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BIOSENSORS

A brief overview of the development of biosensors at the University of Tartu – theory meeting practical applications

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Abstract. This review introduces biosensing research and development activities at the University of Tartu. The biosensor studies were initiated by Jaak Järv and Toomas Tenno at the beginning of the 1980s when a biosensor combining the enzyme glucose oxidase and a cylindrical Clark-type oxygen sensor was constructed in Tartu. Since then, a lot of theoretical research has been carried out in this field, and several original ideas for constructing biosensing devices have been proposed. Different biosensor systems, from single-use immunosensors to reusable enzyme sensors, have been developed and tested for environmental analysis, food analysis, and clinical and veterinary diagnostics. These solutions have a high potential for practical, everyday applications and automated monitoring. Until now, biosensor research at the University of Tartu has resulted in five patents and more than one hundred scientific papers. Although some biosensor developments have been prototyped, none have been commercialized to date.

Keywords: biosensor research and development, University of Tartu, overview, biosensor application.

INTRODUCTION

Caged canaries were the first natural biosensors used in coal mines to detect toxic gases. The cessation of their usual singing served as a timely signal of danger for the miners to exit the mines safely.

According to the generally acknowledged definition, biosensors are devices that measure biological or chemical reactions by generating signals proportional to the concentration of the targeted analyte [1]. The biosensing field is a multidisciplinary research area bridging different scientific disciplines such as physics and chemistry with micro-/nanotechnology, electronics, and information technology fundamentals. In principle, biosensors can be considered as devices consisting of three major integrated components: 1) the *BIO* component responsible for the specific biorecognition of the targeted analyte, 2) the *SEN* part or a transduction element converting the signal of biorecognition reaction into a measurable signal (e.g. electrical, optical, etc.), and 3) the *SOR* or 'Smart Organisation of Results' system, which correlates the measured signal with the concentration of the targeted analyte (Fig.1).

The first biosensor device for measuring glucose levels was proposed over 60 years ago – in 1962 by Leland C. Clark Jr., also known as the 'father of biosensors' and Champ Lyons [2]. This first biosensor combined biochemical material with an electrochemical electrode measuring the ambient oxygen partial pressure. Thirteen years later, in 1975, Yellow Springs Instrument Company released the first commercially successful glucose biosensor, a predecessor of modern glucose sensors used daily by hundreds of millions of people with diabetes for monitoring their blood sugar levels.

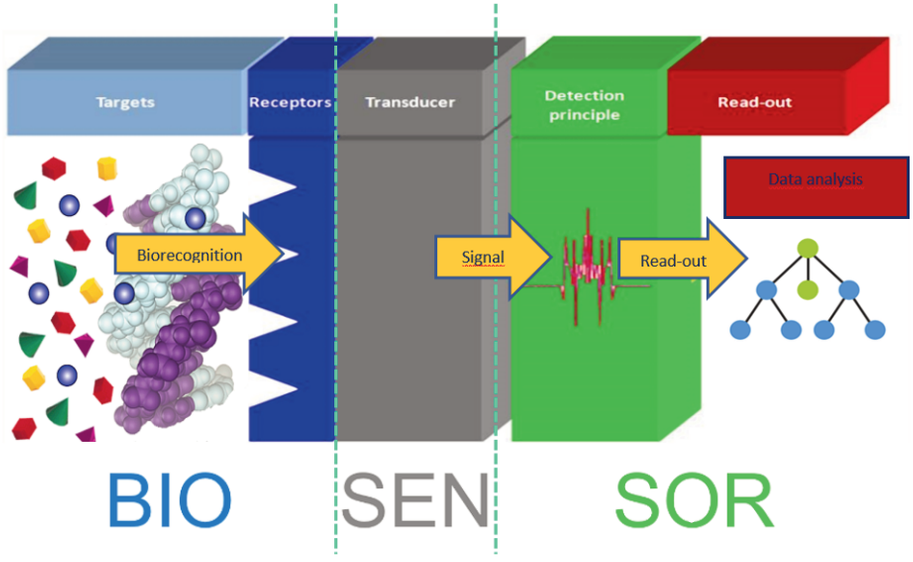

Fig. 1. Biosensor elements.

DEVELOPMENT OF BIOSENSOR THEORY AND BASIC PRINCIPLES AT THE UNIVERSITY OF TARTU

The research on biosensors in Tartu started at the beginning of the 1980s when a discipline of bioorganic chemistry was opened in the Department of Chemistry of the University of Tartu. This field was initiated by Jaak Järv to promote emerging topics in enzyme kinetics and catalysis. The first group of students specialized in bioorganic chemistry in 1980 [3].

On the other hand, electrochemistry had been traditionally a focus field in Tartu from the 19th century. Practical research on the development of electrochemical sensors for detecting O_2, SO_2, NO_2, and other gases with a focus on practical applications of these sensors in the industry started in the 1970s [4]. As a result of these studies, Toomas Tenno and his colleagues proposed a high-quality cylindrical Clark-type oxygen sensor a little more than ten years later. This sensor comprised a Cr/Ni cathode on a perforated Teflon cylinder, an anode consisting of Cd chips, and a thin polyethylene film covering the electrode and allowing the diffusion of dissolved oxygen from the surrounding environment to the cathode [5], thus serving as a perfect platform for the construction and development of biosensors.

The cooperation between the research groups of bioorganic chemistry and electrochemistry initiated the research on biosensors in 1982 (Photo 1).

We started with testing the applicability of the cylindrical oxygen sensor for the construction of biosensors by combining it with the enzyme glucose oxidase, followed by different biosensor set-ups with a soluble or immobilized onto insoluble carrier enzyme. The first carrier to immobilize glucose oxidase was a nylon net cut from customary textile and attached around the oxygen sensor [6]. The following carriers used for the immobilization of glucose oxidase were nylon 6,6 sewing threads coiled around the cylindrical oxygen sensor [7,8] (Fig. 2).

The use of thread-shaped biorecognition elements turned out to be a splendid idea, which was later used for the construction of different biosensors [9–12] and was granted an Estonian patent in 2004 [13]. The use of bio-selective threads allows the construction of biosensors with a required range of work, sensitivity, and selectivity, which can easily be customized within seconds as one can cut appropriate pieces of enzyme-containing thread with a certain amount of enzymes for a specific biosensor or bioreactor [8]. The immobilization of an enzyme onto a thread-shaped carrier also allows us to measure the amounts of bioactive components in the units of length.

Along with the construction of biosensors, more comprehensive studies were started on the signal analysis of the biosensors or the *SOR* component as a thorough understanding of the signal generation is the basis

Photo 1. Jaak Järv (left) and Toomas Tenno (right) are discussing biosensor development in the lab in 2000 (photo: M. Toom).

Fig. 2. Biologically active thread wound spirally around the cylindrical cathode of an oxygen sensor [8].

for biosensor calibration algorithms and the quality of analyses. The first model proposed for the normalized output current $I(t)/I_0$ of the oxygen-electrode-based biosensor considered the kinetics of the ping-pong mechanism of the enzyme-catalysed reaction and the diffusion of substrates from the solution to the electrode membrane [14]. Later, this model was supplemented with an additional factor, characterizing the inertia of the oxygen sensor to describe the lag period at the beginning of the process [15]. This enhanced dynamic model contains three independent complex parameters: the maximum signal change or steady-state parameter A, the kinetic parameter B, and the lag time of the process (τ_s), and it runs as follows [15,16]:

$$\frac{I(t)}{I_0} = A\,exp(-Bt) - 2A\sum_{n=1}^{\infty}(-1)^n \frac{\tau_s}{\frac{n^2}{B} - \tau_s}\left[exp(-Bt) - exp\left(-n^2\frac{t}{\tau_s}\right)\right] + (1-A)\,. \qquad (1)$$

Different aspects of biosensor modelling and the physicochemical meaning of model parameters were analysed in further studies [17]. The primary practical outcome of the modelling studies was the opportunity to determine the biosensor steady-state signal from the transient phase data, eliminating the potential effects of side reactions and minimizing the analysis time. The concept of calculating the biosensor steady-state signal was later successfully used for signal modelling in flow-through biosensor set-ups, allowing to obtain results in only 20 seconds [18], but also for the chemometric approach for simultaneous multiplexed detection of different substrates in mixtures [19]. In addition, studies were conducted on modelling non-stationary processes in amperometric biosensors following processes generated by immobilized microorganisms [20,21].

BIOSENSORS FOR DIFFERENT APPLICATIONS

In the 1990s, the development of biosensors for practical applications was started. Until now, several different biosensors for environmental, food, and clinical analyses have been developed (Fig. 3). These biosensors use a variety of different biorecognition *BIO* elements, from enzymes and microorganisms to antibodies and

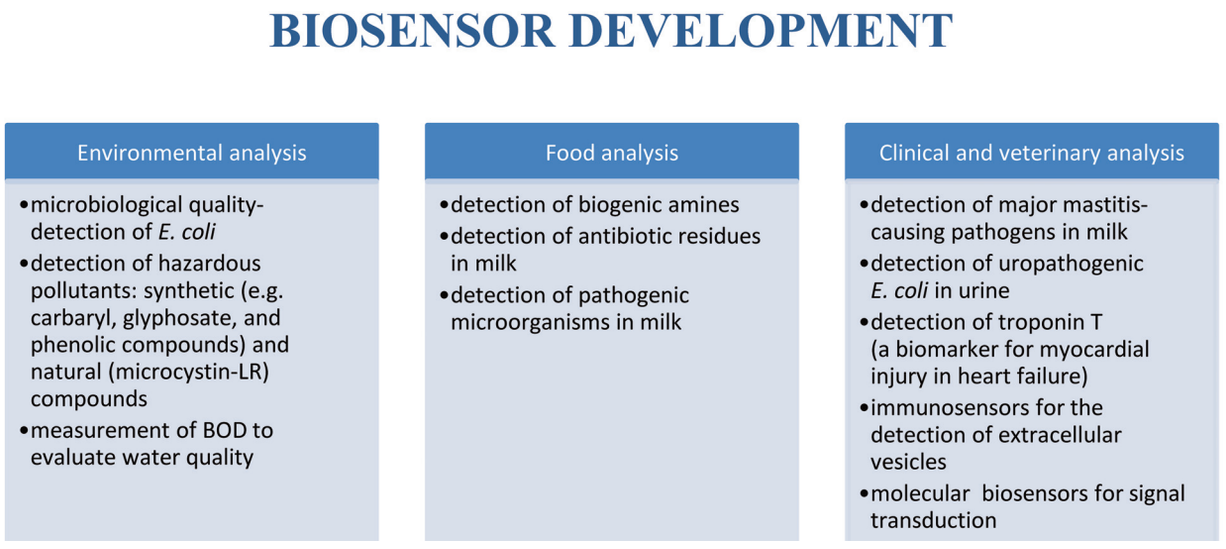

Fig. 3. The application areas of biosensors developed at the University of Tartu.

aptamers. For signal transduction (*SEN*), the earlier developments mainly used a cylindrical membrane-covered oxygen sensor, which has been replaced by optical signal recording systems in recent developments.

In the field of environmental analysis, the main focus of biosensor development has been on the measurements of water quality. Although numerous methods and options exist for assessing water quality, biosensor-based technologies have clear advantages in speed and the possibility for automation and on-site analyses.

To start with, an essential part of biosensor research in Tartu has been the development of biosensors for detecting pesticide residues in natural water. The pesticide biosensors were initially based on using tyrosinase and a cylindrical oxygen sensor, e.g. the carbaryl sensor was measuring the inhibiting effect of carbaryl on tyrosinase activity. However, carbaryl acts as an inhibiting substrate of tyrosinase and, at low concentrations, accelerates the tyrosinase-catalysed oxidation of tyrosine by dissolved oxygen, making the use of one signal parameter insufficient for analysis [22,23]. Thus, the combination of different signal characteristics (steady-state and kinetic parameters) was used to determine carbaryl concentration.

Further studies for pesticide sensors were based on a more specific biorecognition and more complex sensing platforms. For the detection of glyphosate, an immunosensor was proposed for on-site analyses. This biosensor was based on the displacement of glyphosate in its complex with anti-glyphosate antibody by 5-T-g. The biosensor's limit of detection was relatively high, in the millimolar concentration range. However, the obtained detection limit is sufficient to apply the proposed immunoassay for *in situ* glyphosate analysis to timely detect glyphosate pollution in water reservoirs before glyphosate dilution and degradation and ensure relevant safety levels. The method's main advantage is that the sample pre-treatment step of traditional analytical techniques is not required, and the analysis results will be obtained in about half an hour [24–26].

Another immunosensor has been proposed for microcystin-LR (MC-LR) – the most common cyanotoxin found in water during the proliferation of cyanobacteria [27]. As the molecule of MC-LR is relatively small (the molecular weight of MC-LR is 995 Da), a sandwich-type biorecognition system combining a specific anti-MC-LR antibody and an aptamer conjugated with a fluorescence marker for detection was used. The aptamer-based biorecognition was combined with a bead injection analysis system (BIA) and allowed to obtain an MC-LR detection limit of 1.7×10^{-8} mg/mL [28], thus creating a prerequisite for using this biosensor in natural water bodies for water quality monitoring.

For the detection of the microbiological quality of water, biosensors have great potential to replace the traditional microbiological cultivation methods, which require at least 24 hours to obtain results. An *E. coli* immunobiosensor, based on a specific antigen/antibody interaction and BIA platform, was developed and tested for the water analysis of the popular swimming beach in Tartu – Anne Canal [29,30]. To provide the

sensitivity of this biosensor towards different *E. coli* strains, the *E. coli* immunosensor used polyclonal *E. coli* antibodies for bio-recognition and the BIA analysis system for the rapid detection of *E. coli* and the determination of coli index of the bathing water. Although other *Enterobacteriaceae* bacteria generate a measurable signal, this immunosensor has excellent potential for a quick automated *in situ* identification of the presence of *E. coli* if different impacts of different strains and the decomposition of organic material in time are taken into consideration [30,31].

Biochemical oxygen demand (BOD) is a measure of the amount of oxygen required to remove waste organic matter from the water, used for the assessment of (waste)water quality. Microbial biosensors, where live microorganisms are used as a specific 'biorecognition element', have been proposed to assess BOD [32]. The research has continued to develop a rapid tool for assessing wastewater quality as the standard procedure for BOD measurements takes 5 or 7 days [33]. Later studies for developing BOD sensors have focused on more specific biosensor arrays for the targeted assessment of selected industrial wastewater, e.g. wastewater from the dairy industry rich in lactose [34]. In addition, the application of microbial biosensors using two genetically engineered *Pseudomonas putida* strains for the determination of phenolic compounds in the aquatic environment have been studied [35].

The second focus area in biosensor development at the University of Tartu has been food analysis as biosensors have a high potential for directly evaluating food quality in non-laboratory conditions in commonly complex sample matrices.

Biogenic amines (BA) are mainly formed during the decarboxylation process of free amino acids in proteins during ageing. Thus, the assessment of BAs, particularly histamine, putrescine, and cadaverine, is a valuable tool for evaluating the quality and freshness of protein-containing products (fish, meat, cheese, wine, etc.). The BA biosensors, developed in Tartu at the beginning of the 2000s, use the enzyme diamine oxidase for biorecognition and an oxygen sensor for signal transduction [36]. Commonly, the purified enzymes are relatively expensive, so options for separating and purifying this enzyme from pea seedlings [37,38] and its catalytic properties have been studied for proper signal analysis of these biosensors [39]. The main problem of these BA biosensors is their low selectivity, so the measurable biosensor signal in samples, usually containing different BAs, is influenced by most BAs present. In addition to individual BA signals, there are interactions between different BAs, and spermidine acts as an inhibiting substrate at higher concentrations [39]. Tests with the proposed BA biosensor for assessing the quality of Estonian white fish showed that it is a prospective tool for assessing fish freshness; auspicious preliminary results have been obtained in the case of European perch (unpublished data, Helen Sõmerik).

Numerous biosensor studies have been related to milk analysis. Milk and dairy products are among the most essential foodstuffs, and the quality of raw milk is of significant importance from the point of view of human health. When this research was started about 20 years ago, in 2004, one of the main problems in the dairy industry was the presence of antibiotic residues in raw milk. To address this issue, a lactate oxidase-based amperometric biosensor was used to rapidly determine chloramphenicol and penicillin residues [40]. The concentration of antibiotic residuals was determined using the dynamic biosensor model [16], complemented with bacterial respiration as a negative linear time-depending factor [41]. The penicillin biosensor was based on determining glucose concentration in milk, which is highly dependent on the rate of milk lactose hydrolysis, catalysed by enzyme lactate oxidase. This enzyme is produced by normal milk microbiota, while penicillins inhibit its activity. The presence of penicillin in milk resulted in decreased glucose concentrations compared to the ones in high-quality milk. The penicillin biosensor offers a good option for simple and quick on-site assessment of antibiotics, and several studies have been carried out to optimise and improve the antibiotic biosensor [11,42–45]. The proposed algorithm for biosensor signal calculation allowed the elimination of the ageing effect of the enzyme-activated thread for several months [11]. Our antibiotic biosensor array, comprising different enzymes and an oxygen sensor, and the method for simultaneous detection of antibiotic residues and their concentrations in milk with this system have been granted Estonian, European, and Russian patents [46–48]. This biosensor was tested in farms to rapidly detect antibiotic residues and prototyped in 2013 to carry out semiautomatic milk analyses (Photos 2, 3). However, the laboratory prototype was not further commercialized as the current allowed limits for antibiotic residues in milk can be easily determined with various widespread rapid test kits, e.g. Delvotest, Bioeasy Milk Test, etc.

Along with improving the milk quality control system in farms, our research focus has shifted towards detecting pathogenic bacteria in raw milk. The studies on the construction of the immunosensor for the detection of *Staphylococcus aureus*, one of the major mastitis-causing pathogens in cows, were started in Tartu at the beginning of the 2010s, and the first *S. aureus* biosensor was proposed in 2014 [49]. The proposed

Photo 2. Kairi Kivirand (left) and Siim Rinken (right) are testing the antibiotics biosensor in Rahinge farm during milking in August 2010 (photo: T. Rinken).

Photo 3. Toonika Rinken with the prototype of this biosensor in the lab in November 2013 (photo: S. Kinnas).

immune-biosensing system was based on the specific binding of *S. aureus* in automatically renewable microcolumns (microbeads with human immunoglobulin (Ig) G crystallisable fragment (Fc)) of the BIA platform and specific biorecognition of the bound bacteria by secondary antibodies, conjugated with a fluorescence marker. This setup enables the detection of *S. aureus* bacteria in milk within 17 minutes, with a detection limit of 200 CFU mL^{-1}. This is significantly faster than microbiological cultivation, which serves as the 'gold standard' for identifying pathogens in milk and requires 48–72 h, which is too long for separating substandard milk from the production line. Our bioanalytical set-up does not require any pre-treatment of milk as the milk matrix is removed before collection of the biosensor signal [50]. A substantial advantage of the BIA-platform biosensor is that the microscale analyses require only minute amounts of reagents and materials. Thus, disposable microcolumns can be used, which do not require regeneration, and, therefore, the reproducibility of the measurements is excellent.

The next biosensing system proposed was for the simultaneous detection of two different pathogens: *S. aureus* and *E. coli*, which both can be captured to microbeads activated with human IgG Fc fragment (Fig. 4) [50,51]. Such an immunosensing system is a perfect platform for the rapid multiplexed detection of all pathogenic bacteria. In addition to securing the microbiological quality of milk, raw milk also serves as a perfect medium for veterinary diagnostics, e.g. the identification of mastitis-causing pathogenic bacteria. There is an urgent need for such rapid on-site pathogen analysis in farms as about 30% of all dairy cows suffer from mastitis at least once a year [52]. Timely information about mastitis-causing pathogens allows us to specify the selection of antibiotics used to treat animals and sufficiently reduce the required amounts.

The most common mastitis-causing pathogens are *S. aureus*, *E. coli*, and *Str. uberis*, which are responsible for the majority of mastitis cases [53]. Due to these statistics, developing a biosensing system for detecting these three bacteria was started in 2016. In this system, we used commercial polyclonal antibodies for the biorecognition of *S. aureus* and *E. coli*. However, there are no specific antibodies available for *Str. uberis*. An anti-*Str. uberis* antibody was produced against oligopeptides, which were proposed using *in silico* bioinformatic analysis to mimic the adhesion protein of *Str. uberis,* and synthesized [54]. The purified anti-*Str. uberis* antibodies showed high affinity and specificity towards *Str. uberis*, allowing rapid detection of *Str. uberis* in milk with an immunobiosensing system [55].

For the concentration and capture of pathogens from samples onto microcolumns, we have also studied the possibilities of using microbeads, which carry different bioactive compounds, e.g. human IgG Fc fragment or lactoferrin, allowing simultaneous attachment of several targeted bacteria [56]. The proposed immunosensor can be used for automatic routine analysis of milk as the results are obtained in less than 30 minutes. Such speed of analysis allows farmers to start a correct and targeted timely treatment of affected animals. Prototyping of this biosensor is currently underway. For rapid diagnostics of mastitis, we have also

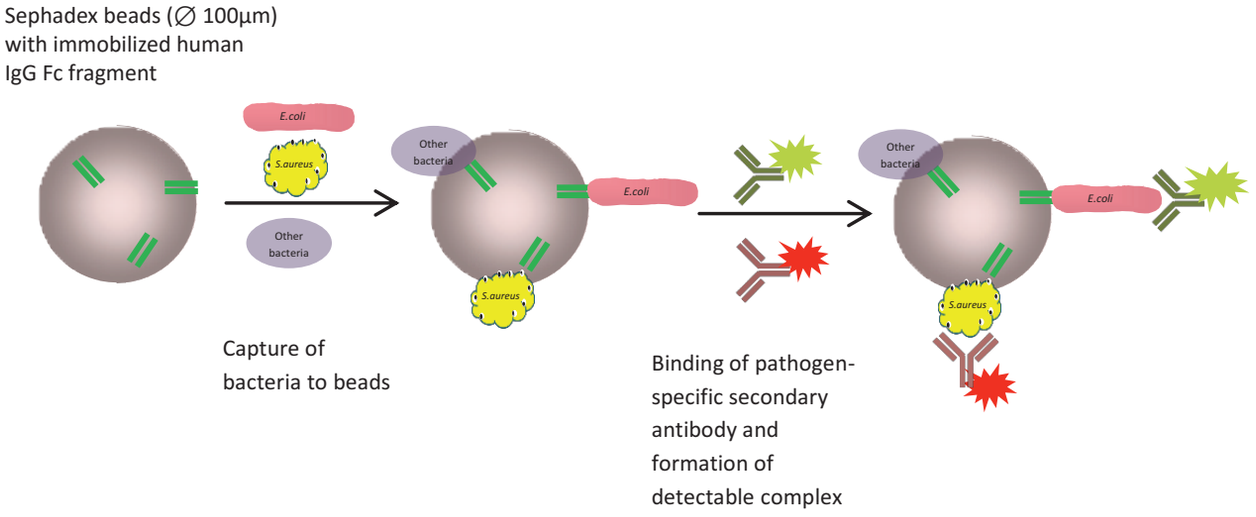

Fig. 4. The principle of capturing and detecting of two pathogens with a BIA-based biosensing system. The sample is injected into the biosensor system where, in the first step, *E. coli*, *S. aureus* and other pathogens that can interact with human IgG Fc fragments are captured to the bio-functionalized beads; in the second step, the captured bacteria are detected with bacteria-specific antibodies conjugated with different fluorescence markers.

proposed a more general solution, where the presence of pathogens in milk is identified with an oxygen sensor following the dynamics of oxygen concentration in milk [57]. This method was also granted an Estonian patent in 2012 [58].

The applicability of the BIA-based immunosensor for clinical diagnostics has also been studied to detect uropathogenic *E. coli* in human urine [59]. This immunosensor provides limits of detection and quantification for *E. coli* under 3 and 5 cells/mL, respectively, in 150 mL urine samples within 17 minutes, indicating its applicability in clinical analyses. The biosensor was also tested in urine samples of patients suffering from urinary tract inflammations, and the results were in good correlation with the qPCR (quantitative polymerase chain reaction) and MALDI-TOF mass spectrometry (matrix-assisted laser desorption/ionization time-of-flight) results [60].

To test new drug candidates, a capacitive immunosensor assay has been studied for the quantitative detection of troponin T in cell culture. Troponin T is a biomarker for myocardial injury in heart failure. The setup allowed us to obtain a linear relationship between the measured signal and troponin T concentration from 1 to 1000 ng/mL, and the limit of detection was calculated to be 1 ng/mL [61]. In order to be applicable for clinical studies, this biosensor needs further improvement regarding its selectivity and sensitivity.

Novel types of immunosensors have been developed to detect proteins displayed on the surface of extracellular vesicles (EV). EVs are membrane-derived particles released by cells into extracellular space and are characteristic of the cells they originate from. It has been suggested that EVs can serve as valuable diagnostic markers [62]. Small size, complex structure, and low concentrations in biological fluids have made EVs a challenging target for their characterization and quantification. The developed total internal reflectance microscopy (TIRFM)-based biosensor assay allows the use of marker-conjugated antibodies for detecting specific membrane proteins on the surface of EVs with single particle sensitivity and determining the co-localization of targeted proteins [63]. The exceptional sensitivity of the sensor system has been achieved using the unique low non-specific binding pegylated glass surfaces developed at the University of Tartu. Currently, this assay is used for multiplex detection of different tetraspanins (CD9, CD63, and CD81), and the implementation of this testing system for assessing the development of embryos in *in vitro* conditions is in progress.

In addition to biosensor-based devices, novel molecular fluorescence-based biosensor systems have been developed for basic biochemical research. These studies focus on the characterization of signalling systems involving G protein-coupled receptors (GPCR) and protein kinases (PK). The fluorescence anisotropy (FA)

based biosensing method enables the characterization of fluorescence probe binding to target proteins [64], allowing monitoring of ligand binding kinetics and the determination of the affinities of competitive ligands essential for screening new drug candidates [65,66].

Another Förster resonance energy transfer (FRET)-based biosensing system has been developed for the detection of intracellular cyclic adenosine 3',5'-monophosphate (cAMP) [67]. This system simplifies and opens new possibilities for determining the biological activity of protein hormones important in signal transduction. For example, the thermal inactivation of gonadotropin hormones, not detectable with antibody-based conventional assay, can be discovered with the help of this biosensor [68]. The FRET-based biosensing system was also used to show that different forms of human chorionic gonadotropin (hCG), synthesized during different stages of pregnancy, show different potency to activate corresponding receptor systems [69].

Based on the obtained results, the molecular biosensing systems have high potential to serve as prospective alternatives to traditional antibody- and animal-testing-based assays used in the pharmaceutical industry and clinical research for quantifying hormonal activity.

CONCLUSIONS

By now, the research and development of biosensors at the University of Tartu has lasted for more than 40 years. During this period, the fundamentals of biosensing, such as signal rising and biosensor calibration, have been thoroughly studied, and numerous analytical solutions have been proposed for the applications of biosensors in environmental analysis, food assessment, and veterinary and clinical diagnostics. Over time, these biosensors have become more complex regarding their construction and signal analysis algorithms. As a result, the selectivity of biosensors and the reliability of the obtained results have increased along with the convenience of their use. The time required for analysis has decreased, and the user can easily detect minute concentrations of a variety of compounds in minutes.

Until now, the novel technologies proposed have been granted five patents. The number of scientific papers published is over one hundred. Hopefully, this journey will continue, and there will be original ideas and new developments to meet the growing demands of modern society.

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Photo: private collection/ Ago Rinken

Toonika Rinken is an associate professor of environmental chemistry and is leading a biosensor development lab at the Institute of Chemistry, University of Tartu, Estonia. She received her PhD degree in chemistry in 2000 at the same university for the modelling and calibration studies of biosensors and has passed professional self-improvement courses in Uppsala and Gröningen. Dr Rinken's research activities are focused on the studies and development of biosensing systems for automatic monitoring along with testing and application of biosensor-based analytical systems.

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Lühike ülevaade biosensorite alasest uurimistööst Tartu Ülikoolis: teooria ja praktilised rakendused

Toonika Rinken

Artikkel tutvustab biosensorite alast teadus- ja arendustööd Tartu Ülikoolis, mis sai alguse Jaak Järve ja Toomas Tenno eestvedamisel. Esimene glükoosi oksüdaasil ja silindrilisel hapnikuanduril põhinev glükoosi biosensor konstrueeriti bioorgaanilise keemia ja elektokeemia töögruppide koostöös neli aastakümmet tagasi, 1980. aastate alguses.

Uuringu algusaastatel pöörati peamist tähelepanu biosensorite teooriaga seotud küsimustele. Nii pakuti välja mitu mudelit biosensorite väljundsignaali kirjeldamiseks ning kalibreerimisgraafikute koostamiseks, mida on hiljem kasutatud biosensoritel põhinevate analüüside läbiviimisel. Nende mudelite kasutamine on võimaldanud oluliselt vähendada analüüsideks kuluvat aega, parandada tulemuste usaldatavust ning määrata üheaegselt mitmeid analüüte nende segudes. Samuti on põhjalikult uuritud võimalusi biosensorite konstruktsiooni optimeerimiseks, sealhulgas niidikujulise kandja kasutamist sensorite bioselektiivse komponendi immobiliseerimiseks.

Väga oluline osa biosensorite alasest uurimistööst on seotud biosensorsüsteemide erinevate rakendustega, mille põhisuunad Tartu Ülikoolis on olnud seotud keskkonna- ja toiduainete analüüsi ning kliinilise ja veterinaardiagnostikaga. Keskkonnaanalüüsi valdkonnas on peamises fookuses olnud vee kvaliteeti määravate näitajate, näiteks bioloogilise hapnikutarbe, *coli* indeksi jt kiireks määramiseks kasutatavate biosensortehnoloogiate väljatöötamine.

Toiduainete analüüsi alal on olnud kaks peamist eesmärki: biogeensete amiinide määramine valku sisaldavate toiduainete värskuse hindamiseks ning antibiootikumijääkide ja patogeensete bakterite määramine toorpiimas. Diagnostilistel eesmärkidel kasutatavate biosensorite arendustööd on olnud suunatud peamiselt veterinaardiagnostikale, eelkõige udarapõletikku ehk mastiiti põhjustavate enamlevinud patogeenide määramisele piimas.

Tänaseks on biosensorite alase uurimistöö tulemuste põhjal avaldatud üle 100 teadusartikli ning antud välja viis patenti uudsete tehnoloogiliste lahenduste ja meetodite kaitseks.