Chapter

Biosensors for Determination of Heavy Metals in Waters

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Abstract

Biosensors are nowadays a powerful alternative to conventional analytical techniques for controlling the quality of not only natural water but also process water used by the food industry during the production process, as well as waste-water prior to release into natural watercourses. The goal is to provide the required quality and safety of water from the standpoint of heavy metal contamination. The basic and most important characteristics of biosensors are high sensitivity, short response time, specificity, and relatively low production cost. Biosensors can detect the presence and measure the content of various toxic substances (pesticides, heavy metals, etc.) not only in water but also in food. Detection of contaminants, primarily heavy metals in water used in food production processes, is a potential area of biosensor application in the food industry. Biosensors can be adapted for direct and continuous (online) monitoring by measuring certain analytes that can affect the quality and safety of water. This chapter will give an overview of the development and application of biosensors in order to control the quality and safety of water from the standpoint of the presence of heavy metals.

Keywords: biosensors, heavy metals, natural water, waste water

1. Introduction

Monitoring of water pollution is very important for the preservation of the environment and prevention of negative impacts that it can have on human health. Therefore, great attention is paid to simplifying procedures for detection and monitoring of pollutants. Heavy metals are particularly dangerous due to their ability to accumulate over time in both plants and animals, as well as in water. For these reasons, there are already developed different methods that determine their concentrations generally in the environment.

Biosensors represent a simple, reliable, and fast solution for monitoring water pollution caused by various heavy metals. The small size of biosensor devices has enabled their in situ application, thus avoiding long-term and sometimes expensive measurements in laboratories.

According to IUPAC, biosensor represents a "self-contained integrated device, which is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is retained in a direct spatial contact with an electrochemical transduction element" [1, 2]. Biosensors allow not only determining the presence and overall biologically

available concentrations of heavy metals in water but also assessing their biological effects, such as toxicity or cytotoxicity, which are sometimes more important than chemical composition information.

2. General characteristics of heavy metals

The term "heavy metals" refers to all metals except Al, Na, Ca, Mg, and K, i.e., to all metals that have a density higher than 5 g/cm^3. It includes a number of physiologically important elements such as Fe, Cu, Zn and Mn, then highly toxic Pb, As, Hg, Cd, Sb, Cr(VI) and less toxic Au, Ag, Mo, Cr(III) and Co [3]. The physiological and toxicological effects of these elements represent a collection of very different mechanisms.

Even at very low concentrations, they pose a threat to the environment and human health, because they are not biodegradable, so heavy metals are the cause of one of the most serious pollution problems. The most important nonessential heavy metals which affect the surface water systems are cadmium, chromium, mercury, lead, arsenic, and antimony [4].

Heavy metals present in pesticides and therapeutic agents are additional pollution sources. Burning of fossil fuels containing heavy metals and increasing industrial applications of metals such as metal galvanizing, paint and varnish industry, and mining and chemical industries are the main source of pollution of water systems by heavy metals.

Heavy metals are transported with waste water at the place of discharge and contaminate water sources downstream from an industrial site. In water, heavy metals have the ability to bind to the surface of microorganisms, from where they are transported inside the cell where they can be involved in chemical reactions and change chemically.

The majority of known techniques can determine the total amount of heavy metal ions. In addition, laboratory techniques that are routinely used for the analysis of metal ions, such as atomic absorption spectrometry, inductively coupled plasma mass spectrometry, anodic stripping voltammetry, and X-ray fluorescence spectrometry, require sophisticated equipment, pretreatment of samples, or qualified operators.

However, today it is known that only certain oxidation states of biologically available metal ions pose the greatest risk to human health and the environment. For example, "Cr(III) is an essential nutrient required in insulin action and sugar and fat metabolism, while Cr(VI) is believed to be highly toxic and carcinogenic" [5].

2.1 Mechanism of heavy metal toxicity

Metals and metalloid ions can be divided into three groups according to their toxicity. The first group includes metals (metalloids) that are toxic at extremely low concentration, such as lead, cadmium, and mercury. "Metals of the second group (arsenic, bismuth, indium, antimony and thallium) are less toxic, i.e., they are toxic only in higher concentrations. The third group includes metals (metalloids) of essential importance, such as copper, zinc, cobalt, selenium and iron, which are necessary for different chemical and biochemical processes in the body, and are toxic only above a certain concentration." Concentration window "of these heavy metals is somewhere between toxic and maximum permissible limits" [6].

Table 1 gives critical concentrations of some heavy metals in natural waters according to EPA [7].

Metal	Max. allowable concentration (μg/ml)
Mercury	0.002
Arsenic	0.5
Lead	0.5
Copper	0.6
Cadmium	0.04
Zinc	5

Table 1.
Critical concentrations of some heavy metals in natural waters according to EPA

The toxic effects of heavy metals can be the result of changes in numerous physiological processes at the cellular or molecular level caused by the inactivation of the enzyme. It can also occur as a result of the blocking of functional groups of metabolically important molecules or by replacing the essential elements and disturbing the integrity of the membrane. A rather frequent consequence of heavy metal poisoning is the production of reactive oxygen species (ROS) due to interference with the transport activities of electrons, especially the chloroplast membrane [8]. This increase in ROS exposes cells to oxidative stress that leads to peroxidation of lipids, biological damage of macromolecules, membrane decay, and DNA splitting [9].

They can penetrate into the organism in elemental form, in salt form, or as organometallic compounds, wherein the process of absorption, distribution, deposition, and elimination depends on the form in which the metal is present. Metals are very toxic because they are either in ionic form or within the compound, soluble in water, and easily absorbed by living organisms [3].

The mobility of heavy metals in water is particularly affected by the pH of water, the presence of hydrated forms of Mn and Fe, the concentration of carbonates and phosphates, as well as the content of organic matter. In addition, if the medium is very acidic and increased redox potential, the mobilization of Cu and Pb occurs, and under the reduction conditions, the hydroxides Mn and Fe are mobilized.

Heavy metals which are mostly the subject of research and monitoring in water and also generally in the environment due to their pronounced toxicity are arsenic, chromium, lead, mercury, and cadmium, while zinc, cobalt, copper, iron, and manganese are also interesting because they belong to the group of essential elements. The level of toxicity for some of these heavy metals is at or slightly above the concentration in which they are naturally found in nature [10]. Heavy metals occur in the environment naturally or as a result of human activities. Natural sources include volcanic eruptions, weathering (acid rock drainage), and discharge into rivers, lakes, and oceans.

Anthropogenic sources of heavy metals have emerged with the development of society. For example, the release of metal from the dishes causes contamination of food and water with metals.

2.1.1 Essential metals

2.1.1.1 Iron

Iron belongs to a group of essential metals and is crucial for a number of synthetic and enzyme processes in the human body. Most of the iron in our body exists as part of the hemoglobin molecule or myoglobin molecule. In addition to the

vital importance it has for most living organisms, iron is potentially toxic at high concentrations. The effect of iron on aquatic organisms and their habitats is mostly indirect. Combined direct and indirect effects of contamination of the aquatic environment cause a decrease in biodiversity and number of fish. In aqueous solutions, the Fe^{3+} ion is in the form of the aqua complex, $Fe(H_2O)_6^{3+}$, which is quite hydrolyzed (hydrolysis starts at pH 1). Hydrolysis of Fe(III) ions depends on the type of ionic environment, temperature, and the presence of other substances. The results of the researches show that the most important chemical types are found in hydrolyzed solution.

2.1.1.2 Copper

Copper is a microelement of outstanding biological importance and is part of essential metabolic pathways. Copper ions play a key role in active centers of oxidoreductases, such as superoxide dismutase (Cu, Zn-SOD), [5], an enzyme important for maintaining a low level of free radicals in the cell, thus protecting biomolecules such as proteins and lipids from the pathological conditions.

Copper deficiency can cause anemia, because insufficient amount of copper causes poor absorption of iron, reducing the number of red blood cells. The lack of copper also reduces the amount of white blood cells and therefore the resistance of the organism to diseases. In general, copper is not considered to be a major ecotoxicological problem, but its widespread distribution and exposure to exhaust gases are certainly the reasons why copper is involved in the structuring of ecosystems. Copper is found in three oxidation states, Cu^{+}, Cu^{2+}, and Cu^{3+}, with the Cu^{2+} form being the most common. The most mobile forms of copper are Cu^{2+} and $CuOH^{+}$. In the aqueous environment, copper is found in three basic forms, as suspended, colloidal, and dissolved. The accumulation of copper in the aquatic environment results in the primary exposure of aquatic organisms. Aquatic organisms can accumulate dissolved copper by direct absorption through the body surface, while colloidal forms of this metal are introduced into the body by ingesting contaminated food.

2.1.1.3 Zinc

Zinc participates in the structure of many enzymes and is an essential element. It is attached to insulin and plays a significant role in the metabolism of nucleic acids and amino acids, DNA replication, and gene expression. However, like all other essential metals, zinc in higher concentrations is toxic to living organisms. Zinc can bioaccumulate in fish, and the degree of bioaccumulation usually depends on the exposure mode, as well as the conditions prevailing in the observed aquatic environment. Conditions that may affect the toxicity of zinc (but also other heavy metals) in the aquatic environment are the content of Ca and Mg, the pH of water, the content of the hydroxide (alkalinity), and the content of dissolved natural organic matter, i.e., humic substances.

2.1.1.4 Cobalt

The required amount of cobalt in the body is about 5 mg for vitamin B12 to avoid anemia. In general, cobalt has low toxicity. Gastrointestinal (digestive tract) absorption of soluble cobalt compounds is estimated to be 25%. However, cobalt is toxic to humans. When cobalt has been used as an additive in beer (for foam stabilization), severe biventricular heart failure and a high mortality rate were observed in heavy beer drinkers [11].

Long-term inhalation of cobalt dust irritates the respiratory tract and can cause chronic bronchitis, and cobalt salts can cause benign dermatosis. Cobalt occurs in oxidation states 0, +1, +2, +3, and +4, and most of its compounds have an oxidation number +2 and +3, of which the cobalt(II) compounds are more stable. Most cobalt(II) compounds have an ionic character (halides and numerous Co(III) complexes). Cobalt is relatively a nonreactive metal. It does not oxidize under dry and humid conditions at normal temperatures. It binds to halogen elements by heating. Cobalt is used in the production of artificial fertilizers and so can be found in higher concentrations in soil and water. It is also used in medicine, in the treatment of anemia that cannot be treated with iron.

2.1.2 Toxic metals

2.1.2.1 Lead

Lead in the environment mainly comes from anthropogenic sources such as combustion of fossil fuels, landfills and fires at landfills, waste industrial sludges, phosphate-based fertilizers, pesticides, and exhaust gases from vehicles.

It is found in the form of sulphates, sulphides, and carbonates. It is considered the leading environmental pollutant and is increasingly endangering the living world, especially the surrounding areas of large industrial plants, frequent roads, and large cities.

The intensity of the adoption of lead depends on its concentrations in soil, soil pH, organic matter content, ratio of cations and anions, and other environmental factors. Human is exposed to toxic effects of lead by consuming food and water that are contaminated with this heavy metal but also by inhaling particulate matter with lead content. Absorption over the skin is only possible for tetraethyl and tetramethyl lead. Lead is rapidly absorbed into the bloodstream and binds to red blood cells in the form of Pb^{2+}, and via blood about 90% is deposited in the bones in the form of $Pb_3(PO_4)_2$. In the case of acidosis (increased acidity), the mobility of lead from the bones in the form of Pb^{2+} which has a toxic effect on the central nervous, circulatory, and immunological systems and kidneys can occur. [10]

2.1.2.2 Mercury

Mercury vapors and organic compounds of mercury are very strong poisons. Harmful substances are released by combustion of fossil fuels, and the risk of pollution threatens also due to increased use of mercury in industry and agriculture [12].

2.1.2.3 Chromium

In its compounds, chromium exists in several oxidation states: from bivalent to hexavalent. In solutions, chromium can occur in trivalent and hexavalent forms. Hexavalent chromium is usually present in the compounds as chromate $(CrO_4)^{2-}$ or dichromate $(Cr_2O_7)^{2-}$ ion. Cr(VI) is toxic due to its high degree of oxidation and easily enters the biological membranes. Therefore, this form of chromium is considered carcinogenic. Because chromium(VI) is toxic, carcinogenic, and mutagenic to living organisms, damages the liver, and causes lung congestion, skin irritation, and the formation of ulcer, it needs to be removed from the wastewater before their release into natural recipients. On the other hand, trivalent chromium, Cr(III), is 300 times less toxic than chromium(VI). Chromium is a vital nutrient for many animal and plant species, but it can also cause allergic reactions on the skin and can be carcinogenic [13].

3. Biosensors

A biosensor is an analytical device consisting of immobilized biological material in direct contact with a compatible transducer that will convert the biochemical signal into a measurable electrical signal. Biomolecules are responsible for specific recognition of the analyte, while the physicochemical converter provides electrical output signal that is amplified by electronic component [14]. Biosensors find application in various areas, from agriculture, food quality control, medicine, army, and control of various processes in the environment. Biosensors can provide quick information about the site of pollution, which is necessary for environmental control and monitoring. In addition, the advantage of biosensors over other analytical methods is their mobility that allows researchers to measure the in situ pollutant concentration and the ability to measure the concentration of pollutants in situ without additional sample preparation. Also, in addition to the determination of specific compounds, they can provide information on their biological effect (e.g., toxicity of a compound).

Due to exceptional performances, including high specificity and sensitivity, rapid response, low cost, relatively small size, and simple operation, biosensors have become an important tool for detecting chemical and biological components and their monitoring for clinical, nutritional, and ecological needs [15].

3.1 General characteristics of biosensors

Biosensors are analytical sensory devices that combine physical and chemical sensing techniques [16, 17]. Their performance is based on direct contact of two elements: biological and physicochemical, whose tight bond is achieved by physical or chemical methods of immobilization. Biological element serves as a receptor (bioreceptor), i.e., for the recognition of particular analyte from the medium of interest, based on the interaction of analyte and bioreceptor. Physicochemical transducer converts the response that occurs as a result of analyte-bioreceptor interaction on their interface into a measurable signal which can be processed and displayed in the form of readable values. For proper biosensor operation, the biological compound has to be immobilized in the vicinity of the transducer, and immobilization can be done either by physical entrapment or chemical attachment. Only small amounts of bioreceptor molecules are required, and they will be repeatedly used for measurements [18].

The displayed values are in correlation with the detected analyte-bioreceptor interactions, i.e., the concentration of a specific analyte or group of analytes in the analyzed sample [4, 16, 17]. General working principle of biosensors is illustrated in **Figure 1**.

Although widely used, conventional analytical techniques require sophisticated instruments and highly trained personnel to conduct operational procedures and

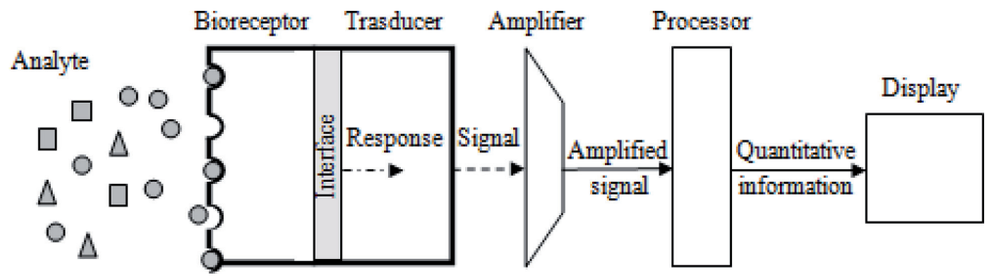

Figure 1.
Schematic illustration of a biosensor general working principle.

sample preparation, which makes them expensive and time-consuming [19, 20], thus not enabling determination of a large number of samples in a short time [21].

The main advantages of biosensors in relation to conventional analytical techniques are possibility of miniaturization and portability of device, reduced requirements for laboratory skills, reduced sample volume and pretreatment [1, 22], assessment of all possible types of analytes, inorganic or organic [23, 24], and possibility of performing single measurements or continuous real-time monitoring of analytes [1, 25]. Biosensors allow estimation of biological effects, e.g., toxicity of specific chemicals, because they can be used to detect their bioavailable concentrations [26].

3.2 Classification and types of biosensors

Biosensors can be divided into classes according to different approaches, among which the two are commonly used—type of biorecognition element (biocomponent, bioreceptor) and type of transduction system in biosensor. Each class of biosensors can be further classified into subclasses (**Figure 2**).

3.2.1 Classification by type of transducer

Based on the principle used in transduction systems, electrochemical, optical, piezoelectric, and thermal biosensors may be distinguished.

3.2.1.1 Electrochemical biosensors

The first proposed and commercialized biosensors were electrochemical biosensors, which is why they are most commonly reported. The basic principle of this class of biosensors is that the interaction between the biomolecule (bioreceptor) and the target analyte results in a chemical reaction that produces or consumes ions or electrons and in turn changes the electrical properties of the analyte solution, such as electrical current or potential. Transducer detects these changes by producing an electrochemical signal which is correlated with the amount of analyte present in the sample solution.

Advantages of electrochemical biosensors include minimal requirements for sample preparation and sensitivity at small sample volumes. It is also possible to perform sample analysis directly, which enables automation. Drawbacks of detections are poor reproducibility and stability [27].

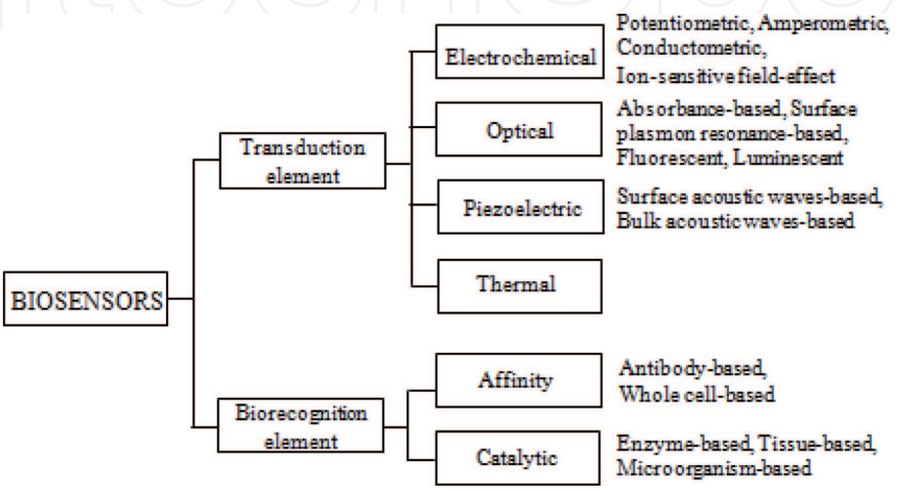

Figure 2.
Schematic illustration of the common classification of biosensors.

Electrochemical biosensors are classified according to the type of measured signal into subclasses: potentiometric, amperometric, conductometric, and biosensors based on ion-selective field-effect transistors (ISFETs). Different measurement principles always require a specific design of an electrochemical cell [21].

Potentiometric biosensors are based on the use of ion-selective electrode (ISEs) at the top of which an ion-selective membrane is placed which is responsible for selectivity to target ions in the presence of interfering ions in the sample. These devices measure the difference between the potential of the working and reference electrodes at essentially zero current, and this difference corresponds to the concentration of the analyte.

Amperometric biosensors are the most widespread class of electrochemical biosensors. Amperometric biosensors are more sensitive and faster than potentiometric but have poor selectivity and are susceptible to the interference of electroactive species that are not of interest [22, 28].

Conductometric biosensors are based on measurement of electrical conductivity in sample solution between two electrodes, as a consequence of the biochemical reaction. Conductometric biosensors operate at sufficiently low driving voltage, are not sensitive to light, do not require the use of a reference electrode, and can be produced using inexpensive technology [23, 24].

Biosensors based on the ion-selective field-effect transistors (ISFET) are the fourth class of electrochemical biosensors, suitable for the direct detection of ions. Change of activity of ions of a sample causes a change in the potential of the gate electrode that is brought into contact with the analyte solution. The change of the electric potential is then measured.

3.2.1.2 Optical biosensors

Optical biosensors are a biosensor class in which the transducer detects optical changes in the input light resulting from the interaction of the bioreceptor and the target analyte, and the amplitude of these changes is in correlation with the concentration of the present analyte in the analyzed sample. Among the significant advantages of these optical devices are insensitivity to electromagnetic interference, small instrumentation, simplicity, and noninvasiveness of measurement, as well as the possibility of application in vivo, since they are non-electrical biosensors. According to the optical configuration, biosensors can be intrinsic or extrinsic. In intrinsic biosensors, the incident light wave is closed in a wave guide or an optical fiber, along which it propagates, but the design of the structure in which the wave is closed is such that it allows the interaction of the wave with the analyte. In extrinsic biosensors, the light wave passes directly through the sample phase and reacts with it, and the optical fiber serves as a means of transmitting the signal.

Absorption-based biosensors are simple and inexpensive devices that allow the determination of concentrations of different analytes, based on the fact that each type of analyte absorbs a certain wavelength of light emitted into the sample. Guiding the light from the light source to the sample and from the sample to the detector can be performed using the same optical fiber or different fibers [29].

Surface plasmon resonance (SPR) biosensors use an optical detection technique where on the interface of metal and dielectrics, the amplified incident light hits the metal surface and excites the electrons, thereby generating electromagnetic waves (plasmons). Plasmon propagation is very sensitive to the changes in the refractive index of the material near the metal surface, which are caused by biomolecular interaction, such as, for example, specific binding of the analyte [30, 31].

Fluorescence-based optical biosensors can directly detect target atoms or molecules by measuring the change in the frequency of electromagnetic radiation emitted by

them. The frequency change is stimulated by the absorption of radiation and the consequent appearance of the excited state of the target species. Detection can also be carried out indirectly, using fluorescence labels or fluorescence energy transfer (FRET).

Luminescence-based biosensors can be classified into chemiluminescent and bioluminescent optical biosensors. Unlike biosensors based on fluorescence, in these sensor devices, the triggered state of the target atoms or molecules is obtained as a result of their exothermic chemical reaction, and while returning to the ground state, the excited species emit light without or with minimal heat. When such a chemical reaction occurs within a biological organism, then it is a bioluminescence.

3.2.1.3 Piezoelectric biosensors

Piezoelectric biosensors are devices in which the biorecognition element is integrated with a piezoelectric material used as a transducer. Among many types of natural and synthetic materials that exhibit a piezoelectric effect, quartz crystals are most commonly used [28, 32] because of their availability, as well as high temperature resistance and chemical stability in aqueous solution. The basic principle of measurement for this type of biosensor is based on the ability of a piezoelectric material to generate electrical potential when deformed under the applied mechanical stress, and vice versa, to elastically deform when exposed to an electric field.

3.2.1.4 Thermal biosensors

Thermal biosensors, also called calorimetric or thermometric, are a biosensor class in which the transducer detects interactions between bioreceptors and analyte resulting in a change of temperature, which is in correlation with the concentration of the analyte. As thermal transducers in these devices, thermistors or thermopiles are used [21, 33]. Some of the advantages of thermal biosensors are detection without the need for labeling of reactants, not requiring frequent recalibration, and no disturbances by electrochemical and optical properties of the sample [21, 34]. In most research papers published about this type of sensor, described experiments were carried out using enzyme-based thermal biosensors, due to the exothermic nature of the reactions catalyzed by enzymes.

3.2.2 Classification by type of biocomponent/bioreceptor

Biocomponent/bioreceptor is responsible for the detection and interaction with the analyte and therefore is a very important part of any type of biosensor. The receptor is responsible for the selective and sensitive recognitions of the analyte, and the energy liberated during the interaction of the analyte and the receptor is converted into an electrical signal that is suitable for measurement. The most commonly used biological elements are enzymes and antibodies. Biosensors can be divided into two main categories: biocatalytic and affinity sensors based on the interaction between biological material and analyte.

Biocatalytic biosensors, also known as metabolism sensors, comprise a biological component that catalyzes the chemical conversion of the analyte with which it interacts and detect the magnitude of the resulting changes such as product formation, reactant disappearance, or inhibition of the reaction, which are correlated with the concentration of the analyte [35]. Affinity biosensors are based on selective interaction between the analyte and the biological component through their irreversible binding, resulting in a physicochemical change detected by the converter.

3.2.2.1 Antibody-based biosensors

Antibodies are proteins produced by the immune system in response to a foreign substance in the body. Also known as immunoglobulins (Ig), they are Y-shaped proteins generated by a type of white blood cells called B lymphocytes (B cells). Their ability to recognize specific molecules makes them suitable for use as biorecognition component in biosensors. During the process of biological recognition, the antibodies bind tightly to antigens forming complexes. There are five classes of antibodies, based on their structure and function: IgA, IgD, IgE, IgG, and IgM. Among them, IgG is the class most frequently used for heavy metal detection, because of their higher affinity and specificity compared to other classes. Antibodies such as monoclonal, polyclonal, or recombinant can be utilized in biosensors. Monoclonal antibodies are homogeneous antibodies, derived from single B cell; thus they all have the same specificity, i.e., to bind to one unique epitope (binding site) on a specific antigen. Unlike monoclonal antibodies, polyclonal antibodies are produced from different B cells against the same antigen and therefore have affinity for various binding sites of that antigen. This feature of polyclonal antibodies results in their stronger binding to the target species, but due to the recognition of multiple epitopes, they have higher potential for cross-reactivity, i.e., specificity for nontargeted antigens with similar structural regions as the targeted one. The production of recombinant antibodies is enabled by genetic engineering. Important properties of antibodies for providing accurate results for detection and measurement using biosensors are high sensitivity and specificity, with minimal cross-reactivity [36].

Different types of approaches have been developed and used for immobilization of Abs onto a sensor surface, such as covalent binding, non-covalent immobilization, and coupling by affinity interactions, because the immobilization is the crucial step which can affect the optimal performance of an antibody-based biosensor [37]. Reaction conditions, such as temperature, pH, and ionic strength, can also affect the activity of the antibodies [38].

3.2.2.2 Enzyme-based biosensors

Enzymes are biocatalysts that catalyze chemical reactions. Their task is to translate the characteristic substance (substrate) into a product. Enzymes are highly selective for the particular substrate which makes them suitable sensor material. Detection mechanism of enzyme-based biosensors is based on activation or inhibition of their activities as a response caused by heavy metals. Usually the metal ion reacts with the thiol groups present in enzymatic structures that result in conformational changes and thus affect the catalytic activity. Different enzymes have been used for the structure of biosensors based on inhibition. Enzymes such as glucose oxidase, urease, glutathione S-transferase, alkaline phosphatase, lactate dehydrogenase, acid phosphatase, and invertase have been utilized to detect metals such as cadmium, lead, copper, mercury, zinc, etc. However, inhibition-based biosensors have an important disadvantage, which is insufficient selectivity because some of the enzymes simultaneously inhibit several metals.

Biosensors based on immobilized enzymes are also used, and they show several advantages compared to free enzymes:

- A thousand times lower consumption of immobilized enzymes.
- Reduced interferences in differential mode.
- No preincubation is required.

- Faster analysis, less than 5 min.

- In the case of reversible inhibition, sometimes reactivation of the enzyme activity is not necessary.

The problem with biosensors based on enzymatic inhibition is that only a few enzymes are sensitive to heavy metals.

3.2.2.3 Protein-based biosensors

Proteins, such as phytochelatins or metallothioneins, can be used as biological components in biosensors when immobilized on the surface of the transducer [39]. The interaction of proteins and metals in the biosensor is realized through the formation of complexes, and the detection technique does not require labeling. The resulting changes in the protein layer are detected by measuring the electrical capacity or impedance by the relevant transducer. Using the protein biosensor enabled the assessment of bioavailable concentrations of heavy metals. In addition, using capacitive sensors, which belong to the class of electrochemical biosensors, it is possible to achieve much higher sensitivity to low concentrations of heavy metals, compared to cell-based devices.

3.2.2.4 Whole cell-based biosensors

Whole cell-based biosensors are based on using biosensing cells, such as microorganisms, plant cells, algae, fungi, protozoa, etc., which can be natural or recombinant [40]. The use of whole cells as biological elements of recognition has many advantages. Whole cell-based biosensors are usually cheaper than biosensors based on enzymes, because the whole cells can be easily cultivated and are easier to isolate and purify compared with enzymes. Whole cells are more tolerant to a significant change in pH, temperature, or ionic strength. A multistep reaction is possible because one cell can contain all the enzymes and cofactors needed to detect the analyte. Biosensors of this type can easily be regenerated or maintained by allowing cells to regrow while working in situ. Preparation of samples is usually not necessary. Compared to enzyme-based biosensors, the disadvantages of these devices are that they are susceptible to interference of contaminants that are not targeted analytes. They also have a relatively slow response, compared to other types of biosensors.

3.3 Application of biosensors in detection and monitoring of heavy metals

The unique biosensor features make them widely applicable in the field of water quality control, from the point of view of detecting and determining the concentration of heavy metals. The use of biosensors for individual or continuous measurements is dependent on the type of biologically active element. Since biological compounds such as cholesterol, glucose, urea, etc. are generally not electroactive, the combination of reactions is needed for obtaining an electroactive element, which leads to a change of current intensity [41]. **Table 2** shows the classification of biosensors based on the recognition component that was utilized for the detection of heavy metals.

A proper immobilization of the biosensing element onto the transducer surface maintains biomaterial functionality while ensuring accessibility of the receptor cells toward analytes and proximity of the bioreceptor and transducer. The factors which determine the choice of a suitable physical or chemical immobilization method

Type of bioreceptor	Analyzed heavy metal	Reference
2A81G5	Cd	[42]
Antibody ISB4	Cd	[43]
12F6	U	[44]
Alkaline	Zn	[45]
Phosphatase	Hg	[46]
Pyruvate enzymes	Cd	[47]
Oxidase	Hg	[48]
Urease	Hg, Ag	
Glutathione S-transferase	Cd, Zn	[49, 50]
Mer R proteins	Hg, Cu, Cd, Zn, Pb	[51, 52]
Metallothionein	Cd, Zn, Ni	[53]
Whole cells and cardiac cells	Hg, Pb, Cd, Fe, Cu, Zn	[54]

Table 2.
Classification of biosensors based on the recognition component that was utilized for the detection of heavy metals

are physicochemical properties of the analyte, nature of the chosen biosensing element, the type of used transducer, and the operating conditions of biosensor. Antibody-based biosensors can be used as an alternative approach for the detection of metal ions, due to antibody features such as high specificity and binding affinity for antigens harmful for the organism. Detection mechanism of these devices is based on antibody-metal ion complex formation. The resulted response of their immunochemical interaction is converted by a transducer to measurable values and processed to readable values. Antibodies are capable for antigen detection in very low concentrations [38], but if their cross-reactivity is high, they can yield false-positive results of an assay of heavy metals in water [55].

A monoclonal antibody that recognizes 16 different metal-EDTA complexes has been produced and evaluated in terms of its binding affinity. The obtained results showed that the antibody has a maximum binding affinity for cadmium and mercury-EDTA complexes. [56]. In the inhibition immunoassay where the measurement of Cd^{2+} in water samples was carried out using monoclonal antibodies firmly bound to the cadmium-EDTA complex, but not to EDTA without metal [42], the biosensor showed satisfactory insensitivity to cations Ca^{2+}, Na^{2+}, and K^{1+} it encountered and achieved a reliable measurement in the presence of 1 mM of excess Fe^{3+}, Mg^{2+}, and Pb^{2+}.

Monoclonal antibodies were used to detect Pb^{2+} without labeling, in a localized surface plasmon resonance-based optical biosensor [57]. The results of the experiment showed that at optimal monoclonal antibody immobilizing conditions, absorbability increased to 12.2% for detecting 10–100 ppb Pb(II)-EDTA complex with a limit of detection of 0.27 ppb.

Kulkarni et al. were the first to develop acid phosphatase-based fluorescence biosensor for the analysis of heavy metal ions Hg^{2+}, Cr^{2+}, and Cu^{2+}. Increased concentration of metal ions resulted in increased enzyme inhibition and therefore decreased fluorescence. The enzyme was stable for more than 2 months at 4°C [58]. They also observed that mixture of heavy metal ions exhibit positive effect on the performance of biosensor.

The urease enzyme has been widely investigated as a possible biocomponent in heavy metal detection biosensors. Urease has been tested single and in combination with other enzymes. Electrochemical biosensor based on urease and glutamic dehydrogenase (GLDH) was developed for detecting heavy metals in water samples [59]. Also, a disposable potentiometric biosensor based on pure urease was developed, with the ability to detect copper and silver at sub-ppm level. For the detection of Pb and

Cd in liquid samples, biosensors based on the combination of urease and acetylcholinesterase (Ache) were developed as a biocomponent with a detection limit of 1 ppb in water samples. It is known that ions of heavy metals inhibit alkaline phosphatase which was used for forming the biosensor with alkaline phosphatase as a biocomponent. It was found that the sensitivity of the developed biosensor to Cd^{2+} and Zn^{2+} was 10 ppb, whereas, with regard to ion Pb^{2+}, there was no significant inhibition.

Two protein-based biosensors were developed on the basis of GST-SmtA and MerR [60] proteins, and their sensitivity and selectivity for heavy metal ions (Cd^{2+}, Cu^{2+}, Hg^{2+}, and Zn^{2+}) were measured using a capacitance transducer. Both types of biosensors have shown high sensitivity, enabling detection of metal ions up to femtomolar concentration.

Capacitance protein-based biosensor using synthetic phytochelatins (ECs) was developed for the detection of heavy metal ions (Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, and Zn^{2+}), and the results of the experiments showed a lower sensitivity for all metal ions except for Zn^{2+} compared to systems based on SmtA and MerR, which can be explained by conformational changes in the protein, taking into account that the change in capacitance is function of the resulting change in protein conformation [51].

In cell-based biosensors, bioelement is fused with reporter gene. The detection mechanism is based on the activation of the reporter gene upon the contact between bioreceptor and target analyte, yielding an output measurable signal that is a correlation with bioavailable concentration of heavy metal.

Various cell-based biosensors have been used for the detection of heavy metals in water due to their ease of production and field testing, the ability to perform fast single measurement, as well as continuous measurements, and the ease of identifying bioavailable concentrations of toxicants that allows estimation of effects that heavy metals have on living organisms.

The advantage of bacterial cells is resistance to environmental conditions that could destroy the sensory element if exposed to them, supplying it with a relatively stable environment. Due to specific metabolic pathways used in microorganisms, compared to isolated enzymes, microbial sensors have the potential for more selective analysis of heavy metals which cannot be measured by simple enzyme reactions [61].

In order to be available for any sensing mechanism that is based inside the cell, there is a need for analytes to be able to enter the cell via diffusion, nonspecific uptake, or active transport. Alternative approaches are implemented in the cases when membrane permeability for an analyte is not sufficient. These approaches include allocation of the recognition element to the outside of the cell or the introduction of an appropriate transport mechanism for importing the analyte [61].

A large number of studies in which performances of whole cell-based biosensors were tested have utilized electrochemical and optical transducers. For detection of heavy metal ions (Cd^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+}, Pb^{2+},and Zn^{2+}) at concentrations of 10μM, a mammalian heart cells-based biosensor was developed [54], with excellent performance in terms of frequency selection, amplitude and duration of detection within 15 min.

Biosensor, based on immobilized engineered bacteria *Alcaligenes eutrophus* (AE1239) and optical transducer, was utilized for monitoring the bioavailable copper ions in synthetic water samples, wherein the lowest limit of detection was 1 μM [62].

4. Conclusions

Biosensors have a very wide range of applications, from environmental monitoring, food safety, detection of various diseases, use in artificial implantable devices such as pacemakers to the detection of drugs.

Application for pollution monitoring requires the biosensor to work from several hours to several days. Such biosensors are a tool for "long-term monitoring." Whether it is a long-term follow-up or analysis of individual shots, biosensors are used as technologically advanced devices both in settings with limited resources and in sophisticated medical settings.

Considering the complex and critical situation in the field of environmental protection, and the state of natural waters from the aspect of pollution with heavy metals, and taking into account the toxicity of heavy metal ions, it is necessary to continuously work on finding new efficient techniques for their detection. Conventional analytical techniques can no longer satisfy the needs of constant monitoring and frequent field analysis of water because they are expensive, often with bulky equipment and a long analysis time, and require well-trained analysts. Biosensors can be used to overcome the limitations of conventional methods. In the future, designing a biosensor with the appropriate material will surely help in the selective identification of metal ions not only from water but also from any other matrix.

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