares), D-AMP (closed triangles) and L-AMP (open triangles). Δr is a difference between the measured anisotrophy in the presence and in the absence of analyte (Perrier et al., 2010).

Aptamers are increasingly being used as chiral selectors in separation techniques as capillary electrophoresis or HPLC. Recently new aptamers for different specific molecular targets are selected. Some of them posses enantioselective properties for example for D-peptides (Michaud et al., 2003), histidine (Ruta et al., 2007a), arginine (Ruta et al., 2007b; Brumbt et al., 2005), thalidomide (Shoji et al., 2007) or ibuprofen (Kim Y. S. et al., 2010). These aptamers can potentially be used to construct chiral biosensors. Despite of successful chiral separation by aptamer modified stationary phase (Ravelet et al., 2005) or aptamers

based capillary electrophoresis there still exists deficiencies in the understanding of the molecular basis of their chiral recognition. In (Lin P. H. et al., 2009) authors study the binding mechanism of DNA aptamers with L-argininamide by spectroscopic and calorimetric methods.

5. Conclusion

The design and optimization of sensors based on the use of active biological materials, biosensors and immunosensors for rapid, selective and sensitive determination of chiral compounds seems to be an extremely promising direction of development. As it was presented to the construction of such sensors a different detection methods may be involved. Guideline in the selection of biologically active material can be results of research conducted by separation methods using chiral antibodies or aptamers. Especially development of aptasensor which are a relatively new technique seems to be promising. The number of available biological active materials suitable to the construction of biosensors could be increased by enzyme screening and protein design. It is quite possible that with very well optimized enantioselectivity, stability and reproducibility biochemical sensors may become in the future valuable instruments for quick control of chiral purity for biotechnology and pharmaceutical industry.

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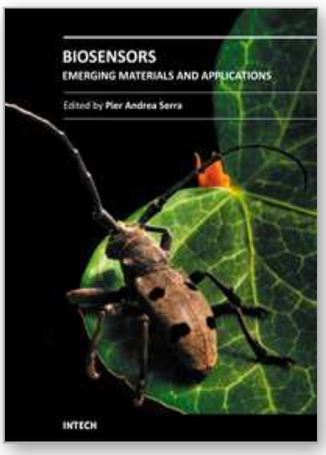

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A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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