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Review

The enzyme thermistor—A realistic biosensor concept. A critical review

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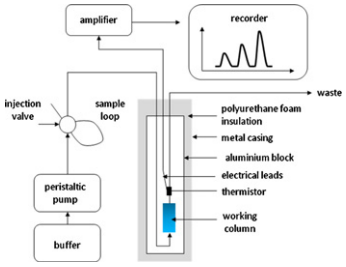
HIGHLIGHTS

- Major principles and features of enzyme thermistor are described.
- Different types of enzyme thermistors are overviewed and compared.
- Applications of enzyme thermistor for determination of various analytes are presented.
- Advantages and drawbacks of the analytical method are highlighted.

GRAPHICAL ABSTRACT



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ABSTRACT

This review describes principles and features of thermal biosensors and microbiosensors in flow injection analysis. Examples are given that illustrate the great versatility and excellent operational stability offered by thermal biosensors. The examples are mostly from work with the original type of enzyme thermistor operating with an enzyme column, but there will also be work described involving miniaturised devices including thermal lab-on-chip constructions and other types of sensing materials, such as MIPs (molecularly imprinted polymers) for both affinity and catalytic reactions. Several recently presented thermal biosensor concepts are reviewed including a thermal–electrochemical hybrid sensor for lactose based on immobilised cellobiose dehydrogenase. Another recent method is the determination of fructose using a fructose-6-phosphate kinase column. Operation with complex sample matrices such as blood, plasma and milk and how to avoid non-specific temperature effects are considered.

Contents

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Bengt Danielsson received his PhD in biochemistry 1979 at Lund University (Sweden) and was running a research group there during many years specialising in biomedical analysis. A notable development was the enzyme thermistor, a thermometric biosensor concept featuring great versatility and operational stability making it particularly useful for practical applications in biomedical and environmental monitoring and bioprocess control. Current interests include bioaffinity interactions, pattern recognition, glycosystems, and nanoscience. Since 2008 he is involved in developing various upstart companies, such as the bio-incubator Acromed Invest AB in Lund and similar activities in China and India.

Maria Yakovleva was born in 1985 in Moscow, Russia. In 2007, she received her MSc in pharmacy at I.M. Sechenov First Moscow State Medical University (Russia). She was working with enzyme thermistors, surface plasmon resonance, quartz crystal microbalance and atomic force microscopy at the Department of Pure and Applied Biochemistry at Lund University (Sweden) until 2011, when she started her PhD at the Department of Biochemistry and Structural Biology at the same university, under supervision of Prof. Lo Gorton. She is currently working on development of amperometric biosensors and on characterisation of redox enzymes using amperometry and mass spectrometry.

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1. Introduction

Inspired by the first enzyme electrode presented by Clark and Lyons 1962 and microcalorimetric studies on enzyme reactions several research groups in the 1970s proposed analytical devices that we today would call thermometric biosensors [1]. A common feature was the use of immobilised enzymes for improved economy and analytical performance. Over the years several of these concepts were abandoned and some others appeared. A few of these constructions are still produced and finding ever new applications. One such device is the Enzyme Thermistor (ET) developed in Lund (Sweden) and studied in many applications and application areas [2]. Reasons for the continuous interest in the technique are its unsurpassed versatility and generally superior operational stability [3]. The ET is usually based on an immobilised enzyme column (also called in the literature "immobilised enzyme reactor" or "IMER") placed in a flow microcalorimeter run as a flow-injection analyzer. Since the temperature sensor (usually a thermistor) does not need to be placed directly in the flow, fouling of the sensor can be avoided and transducer drift eliminated. On the other hand, many have been concerned about lack of specificity in the temperature measurement; there is no direct way to discriminate between specific and non-specific heat changes. This problem may have discouraged some of the early or potential users of enzyme calorimetry as an analytical technique. By careful experimental design, however, most of these disturbances can be avoided. It is often a question of mismatch of pH or ion concentrations between the sample and the running solution, which usually can be fixed by dilution or masking. In the worst cases an inert reference column can be added for differential measurements that compensate for nonspecific effects. The use of a reference could certainly be helpful in many other biosensor determinations in which high specificity is often taken for granted.

In the following examples will be given that illustrate the great versatility of thermal biosensors. The examples are mostly from work with the original type of ET operating with an enzyme column, but there will also be work described involving miniaturised devices including thermal lab-on-chip constructions and other types of sensing materials, such as MIPs (molecularly imprinted polymers) for both affinity and catalytic reactions.

2. Working principle of enzyme thermistors

The evolution of heat is a general property accompanying biochemical transformations [4]. The total heat evolution is proportional to the molar enthalpy change and to the total number of moles of product molecules created in the reaction:

$$Q = -n_P(\Delta H) \quad (1)$$

where Q – total heat, n_P – moles of product, and ΔH – molar enthalpy change. The resulting temperature change (ΔT) is

Table 1
Molar enthalpy changes for some common enzyme reactions.

Enzyme	Substrate	$-\Delta H$ (kJ mol^{-1})
Catalase	Hydrogen peroxide	100
Cholesterol oxidase	Cholesterol	53
Glucose oxidase	Glucose	80–100
Hexokinase	Glucose	75[a]
Lactate dehydrogenase	Sodium pyruvate	62
NADH dehydrogenase	NADH	225
β-Lactamase	Penicillin G	115[a]
Trypsin	Benzoyl-L-arginineamide	29
Urease	Urea	61
Uricase	Urate	49

[a] In Tris buffer (protonation enthalpy −47.5 kJ mol^{-1})

dependent on the total heat capacity (C_S) of the system including the heat capacity of the solvent.

Thus the change in temperature recorded by the thermal biosensors can be defined by:

$$\Delta T = -\frac{(n_P \Delta H)}{C_S} \quad (2)$$

The enthalpy changes for enzymatic catalysis is around −10 to −200 kJ mol^{-1} (see Table 1), which is adequate for the determination of the substrate concentrations at clinically interesting levels for a range of metabolites including free and esterified cholesterol [5], ethanol [6], glucose [7], lactate [8], oxalate [9], triglycerides [10] and urea [11]. Most of them can be determined in the concentration range down to 10 μM. A partial list of analytes and the corresponding enzymes is shown in Table 2.

Thermometric detection is especially advantageous when multiple reactions are involved, since it is the sum of all the reaction enthalpies that determines the sensitivity of the assay [12,13]. Thus, in the case of oxidases, it is recommendable to co-immobilise catalase, which consumes the hydrogen peroxide produced in the oxidase reaction. This highly exothermic reaction doubles the sensitivity and reduces the deleterious effects that hydrogen peroxide has on enzyme activity. Furthermore, it reduces oxygen consumption thereby increasing the linear range of the oxidase reaction (see the reactions below):

Glucose oxidase

$$\text{D-Glucose} + H_2O + O_2 \rightarrow \text{Gluconic acid} + H_2O_2 + \Delta H_1 \quad (1)$$

Catalase

$$H_2O_2 \rightarrow H_2O + 1/2O_2 + \Delta H_2 \quad (2)$$

As seen in Table 1, the high protonation enthalpy of certain buffers like Tris can be utilised to enhance the total enthalpy of proton-producing reactions. The gain in enthalpy change by selecting the most suitable buffer can be as much as ca 50 kJ mol^{-1}. It was also demonstrated that small amounts of organic solvents (around 5%, v/v) present in the aqueous buffer can be used to up to double the registered temperature changes by increasing the total enthalpy change in the reaction. Two–three-fold enhancement of sensitivity can be obtained in neat organic solvent, which can be attributed to the lower heat capacity of the solvent [14]. Finally, the concept of enzymatic recycling of an analyte in order to increase the sensitivity has been applied in a number of studies [15,16].

3. Biorecognition elements in thermistors

In recent years several simple, inexpensive devices for the determination of glucose, urea and other compounds have been introduced. They are generally flow systems based on combining thermometric detection with immobilised enzymes. Enzymes showed to be ideally suitable as biorecognition elements for thermometric measurements due to a number of their useful properties. First, enzymes exhibit high substrate specificity allowing determination of a desired component even when a number of structurally related compounds are present in the sample. The other important property of enzymes is that they can be easily immobilised on a solid support. This approach consumes less enzyme or highly purified protein and also increases the enzymatic stability against heat, pH and organic solvents. In combination with a suitable support immobilised enzymes can be used repeatedly for as long as they remain active, thereby minimising costs of analysis and making it economically feasible to operate in a continuous mode [17].

The disadvantage of surface-bound enzyme is that it usually exhibits slightly lower activity compared to solute state of the same enzyme and is greatly dependent on several factors. The choice of coupling technique, the properties of the support material and the structural characteristics of the enzyme can have a pronounced effect on the resulting catalytic performance of the surface-bound enzyme [17]. As a general rule it is important that the functional groups used for immobilisation are not involved in the catalytic activity of the enzyme. The properties of the support material used for immobilisation are also important. The pores should be large enough for the enzyme to be immobilised within the support matrix and for the substrate to easily access the catalytic site of the surface-bound enzyme. Structural information about the enzyme,

Table 2
Determination of various analytes using enzyme thermistors.

Analyte	Enzymes	Linear range (mM)
Ascorbic acid	Ascorbate oxidase	0.01–0.6
ATP (or ADP)	Pyruvate kinase + hexokinase	10 nM[a]
Cellobiose	β-Glucosidase + glucose + GOx/catalase	0.05–5
Cephalosporins	Cephalosporinase (β-lactamase)	0.005–10
Creatine	Creatinase + sarcosin oxidase + catalase	0.1–5
Creatinine	Creatinine iminohydrolase	0.01–10
Cholesterol	Cholesterol oxidase + catalase	1–8
Cholesterol ester	Cholesterol oxidase + cholesterol esterase	0.01–2
Choline	Choline oxidase + catalase	0.03–0.5
Ethanol	Alcohol oxidase + catalase	0.0005–2
Fructose	Hexokinase + fructose-6-phosphate-kinase	0.5–6.0
Glucose	Hexokinase	0.01–25
Glucose	Glucose oxidase + catalase	0.0002–1 (75[b])
Glutathione	Glutathione sulfhydryl oxidase + catalase	0.5–10
Glycerol	Glycerokinase	0.005–1.1
Lactose	Cellobiose dehydrogenase	0.05–30
L-Lactate	Lactate-2-mono-oxygenase	0.0005–2
L-Lactate	Lactate oxidase + catalase	0.0002–1
L-Lactate (or pyruvate)	Lactate oxidase + catalase + lactate dehydrogenase	10 nM[a]
NADH	NADH dehydrogenase	0.001–1
APEE	Trypsin	0.01–10
Oxalic acid	Oxalate oxidase	0.005–2
Oxalic acid	Oxalate decarboxylase	0.1–3
Penicillin G	β-Lactamase	0.005–200
Penicillin	Penicillin acylase	0.02–200
Pyrophosphate	Pyrophosphatase	0.1–20
Pyruvate	Lactase dehydrogenase	0.01–1
Sucrose	Invertase	0.05–100
Triglycerides	Lipoprotein lipase	0.1–5
Urea	Urease	0.005–500 1–200
Uric acid	Uricase	0.05–4

[a] With substrate recycling.
[b] With benzoquinone as electron acceptor.

such as functional groups available for immobilisation and the size of the enzyme can therefore be useful for obtaining the maximum activity of the bound enzyme within the support matrix. However, each enzyme is unique in its characteristics and an empirical approach is always required to obtain and maintain the maximum activity of the support-bound enzyme [18].

The enzymes are the most commonly utilised biorecognition elements in ETs. However, it is not always possible to isolate the active enzyme or the response is produced in a cascade enzymatic reaction. In this case the whole cells, organelles or tissue slices can be used as a useful alternative [19]. Svitel et al. [20] reported on a microcalorimetric biosensor for the determination of citrate based on immobilised *Enterobacter aerogenes* cells. The method was proposed as an alternative to previously developed biosensors based on isolated citrate lyase (used as a biorecognition element) and allowed to eliminate problems associated with the rapid deactivation of isolated enzyme during the catalytic reaction [21]. However, the *E. aerogenes* cells were less specific than the pure enzyme and responded to a number of interfering compounds that are usually present in food samples (glucose, fructose and sucrose). Such interfering problems associated with lower specificity are generally applicable to all assays based on whole cells and can be abated by introduction of the split-flow technique with a reference column in the biosensor device construction (see Section 5).

3.1. Immobilisation of biorecognition elements and support materials in ETs

The physical and chemical property of the support, on which the enzyme is immobilised, has a crucial role for retaining the catalytic activity of the enzyme. When choosing the support material some basic criteria should be fulfilled: (1) the support must possess mechanical stability high enough to withstand physical stress; (2) it should give constant and good flow properties; (3) the support material must not have any catalytic activity or tendency to interact with the sample that would disturb the measurements of the enzymatic reaction [22]. The carrier material generally used is controlled pore glass (CPG) which is available is different pore and particle size ranges and with different ligands. CPG offers high enzyme-binding capacity, good mechanical, chemical and microbial stability and simple immobilisation chemistry [23]. Other materials that have been used include Sepharose CL-6B or CL-4B and Eupergit. The mechanical stability of Sepharose, however, is not as high as that of CPG, it can be used only at flow rates below ca 1.5 cm min^{-1} or the column will rapidly become clogged.

When applying crude solutions to the ET systems, as for example in analysis of sera in clinical samples, in the analysis of samples from microbiological fermentations or of waste water samples in environmental control, it may be advantageous to use nylon tubing for immobilisation of biorecognition element. In that case the biorecognition element (such as enzyme) can be covalently attached to the nylon with glutaraldehyde after treating the nylon tubing with dimethylsulphate followed by derivatisation with polyethyleneimine [22].

Reticulated vitreous carbon (RVC) is a commonly utilised support material for the construction of hybrid biosensors where an electrochemical measuring principle is combined with biocalorimetry [16,24–26]. The enzyme column in hybrid biosensors can consist of a platinum foil attached to the inner wall of a stainless steel tube. The column is packed with RVC – an open pore foam with a honeycomb-like structure, and poro size varying between 100 and 200 μm. It can be crushed into smaller pieces in order to increase surface area and then coated with chemically polymerised pyrrole for better electrical conductivity. The enzyme is adsorbed onto the conductive matrix which makes the entire column function as an enzyme reactor, a working electrode and thermal transducing element [16]. The drawbacks of using (pyrrole)-coated RVC are its low binding capacity compared with other support materials and possible leakage of the adsorbed enzyme from the enzyme column. This can be avoided by combining the RVC with other support material on which enzyme can be covalently immobilised. Khayyami et al. [26] reported new support material which has several favourable properties such as high binding capacity and good flow properties. The composite consisted of RVC which was filled with agarose gel. The enzyme was then covalently coupled to the agarose matrix through the simple cyanogen bromide activation.

Ramanathan et al. [25] demonstrated immobilisation of glucose oxidase (GOx)/catalase on RVC surface using silica sol–gel as a binder. The porous nature of sol–gel increased enzyme loading, while the RVC skeleton acted as a mechanical support.

Resent work conducted in the authors laboratory concerns application of ceramic hydroxyapatite as a new carrier material for immobilisation of enzymes [12]. Hydroxyapatite is an easily accessible and inexpensive chromatographic matrix, which allows reversible adsorption and desorption of enzymes, thus eliminating any need for covalent immobilisation or chemical cross-linking. Salman et al. reported determination of glucose in a commercial soft drinks, honey and serum by GOx and catalase, immobilised on the surface of hydroxyapatite. The reliability of the sensor was studied under different experimental conditions, indicating that the linear range was slightly reduced in comparison with the previous studies where GOx/catalase were covalently immobilised on CPG. However, the biosensor showed excellent operational stability (more than 150 samples) and guaranteed storage stability for three months.

The immobilisation technique should also be taken into consideration when designing the ETs. In order to ensure good operational stability a large excess of enzyme is usually immobilised which leads to almost complete consumption of the amount of substrate injected. The standard conditions are to use enzymes covalently attached to amine-CPG with a pore size in the range 500–2000 Å and particle size around 80 mesh (0.18 mm) activated with glutaraldehyde. The Schiff's base formation between the aldehyde group and the amine group of the enzyme is obtained under mild conditions in accordance to those required for the optimal catalytic activity and stability of the enzyme [27].

For cell immobilisation different procedures can be used. Cells can be entrapped within a three-dimensional lattice of 15% polyacrylamide [28] or immobilised through the biospecific interaction between glycoproteins on the cell membrane and lectins [29]. It is also common to use entrapment in ionotropic hydrogels (Ba/Ca-alginate or -pectate) [20,28,30] and gelatin beads [31,32]. The operational stability of hydrogel-entrapped cells was initially limited by the gel decomposition in the presence of chelating anions (phosphate or citrate) or due to pH changes [33]. However, it was shown later that the mechanical properties and stability of the hydrogels can be improved by treatment of the gel with glutaraldehyde and polyethyleneimine [34].

4. Thermal transducers

In most practical applications thermistors have been used as temperature transducers since they are highly sensitive, relatively inexpensive and easy to incorporate in the measurement circuitry [35]. Thermistors used here are resistors made of beads (down to 0.1 mm size) or small discs of metal oxides with a high negative temperature coefficient in the order of −4% $degree^{-1}$. This makes it easy to measure (differential) temperature changes < 10^{-5} degrees with a Wheatstone bridge. A drawback of the present constructions is that a small electric current needs to be passed through the

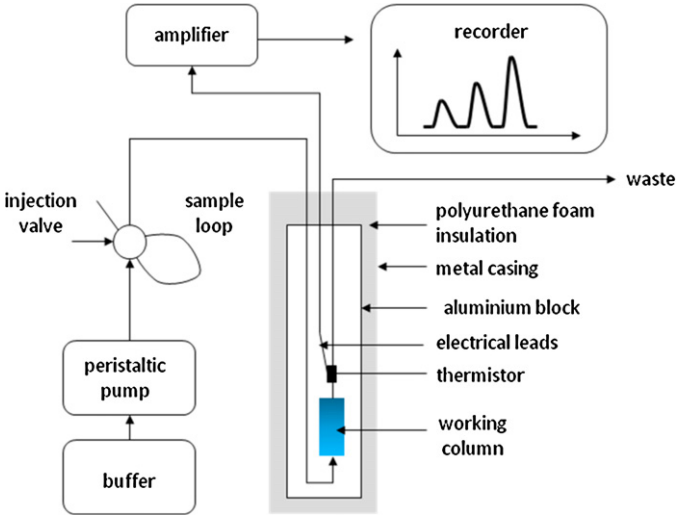

Fig. 1. Schematic enzyme thermistor design.

thermistor for the measurement, which heats it up a little and makes it flow sensitive. In fact thermistors are used as sensors in flow metres for gases and liquids. Here this necessitates a pulse-free flow that requires some consideration in the choice of pumps. A mechanical pulse damper can also be helpful, but for normal measurements a good peristaltic pump is sufficient if it can be equipped with long-lasting pump tubing, preferably made of marprene or neoprene. Other types of thermal transducers have also been used, such as thermopiles that are less flow sensitive and film thermistors that can be integrated on chips in lab-on-chip designs [36–38]. In most of the works described here a small thick-film type of thermistor has been used mounted on a thin gold tube at the outlet of the reaction column. In this way the transducer is protected from fouling from the solutions passing through the column, which makes the transduction mechanism drift- and calibration-free.

5. Instrumentation

5.1. The conventional thermometric devices

The ET used in many studies cited here was first described by Danielsson and Mosbach [2]. In initial studies different types of simple Plexiglas constructions containing the immobilised enzyme column were used. These devices were thermostated in water baths [39]. Later improvement of the sensitivity was achieved by development of ETs where the water baths were replaced by a carefully temperature-controlled metal block which contained the enzyme column [39]. Although clearly useful, these devices still could not provide high sensitivity due to escape of evolved heat to the surroundings. A solution to that problem entailed the use of flow-through systems, because these allow evolved heat literally to be transported to or along the thermal probe, thus minimising heat losses. Combination of enzyme catalysis with FIA system offered many attractive features to biosensor analysis such as high reproducibility and throughput. An obvious additional advantage gained by FIA arrangement was the possibility for continuous analysis.

Typical instrumentation of an ET device is presented in Fig. 1. Surrounded by a thick insulating layer of polyurethane foam is a thick-walled aluminium block which can be thermostated at 25, 30 or 37 °C with a stability of at least ±0.01 °C [39]. Inside is a second aluminium cylinder with cavities for two probes. The key element of the system is a small thin-wall plastic column (0.2–1.0 mL) containing enzyme immobilised on a solid support, such as CPG. The columns are attached to the end of probes which can be mounted with two different columns for measuring two different analytes, or with one enzyme column and one reference column, that is, split flow. Before entering the column, the solution passes through a thin-walled acid-proof steel tube (i.d. 0.8 mm) which serves as a heat exchanger. The purpose of this arrangement is to minimise temperature fluctuations in the solution passing through the column. At the top of the columns small thermistors are fixed to a short piece of gold capillary with heat-conducting, electrically insulating epoxy resin. They are connected to a Wheatstone bridge with a maximum sensitivity of 100 mV for a temperature change of 0.001 °C. Commonly used full-scale sensitivities are in the 10–50 m°C range. This permits determination in the 0.01–100 mM range for most reactions [39]. Buffer is continuously pumped through the whole system by peristaltic pump with a flow rate of 0.5–2 mL min^{-1}. Typically used flow rate is 1 mL min^{-1}, which allows one assay per two minutes throughput. With a 6- or 10-port injection valve the sample volumes in the range of 0.1–1.0 mL or smaller can be introduced into the system. The resulting temperature peak is used as a measure. The peak heights, as well as the area under the peak and the ascending slope of the peak, proportional to the enthalpy change, corresponds to a specific substrate concentration [40].

In order to improve baseline stability and insensitivity to non-specific heat production caused by dilution or interactions with the enzyme support, a split-flow ET arrangement can be applied. In this case two columns in two separate flow lines are used: one containing the immobilised enzyme, another – the reference column, containing support material only or, if required, inactivated enzyme. Nonspecific effects are assumed to be equal in the two columns, so that the differential temperature signal recorded by the two thermistors at the outlets of the columns represents the true signal originating from the enzymatic reaction. This approach is especially useful when dealing with industrial on-line monitoring in fermentations. The split-flow arrangement in this case helps to eliminate unspecific heat production associated with the complex matrix of fermentation broth [41]. The split-flow concept is also useful when dealing with complex biological matrixes such as blood. Satoh and Teramura reported a rapid determination of total cholesterol with a split-flow ET in human serum samples. It was shown that measurements can be performed without any sample preparation such as extraction of the lipids and none of the serum components have any inhibitory effect on the temperature response [42].

5.2. Mini/micro thermometric devices

5.2.1. Portable (mini-) thermometric devices

Advances in IC technology and micromachining of liquid filters, transducers, microvalves and micropumps on chips made it possible to construct miniaturised thermometric systems [40]. The design and development of various mini/micro formats of microcalorimetric devices are described below.

The first version of miniaturised ET was scaled down to about 1/5 times. Small plastic devices (50 mm × 15 mm) with 1.5 mm × 15 mm GOx/catalase column have been designed for monitoring of glucose in whole blood samples [43]. Due to high sensitivity, small dimensions, modest buffer consumption and good operational stability this device is suitable for portable use, for instance for home monitoring of glucose in diabetes [43]. A major limitation factor was the mechanical obstruction of carrier material by the particulate matter [40]. To circumvent this problem two successful approaches have been applied. In the first approach, small dialysis/filtration unit was included into the construction to remove blood cells. This resulted in a linear range of 0–25 mM glucose, which is adequate for diabetes monitoring. Alternatively, a superporous agarose material was employed as enzyme

carrier. This material allows a large number of whole blood samples to be injected without any sign of clogging. Feasibility studies showed that these pocket-size biosensors could perform personal monitoring on 1 μL blood samples with 10 times better precision than current electrochemical personal devices. About 200 whole blood samples could be analysed without need for recalibration. The quality of data and speed of analysis (up to 1 samples per minute) would allow for safe automatic insulin administration.

A portable ET system (a bio-thermochip) for the detection of glucose in urine in the concentration range of 1–4 mM has been developed by Shimohigoshi et al. [44]. The miniaturised device consisted of a thermistor head surrounded by a silicone tube which served as a support for enzyme immobilisation. GOx/catalase and bovine serum albumin (BSA) were immobilised around the two bead thermistors and the temperature difference between GOx/catalase- and BSA-immobilised reference thermistor was measured in an insulated cell. The incorporation of the silicon frame around the bead thermistor allowed to reduce the heat loss to the surroundings, to enhance the enzyme loading, and to physically protect the enzyme layer.

5.2.2. Microfabricated (micro-) thermometric devices

A further step in miniaturisation was achieved by integrating the ET on a 14 mm × 6 mm × 0.4 mm silicon chip with the enzyme directly immobilised in micro-channels (total volume of 0.02 μL) etched into the surface of a silicon wafer [40,45]. In order to investigate the properties of the silicon based thermal micro-biosensor, penicillinase and catalase were immobilised and results were compared with these for conventional ET. This construction not only provided a flow channel with small dimensions, but also allowed re-immobilisation of the enzyme and reduced the heat losses from the cell. However, the sensitivity and LOD were reduced due to low enzyme loading.

Another approach in designing thermal micro-biosensors is to immobilise enzyme directly onto the surface of thermistors [46] or thermopiles [36,38]. Fulton et al. constructed a thermal enzyme probe with urease immobilised on the surface of one of a pair of thermistors, which were placed in an open-flow channel in a small stainless steel cylinder, comprising a flow-through cell. Under reported conditions it was possible to achieve the concentration resolution of about 0.1 mM urea [46]. Such arrangement permits avoiding temperature fluctuations and non-specific heat production due to dilution, mixing, etc. Therefore, it may not be necessary to use reference column or split flow to discriminate effectively between the desired signal and the deleterious effects. Nevertheless, the thermistors are sensitive to flow variations. Additionally, in this construction the sensitivity is greatly reduced because of the heat loss by convection.

In comparison to enzyme-coupled thermistors, devices employing thermopiles provides higher signal-to-noise ratio and baseline stability [37,38]. Since thermopiles are by nature responsive to temperature differences rather than absolute temperatures, they have an enhanced ability to reject common-mode thermal signals. Towe et al. reported on a thermoelectric enzyme sensor with co-immobilised GOx and catalase for measuring of blood glucose. The thermopile consisted of 50 couples of bismuth and antimon connections deposited by evaporation onto a thick Mylar sheet, which was inserted into a silicon tube (3 mm in diameter). The sensitivity for glucose was $34 \pm 2\,\mathrm{nV\,(mg\%)^{-1}}$ and the linear response covered the region of 328 mg% (5 mM glucose corresponds to 90 mg/100 mL or 90 mg%) [38].

5.2.3. Integrated thermal biosensor arrays

There is a strong demand, especially in the intensive care unit and the operation theatre, for miniaturised multiple-analyte sensing devices that are able to measure simultaneously a set of physiological parameters in vivo or in vitro and that are fast, accurate, cheap, and, of course, reliable [47]. These devices might find additional applications in the field of environmental monitoring and bioprocess control, bedside clinical analysis. Series of studies have been reported on devices for the multiple analytes determination [47,48]. They are based on multichannel or split-flow systems in combination with electrochemical or optical detection. The maintenance of balanced flow rates between individual flow channels in these systems proved to be difficult. This is due to physical, biological and chemical variations between channels [49]. In addition, electrochemical and optical interferences from other reactions on electrode and optical transducers cause erroneous signals. Further, the sensitivity of these devices is dependent on the applied potential or the wavelength [40] required for detection of each particular analyte. Due to complexity of the measuring systems and careful control, which is required for their operation, there is still a strong necessity for alternative devices. In that case, ET seems to be a promising tool for simultaneous determination of various substances because it utilises re-usable enzyme catalysis and a universal detection principle, namely heat [49]. Therefore, measurement conditions can be dramatically simplified while minimising interferences and facilitating automation of the analysis [40].

Development of an integrated thermal biosensor array for the simultaneous determination of several analytes has been demonstrated by the authors group [40,49,50]. The principle upon which these similar devices are based is shown in Fig. 2 [40]. They consist of a single flow micro-channel, fabricated on a surface of a quartz chip. The micro-channel is serially partitioned into four distinct reaction/detection zones. Each zone includes one immobilised enzyme and one pair of film-thermistors. A unique advantage of the designed device is that it provides possibility to measure different analytes under identical conditions. In addition, application of micromachining and IC technologies is of benefit for the manufacture of uniform and cheap thermal transducers with flexible shape, size, resistance, as well as delicate microstructure on the chips. The good chemical insulation of the transducers from the flow stream eliminates interference from the reactants on the transducers, and the intrinsic stability of the transducers obliterated the need for frequent recalibration of the sensor. The construction provides convenient access to the microchip for repeated replacement of the enzyme matrix.

Determination of glucose, lactate, urea and penicillin was performed [49] using an integrated thermal biosensor array where lactate oxidase, GOx, urease, β-lactamase were separately immobilised on N-hydroxysuccinimide (NHS)-activated agarose beads, filled into the individual zones in the micro-channel. For the lactate and GOx reactions, catalase was co-immobilised together with these enzymes to generate additional heat and recover a portion of the oxygen which extend their linear ranges. The temperature changes were registered with respect to the individual reactions catalysed by the respective enzyme matrices. Linear ranges up to 40 mM urea and glucose, 20 mM penicillin and 10 mM lactate were obtained using 20 μL sample loop.

A very recent alternative concept of thermal sensor arrays for real-time, two dimensional temperature detection of enzyme catalysed reactions involves miniaturised CMOS technology (complementary metal-oxide semiconductor) [51]. The CMOS technology is more sensitive than conventional thermoelectric techniques and can be used for dense arrays, but at this early stage of development the sensitivity is very low with a glucose detectability of only about 100 mM – the most sensitive thermal biosensors are one million times more sensitive.

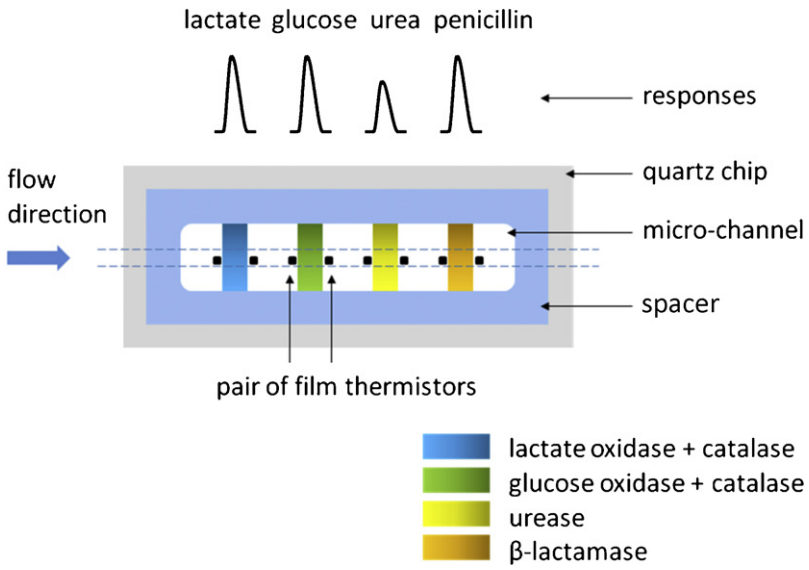

Fig. 2. Principle design of a thermal biosensor array for simultaneous flow injection determination of lactate, glucose, urea, and penicillin.

5.3. Hybrid biosensors

Generally, biosensors can be classified according to the mode of signal transduction as electrochemical, optical, thermal and piezoelectric sensors. The choice of the biological material and the adjusted transducer depends on the properties of each sample of interest and the type of physical magnitude to be measured [52]. Consequently, two statements can be defined: first, there is no universal type of sensor which can be applied for any kind of sample, and the second, each type of sensor has its own merits and drawbacks. Therefore, one of the attractive directions in future development of thermal biosensors is hybrid sensors, which combine different detection principles. Creating hybrid sensor allows retaining advantages of different measuring techniques and diminishing their drawbacks.

Several studies on hybrid biosensors for simultaneous monitoring of electrochemically generated current and the thermal signal produced in association with enzymatic catalysis were reported in the literature [7,16,24,26,53]. Xie and co-workers fabricated and demonstrated a device, in which the enzyme column was constructed of platinum foil filled with RVC, on which an enzyme (GOx) and a mediator (ferrocene) were immobilised/adsorbed (Fig. 3). The voltage was applied between working electrode (RVC) and counter electrode (platinum foil) to maintain the electrochemical reaction. The current generated from the reduction of ferrocene was registered by a simple potentiostat connected to the column. The temperature changes associated with the enzymatic oxidation of glucose were registered by a pair of thermistors attached to the inlet (reference thermistor) and outlet (measurement thermistor) of the column. In order to demonstrate the feasibility of the hybrid biosensor, it was applied for determination of glucose. By using this method, a linear range of glucose concentration up to 20 mM was achieved.

A similar device was employed in another study for the determination of catechol [16] (Fig. 4). As a catechol containing sample was injected into the system, it was catalytically oxidised to 1,2-benzoquinone by immobilised tyrosinase, which was subsequently oxidised by oxygen to its original form. Produced 1,2-benzoquinone was reduced to catechol on the RVC electrode surface. The thermometric and amperometric signals were detected simultaneously. The electrochemical recycling of the substrate, demonstrated in this study, enabled increase in sensitivity of the device. An additional benefit of such a hybrid biosensor lies in the fact that the thermometric signal can serve as a the reference for electrochemical measurements.

Future improvement in feasibility of the hybrid sensor was achieved by incorporating the column with composite of RVC and superporous agarose or sol–gel [7,26]. The combination introduced several favourable properties. Agarose and sol–gel possess higher binding capacity for the enzyme than RVC itself. Therefore, higher sensitivity for the analyte of interest can be achieved. Additionally, higher flow rates can be applied [26].

A recent example of the use of a hybrid biosensor for the investigation of enzyme properties can be found in [53]. In this work immobilised cellobiose dehydrogenase was used in a combined thermometric and amperometric biosensor to measure lactose in milk. Highly reproducible linear response was obtained for lactose between 0.05 mM and 30 mM (Fig. 5). The assay time was two minutes and no special sample treatment was necessary except dilution of the milk samples. *p*-Benzoquinone was used as mediator to generate an amperometric signal. The thermometric and amperometric results were in good correlation and could so to say be used as reference for each other. In addition the system showed good stability (at least two months at room temperature).

Furthermore applications of the ET have been demonstrated in hyphenation with optical biosensor techniques for studying mixtures of contaminants such as heavy metals and pesticides [54]. Using the combined setup the mixture of metals such as arsenic and organophosphate pesticides could be determined sequentially. Butyryl cholinesterase was exploited for the determination of inhibition due to arsenate and organophosphates in an external column. The choline oxidase/catalase column was deployed for measurement of choline using ET as well as optical detection using CCD camera.

6. Enzyme thermistor applications in food analysis

There are numerous ET applications reported from different laboratories showing the great versatility of thermal biosensors. Table 2 is a representative list of measuring ranges found for a number of analytes [3,55]. Below examples of analytical applications in food and environmental technologies are given to provide some further insight in the practical use of thermal biosensors. Recently

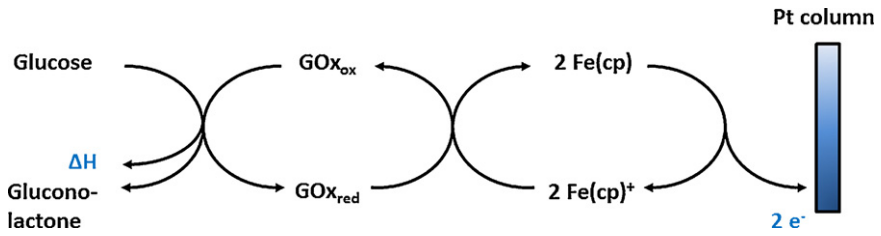

Fig. 3. A ferrocene-mediated hybrid glucose biosensor illustrating the hybrid biosensor principle. The enthalpy change of the reaction can be measured with a thermistor and the oxidation current with a potentiostat.

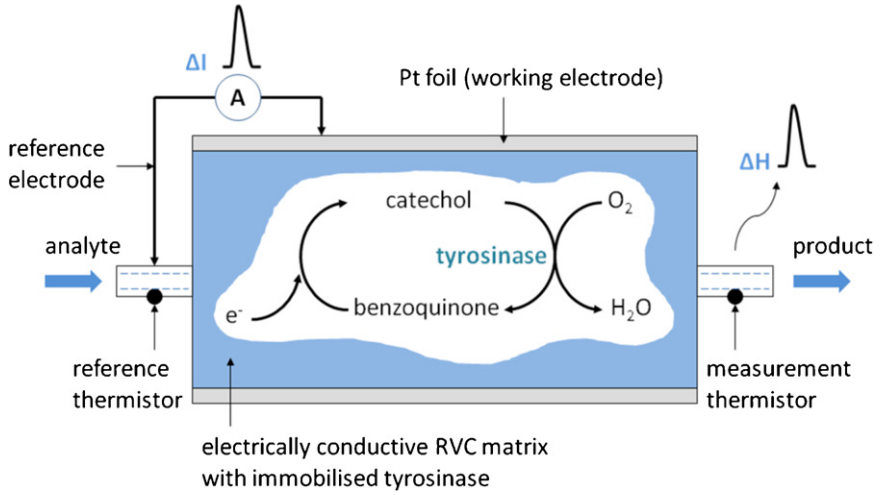

Fig. 4. Simultaneous thermal and electrochemical determination of catechol with a hybrid biosensor using a conducting tyrosinase column.

a novel calorimetric biosensor for the determination of fructose in syrups was demonstrated [56]. The unique feature of this sensor is its ability to selectively measure fructose in the presence of glucose. The biosensor utilises the selective phosphorylation of fructose-6-phosphate (F6P) in the presence of glucose-6-phosphate by the enzyme fructose-6-phosphate-kinase (F6PK). This is the first report utilising the enzyme F6PK for a fructose biosensor. The biosensor facilitates simultaneous measurement of glucose as well as fructose by deploying immobilised HK and F6PK columns in sequence using a flow injection system. A practical set up was presented for the determination of fructose in commercial syrup samples with advantages such as high selectivity, short analysis time and low reagent consumption. The reported excellent operational stability and the shelf life over three months strengthens the benefits of calorimetric technique over other reported fructose biosensors that in most cases have resorted to expensive and less stable enzymes.

Very recently, a practical application of a FIA-ET based biosensor was demonstrated in the determination of urea in adulterated milk [11]. The technique is very simple to perform, and is economical and highly stable. An important feature is the lower detection

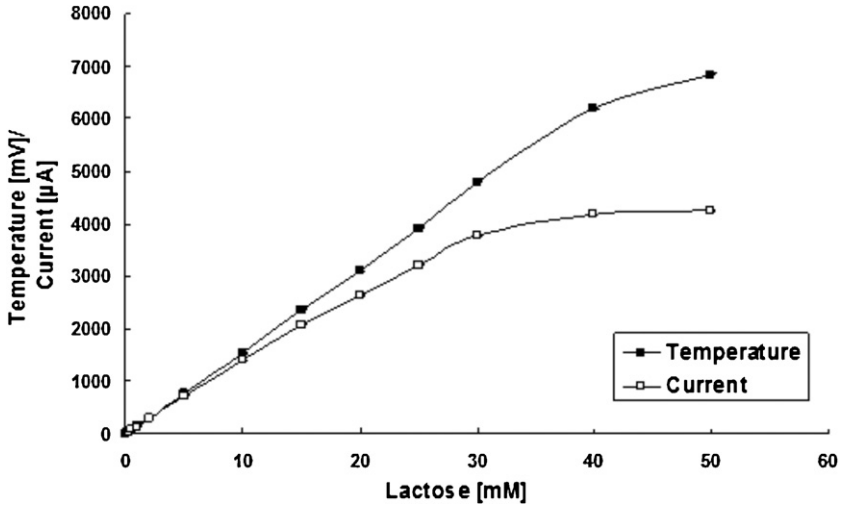

Fig. 5. Thermometric and amperometric responses to 50 µL lactose samples measured with an enzyme column containing 50 U of immobilised cellobiose dehydrogenase. As mediator *p*-benzoquinone was used.
From [53].

limit of 0.1 mM urea in milk and a dynamic range of detection of 1–200 mM. This makes the technique more flexible to adapt to different levels of contamination or adulteration. The immobilised urease column has been reported to be stable for more than 180 days. The method was cross-validated against a commercial kit. The parameters such as detection range, analysis time, sensitivity, sample throughput and cost per sample were evaluated and thus confirmed to be suitable for routine analysis in dairy industry.

7. Enzyme thermistors in bioprocess monitoring

Monitoring of biotechnological processes is usually performed in off-line mode while on-line monitoring would be preferable for better control and automation. Owing to the complexity of biotechnological media it is usually not possible to apply conventional analytical techniques for determination of the desired compound in on-line mode without sample preparation. Additional problems are associated with the operational difficulties such as high humidity, large temperature variations and ambient temperatures up to 40 °C, continuous vibrations, water and stream release [57]. For on-line monitoring of bioprocesses ETs can be successfully applied due to their selectivity and superior operational stability [30].

Many examples demonstrating the application of ET for monitoring of various biotransformations have been described in literature [30,41,58,59]. Scheper et al. developed a four-channel ET system suitable for simultaneous monitoring of four different compounds in a complex fermentation medium. ET was used for determination of mono- and disaccharides (glucose, sucrose, maltose and fructose) with immobilised enzymes during a cultivation process over a period of 300 h in a fully automated and computer controlled system. The results obtained for glucose concentration during fed-batch production of cephalosporin C were initially deviating from these obtained with off-line monitoring of glucose using a YSI glucose analyser due to unspecific interaction of fermentation medium with the carrier material (Eupergit C) used for immobilisation of specific enzymes (GOx/catalase). Inclusion of an additional dialysis module into the system and blocking of unspecific sites on Eupergit C with glycine prior to analysis allowed obviating this problem. To demonstrate the potential of the method whole cells of *Saccharomyces cerevisiae* were immobilised in calcium alginate in order to measure amount of assimilable substances which sometimes might be interesting for characterisation of the whole fermentation procedure [30,59].

An interesting application of ET for the determination of enantiomeric compounds in aqueous and organic phases have been proposed by Scheper and co-workers [60]. The sensor utilised an ET unit with two channels leading to separate columns in which two enzymes were immobilised on a polymer resin. The measurement principle was based on the fact that one enzyme hydrolyses only one enantiomer while the other converts both. It was thus possible to determine the concentrations and ratio of *L* and *D* enantiomer in organic solvent-containing media. The method opens an opportunity to use the ET for on-line monitoring and control of enzymatic enantioselective production of pharmaceuticals in organic solvents for which time-consuming and costly chiral HPLC is currently utilised [61].

8. Apoenzyme-based thermistors for determination of heavy metals

A separate unique group of calorimetric biosensors is based on flow-injection analysis in combination with metalloenzymes for determination of submillimolar concentrations of heavy metals [62]. Metalloenzymes are the group of enzymes which have heavy metal ions coordinated into their active centre. The metal ions are intrinsic and can be reversibly removed from the catalytic site of the enzyme by strong chelating agent. Obtained metal-free enzymes (apoenzymes) lack catalytic activity but can be positively reactivated by exposure to a metal-containing sample. Thus, the amount of metal trapped in the catalytic centre on the enzyme can be estimated by measuring the activity of the reactivated enzyme.

Apoenzyme thermistors were utilised for determination of trace amounts of copper(II) in human blood sera using immobilised ascorbate oxidase [63] or galactose oxidase [64] and cobalt(II) and zink(II) using either immobilised alkaline phosphatase [63] or bovine carbonic anhydrase [64]. There was a significant agreement between the results obtained using apoenzyme thermistors and other well-established methods such as atomic absorption spectrometry and amperometry. Though more sensitive and rapid results can be achieved with amperometric determination of the metal ions, the apoenzyme thermistor owns remarkable long-term stability as well as low-priced instrumentation and assay.

9. MIP-based thermistor

The ET instrumentation is also very useful in molecular imprinting studies, since binding and unbinding as well as catalytic reactions usually are associated with enthalpy effects [65]. This makes it possible to follow these interactions in a label-free way. By the normal procedure to apply molecularly imprinted polymers in analysis the support material is integrated with binding sites so there is no need for an immobilisation step [66].

Molecular imprinting polymers (MIPs) have been developed as artificial receptors in order to mimic the active sites or binding sites of enzymes, antibodies, and natural receptors. The usual way to produce MIPs is to employ the target molecule (or a similar derivative of it) as a template around which interacting and cross-linking monomers are arranged and co-polymerised to form a block that contains a number of cavities each with a template molecule inside. After polymerisation, the polymer block is ground into small particles of a size suitable for the application and the template removed. The binding sites that have been imprinted on the polymer are complementary to the template in size, shape, and position of the functional groups, and are capable of selectively rebinding to the template or the target molecule. Compared to biological recognition elements, MIPs offer high robustness and ability to work in a harsh environment [67]. A large number of potential applications, including chromatography, enzymatic catalysis, solid-phase extraction, and sensor technology, has been intensively developed for this class of materials [68].

It is a general problem to detect binding between the MIPs and the target molecule. Thermal monitoring of the binding enthalpies offers, however, a label-free possibility for the characterisation of MIP catalysis and binding. Combination of calorimetric transducer – the thermistor – with analyte recognition by a catalytically active MIP was first demonstrated by Scheller and co-workers [65]. Adsorption/desorption of a substrate (phenylacetate) to MIP and non-imprinted NIP were reflected by concentration-dependent heat signal. Additionally, the results demonstrated that there are two different types of recognition sites in the MIP, which can be distinguished by the difference in heat generation. Thus, a MIP thermistor enables two possibilities: determination of the target molecule and characterisation of the selectivity and binding properties of MIP. Two following studies on interaction of fructose (Fig. 6) and fructosyl valine with specific MIP were reported [66,69].

The concept was extended to combination of MIP with immobilised enzyme [70]. The MIP-tyrosinase reactor was incorporated into the conventional flow-through ET. The measurements were based on the hydrolysis of substrate (phenylacetate) by catalytically active MIP to acetic acid and phenol, and further oxidation

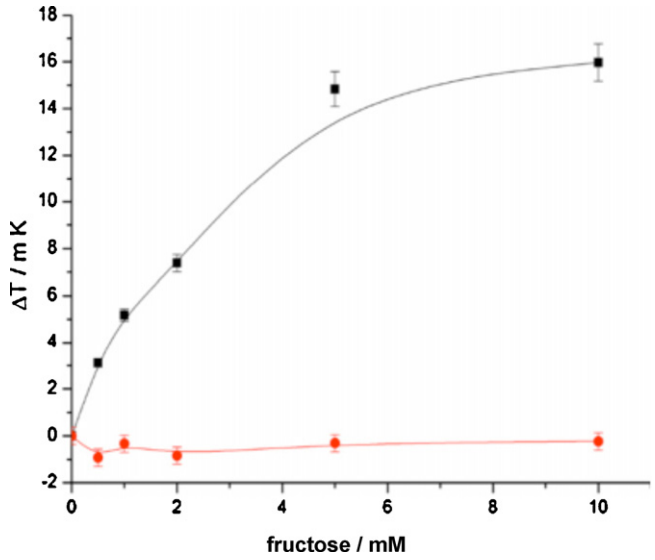

Fig. 6. Temperature changes registered for different fructose concentrations using a MIP column (squares) and control polymer (circles).
From [66].

of released phenol to *o*-benzoquinone by immobilised tyrosinase. The sequential substrate conversion resulted in a combined heat generation that was five times larger than from the polymer alone.

10. Thermometric enzyme-linked immunosorbent assay (TELISA)

Thermometric enzyme-linked immunosorbent assay (TELISA) is based on the previously described enzyme-linked immunosorbent assay (ELISA) but utilises heat production from the enzyme label measured using an ET unit [4]. Two measuring formats used in TELISA have been reported in the literature. One of them is based on direct competitive immunoassay [71–73] where unlabelled antigen (analyte) competes with the labelled antigen for the limited number of the antibody binding sites. The amount of the enzyme (e.g., catalase or peroxidase) bound to the antibodies is then measured with the ET unit by injecting a pulse of a substrate. In a final step the bound antigen is released from the immunosorbent by washing buffer preparing the column for a new measuring cycle. In the direct competitive format of TELISA the signal produced is reversibly proportional to the concentration of analyte.

Scheper et al. [74] established a simple TELISA based on "sandwich" principle for determination of various IgGs. The system utilised protein A-immobilised column which binds IgGs containing in a sample. In the following step protein A-bound IgGs are labelled with a fusion protein from protein A and β-galactosidase forming a "sandwich". The amount of label is then measured by injecting a pulse of a substrate (lactose) which is converted into glucose and galactose by the fusion protein. By switching the valve the flow is directed into a different column (ET column) where the heat generated during enzymatic conversion of glucose is measured. In a final step the "sandwich" is eluted from the surface by a washing buffer and the protein A-immobilised column can then be used for a new measuring cycle. The signal produced in "sandwich" format of TELISA is directly proportional to the concentration of IgGs present in a sample. The schematic representation of TELISA procedure for both direct competitive (A) and "sandwich" (B) assays is illustrated in Fig. 7.

TELISA offers a variety of advantages compared with conventional ELISA. First of all, it can be used for determination of large compounds in complex matrixes. Utilisation of spectrophotometric detection principle (generally employed in conventional ELISA), when dealing with fermentation broth, blood samples or hybridoma cell media, becomes difficult as many other compounds present in the matrix may have unwanted optical properties and contribute to the signal. This can be avoided when thermometric detection principle is employed. Secondly, the TELISA provides much wider choice of enzymes available for labelling since heat is produced in all enzymatic reactions. Borrebaeck et al. described determination of human serum albumin in serum using catalase as marker enzyme [71]. Catalase is characterised by an extremely high turnover number leading to increased sensitivity of the system. Finally, the immunosorbent in TELISA can be used repeatedly when applying proper reagent for dissociation of antigen/labelled antigen-immobilised antibody complex and no separation step is needed.

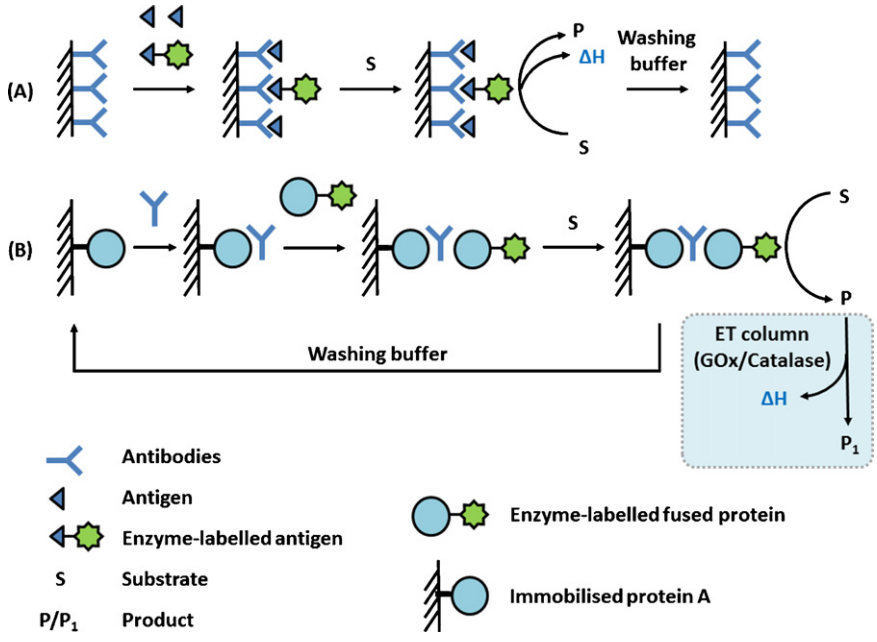

Fig. 7. Schematic representation of TELISA procedure. (A) Direct competitive TELISA; (B) "Sandwich" TELISA. Explanation in the text (Section 10).

About 10–15 min is required for one assay cycle in TELISA since washing steps should be performed. That makes TELISA a low throughput technique. The sample capacity and sensitivity of the technique is lower than that for other established immunoassay methods (radioimmunoassay (RIA) and ELISA). Nevertheless, the method is optimal for monitoring the fermentation procedures employing high concentrations of analyte in small sample series when fast results are desired.

11. Conclusions

In our opinion the enzyme thermistor is a very realistic biosensor concept. It is truly universal; it works with exothermic as well as endothermic reactions. The instrumentation and procedures involved are relatively simple, direct and applicable to reactions involving enzymes, proteins, whole cells, MIPs etc. The assays do usually not require additional reagents and are not sensitive to colour or turbidity of the sample. The temperature transducer is not in direct contact with fluid streams which eliminates transducer drift and makes the technique very robust and useful for high-precision bioprocess and biomedical monitoring. The columns allow high enzyme loading offering excellent operational stability and long storage times. These features make process control based on ET measurements a realistic duty. The thermal biosensor technique is scalable and there are excellent possibilities for miniaturisation, for instance for personal monitoring of blood glucose. The technique could be extended to multiparameter analysis and it works well with both organic and aqueous solutions. The hybrid biosensors which are based on combined calorimetric and electrochemical detection principles retain the advantages of both techniques and at the same time make them insensitive towards electrical or optical interferences. The main drawbacks include non-specificity, as this is dependent only on the underlying reaction, and a somewhat more complicated instrumentation than for some other techniques. In recent time, however, there has been an increasing interest in thermal biosensors, not least for routine measurements. In a laboratory where measurements of some common analytes like, glucose, lactate, and ethanol are more or less frequently coming up, an ET with a number of enzyme columns stored in the refrigerator could be a very practical and economical alternative. Change of analysis is mainly a matter of selecting the right column for the task. The calibration is usually valid for long and may only need a one-point control from time to time.

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