Chapter 30

Lipase and Phospholipase Biosensors: A Review

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Abstract

Recent advances in the field of biology, electronics, and nanotechnology have improved the development of biosensors. A biosensor is a device composed of a biological recognition element and a sensor element. Biosensor applications are becoming increasingly important in areas such as biotechnology, pharmaceutics, food, and environment. Lipases and phospholipases are enzymes which have been used widely in food industry, oleochemical industry, biodegradable polymers, detergents, and other applications. In the medical industry, lipases and phospholipases are used as diagnostic tools to detect triglycerides, cholesterol, and phospholipids levels in blood samples. Therefore, the development of lipase and phospholipase biosensors is of paramount importance in the clinical area. This chapter introduces the reader into the preliminaries of biosensor and reviews recent developments of lipase and phospholipase biosensors.

Key words: Biosensor, Lipase, Phospholipase, Nanotechnology, Monitoring, Clinical applications

1. Introduction

The first biosensor was developed in the 1960s by Clark and Lyons (1). This biosensor called "enzyme electrode" consisted of a glucose oxidase enzyme coupled with an oxygen electrode. Since then, the biosensor market has widely grown in the fields of biotechnology, environment, medicine, and military applications (2). In food industry, biosensors have been used for quality control, for determining composition, contamination of primary materials and processed foods (3). In fermentation processes the availability of online measurements of key variables such as substrates and product concentrations had allowed improving and controlling their products (4). Environmental applications of biosensors include determining contaminants such as pesticides, metals, pollutants like dioxins, phenols, and polycyclic aromatic hydrocarbons (5, 6). The most important biosensor market is related to clinical and pharmaceutical applications. These devices have been used as diagnostic tools for monitoring glucose (7), urea (8), and ethanol (9) among other

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important medical applications (10). Detection of pathogenic microorganisms is a research area for food industry and bioterrorism detection and prevention (11).

Enzymes have been extensively employed as a biological recognition element in biosensor design because they present some advantages such as high selectivity, fast response, some of them are commercially available or can be isolated and purified from diverse sources (12). Lipases and phospholipases are enzymes that catalyze the hydrolysis of fats, oils, and phospholipids, respectively. Lipase production is important since they had many applications such as detergents, agrochemicals, biodegradable polymers, textile industry, oleochemical industry, cosmetics and flavors, and resolution of racemic mixtures (13). Phospholipases are related to food and oil industries (14). Lipases are important drug targets or marker enzymes in the medical sector. They can be used as diagnostic tools and their presence or increasing levels can indicate certain infection or disease such as high triglycerides or pancreatitis (15). Advances in nanotechnology and biology have improved biosensor technology allowing more specific and miniature devices. In addition, it is estimated that in the year 2012 the biosensor market for medical diagnosis will reach to US $6.1 billion (16). Thus, lipase and phospholipase biosensor development for diagnosis is relevant and still is an open issue; particularly for clinical and pharmaceutical applications.

This review describes the developments and applications of lipase and phospholipase enzymatic biosensors. The chapter is organized as follows: Subheading 2 describes the generalities in biosensor design and Subheading 3 centers on enzymatic biosensors. Subheading 4 depicts lipase and phospholipase biosensors and finally Subheading 5 states conclusions and new trends in biosensor design.

2. Biosensor Preliminaries

2.1. Biosensor Definition

An electrochemical biosensor has been defined as a "self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative information using a biological recognition element retained in direct spatial contact with an electrochemical transduction element" (17). In general, a biosensor is a device contained in a small package, where the biological recognition element is in direct contact with the transducer. The biological recognition system translates biochemical information, usually an analyte sample, into a chemical or physical output signal with a defined sensitivity. The recognition system provides the sensor with high selectivity for the analyte to be measured and does not recognize other analytes. Biological recognition elements or bioelements

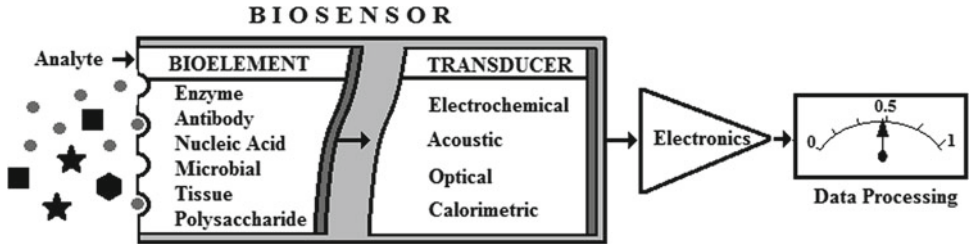

Fig. 1. Structure of a biosensor.

include: enzymes, antigens, antibodies, nucleic acids, receptors, cells, and cell organelles. The transducer takes the output signal from the biological recognition system and converts it into a measurable signal, usually an electric signal. The transduction element could be: optical fibers, piezoelectric devices, crystals, and various types of electrochemical electrodes. The principle of detection in a biosensor is the specific binding of the analyte of interest to the biorecognition element immobilized on a suitable support medium. The specific interaction results in a change of physical–chemical properties such as: pH, electron transfer, mass, and heat transfer which are detected and measured by the transducer (18). Figure 1 shows the structure of a biosensor.

2.2. Biosensor Classification

Biosensors may be classified based on their transducers type, such as acoustic, optical, calorimetric and electrochemical. The acoustic biosensor is based on the use of piezoelectric crystals. Crystals can be designed to vibrate at a specific frequency when an electrical signal excites them. The frequency of oscillation is therefore dependent on the electrical frequency applied to the crystal as well as the crystal mass. Therefore, when the mass increases due to binding of chemicals, the oscillation frequency of the crystal changes and the resulting change can be measured electrically and used to determine the additional mass of the crystal (19). In optical biosensors, the output signal is light, where energy of the electromagnetic radiation measured can provide information about changes in the local environment surrounding the analyte, its molecular vibrations or the formation of new energy levels. This information may be determined with many different types of spectroscopies such as: absorption, fluorescence, phosphorescence, Raman, etc. (20). A calorimetric biosensor is based on combining enzymes with temperature sensors. Thermal detection biosensors are based on the absorption or production of heat that changes the temperature of the medium when a reaction takes place. When the sample comes in contact with the enzyme, the heat reaction of the enzyme is measured. The heat produced or absorbed is proportional to the molar enthalpy and the total number of molecules in the reaction (2). The last classification is given by electrochemical biosensors which will be discussed in more detail in next section.

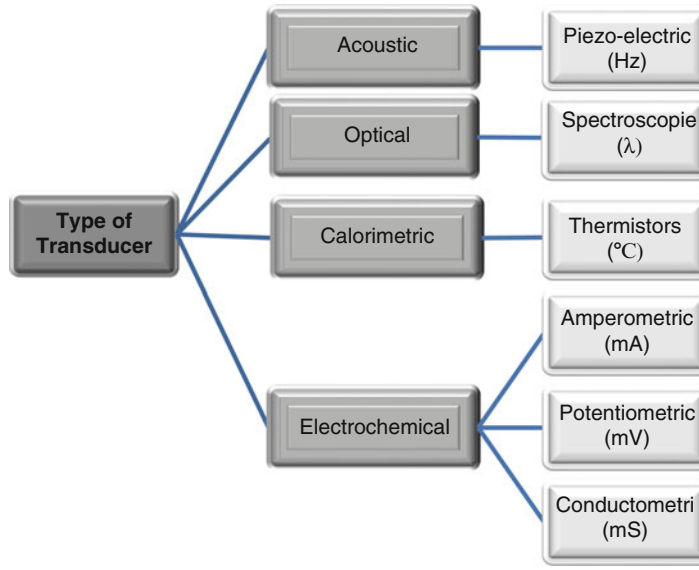

Fig. 2. Classification of the main types of biosensors.

2.2.1. Electrochemical Biosensors

The electrochemical biosensor is the most used for the analysis of different type of substrates (21). In electrochemical biosensors enzymes are commonly employed as the biological recognition element in which analytes are detected by the physicochemical transducer providing a measurable signal (22). Electrochemical biosensors are divided into amperometric, potentiometric, and conductometric according to the way they generate a signal. Figure 2 shows the main class of biosensors.

Amperometric biosensors are based on measuring of the current resulting from an electrochemical reaction. It is usually performed by maintaining a constant potential at a Pt or Au electrode. The resulting current is directly correlated to the bulk concentration of the electroactive species or its production or consumption rate within the adjacent biocatalytic layer (17). Potentiometric measurements involve determination of the potential difference between an indicator and a reference electrode and are based on ion-selective electrodes (ISE) and ion-sensitive field effect transistors (ISFET) (17). The transducer may be an ISE which is an electrochemical sensor based on thin films or selective membranes as recognition elements (23). An ISFET is composed of an ion-selective membrane applied directly to the insulated gate of the field effect transistor (FET). In a potentiometric biosensor, ISFETs are coupled with a biocatalytic layer and are usually called enzyme field effect transistors (ENFETs) (24). In a conductometric biosensor, the biological and chemical changes in the conductance between a pair of metal electrodes in a bulk solution are measured (25).

2.2.2. Bioaffinity and Catalytic Sensors

Biosensors could also be classified as bioaffinity and catalytic sensors. Bioaffinity biosensor operation is based on interaction of the analyte with macromolecules that have been isolated from their original biological environment or engineered. There is no consumption of analyte by the immobilized biocomplexing agent since equilibrium is usually reached. Typical receptors used in bioaffinity biosensor include antibodies and nucleic acids. These biosensors can be used to detect genetic material of microorganisms, for the presence of pathogens or pesticides or any type of substances that may cause an immune response (26). Catalytic biosensors are based on a reaction catalyzed by macromolecules where continuous consumption of substrate is achieved by the immobilized biocatalyst incorporated into the sensor. Typical receptors used in catalytic biosensor include enzymes, whole cells, and tissues (17).

2.3. Biosensor Requirements

Biosensor performance may be appraised on sensitivity, response times, dynamic ranges, robustness, and simplicity of fabrication. Recent advances in technology involved in biosensor design and construction has allowed overcome the technical difficulties that initially these devices presented for use in industry such as: to be inexpensive, sensitive, accurate, and easily manufactured at high rates (27). Some important characteristics that biosensors may have include the following: high selectivity and sensitivity, accuracy and repeatability, reliable, fast response, long lifetime with a low price, and being miniaturizable (26). The biosensor must be sufficiently selective to interact exclusively with the compound of interest and not with others bearing similar properties; this is achieved through highly specific recognition elements. The biosensor must have enough sensitivity to detect concentrations of certain analytes. Sensitivity and linear range are a function of the physical design and the molecular recognition element (28). The biosensor has to be reliable so it cannot be altered by the sample, should be insensible to temperature, electrical noise, or other environmental interference. Some industrial processes will require fast response from the biosensor in order to rapidly get rid of contaminated or damaged products. Therefore low cost, long lifetime, and reusability are other desired characteristics. The size of the device is another important factor; advances in electronics and nanotechnology have allowed miniaturizing, increasing the field application of these devices.

3. Enzyme Biosensors

Enzymes are proteins that catalyze chemical reactions in living organisms. In a reaction catalyzed by an enzyme, substrate binding occurs in a specific region of the enzyme called the active site, comprising a binding site and a catalytic site. An enzyme biosensor is an analytical

device that combines an enzyme with a transducer to produce a signal proportional to target analyte concentration. This signal can result from a change in proton concentration; release or uptake of gases, such as ammonia or oxygen; light emission, absorption, or reflectance; heat emission; and so forth, brought about by the reaction catalyzed by the enzyme. The transducer converts this signal into a measurable response, such as current, potential, temperature change, or absorption of light through electrochemical, thermal, or optical means. This signal can be further amplified, processed, or stored for later analysis (29). Advantages of enzymatic biosensors include high selectivity, fast response, possibility of regeneration, simplicity involved in constructing the devices, and low costs (30). Drawbacks will include sensitivity to environmental conditions, limited lifetime, and inhibition by substances in the sample (26).

Clinical-based enzyme biosensors may be classified as *off line*, *in vivo*, and *on line* detectors (10). In *off line* detectors, biological samples are put in the measuring chamber of the biosensor where the concentration of the analyte of interest is measured; commercial glucose belongs to this type of detectors. The entire process lasts only few seconds and these devices are disposable (31). *On line* detectors are coupled with a flow line coming from a sampling device in contact with the biological sample. *In vivo* detectors are biosensors implanted in the patient body and continuously read the concentration of the analyte of interest.

3.1. Immobilization Methods

Immobilization is a key stage in biosensor design and is the basis to achieve the interface between the biological material and the transduction system. Biosensors are usually designed with high enzyme load to ensure sufficient biocatalyst activities, and the enzymes are provided with an appropriate environment to sustain their activities (32). Additionally, the stability of the enzyme in the biosensor is of paramount importance. In order to obtain stable activity for long time, enzymes are fixed on suitable solid supports. The process is known as immobilization. Immobilization means associating the biocatalysts with an insoluble matrix, so that it can be retained in proper reactor geometry for its economic reuse under stabilized conditions (33). The immobilization method will depend on the biological recognition element, the type of transducer, the physicochemical properties of the analyte, and the operating conditions. The improper strategy of selection for the development of immobilization technique can cause damage to the conformation of biomolecules leading to the inactivation of biomolecular activity (34).

Immobilization methods include adsorption, covalent binding, cross-linking, and entrapment (see Figure 3). With the adsorption method, the biomaterial is attached to the surface of the sensor by van der Waals forces, hydrophobic forces, hydrogen bonds, and ionic forces. Organic substances like charcoal, silica gel, clay and

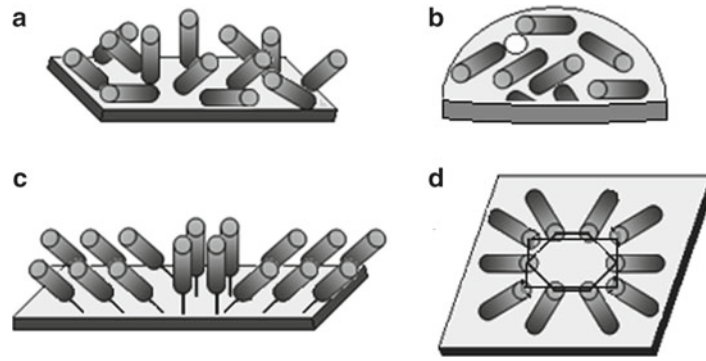

Fig. 3. Immobilization method illustrations (**a**) adsorption, (**b**) covalent binding, (**c**) entrapment, and (**d**) cross-linking.

inorganic substances as cellulose, starch, and collagen are used to immobilize enzymes with this method on the biosensor. In covalent binding the sensor surface is treated as a reactive group to which the biological materials can bind (33). It requires mild conditions under which reactions are performed, such as low temperature, low ionic strength, and pH in the physiological range (32), and their cost is expensive (35). Substances as agarose, cellulose, and resins are commonly used for immobilization in this method. In the cross-linking method, biological material is chemically bound to solid supports or cross-linking agents as glutaraldehyde and hexamethyl diisocyanate. This process is known as coreticulation, since it creates complex matrices that make multienzyme immobilization possible (36). In the entrapment method a semipermeable membrane separates the analyte and the bioelement where the sensor is attached to the bioelement. The porous entrapment scheme is based on forming a porous encapsulation matrix around the biological material that helps binding it to the sensor (33). Substances such as gelatin, collagen, alginate, and polyacrylamide have been commonly used for immobilization in the entrapment method.

3.2. Nanotechnology

Nanotechnology plays an important role in biosensor design. Nanotechnology involves the study, manipulation, creation, and use of materials, devices, and systems typically with dimensions smaller than 100 nm (37). Electrochemical biosensors incorporating enzymes with nanomaterials are new materials with synergistic properties originated from the components of the hybrid composites. Biosensors based on nanotechnology have an excellent scenario as a new generation of bioelectronic devices with high sensitivity and stability (38). Nanoscale materials have been used to achieve direct wiring of enzymes to electrode surface, to promote electrochemical reaction, to impose nanobarcode for biomaterials, and to amplify signal of biorecognition event (39). Commonly used nanomaterial for enzyme biosensors are carbon nanotubes

(CNT) and gold. CNT are graphite sheets rolled up into a nanoscale, which have diameters ranging between fractions of nanometers and tens of nanometers and lengths up to several centimeters with both their ends normally capped by fullerene-like structures (40). Gold as a precious metal shows high catalytic capability for many organic reactions. Therefore, metal nanoparticles have been used to design biosensors to catalyze biochemical reactions. In addition, nanoparticles behave in the reaction medium as conventional homogeneous catalysts, but can be easily recovered after the reaction (37).

4. Lipase and Phospholipase Biosensors

4.1. Lipases

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of fats and oils (41) as long-chain mono-, di-, triacylglycerols (TAGs), phospholipids, and cholesterol esters. The main reaction occurs at the lipid–water interface. This characteristic distinguishes lipases from other hydrolytic enzymes and makes them unique target proteins with regard to selectivity and inhibition (42). Titrimetric, colorimetric, fluorimetric, turbidometric, chromatographic, radiometric, enzymatic, physical, and immunological procedures may be used to detect lipase activity (43). Existing methods to control the lipase activity are not yet suitable for non-purified samples and for large-scale analyses due to their cost and time requirements (44). Therefore, specific devices like biosensors are suitable as an alternative for lipase activity detection (21). A biosensor should be distinguished from analytical systems such as high performance liquid chromatography (HPLC) or flow injection analysis (FIA) that incorporates additional separation steps or hardware. HPLC or FIA systems may incorporate a biosensor as a detecting device; however, a FIA system containing a reagent reservoir, an enzymatic or immunological reactor, and, downstream, an electrochemical sensor is not a biosensor (17).

Diverse biosensors have been reported to detect lipase activity. A surface acoustic wave was developed to detect pancreatic lipase activity (45). The sensor was based on the change in conductance of the solution caused by the release of fatty acid. Triolein was used as substrate. A linear relationship between frequency response and enzyme concentration was obtained. An amperometric sensor for detecting lipase activity based on glycerol dehydrogenase/NADH oxidase was reported in ref. 46. A Prussian Blue modified screen-printed electrode was selected as substrate for the immobilized-enzyme systems. Diverse parameters such cofactor and coenzyme concentrations, pH effect, response time, and storage stability were evaluated and optimized. In spite of the importance of determining

lipase activity, most lipase biosensors are related in using lipases for determining other compounds. A lithium niobate piezoelectric crystal-based surface acoustic wave sensor connected to a couple of parallel Pt electrodes in a detection cell was used to detect dimethoate, an organophosphate insecticide (47). A lipase solution was put in the detection cell. The surface acoustic wave sensor was used to record the change in resonance frequency with time with a linearity of 0.167–1.34 μg/ml dimethoate and detection limit of 81 ng/ml dimethoate.

Lipases are important drug targets or marker enzymes in the medical sector. They can be used as diagnostic tools and their presence or increasing levels can indicate certain infection or disease (15). High concentration of cholesterol can cause heart disease, hypertension, arteriosclerosis, coronary artery disease, cerebral thrombosis (34). High concentration of triglycerides can cause hyperlipidemia which has been associated with several disorders including diabetes mellitus, liver obstruction, and endocrine (48). Detection of triglycerides and cholesterol is of extreme importance in order to prevent unwanted disorders. The development of lipase sensors has been strongly focused on devices for the detection of triglycerides and cholesterol. The basic concept is to utilize a lipase to generate glycerol from triacylglycerol and quantify the released glycerol or alternatively the non-esterified fatty acid by chemical and enzymatic methods, enabling physicians precisely to diagnose patients with cardiovascular complaints (49).

4.1.1. Triglycerides

The production of fatty acids by lipolysis under specific reaction conditions may result in pH changes (50). A pH ISFETs sensor and a microreactor containing silica gel beads with surface immobilized lipase were used to detect triglycerides (51). Immobilization methods given by chemical bond to the surface of glass beads coated with keratin and adsorption onto nitrocellulose sheets had satisfactory behavior. The highest sensitivity was obtained for tributyrin (0.478 pH/mM for concentrations <4 mM), while the widest linear range was obtained for triacetin (up to 30 mM). Enzyme solution-oxidized porous silicon–crystalline silicon structure was used to detect changes in pH during the hydrolysis of tributyrin as a shift in the capacitance–voltage characteristics. Porous silicon, prepared from p-type (100) crystalline silicon was thermally oxidized and used to immobilize lipase, an ezyme, which hydrolyzes triglycerides resulting in the formation of fatty acids. This causes a change in the pH of the solution (52). Glycerol and triglycerides were determined in an amperometric biosensor by means of glycerol dehydrogenase and lipase. Glycerol dehydrogenase was adsorbed on the electrode, entrapped in gelatin, immobilized in polylysine gel orb organic salts. Sensitivity of the electrodes varied from 2 to 9 nA/mM glycerol with steady state. NADH was produced by the catalytic oxidation of glycerol in the presence of

glycerol dehydrogenase immobilized on the surface of an electrode (53). Glycerol and triacylglycerol were monitored with a flow-injection enzymatic system. The best approach to measure glycerol and triacylglycerols was with the connection of a lipase column and a silica-fused capillary column co-immobilized with glycerokinase and glycerol-3-phosphate oxidase. Lipase helped the breakdown of triacylglycerol to yield free fatty acids and glycerol. The proposed biosensor exhibited a flow-injection analysis peak response of 2.5 min and a detection limit of 5×10^{-5} M, with linear working ranges from 10^{-4} to 10^{-2} M for glycerol and from 10^{-3} to 10^{-2} M for triacylglycerol. The enzyme reactor was stable up to 2 months in continuous operation, and it was possible to analyze up to 15 samples per hour (54). Amperometric triglyceride biosensor was designed based on commercial lipase, glycerol kinase, and glycerol-3-phosphate oxidase was co-immobilized onto egg shell membrane through covalent coupling (55). The biosensor showed optimum response within 10 s. The current was proportional to concentration of triglyceride in the range 0.56–2.25 mM. The minimum detection limit of the method was 0.28 mM. The biosensor could be used 200 times in a period of 70 days. Biosensor was still hampered by the awkward nature of the detection method requiring different enzymatic reactions; nevertheless, amperometric biosensors have the best perspectives out of all electrochemical biosensors (21).

With the advances in nanotechnology, it has become possible to design more sensitive and specific devices. For example a triglyceride potentiomentric biosensor based on immobilizing enzymes in an ISFET. The new approach of this bionsensor consisted in using magnetic nanoparticles to immobilize the enzymes over the ISFET gate surface (56). Enzymes immobilized could be conveniently retained on the ISFET gate surface by providing a laboratory magnet underneath the gate. Tributyrin, trioctanoate, and triolein were detected in the range of 5–30 mM, with fast response (<5 min). The biosensor could be used for several days without any loss of linearity. Additionally the measurements could also be made in small volumes (0.2 ml) reducing the requirements of large sample volume. Another version of this biosensor was reported and includes nanocomposite film comprising of polyaniline and single-walled carbon nanotubes (SWCNT) mounted onto indium-tin-oxide-coated glass plate using electrophoretic technique. In this case glycerol dehydrogenase and lipase were immobilized via *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide. Response of the biosensor states that tributyrin could be detected in the range 50–400 mg/dL, with a response time of 12 s, high sensitivity as 4.28×10^{-4} mA/(mg·dL) and storage stability of about 13 weeks (57). Lipases for triglyceride detection were immobilized in sol–gel derived Nano-CeO_2 film (35 nm). The lipase/nano-CeO_2/indium-tin-oxide biosensor shows linearity as 50–500 mg/dL, low detection limit of 32.8 mg/dL, response time of 20 s, shelf life of 3 months, and sensitivity of 0.195 mA/mg·dL·cm^2 (58).

Triglycerides were determined with a biosensor where the detection method was based on Fast Fourier Transform (FFT) in a flow injection system. Lipase and glycerol dehydrogenase enzymes were immobilized on CeO_2 nanoparticles and multiwall CNT. Under optimal detection conditions, the linear response was in the range of 1–100 mg/L with detection limit of 0.5 mg/L (59). A biosensor to detect diglycerides hydrogen peroxide, formed through the enzymatic breakdown of DG via lipase, glycerol kinase, and glycerol 3-phosphate oxidase, was electrochemically oxidized at an applied potential of +0.5 V in Ag/AgCl reference electrode. The current generated was proportional to diglyceride concentration in range of 0–25 μM in phosphate buffer and bovine serum media (60). The performance of two sensors to detect tributyrin was compared. The electrolyte–insulator–semiconductor capacitor (EISCAP) had limited sensitivity of the EISCAP was limited to less than 500 μM whereas the microcantilever, a micromechanical device, was capable of detecting 10 μM of tributyrin levels (61).

4.1.2. Cholesterol

The cholesterol esterase (ChEt) also known as bile salt-activated lipase catalyzes the hydrolysis of dietary cholesterol esters, triacylglycerols, phospholipids, and vitamin esters via a serine protease mechanism (34). Several biosensors for cholesterol detection using a mixture of ChEt and cholesterol oxidase (ChOx) have been reported. A voltammetric and chronoamperometric modified carbon biosensor was used for the direct determination of cholesterol. Cholesterol ester hydrolase and ChOx were used as sensing elements. The current generated was proportional in the range 1.0×10^{-6}–1.5×10^{-4} M, with a response time of 30 s (62). Three enzymes: ChEt, ChOx, and peroxidase combined with flow injection potentiometry were used to detect cholesterol. The ChEt converts esterified cholesterol to free cholesterol, which is then oxidized by ChOx with hydrogen peroxide as product. A fast response of 30 s was observed for this sensor, with a linear slope between 0.05 and 3.0 mM (63). A patented device for cholesterol detection is described in ref. 64. The biosensor consisted of a feeder strip where the sample is put and easily transferred to the detection zone. An amperometric technique was used to detect the cholesterol/ChOx reaction. Linear responses in the range 2.81–13.0 mM were achieved for this device. ChEt and ChOx were immobilized individually and co-immobilized on arylamine glass beads through diazotization (65). Similar to the other reported methods cholesterol ester is hydrolyzed by ChEt to free fatty acid and cholesterol, which is oxidized by ChOx to cholestenone and H_2O_2. The lower detection limit of the method was 42.8 mg/L for individually immobilized enzymes and 21.4 mg/L for co-immobilized enzymes. The biosensor could be used 300 times without losing activity. A crystal sensor to measure in real time cholesterol concentration in buffer and serum using ChE, ChOx, and horseradish peroxidase (HRP) was developed in ref. 66. Linear range between 50 and

300 μM, and 25 and 400 μM was found for free cholesterol and low density lipoprotein cholesterol, with a response time of less than 25 min. Authors claim that the biosensor could be used for cholesterol determination in clinical samples. A method was developed for total serum cholesterol determination employing ChEt, ChOx, and peroxidase co-immobilized onto alkylamine glass beads through glutaraldehyde coupling with a conjugation yield of 2.3 mg/g of support and 76% retention of initial activity. The minimum detection limit of the method was 50 mg/dL with linear response between A_{520} and cholesteryl acetate concentration ranging from 5 to 50 mg/dL (67). A biosensor based on an oxygen electrode with gold cathode and Ag/AgCl anode electrode was developed for cholesterol detection. ChEt and ChOx were immobilized by cross-linking with glutaraldehyde and Bovine Serum Albumin. The sensor presented linearity in the range of 2–50 mg/dL and could be used over 30 times. The biosensor was used to determine cholesterol in food samples such as egg and meat (68). A biosensor made of a polyvinyl chloride beaker was chemically modified for covalent immobilization of cholestryl esterase and ChOx using glutaraldehyde as a coupling agent. A fabricate enzyme reaction beaker acts as an amperometric electrochemical cell for three electrode-based system for measurement of serum total cholesterol. The sensor response was of 20 s. The reaction cell lasted over 100 days and lost 50% of its initial activity during its regular use of 200 times (69). A chronoamperometric biosensor determining concentrations of esterified and free cholesterol was developed using homemade potentiostat and disposable strips immobilized with Fe_3O_4, ChOx, and ChEt. The sensor was based on the detection of the reduction signal of hydrogen peroxide generated in two enzymatic reactions. The sensing device had a linear response over the range of 100–400 mg for cholesteryl oleate and a detection limit of 19.4 mg/dL (70).

A wireless biosensor system to continuously monitor the total cholesterol concentration in fish (Nile tilapia, *Oreochromis niloticus*) was reported in ref. 71. ChOx and ChEt were immobilized using glutaraldehyde on sensor built with Pt–Ir wire ($\varphi = 0.178$ mm) as the working electrode and Ag/AgCl paste as the reference electrode. The sensor had a linear output correlated with the cholesterol level in the range of 2.65–403 mg/dL and could be used over 40 h monitoring total cholesterol levels in free-swimming fish in an aquarium. An amperometric biosensor by entrapment immobilization of ChEt and ChOx onto conducting polypyrrole was developed to detect cholesterol. The biosensor had linear response from 1 to 8 mM and shelf life of 4 weeks with about 15 estimations of cholesterol. The sensitivity of the electrode was 0.15 μA/mM (72). An amperometric biosensor based on sensing and reference electrodes in simultaneous contact with an integrated reagent layer was developed to detect total cholesterol. The integrated

Table 1
Description of various lipase biosensors

Analyzed substance	Type of biosensor	Linearity	Sensitivity	Reference
Dimethoate	Acoustic	0.167–1.34 μg/mL	81 ng/mL	(47)
Glycerol	Electrochemical amperometric	2–9 nA/mM		(53)
Triglycerides	Electrochemical amperometric	0.56–2.25 mM	0.28 mM	(55)
Triglycerides	Electrochemical potentiometric	5–30 mM		(56)
Triglycerides	Conducmetric	25–300 mg/dL	2.59×10^{-3} 1/(kΩ·mg·dL)	(74)
Triglycerides	Electrochemical amperometric (CNT)	50–400 mg/dL	4.28×10^{-4} mA/mg·dL	(57)
Triglycerides	Electrochemical Sol–gel	50–500 mg/dL	32.8 mg/dL	(58)
Triglycerides	Fast Fourier Transform	1–100 mg/L	0.5 mg/L	(59)
Cholesterol	Chronoamperometric	1.0×10^{-6}–1.5×10^{-4} M	9,500 mA/M	(62)
Cholesterol	Chronoamperometric	0.05–3.0 mM	60.2 mV	(63)
Cholesterol	Electrochemical amperometric	2.81–13.0 mM	0.255 mA/M	(64)
Cholesterol	Acoustic	50–300 μM		(66)
Cholesterol	Chronoamperometric	100–400 mg/cholesterol	19.4 mg/dL	(70)
Cholesterol	Potentiostat	2.65–403 mg/dL		(71)
Cholesterol	Electrochemical amperometric	1–8 mM		(72)
Cholesterol	Electrochemical amperometric	50–500 mg/dL	50 mg/dL	(73)

reagent was formed by ChEt, cholesterol dehydrogenase, coenzyme, redox mediator, surfactant, stabilizer, filler, and at least one aqueous thickening agent. The sensor had a linear response of 50–500 mg/dL cholesterol acetate and a minimum detection limit of 50 mg/dL (73). Table 1 shows a brief description of lipase biosensors.

4.2. Phospholipases

Phospholipases are enzymes that hydrolyze phospholipids (14). Phospholipids are present in all living microorganisms as a major component of all biological membranes. Phospholipases are important markers in pancreas and coronary diseases (75, 76). Diverse biosensors to detect phospholipids are reported in the literature. A biosensor to detect phospholipase D activity in rapeseed was developed. A Clark-type oxygen sensor was used to co-immobilize choline oxidase and catalase using glutaraldehyde and cyclohexyl

isocyanide. The sensor had a linear response in the range 3.34×10^{-3}–0.167 mM and was used for 600 assays over an 18-month period under different pH conditions (77). A biosensor to monitor phospholipids in serum samples was developed. Phospholipids are hydrolyzed by the enzyme phospholipase-D to choline which is analyzed by a fiber-optic biosensor based on choline oxidase and oxygen transduction. The linear range for the biosensor was 0.08 ± 3.00 mg/ml phosphatidylcholine (78). Another choline biosensor based on the determination of H_2O_2 generated at the electrode surface by the enzyme choline oxidase was developed. This biosensor was used to detect choline released from phosphatidylcholine by phospholipase D. Choline oxidase retained onto a HRP was immobilized in a solid carbon paste electrode. Amperometric detection of choline oxidase was realized at an applied potential of 0.0 mV versus Ag/AgCl. The sensor has a linear concentration range of 5.0×10^{-7}–7.0×10^{-5} M, with a detection limit of 1.0×10^{-7} M (79). Amperometric detection of choline was realized at an applied potential of –0.2 V in a saturated calomel electrode in 1/15 M phosphate buffer solution (pH 7.4) with a linear response range between 5.0×10^{-6} and 6.0×10^{-4} M choline and a response time of 15 s. Choline oxidase and HRP were cross-linked onto a HRP-immobilized carbon paste electrode (80). Quantitative determination of total, free, and phosphatidyl-bound choline in milk and a dietary supplement using phospholipase D packed bioreactor coupled to a choline oxidase-based amperometric biosensor is reported in ref. 81. The response for choline and phosphatidyl choline was linear up to 0.5 and 1 mM, respectively, and the detection limits were 0.02 and 0.03 mM, respectively.

A panel of monoclonal antibodies was raised against recombinant bovine p85a to probe its multidomain structure in relation to function using a BIAcore biosensor instrument. Phosphatidylinositol 3-kinase, which generates putative novel second messenger phospholipids, is a heterodimer composed of regulatory adaptor 85-kDa and catalytic 110-kDa subunits. Epitopes for nine monoclonal antibodies were mapped in relation to the main structure of p85a using recombinant protein fragments expressed in bacteria. Real-time binding experiments using phospholipid-containing vesicles showed that p85a by itself could specifically bind certain phospholipids (82). A capacitive biosensor was used to determine phospholipase activity. The sensing electrodes have a structure $Au/S(CH_2)_{17}CH_3$/substrate/electrolyte. Hydrolysis of the substrate by phospholipase A_2 leads to the formation of water-soluble products from the insoluble substrate. Desorption of these products into aqueous phase increases the electrode capacitance. The detection limit of the biosensor is 0.5 ng/mL (500 μU/mL) (83). A sensor was used to study the role of Ala426 and Lys438 of phospholipase D from *Streptomyces septatus* TH-2 (TH-2PLD) in substrate recognition. Liposomes were captured on the surface of a Sensor Chip L1 (Biacore AB) as "ligand". By substituting Ala426

and Lys438 with Phe and His, respectively, the inactive mutant showed a much stronger interaction with phosphatidylcholine and a weaker interaction with phosphatidylglycerol than the inactive TH-2PLD mutant. It was demonstrated that Ala426 and Lys438 of TH-2PLD play a role in sensing the head group of phospholipids (84). A biosensor for quantification of lipoprotein-associated phospholipase A2 was achieved using iridium-modified carbon-based biosensor. The biosensor was based on the hydrolysis of short-chain carboxylic esters, which produced organic acids and alcohol. The alcohol was then oxidized by alcohol oxidase producing hydrogen peroxide, which was detected electrochemically and was quantified using the iridium-modified carbon-based biosensor. The detection of the lipoprotein-associated phospholipase A2 was linear in the concentration range 0–150 U/ml, with a sensitivity of 1.45 nA/U in bovine serum (85). A liquid crystal-based sensor for real-time identification of phospholipase-like toxins was also developed. The sensor can selectively identify beta-bungarotoxin, when it hydrolyzes a phospholipid monolayer self-assembled at aqueous liquid crystal interface, through orientational responses of liquid crystal. As a result, optical signals that reflect the spatial and temporal distribution of phospholipids during the hydrolysis can therefore be generated in a real-time manner. The sensor is sensitive and requires less than 5 pg of beta-bungarotoxin for the detection (86). By integrating enzyme triggered liposome release with controllable Au nanoparticles aggregation it was possible to develop a sensor system which makes use of a natural non-immobilized lipid substrate to detect phospholipase activity. With this sensor, enzyme concentrations as low as 700 pM were detected in real time (87).

The Förster resonance energy transfer (FRET) sensor is capable of measuring intracellular phospholipase A2 in living cells and small organisms. The probe was modified to detect particular isoforms. Arachidonic-acid-mimicking fatty acids were prepared by copper-mediated coupling reactions. Probes with a single double bond in the 5-position exhibited favorable substrate properties for secretory phospholipase A2. In vitro experiments with the novel unsaturated doubly labeled phosphatidylethanolamine derivatives showed preferred cleavage of the sensor by the physiologically important group V sPLA2 (88). A bionsensor named phosphatidic acid indicator (Pii) was developed based on the principle of FRET. In the biosensors a lipid binding is sandwiched with fluorescent proteins which are tagged with the plasma membrane-targeting sequence of K-Ras. The addition of synthetic phosphatidic acid or the activation of phospholipase Dor diacylglycerol kinase at the plasma membrane changed the level of FRET in Pii-expressing cells, demonstrating the response of phosphatidic acid indicator to phosphatidic acid. The biosensor detected divergent phosphatidic acid content among various cell lines as well as within one cell line and even phosphatidic acid distribution within the cell (89). Table 2 shows a brief description of phospholipase biosensors.

Table 2
Description of various phospholipase biosensors

Analyzed substance	Type of biosensor	Linearity	Sensitivity	Reference
phospholipase D	Amperometric	3.34×10^{-3}–0.167 mM		(77)
Choline	Amperometric	5.0×10^{-7}–7.0×10^{-5} M	1.0×10^{-7} M	(79)
Choline	Amperometric	5.0×10^{-6}–6.0×10^{-4} M		(80)
Phospholipase	Electrochemical	0–150 U/ml	1.45 nA/U	(85)

5. New Trends in Biosensor Design

Definitively, biosensors have demonstrated to be an excellent alternative or complement to classic detection systems such HPLC and FIA. The reduced size of biosensor has allowed developing commercial portable devices. Electrochemical nanobiosensors, incorporating enzymes with CNT and other nanomaterials, will keep improving the specificity and sensitivity, response times, dynamic ranges, robustness, and miniaturization. Lipase and phospholipase biosensors will be centered to clinical and pharmaceutical applications. Market for in vivo biosensors implanted in the patient body continuously reading the concentration of the analyte of interest will increase. Lab on chip is around us and surely we will hear more frequently about lipase and phospholipase biosensor applications in several knowledge areas different from medicine.

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