Prasun Kumar · Sandip Kumar Dash ·
Subhasree Ray ·
Shahila Parween *Editors*

Biomaterials-Based Sensors

Recent Advances and Applications

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Dedicated to our teachers

Foreword

Rapid medical techniques and diagnostics advancements have led to customized solutions for any given medical condition. Various polymers are being designed and developed based on their biodegradability, biocompatibility, and other properties to suffice the growing needs. For diagnostic purposes, a simple-to-use kit has always attracted attention from medical as well as commercial purposes. The spread of Covid-19 infection has shown us the utility of rapid viral detection kits in containing the viral spread in the community. Consequently, the extensive use of such kits also poses a threat to the environment. Therefore, it is imperative to use biomaterial-based sensors not only for medical diagnostics but in other areas as well. In the past decade, there have been a lot of efforts to use cellulose, polyhydroxyalkanoates, chitin, and other functionalized polymers for biosensor applications. The rising ecological concerns have enticed alternative polymers to be used in diagnostics. Yet, there are very few reference books covering this aspect. I have great pleasure in presenting this book to the readers, who I am confident would find it a very valuable and unique source of information. This book has many innovative features that I believe are noteworthy. Dedicated chapters on "Self-assembly and Fabrication of Biomaterials," "Nanohybrid Materials," and "Electrochemical Sensors in Agriculture and Veterinary Applications" are appreciable. Typically, such content is scarce in other foundational texts, and it is very helpful to have this type of review in this book as it sets the stage and establishes the context. With their vast experience and expertise, the editors of the book are among the leading researchers in the area of biosensors, who I would say are among the best to do this job. All the editors have been selective in bringing out this book as a unique compilation of themes oriented towards applications of biomaterials as sensors. This book comprehensively addresses the challenges and opportunities in the area of biosensors. I believe the content of this book has addressed recent advancements in this area with relevant illustrations/figures. I wholeheartedly endorsed this unique work by esteemed scholars and compiled by an experienced editorial team. This book will be equally beneficial for researchers, students, academicians, and professionals working in the area of biosensors. I compliment the editors and the authors of the various chapters for their valuable contributions.

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Ashok Kumar

Preface

Biomaterials are substances of biological origin that can be engineered to act alone or in association with other biological molecules for versatile applications, including clinical diagnosis, screening, and therapy. Miniaturization of devices and tools is one of the prime focuses of researchers nowadays through the use of nanoparticles, quantum dots, alloys, nanosheets, multi-walled carbon nanotubes, and graphene. These materials are further biofunctionalized through surface engineering to enhance antimicrobial, antifungal, and anti-cancer properties. The high surface area-to-volume ratio of these materials enables their fabrication onto different transducers to develop biochips and biosensors for multi-dimensional applications. The last decades have seen unprecedented activities in developing biosensors and other miniaturized analytical devices for detecting, quantifying, and monitoring numerous chemical and biological compounds. Considerable efforts were made toward the development of simple, quick, economical, sensitive, and specific diagnostic and screening devices to replace conventional methods. A biosensor generally consists of at least two functional components: a molecular recognition element (receptor) that selectively interacts with its target analyte (e.g., ions, DNA, antibodies, cells, and microorganisms) and a physicochemical transducer. The latter converts the bio-recognition information into a measurable quantity, being an electrochemical, electrical, optical, magnetic, mass-sensitive, or thermal signal. Since biological analytes are often hard to detect purely based on their intrinsic physical properties, biosensors often require labels such as enzymes and fluorescent or radioactive molecules attached to the targeted analyte. As a result, the final sensor signal corresponds to the number of labels representing the number of bound target molecules. The biomaterials conjugated to DNAs, proteins, and biopolymers to be used for drug delivery, pathogen detection, and pollution mentoring from different environmental samples. Biomaterials-based sensors can also be a magnificent tool for detecting different chemicals, explosives, and harmful gases as a precautionary measure for defense and safety issues. This book, *Biomaterials-Based Sensors: Principles, Design, and Applications,* has been written by experts in this area. Readers of this book will learn about various biomaterials and their potential in sensing applications. The idea of putting biomaterials as sensors into a single book comes from the concern over non-degradable or expensing base materials. There are a lot of non-biodegradable wastes being generated every year, and with the

increasing demand for sensors in various fields, biomaterial-based sensors would provide eco-friendly support. This aspect is rarely addressed in any sensor book. Here, we tried to keep topics that cover the basic principles and design of bio-based sensors followed by topics pertaining to unique applications. Chapters in this book cover the basics of biosensors, various methods used to synthesize different biomaterials, characterization, and their functionalization. The attempts made to fabricate these functionalized materials onto different transducers as biochips and biosensors for diagnosis and screening of diseases and drug delivery against various diseases will be covered in the book. The potential application of these biomaterials-based sensors in the field of agriculture, veterinary, and biomedical sciences are also discussed in this book. This book attempts to provide knowledge on the past, present, and future perspectives of biomaterials-based sensors in diversified areas to cover a large group of readers and researchers interested in this field. Finally, we would like to acknowledge the support from the contributing authors and suggestions received from the editorial office at Springer, Emmy Lee, and Ejaz Ahmad. None of this would have been possible without the contributions of all the authors, coworkers, and collaborators who accepted our invitation.

Cheongju, Republic of Korea — Prasun Kumar
Odisha, India — Sandip Kumar Dash
Greater Noida, India — Subhasree Ray
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About the Editors

Prasun Kumar, Ph.D. holds a Ph.D. in Biotechnology from CSIR-Institute of Genomics and Integrative Biology, Delhi, India. He is presently working as a Scientific Officer at DBT-IOC Center for Advanced Bioenergy Research, Faridabad. He has over 7 years of experience in applied microbiological research including about 2 years of experience in industrial R&D. His main areas of research are biopolymers, microbial biodiversity, bioenergy, microbial biofilms, quorum sensing, quorum quenching, and genomics. His present research is oriented towards valorizing lignocellulosic biowastes into value-added products such as biopolymer, 2G ethanol, bioenergy, and antibiofilm compounds. To his credit, there are over 34 articles in SCI journals, 5 books, and 11 chapters with international publishers. He has been serving the scientific society by reviewing articles for several SCI journals and delivering guest lectures. Publons awarded him the peer review award in the year 2018. He also serves as the editorial board member of a few international journals.

Sandip Kumar Dash, Ph.D. received Ph.D. in Zoology from CSIR-IGIB, Delhi. He is currently working as an Assistant Professor at the Department of Zoology, Berhampur University, India. He has over 10 years of research and teaching experience. His research interest includes DNA biosensors and carbon nanotubes for various biotechnological applications. He has published more than 15 research works in SCI journals. He is also leading research projects and is a member of several committees at Berhampur University. Dr. Sandip also serves the scientific society by delivering guest lectures and reviewing scientific articles for SCI journals. He is

also a life member of various national and international societies. His works on bacterial meningitis have received significant attention in the community. His current research deals with the utilization of multi-walled carbon nanotubes/manganese dioxide composites for biomedical applications.

Subhasree Ray, Ph.D. is currently working as an Assistant professor at Sharda University, Greater Noida, Uttar Pradesh, India. She earned her Ph.D. degree from CSIR-IGIB, Delhi in 2018. She received the prestigious CSIR-SRF fellowship. Her main research work was focused on the production of biopolymers from waste biomass. After Ph.D., she joined as a postdoctoral researcher at Chonnam National University, and the University of Seoul, South Korea. Here, her main focus was the anaerobic digestion of food wastes for methane production. She also studied methanogenesis at a 4000 L pilot-scale plant. After the successful completion of 1 year, she joined another project at Yeungnam University, South Korea. During that period, she worked on several fungal toxins and their inhibition from fermented food. She also worked on biofilm inhibition of pathogenic organisms by natural bioactive compounds. To her credit, she has 16 research papers published in peer-reviewed journals and 8 book chapters. In addition, she is a life member of various scientific societies and also a member of various committees at Sharda University for Graduate and Undergraduate programs.

Shahila Parween, Ph.D. has acquired her Ph.D. (Biological Sciences) and high-quality postdoctoral studies at two flagship research institutes of the Council of Scientific and Industrial Research (CSIR), Government of India, namely the Institute of Genomics and Integrative Biology (IGIB) and the Centre for Cellular and Molecular Biology (CCMB), respectively. Currently, she is working as a Research Scientist at MNR Foundation for Research and Innovations (MNR-FRI), MNR Medical College and Hospital, Telangana, India. She has a strong experience of more than 10 years in biosciences and biomedical research which is evidenced by her 15 plus publications in internationally leading journals, and successful prototype developments for

lipid profiling, on-farm mastitis detection, early cancer detection, whole blood glucose monitoring, and ultra-fast ELISA. Her research interests focus on microfluidics, point-of-care detection, development of affordable diagnostic methods, nanomaterial for biosensing, biopolymers, biosensors, chemical activation of biopolymers, immobilization techniques, medical devices, image-based diagnostic assay, and immunoassay methods. Apart from research, she has a good experience in incubation and entrepreneurship and had conducted Gandhian Young Technological Awards 2020 (GYTI-2020) and Biotech Innovation Ignition School (BIIS) organized by SRISTI-BIRAC. She has successfully conducted various national-level workshops/conferences in close association with BIRAC, DBT, Govt. of India. She is also a life member of various scientific associations and a reviewer of several scientific journals.

Part I

Introduction

Biomaterials and Biopolymers for the Development of Biosensors

M. Luz Scala-Benuzzi, Sofía V. Piguillem Palacios, Eduardo Andrés Takara, and Martín A. Fernández-Baldo

Abstract

Currently, the great diversity of biomaterials and their wide application field have expanded the definition of these materials. A biomaterial is a substance designed for the course of any diagnostic or therapeutic procedure used in veterinary or human medicine, a definition that we will take into account throughout this chapter. This new science field is continually researched and expanded, year after year, increasing the potential of biomaterials. These materials are classified according to biocompatibility and interaction with the tissues (natural, ceramic, and composite materials), mechanical load bearing (metals), or flexibility required (biopolymers). Biomaterials are incorporated as biosensing platforms generating devices with high sensitivity and specificity, rapid detection, besides being portable, economical, and easy to use. Among biomaterials, biopolymer-based materials have been positioned as excellent options for biosensor development, due to their unique advantages, such as environment-friendly manufacturing methods, biocompatibility, and biodegradability. This chapter introduces different biomaterials used for biosensor development, to finally describe a type of biomaterial, such as biopolymers and their applications.

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Biomaterials · Biopolymers · Platforms · Biosensors · Immobilizations

1 Introduction

In recent decades, materials science has enormously evolved due to its implication in industry, everyday life, as well as in the development of new materials in scientific research. Scientific advances that integrate different disciplines, such as biology, chemistry, engineering, and physics, result in materials that are increasingly ecological, environment-friendly, robust, and with many other properties that depend on the application field. These materials can include nanomaterials [1], porous materials [2], semiconductor materials [3], topological materials [4], and the ones that will be discussed in this chapter: biomaterials.

The biomaterial definition has changed over time, adapting to its practical purposes; furthermore, its classification involves interesting points of view that entail a study of the materials' origin as their incorporation into science. No less important are the different synthesis methods, since it is there where new materials emerge and where enormous scientist inventiveness is applied. Broadly, top-down and bottom-up fabrication techniques predominate, but they can be classified according to the types of bonds among the compounds that form the biomaterial (based on covalent bonds or self-assembled materials). However, biomaterials such as metals, ceramics, and alloys are being replaced by biopolymers, and this is not only because of their abundance but also, they are ecological and eco-friendly. These kinds of properties are currently receiving a lot of attention due to the negative impact that nanomaterials are having on the environment.

In this chapter, a summary of biomaterial definition and classification is presented, going through the different synthesis methods, and reaching the use of biopolymer-based biomaterials. Moreover, the incorporation of different biomaterials in biosensors as an interesting application is mentioned due to biomaterial offers a versatile opportunity to turn these devices into excellent detection tools. These modified devices are used to detect and quantify analytes for various purposes, used in a practical, reliable, and economical way [5, 6]. Finally, the conclusions and perspectives for the future will be explained.

2 Biomaterials

2.1 Definition and Features

Specifically, science is dynamic; it changes its resources to obtain information, its research focus, its ways of applying technology for the society's benefit, and as is well-known, it is complicated to reach just one word to define something that has

been investigated in different parts of the world, and with the same meaning for everyone.

There have been many attempts to define "biomaterial"; even today, works are being published with new definitions that authors consider appropriate for their research. This difficulty makes biomaterial a kind of polysemic word.

If we pay attention to the etymology of the word, "bio" means life or that is related to living beings, and "material" is related to the main component of bodies, susceptible to all kinds of forms and changes, characterized by a set of chemical properties, perceptible by the senses. But each word is loaded with its meaning in the corresponding field of science, and that is why in 1987, a consensus conference on definitions in biomaterial science was held. In this conference, a biomaterial was defined as a nonviable material applied to a medical device, designed to interact with "biological systems" [7]. Although this definition has been used for several years, the concept of "nonviable" had to be rethought until in 1999 a materials science dictionary introduced a new idea of biomaterial as a material that can interface with biological systems to evaluate, treat, augment, or replace any tissue, organ, or function of the body [8].

This chapter will emphasize this definition and discuss how biomaterials have been specially incorporated as immobilizing platforms in detection devices. There are essential characteristics that make biomaterials so interesting for scientists and industries. The main one is their biocompatibility, which implies that they do not have toxic effects on the biological systems with which they interact [9]. Biodegradability is another and no less critical property, that is, the resistance that the material opposes to being decomposed into its constituent chemical elements by microorganisms' action [10]. Currently, the massive use of this type of materials is required since the use of nonrenewable sources and the uncontrollable production of materials that take centuries to decompose lead the planet to suffer regrettable catastrophes. Because of that, in addition to being relatively new materials in materials science, they are continually evolving with the help of multiple disciplines to improve their characteristics such as robustness, rigidity, ductility, and other qualities applying nanostructured engineering [11].

2.2 Classification

In this section, biomaterials will be classified from various approaches. Firstly, they are classified according to their evolution over time, following a line through the years highlighting its properties.

As shown in Fig. 1, in the 1950s, the so-called bio-inert biomaterials began to be developed, which do not give rise to any reaction when interacting with living systems. Among them, the most abundant are ceramics. Depending on their composition and purpose, they can be made of alumina, zirconia, silica glass, leucite, and lithium glass ceramics [12]. Due to their ionic or covalent bonds, these bioceramics were hard and fragile, and their primary purpose was to coat prostheses and all kinds of implants. With the improvement of ceramics, it was even possible to build

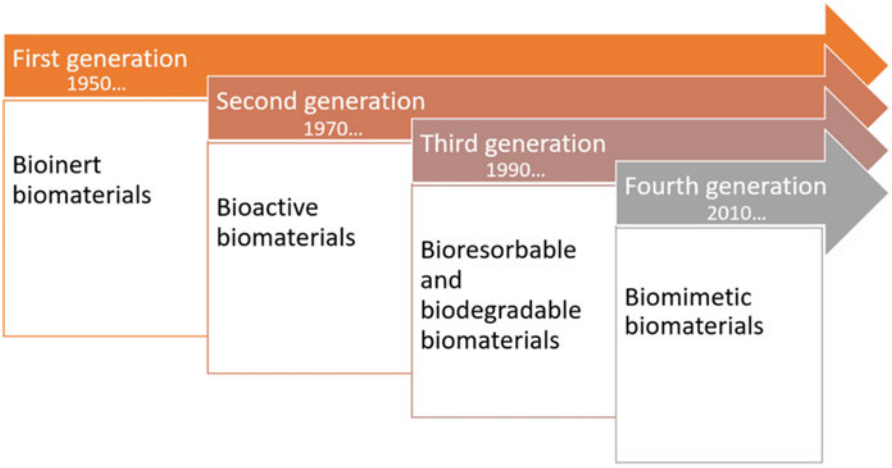

Fig. 1 Classification of biomaterials through their development over time

biosensors made of multilayers of this biomaterial, for example, Achmann et al. developed this kind of device for gas detection [13].

Years later and while the improvement of synthesis techniques was continuing, bioactive biomaterials arose, which, contrary to bio-inerts, interact with living systems. Among various bioactive materials, we can mention bioactive glasses [14] developed mainly in the dental prostheses field, but they were also used in biosensors, for example, for hydrogen peroxide determination [15]. Although glass has been used as a tool by man since ancient times, it was not until the 1970s that it was discovered that the glass surface contains chemical groups capable of interacting with biological matter. Over the years, the glass composition has been modified to interact more naturally, that is why its composition is based on SiO_2, Na_2O, CaO, P_2O_5, B_2O_3, and, thus, all purposes are achieved. Furthermore, with the introduction of sol-gel methods, it has been possible to achieve a SiO_2 with purity higher than 90% and to incorporate OH^- groups in its internal pore structure, promoting the development of nanostructured and mesoporous materials [16].

With the challenge of improving the properties, in the 1990s bioactive biomaterials have been fabricated that were also bioresorbable and biodegradable [17]. This development arises from environmental needs and to improve the quality of medical processes for patients. On the one hand, the biodegradable condition involves that these materials can decompose into different natural by-products such as gases, biomass, and water under ambient conditions. On the other hand, the bioabsorbable property establishes an advantage for the use of these materials in medical implants that do not require a second surgery to remove them because they manage to be metabolized to join the body's natural fluids. These compounds can also be found in biosensors, for example, for alkaline phosphatase delivery [18].

A promising kind of biomaterial is currently being studied, innovated, and developed, which has already begun to be used for various purposes. They are the

biomimetic biomaterials, and as a definition, it is a type of material that has been inspired by biological nature to imitate it [19]. In biosensors based on this biomaterial, biomimetic interfaces such as skin or ingested sensors represent a new revolution in the development of these devices. Because they intervene in a noninvasive way, monitoring, in many cases, the evolution of certain diseases within the human body and, even in some cases, providing treatment. Biomimetic biomaterials mimic the functioning of biological systems, so they can specifically respond to physiological stimuli.

Another approach to classify them is according to their origin, and they are divided into synthetic and natural biomaterials [20]. Among the synthetic ones, a large group belongs to synthetic bioceramics, in fact, there is a subclassification in almost bio-inert, bioactive, and resorbable ceramic. In the first case, they are very stable substances such as alumina (Al_2O_3), zirconium dioxide (ZrO_2), and titanium dioxide (TiO_2), which due to their incredible resistance can be used as charge carriers in the human body. In the second case, they are those obtained by sol-gel already mentioned above. In the latter case, we can mention, for example, amorphous calcium phosphates, calcium sulfate, hydroxyapatite, and a-/b-tricalcium phosphates [21]. Other important synthetic biomaterials are biopolymers; they can be obtained under controlled conditions and thus achieve all the properties that natural ones have at a disadvantage. Mainly, properties such as biodegradability are controllable when these materials are synthesized from well-known monomer units. Furthermore, toughness and robustness can be adjusted as desired with additional components that provide these characteristics. Within this group, polyhydroxyesters, including polylactic acid and poly (glycolic acid), as well as poly (lactic-co-glycolide) copolymers can be mentioned.

Natural biomaterials refer to those materials that, due to their origin, have excellent biocompatibility, biodegradability, are versatile, and are renewable. Because of this, the researchers try to incorporate them into new developments. However, they have some impediments, such as the difficulty of obtaining them in high purity, their poor robustness, and toughness, and they have very rapid biodegradation. In turn, they can be classified depending on the type of protein and polysaccharide involved. Commonly used natural protein-type polymers include, for example, collagen, gelatin, and silk proteins. Other examples of natural polysaccharides can be cellulose, hyaluronan, dextran, agarose, chitosan, and alginate. Moreover, polyester-type polymers produced by bacteria, such as poly (hydroxyalkanoates), are also included in the naturally biodegradable polymers classification.

Polysaccharides have a very important role in biosensor fabrication, since, due to their biological nature, they are macromolecules with very good behavior for biological recognition and stimulation [22]. In addition, water-insoluble polysaccharides have excellent structural rigidity; therefore, they are used as protection against severe adverse conditions.

But in order to face each type of biomaterial and understand its multifunctionality, it is necessary to study the way in which they can be made

and/or purified, a question that is fundamental for scientists and that by its fruit, entire societies can take advantage of these noble materials.

2.3 Biomaterial Synthesis Methods

One of the important issues in a study of a material is synthesis. The biomaterials can have a natural origin or be synthesized. The main problem to classify the synthesis methods is that "biomaterials"are not a kind of material with a well-defined structure. In the classification of these materials, metallic nanoparticles, hydroxyapatite ceramics, or microorganisms can be found as virus coated with a polymer [8].

Despite that, there is a general characteristic in biomaterial preparation which is the predominance of bottom-up techniques over top-down ones. This is due to, in both natural and synthetic biomaterials, the chemical components are building blocks [16]. In order to classify the synthesis methods, it can be said that these materials can be substances based on covalent bonds or self-assembled materials [23]. It is important to clarify that polymer-based biomaterials will not be discussed in this section because it will be a central topic later.

2.3.1 Biomaterials Based on Covalent Bonds

In this category, the materials resulting from a procedure that involves a chemical reaction are included. The list of this kind of biomaterials is far too long, here some of the methods are briefly described.

Metal nanoparticles: among the wide variety of nanoparticles (NPs), gold and silver nanoparticles are the most applied for biomaterials. Au NPs are generally obtained from a hydrogen tetrachloroaurate (III) ($HAuCl_4$) aqueous solution. The well-knownTurkevich method is based on the $HAuCl_4$ reduction by a reducing agent/stabilizing ligand, such as sodium citrate [24]. Most of the reported methods for Au NPs synthesis have proposed a modification of the Turkevich method. One of these methods is the one developed by Frens [25], who demonstrated the diameter size control by changing the reagents ratio: a higher ratio between the trisodium citrate and gold gives a smaller particle size. Therefore, the Frens method represents a simple path to obtain controlled-diameter Au NPs.

Even though the aqueous phase synthesis methods are the most common, there are reported procedures in organic phase. The Brust-Schiffrin method uses two phases (water-toluene) to carry out the reduction of AuCl4 by the reductant sodium borohydride in the presence of dodecanethiol [26], through this method a 1–3 nm AuNPs thiol coated are obtained. Similar methods have been used for the preparation of silver nanoparticles and gold-silver alloys by making use of these two-phase solutions that reduce gold and silver from AuCl4 and AgBr2 to obtain nanoparticles encapsulated in alkane monolayers [27].

Due to some reagents and solvents that could generate a problem in the biological applications of Au NPs, biological syntheses have been developed. These methods are based on the use of components like carbohydrates, lipids, nucleic acids, or

proteins produced in nature, and have the advantages of being clean, eco-friendly, nontoxic, and with low cost of production [28].

An important approach for biomaterial synthesis is that Au- or Ag-based NPs can be easily functionalized with biomolecules which contain thiols, amines, or even phosphine moieties. Thus, nanoparticles with amino acids, peptides, proteins, miRNAs, and DNAs are effectively obtained for different applications.

Other important biomaterials are those based on magnetic NPs, mainly for biomedical applications. The wet chemical routes are the most common ones for iron oxide synthesis; frequently, Fe^{2+} and Fe^{3+} aqueous salt solutions are co-precipitated by a base addition. The NPs size and shape depend on various reaction conditions, such as the iron salt used as precursor, pH, Fe^{2+}/Fe^{3+} ratio, among others [29]. Moreover, these magnetic NPs are modified with organic or inorganic molecules in order to prevent them from possible oxidation as well as from agglomeration.

In relation to this kind of NPs are also reported biosynthesis [30]. Parandhaman et al. proposed an eco-friendly synthesis of a hybrid nanobiomaterial, in the first step a biomineralization process was developed to synthesize magnetic NPs using Shewanella algae coated with the polymer chitosan, and in the second step such surface was modified with gold and palladium NPs by in situ reduction of metal ions. [31].

Carbon-based materials: Carbon-based materials have drawn attention to biomaterial development due to their excellent chemical and thermal stability, and great electrical and optical properties. Among them, graphene and carbon nanotubes (CNTs) are the most applied in this field.

Graphene can be synthesized either by chemical vapor deposition (CVD) where high-yield pristine graphene sheets are obtained or by other synthetic methods, such as Staudenmaier and Hummers methods, where graphene oxide is obtained. The first mentioned method employs heterogeneous catalytic reactions between metallic substrates, such as Ni, and some reducing agents, such as hydrocarbon gases, always under high temperature and vacuum conditions. On the other hand, the used methods consist of a graphite oxidation, using either the Hummers ($KMnO_4$ and $NaNO_3$ in H_2SO_4) or the Staudenmaier ($NaClO_3$ in H_2SO_4 and fuming HNO_3) method, followed by mechanical exfoliation, the final step is the reduction of the graphene oxide by chemical, electrochemical, thermal, or other reduction.

Usually, the graphene-based biomaterials can apply no-modified graphene or graphene composites: graphene-polymer or graphene-nanoparticles composites.

For the CNTs preparation high temperature techniques, such as discharge or laser ablation, had been used. However, low-temperature CVD techniques are the most widely used to synthesize CNTs because characteristics such as purity, density, length, and diameter can be controlled more precisely [32]. Moreover, Jasti and Bertozzi [33] have proposed a bottom-up synthesis method, in which the chirality can be controlled. This method is based on two steps: first, the aromatic macrocyclic templates synthesis and secondly, the extension of these templates to longer CNTs by polymerization reactions.

The use of CNTs as biomaterials implies their functionalization by means of two different strategies. CNTs can be covalently modified with chemical groups by binding on the side walls of the tubes or at the ends, or such modification could be non-covalent adsorption on the surface of the tubes with various functional molecules.

Sol–gel-derived biomaterials: The formation process occurs through hydrolysis and condensation reactions of metal or silicon alkoxides by which a colloidal dispersion (sol) evolves until the formation of a dense or gel-like inorganic structure of the desired material. Hybrid materials produced by the sol–gel synthesis method combine and enhance the advantages of organic and inorganic properties [34].

Among the materials produced by sol–gel route, silicon-based ones are the most applied in biomaterial development. Various modifications of these materials with various species of antibodies, enzymes, polysaccharides, proteins in general, nucleic acids, and multiple biomolecules for different purposes have been reported [35]. Furthermore, the possibility of biopolymer incorporation in the silicon material matrix during the sol–gel process has been demonstrated [36, 37]. Allow to obtain interesting and novel bio-nanocomposites. Mesoporous TiO_2 films or nanoparticles, obtained by sol–gel route, are also attracting considerable attention due to their good biocompatibility.

Two-dimensional (2D) materials [38]*:* 2D nanomaterials have attracted attention as biomaterials due to their particular chemical and physical characteristics. These materials could be used as pristine nanosheets, with or without surface modification, or nanosheet-nanoparticle composites. Some examples of 2D materials applied as biomaterials are graphene, transition metal dichalcogenides (TMD), metal nanosheets, boron nitride (BN), black phosphorus (BP), layered double hydroxide (LDH), and composites based on 2D nanomaterials. A difference between 2D materials and the other descriptive materials is that the frequently used synthesis are top-down techniques.

TMDs and 2D BNs are typically obtained by exfoliation from bulk forms, which can be carried out by various exfoliation methods, such as liquid exfoliation, mechanical exfoliation, molecule-assisted exfoliation, such as surfactants or polymers, alkali intercalation and exfoliation, ion intercalation and exfoliation, among several other methods. Besides, there have been reported bottom-up techniques based on CVD or hydrothermal synthesis.

Metal nanosheets could be synthesized using a template-directed crystal growing route. For example, 2D gold nanosheets have been prepared under a mild UV photoreduction and thermal reduction of gold salt with the polymer P123 as a template.

Finally, for the LDH preparation the co-precipitation of $M(OH)_3$ and $M(OH)_2$ is the most used synthesis method. Also, some anions could be intercalated in the layers incorporating them during the precipitation process.

2.3.2 Self-Assembled Biomaterials

The synthesis methods based on self-assembly consist of the spontaneous association of molecules into one or more supramolecular structures. This procedure is

driven by non-covalent bonds which can include hydrogen-bridge bonds, electrostatic, and van der Waals forces [23]. By this method, the desired 3D structure can be produced from synthetic polymers or natural molecules, such as peptides. These self-assembled biomaterials have often been applied in tissue engineering for drug and cell carriers. For this reason, we are not going to expand on this topic because this chapter focuses on biomaterials applied to chemical sensors.

2.4 Application: Biomaterials-Based Biosensors

Biomaterials have a large number of specific or industrial applications, ranging from drug delivery, the textile industry to the development of sensors to diagnose or monitor the presence of various analytes. Specifically, the interest of this chapter is the application of these materials in biosensor development. The IUPAC defines "biosensor" as a "device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals" [39]. Therefore, a traditional biosensor can be defined as an analytical transducer that can convert a biological input response into a processable and quantifiable electronic output signal. A biosensor consists essentially of the following components: a) a biological recognition element (called "bioreceptor") that interacts with the target analyte and grants selectivity to the sensor; b) an interfacial structure that contains the reaction sites and generates the electrical signal that can be captured by; c) a transducer to receive, amplify, and enhance the signal, so that it finally converts the interaction into an analytical signal; and d) devices for the digital processing and subsequent analysis of the data, where it usually also has a human interface display unit [40].

In comparison to other sensors, biomaterials-based biosensors offer the advantage of covering some issues such as nonspecific binding, strong affinities, and complex selections. Allowing to obtain biosensors with high efficacy, sensitivity, selectivity, and biocompatibility, which can be applied for rapid, specific, and sensitive detections.

The biosensor design and development using various biomaterials consist of an interdisciplinary approach. In the last few years, a great effort has been made to improve biomaterial development for their application both as a bioreceptor and as a transducer.

In the first strategy mentioned above, the use of biomaterials in the bioreceptor, they can achieve catalytic recognition at a high level for its specific binding or interaction with the biological or chemical species. Moreover, in biosensor development, these materials can be used as bioreceptor-supporting material. In this option, it is important that the material does not interact with the analyte, there must be no nonspecific binding and other physical-chemical interactions must be very low or nonexistent. In most of the cases that use this strategy, the advantages achieved are the improvement of the selectivity and sensitivity, accompanied by an increase of the immobilization surface area.

Table 1 Comparison of biomaterials-based biosensors

Reference	Applied biomaterials	Sensor type according to determination method	Biosensor analyte
Retout et al. [41]	Peptide-functionalized gold nanoparticles	Colorimetric sensor	Protein biomarkers (oncoprotein Mdm 2)
Zhu et al. [42]	Gold nanoparticles functionalized with aptamers.	Colorimetric sensor	*Escherichia coli* lipopolysaccharides
Zhao et al. [43]	CeO_2 dispersed on TiO_2 nanotube	Colorimetric sensor	H_2O_2 and glucose
Viter et al. [44]	Antibodies against *salmonella* immobilized on TiO_2 nanoparticles	Optical immunosensor	*Salmonella*
Zanganeh et al. [45]	Amine-functionalized carbon nanotubes conjugated to folic acid molecules	Impedance sensor	Lung cancer cells
Dervisevic et al. [46]	Urease immobilized on polyamidoamine grafted multiwalled carbon nanotube	Amperometric sensor	Urea
Mansouri Majd and Salimi [47]	Multiwalled carbon nanotube/aptamer conjugated onto reduced graphene oxide nanosheets	Aptasensor	Ovarian cancer antigen (CA125)
Yoon et al. [48]	Amine-modified MoS_2/graphene oxide/myoglobin hybrid material	Amperometric sensor	NO
Khetani et al. [49]	Polyethylenimine-modified graphene oxide with immobilized antibodies	Electrochemical impedance spectroscopy-based immunosensor	Glial fibrillary acidic protein
Cui et al. [50]	Aptamer@Fe_3O_4@graphene quantum dots@MoS_2 nanosheets	Magnetic fluorescent sensor	Circulating tumor cells

The other strategy in the biosensor design is the incorporation of biomaterial in the transducer. The main goal is the detection signal improvement. Thus, here it is important that biomaterial properties depend on the type of sensor developed, which can be colorimetric, fluorescence, voltametric, amperometric, piezoelectric, among others.

In Table 1, the most relevant and exemplary biomaterials-based biosensors for the last 5 years are presented.

3 Biopolymers as Biomaterials

As mentioned above, the applications in which biomaterials were used a decade ago are very different from today, and although medicine still uses implantable devices, science has already given rise to new technologies for drug delivery, genetics, organ printing, cell therapies, and new detection or diagnostic systems that also use nanomaterials [51, 52]. These technological advances generated the development of numerous biomaterials, particularly in the field of polymers.

The integration and advances in polymer study have allowed controlled and functional architectures, which have further improved and expanded significantly the range of possible biomaterials [53]. Due to these, in biosensors development, polymeric biomaterials are rapidly replacing other biomaterial classes, such as metals, alloys, and ceramics, in the development of biosensors [54, 55]. Polymers can be classified according to the method of production as synthetic or natural. Synthetic polymers can be produced by homopolymerizations or copolymerizations (or terpolymerizations) of conventional monomers to achieve biomaterials with quite different properties. It is possible to produce polymers with entities specifically hydrophilic or hydrophobic, even biodegradable repeating units or multifunctional structures can be manufactured. Synthetic biomaterials are generally biologically inert, despite that, they have more predictable properties and batch-to-batch uniformity. These have given rise to synthetic biomaterials with very specific properties, promoting the development of novel biosensors [56]. However, natural polymers have appealed significant attention owing to their natural abundance, availability, and mainly for being friendly with the environment. Besides, these biomaterials have excellent properties such as nontoxicity, biocompatibility, biodegradability, flexibility, and renewability which increases the interest in their application in the scientific field.

The natural polymers produced from living matter are found in the field of biopolymers. The prefix "bio" means they are biodegradable materials present in living beings. Biopolymers can be obtained from different sources: 1- available in nature in the form of polysaccharides, exopolysaccharides, and proteins (to name a few, we mention cellulose, alginate, chitin); 2- by microbial fermentation (PHB, PBS), and 3- from biomass (e.g., PLA) [57].

Mentioned first, natural biopolymers, are those polymers available in nature, and are typically derived from biological sources such as microorganisms, vegetables, and animals. These biopolymers can contain different monomeric units, developing hierarchical structures, which dwell as heteropolymers. Out of their primary structures, these biopolymers develop secondary structures and, at times, even acquire a tertiary structure [57, 58]. Depending on the monomer units, these polymers can be classified into three main categories: (1) polynucleotides (polymers consisting of nucleotide monomers, such as RNA, DNA); (2) polypeptides (polymers of amino acids, e.g., hemoglobin); and (3) polysaccharides (polymeric carbohydrates, such as chitosan, cellulose, alginate, gum) [58].

3.1 The Special Case of Polysaccharides

Among the great diversity of natural biopolymers currently under investigation, we will focus our attention on polysaccharides. These polymers are considered vital bio-macromolecules for all living organisms. They are structurally comprised of homo or hetero monosaccharides and uronic acids connected with glycosidic linkages [59]. Polysaccharides have several advantages, mainly due to their inherent biological nature. For example, these biopolymers are biocompatible, respond to biological stimuli, and have great flexibility to adapt to complex biochemical environments. Also, they exhibit good material properties such as mechanical strength, rigidity, and durability. Another quality of these biopolymers is the great variety of functional groups present, such as hydroxyl, carboxylic, amino, and amines. These functional groups endow polysaccharides with multiple functionalities, such as selective adsorption, abundant active sites, and a high surface area [59]. In addition, the functional groups are responsible for the strong interaction that they exhibit with water, generating high mobility of the polymer chains. This makes the polysaccharides soluble or insoluble in water. For example, chitosan, alginate, and tragacanth gum are soluble while cellulose, a highly hydroxylated polysaccharide, has a strong interaction with water, leading to a large holding capacity but is not water-soluble [60]. Besides, depending on the production method, chains with different lengths and/or variations in their conformations can be found for the same polysaccharide. Furthermore, they can have a net positive, negative, or neutral charge and can be modified with the change of pH. These changes generate modifications in the properties, increasing the great diversity of options that these biopolymers offer [61].

Among the polysaccharides soluble in water, chitosan (CH) and alginate (ALG) are widely studied and have potential in the development of biomaterials. First, CH is a linear polysaccharide consisting of (1,4)-linked 2-amino-deoxy-β-d-glucan, a deacetylated derivative of chitin. The variation of the synthesis conditions generates modifications in the chain length and the degree of deacetylation of the precursor material. The amino group -NH_2 can be protonated and readily form electrostatic interactions with anionic groups in an acid environment and has an excellent film-forming ability [62]. Second, alginates are a family of linear unbranched polysaccharides containing varying amounts of 1,4′-linked β-d-mannuronic acid and α-l-guluronic acid residues. The residues are arranged in a pattern of blocks along the chain and may vary widely in composition and sequence. These homopolymeric regions of β-d-mannuronic acid blocks and α-l-guluronic acid blocks are interdispersed with regions of alternating structure (β-d-mannuronic acid–α-l-guluronic acid blocks). The physical properties of alginates are determined by their molecular weight and by the composition and length of the sequences. These molecular variations are related to the organism and tissue from which the alginates are isolated [63].

On the other hand, a water-insoluble linear polysaccharide widely studied and used in different fields of application is cellulose. This polysaccharide is a non-branched macromolecule containing a chain of variable length of 1–4 linked

β-d-anhydroglucopyranose units. The high affinity with water molecules is due to high intramolecular bonding and less intermolecular hydrogen bonding. Being these interactions responsible for the great diversity of properties that can present the cellulose [64].

Therefore, polysaccharides represent a formidable tool with a great variety of structures and diverse interactions that have made it possible to obtain various biomaterials with a wide range of properties. These qualities mentioned have led the scientific community to focus its attention on the study of polysaccharides as base-biomaterial in the development of novel biosensors with high sensitivity and specificity.

3.2 Biopolymer-Based Biosensors

As mentioned in Sect. 2.4, in most of the biosensors, the detection is carried out when a target analyte selectively binds with an immobilized biomolecule in the device platform. It has been widely known that biosensors' performance mainly depends on the immobilized bioreceptor properties, which can include their size, biological activity, and binding capacity and selectivity with the analyte. Furthermore, these bioreceptor characteristics can be modified according to the substrate to which it is immobilized. This can occur due to the interaction between bioreceptor and substrate surface can add some kinetic and physicochemical properties. Also, improving the immobilization methods is one of the mechanisms that can significantly enhance biosensor specificity and sensitivity [65, 66]. The application of biopolymers in this field has called attention due to their great properties for biomolecule immobilization.

In order to improve the biosensor analytical performance, the use of biomolecules in their natural environment, such as polysaccharides, supplies some advantages. Of which the greater catalytic activity stability and protection against inactivation can be named as some of the most important. Anusha et al. proposed a biosensor based on the glucose oxidase (GOx) immobilization in chitosan nanoparticles, which were synthesized from squid gladius. These authors report that GOx cast over chitosan promotes the electron transfer of enzymatic reaction, enhances the amperometric responses, provides a friendly environment for immobilization of enzyme, and enhances the catalytic activity towards glucose [67]. Therefore, polysaccharides act as excellent support for biomolecule immobilization which is crucial because it corresponds to the part of the device that confers selectivity. Nevertheless, despite the extensive employment of polysaccharides for several biomolecules' entrapment, some drawbacks are present. For example, low mechanical strength and active surface exposed for retention are frequently associated with the loss of biomolecules from the biosensor device during its use. To solve the mentioned drawbacks, polysaccharides can be applied together with some biocompatible materials, which can be other polysaccharides or synthetic polymers. Recently, research has started combining polysaccharides with conventional nanomaterials to form nanomaterial composite, allowing the enhancement of polysaccharides properties. Shukla et al.

fabricated a glucose biosensor based on ZnO/CHIT-g-PVAL core-shell nanocomposite. The capacity of the proposed core-shell nanocomposite to entrap glucose oxidase is a main characteristic in the design of this biosensor [68].

The biomaterials-based polysaccharides can be used for enzyme immobilization in different structures, such as films, capsules, and hydrogels, among others. This allows to classify the retention of biomolecules in polysaccharides into two large groups, physical or chemical immobilization which is the most judicious and appropriate immobilization method influenced by the enzyme nature, the transducer, and the applied detection technique. These two types of methods have their advantages and disadvantages. [69].

The physical retention method is simple and inexpensive but has the disadvantage that low surface coverage is obtained with poor uniformity. Using this method biomolecules, mediators, and additives can be deposited in a one-step method onto the same device zone. Therefore, when this kind of method are carried out, biomolecules are immobilized in the polysaccharide matrix with no modification of it and preserving its biological and catalytic activity. The polymeric networks permit substrate and product transports thus increasing their stability and allowing their long-lasting application. Tucci and collaborators report a novel artificial biofilm made of photosynthetic microorganisms. It was applied for amperometric biosensor fabrication, where the direct inhibition of the generated photocurrent was measured. These authors immobilized bacterial cells on carbon-felt electrode. Alginate was used as a polymer matrix for the biomolecule and p-benzoquinone, redox mediator, retention. This biosensor development comprises only a few simple steps, avoiding the purification of subcellular molecules or polymer synthesis [70]. Nevertheless, weak or covalent interactions can lead to the biomolecule leaching from the polymeric matrix, which can be reflected in a catalytic activity reduction over time. Besides, a low catalytic activity may be due to poor diffusion of substrates and products through the polysaccharide matrix. However, the variation of the polysaccharide's molecular weight can be used to solve this drawback, since it modifies the retention and diffusion properties in the polymer matrix [60]. For example, a solution obtained from a high-molecular weight alginate is very viscous and/or gelatinous, which is, in general, an unsuitable property for biomolecule encapsulation. Other common methodologies for physical retention of biomolecules are adsorption, electrostatic immobilization, and electrochemical doping. The first one commonly does not require support functionalization, due to this the adsorption-based method is one of the easiest ones. Furthermore, the biomolecule activity is not affected during the immobilization process. The retention of biomolecules on the surface is related to weak bonds such as Van der Waal forces and/or hydrophobic interactions.

The chemical immobilization method is based on covalent binding of biomolecules to biopolymeric supports, and it is widely used in the development of numerous enzymes or immune biosensors. This binding is performed by functional groups present in the biomolecule, but which do not participate in an essential way in its catalytic activity. In order to generate a covalent binding between a biomolecule and a polysaccharide, usually, the first step includes a reaction of the

polymer with a multifunctional reagent, such as glutaraldehyde and carbodiimide. Then, the biomolecule reacts with the activated polymer. Biomolecule immobilization can be carried out on the transducer surface without modification, or in a thin membrane deposited onto the transducer. Among these methods, the crosslinking of enzymes using glutaraldehyde to immobilize these biomolecules in polysaccharides is a widely applied method in biosensor development. This is due to the great abundance of functional groups present in polysaccharides, its simplicity to perform, and the resulting strong chemical bonding. The main disadvantage of this method is that, during crosslinking, there is a possibility of activity losses because of distortion of the active enzyme conformation or active site chemical alterations [71]. Buk et al. developed a novel electrochemical biosensor based on an alginate hydrogel. This acted as an immobilization matrix for the GOx and Copper (II) oxide (CuO) nanoparticles. The GOx and CuO nanoparticles, covalently immobilized, provide significant catalytic properties to the hydrogel. Also, the addition of these materials provides stability to the hydrogel [72]. Qasemiet al. reported the immobilization of CdTe quantum dots (QDs) and GOx enzyme on a novel platform made of tragacanth gum nanohydrogel for biosensor development. The immobilization of QDs and GOx inside the nanohydrogel, with good operational stability and excellent dispersion, is an important innovation [73]. Barathi et al. fabricated a novel flexible biosensor by electrode modification with chitosan-crafted mesoporous carbon as a platform for glucose oxidase immobilization. In this platform, there is no significant modification in CH original properties and CH becomes firmly trapped on the electrode surface. These authors mention that CH improves electron transfer on the electrode surface due to the good intermolecular interaction with biomolecules and adequate dispersion of mesoporous carbon [74].

3.2.1 Cellulose as Supporting Material for Biosensing

Another important application is the development of portable cellulose paper-based biosensors (PBBs) and microfluidic cellulose paper-based analytical devices (μPADs).

Cellulose is one of the most abundant renewable biopolymers. It has excellent physicochemical properties, such as high stiffness and strength, high sorption capabilities, good thermal stability, and biocompatibility [75]. Among biopolymers, this one arises as an interesting and with great potential material for several applications due to the cellulose properties, the cellulose-based papers, and the nanocellulose which incorporates novel characteristics [76]. Like the biomaterials described above, cellulose papers can have different properties due to their structure and purity depending on their origin: bacterial cellulose [77], cotton cellulose, and cellulose extracted from different types of wood [78], among others. Moreover, these papers can be modified by the adsorption of surfactants or polymers, in their raw form or after the paper formation, modifying some features such as porosity, mean channel size, hydrophobicity/hydrophilicity, filtration capacity, and density. These modifications can also change the charge, active sites, and the possibility of generating hydrogen bonding of the polymeric matrix. Therefore, according to the needs required in the various applications, the physicochemical paper properties can

be easily modified [79]. Furthermore, cellulose papers have other interesting advantages, such as ease of functionalization, biodegradability, biocompatibility, and low cost. These qualities mentioned have made cellulose a promising platform for the development of PBBs and μPADs.

In PBBs, the cellulose acts as a highly porous rigid or semi-rigid support for the immobilization of biomolecules. For this reason, PBBs are becoming cost-effective diagnostic tools for important biomarker or metal ion sensing. Also, these biosensors can be modified with nanomaterials and nanostructures in order to improve some analitycal parameters such as sensitivity, selectivity, dynamic range, and limit of detection, among others. Mahato et al. fabricated a paper-based biosensor for colorimetric detection of alkaline phosphatase in milk samples. For this purpose, the authors covalently immobilized specific antibodies onto the paper surface. The analyte determination was carried out by a digital image colorimetry-based technique integrated with smartphones. [80].

In μPADs, the cellulose paper acts as a support for reagents and analytes transport to the reaction zones and as an anchoring platform for biomolecules in the reaction zone. The μPADs often include multiple printed electrodes and/or multiplex sensors. Generally, the μPADs have similar principles and properties to PBBs however the multiple routes to the reaction zone that these devices possess generate a more complex architecture. Although these devices have similar advantages to those of PBBs, they also have the quality that they usually require smaller sample and reagent volumes which generates less waste. For this reason, μPADs are becoming particularly useful devices that offer versatility and simplicity. Fava et al. proposed a microfluidic paper-based device for multiplexed analysis with electrochemical detection. This work presents advantages, such as no need to use a wax printer for device fabrication, possibility of large-scale production, and the potential application for clinical sensing with safe and short-time assays [81]. Xing et al. developed a paper-based microfluidic aptasensor for labelless electrochemical detection of programmed death-ligand 1 (PD-L1) in human fluids. This ligand for programmed death is considered a reference when it comes to therapeutic biomarkers, and that is why an inexpensive and rapid method is necessary to detect it in body fluids. The aptasensor was based on a reaction cell and a three-electrode system, and the determination was carried out by differential pulse voltammetry. The PD-L1 aptamer was used as a biorecognition molecule [82]. Yakoh et al. designed a 3D sequential microfluidic paper-based analytical device based on the sliding strip concept. This platform stores reagents in a mobile part. Then, these reagents are sequentially transported to the detection zone with a single introduction of a carrier buffer. In addition, the authors suggest that this kind of device can be applied to a wide variety of electrochemical detections without losing the analysis simplicity [83]. Tian et al. reported a new form of paper-based biosensor for early cancer detection with hierarchically assembled nanomaterials and MOF-enhanced bioprobes for the simultaneous detection of microRNA-141 and microRNA-21 [84]. The PBBs and μPADs can be easily operated, do not require highly trained personnel, and have fast response and portability. So, these devices have a great potential as alternative low-cost devices for biomarker detection, cancer screening, and point-of-care

testing. Despite that, there are some problems in the applications of paper-based devices for biosensors, such as maintaining biomolecule activity. In addition, for the long-term storage of these devices, protection against moisture accumulation is important.

Therefore, polysaccharides display remarkable advantages in terms of stability because they generated a biocompatible environment and they improved biocatalytic performance of the biomolecules. These biomaterials provide good adhesion to the surface and adequate biological material dispersion in the polymeric matrix. These are some of the advantages that make it a powerful tool for the invention of novel biosensors.

4 Conclusion and Future Perspectives

In this chapter, several topics related to biomaterials and biopolymers have been analyzed, from their definitions and properties, through their different classifications and detailing their applications, especially in biosensors.

It can be stated that the biomaterial definition has been changing over time as it has been adapted to the practical purposes given to these materials. But we have highlighted the definition that establishes a biomaterial as "a material intended to interact with biological systems to evaluate, treat, augment or replace any tissue, organ or body function." Moreover, different approaches to their classification were discussed, according to their evolution over time and according to their synthetic or natural origin. As with any new material, researchers focus their attention on different synthesis methods to achieve better material qualities. In biomaterials, there is a general characteristic in their preparation, which is the predominance of bottom-up over top-down techniques. Also, the prepared biomaterials differ according to ones based on covalent bonds or self-assembled ones. From this point of view, great advances have been achieved in the preparation of biomaterials with specific functionalities. However, there are some issues that need to be solved, such as a greater understanding of the structure–property relationship, interactions at the matrix/filler interface, well-controlled fabrication processes, and cost-effective, large-scale, eco-friendly fabrication techniques. For this reason, we consider that computational approaches could be integrated with experimental studies to facilitate understanding of the system and overcome these disadvantages.

Regarding the large number of applications in which biomaterials can be incorporated, we have focused on their high potential to be used as sensing platforms for the fabrication of biosensors. These are generating portable and inexpensive devices with high sensitivity and specificity, and rapid detection. Several devices have been fabricated with very interesting properties such as biodegradability, flexibility, and biocompatibility. In particular, the use of biopolymer-based materials for device fabrication adds an important quality, which is the presence of abundant and easily modifiable functional groups. This allows a wide range of possibilities for the fabrication of sensors with new functionalities and tunable properties. Moreover, the use of biopolymers in sensor fabrication is of great importance to reduce

pollution and enhance their applications in eco-friendly and sustainable sensors. However, there are some drawbacks of using biopolymers, such as the difficulty of them in maintaining bioactivity, their thermal or/and optical instability, their solubility in electrolytes, and basic understanding of the transport mechanism in biopolymer molecules. Consequently, it is necessary to concentrate efforts on overcoming this problem.

Due to the abundance and great variety of biomaterials, in addition to the excellent properties which were described in this chapter, this is a wide field of development with great potential. Therefore, future research on biomaterials and biopolymer-based materials could be of great importance in biosensor applications in relevant areas such as chemistry, electronics, medicine, and agriculture. Materials science is increasingly aiming at their use as raw materials in everyday products and society is beginning to see this new way of caring for our planet by using more eco-friendly objects.

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Part II

Development of Nanomaterials and Biocomposites as Sensors

Nanobiohybrid Materials for Development of Biosensors

Jinho Yoon, Hye Kyu Choi, Minkyu Shin, Joungpyo Lim, and Jeong-Woo Choi

Abstract

Various types of biosensors have been reported for detecting the occurrence of various health problems and fatal diseases. However, previously developed biosensors are limited related to sensitivity and selectivity, for precise detection of the harmful or indicator molecules. As such, the development of novel biosensors with high sensitivity and selectivity is crucial to improve disease prevention through diagnosis. The use of nanobiohybrid materials is one of the promising strategies to achieve this and has been widely studied for application in biosensors. Nanobiohybrid materials overcome the limitations of other biosensors through the synergistic effects derived from its composition of biomaterials and nanomaterials. This chapter includes categorized sections describing novel biosensors based on nanobiohybrid materials that include biomaterials, nanomaterials, and nanocomposites.

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1 Introduction

The development of biosensors is one of the most important in the biological and medical fields for the detection of specific molecules in the body to treat health diseases [1, 2]. Biosensors are the analytical detection platform capable of interacting with target molecules using sensing probes that have specific binding or reaction properties with target molecules [3, 4]. Numerous biosensors have been developed to detect important biological components that affect living organisms and can induce fatal harm and the occurrence of diseases such as cancer [5, 6]. To develop these biosensors, various biomaterials like enzymes and antibodies have been employed as sensing probes that utilize inherent characteristics from each biomaterial [7, 8]. However, biomaterials have limited sensitivity and selectivity and overcome the limitations of biomaterials using methods such as the recombination technique [9, 10]. Nanobiohybrid materials have been a popular area of study due to their huge potential for biosensing applications [11]. These materials are created through an elaborate combination of biomaterials and nanomaterials at the nanometer level, and the synergistic effect of the properties of these components may enable the development of outstanding biosensors [12, 13]. The need to introduce nanobiohybrid materials into the production of more advanced biosensors has become increasingly important in recent years. This technology is crucial for preventing widespread fatal diseases and providing early, effective medical treatment, especially in circumstances such as the COVID-19 pandemic [14, 15].

This chapter discusses different biosensors that are based on nanobiohybrid materials. The first section covers biomaterials that act as sensing probes in nanobiohybrid materials. Next, various nanomaterials utilized to develop nanobiohybrid materials are described. These are categorized as metal, carbon, transition metal dichalcogenide (TMD) nanomaterials, or upconversion nanoparticles (UCNP). Next, nanobiohybrid materials developed by combining biomaterials and nanomaterials are discussed to be applied for biosensors. Lastly, this chapter describes selective overview of recent studies on the strategies for characterizing novel nanobiohybrid materials-based biosensors with a selective overview of recent studies (Fig. 1). In particular, we discuss the three types of biosensors based on electrochemical, fluorescent, and surface-enhanced Raman spectroscopy (SERS) techniques.

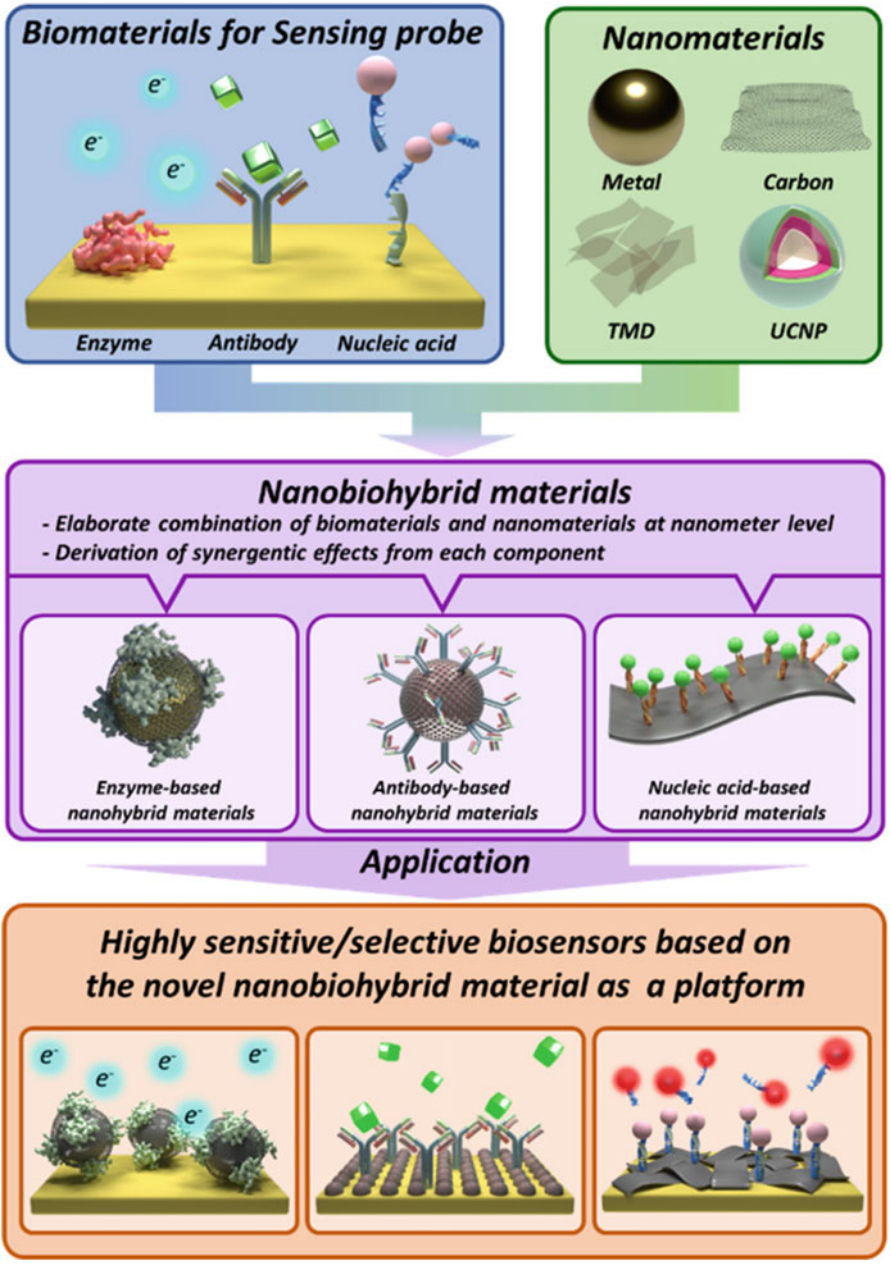

Fig. 1 Nanobiohybrid materials composed of biomaterials and nanomaterials used to develop biosensors

2 Biomaterials for Nanobiohybrid Materials

Numerous materials can be utilized as the sensing probes of biosensors, including biomaterials and inorganic materials such as metal oxides. However, biomaterials have unique advantages that make them suitable for this application. Biological components of the human body can interact with certain molecules in an extremely sensitive manner at the nanoscale level, depending on their original roles, and this characteristic can be utilized to create superior sensing probes for biosensors [16, 17]. When using biomaterials as sensing probes, it is important to select an appropriate biomaterial to achieve accurate and rapid measurement of the target molecule. For example, metalloenzymes are suitable for electrochemical biosensors because of their redox properties, and nucleic acids are suitable for RNA biosensors because they can react with specific RNA sequences of RNA viruses [18, 19]. This section describes the most widely used types of biomaterials used as sensing probes in biosensors: enzymes, antibodies, and nucleic acids.

2.1 Enzyme

Enzymes are molecules in living organisms that can act as biocatalysts to convert substances into specific products [20, 21]. Since almost all metabolic reactions in living organisms are processed through enzymatic catalytic reactions, there are diverse enzymes capable of reacting with specific substances, depending on their role [22, 23]. The specificity of these biochemical reactions makes enzymes suitable for sensing probes. Enzymatic biosensors are one of the oldest types of biosensors that have been studied, and they continue to be a major area of research [24].

Depending on the type of enzyme and the additional components involved in the reaction, a myriad of target molecules can be detected by enzymatic biosensors. Among the various types of enzymes, metalloenzymes are the most suitable for sensing probes in electrochemical biosensors due to their structure and characteristics [25]. Metalloenzymes contain metal ions that can react with target molecules while also serving as the redox signal generator [26]. Representative examples include myoglobin (Mb) and hemoglobin (Hb), which have iron ions that can detect hydrogen peroxide (H_2O_2), a reactive oxygen species capable of damaging cells [27, 28]. Various other enzymes have been studied to develop biosensors to detect molecules such as glucose and lactate, which have important effects on the body. For instance, glucose oxidase (GOx) or lactate oxidase (LOx) has been used as sensing probes to detect glucose and lactate. The reaction of these enzymes also generates electrochemical signals that can be readily measured by electrochemical techniques such as amperometry [29, 30]. As shown in Fig. 2a, GOx and ferrocene (Fc) modified on a carbon electrode could detect glucose through an enzymatic reaction. The electrons generated from that enzymatic reaction and the related Fc response were easily measured by electrochemical analysis.

Although enzymatic biosensors mostly use electrochemical techniques, other effective techniques include fluorescent and SERS [31]. For example, when the

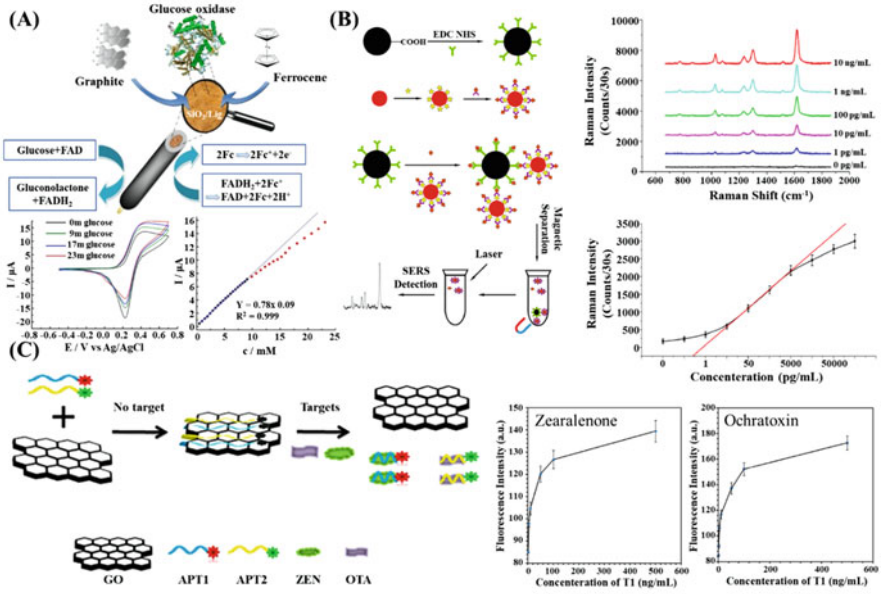

Fig. 2 (**a**) Detection of glucose using GOx and Fc modified on a carbon electrode through enzymatic reactions and glucose measured by electrochemical analysis (From Jędrzak et al. [29], with permission, copyright (2017) Elsevier), (**b**) scheme of SERS-based immunoassay using magnetic beads and GNP for detection of Chl and SERS spectra and relationship between the intensity and Chl concentration (From Yang et al. [45], with permission, copyright (2016) Elsevier), (**c**) fluorescent aptasensor using GO to detect zearalenone and ochratoxin and calibration plots of fluorescence emission from zearalenone and ochratoxin. (From Wang et al. [67], with permission, copyright (2020) Frontiers Media S.A.)

enzymes like creatinase, urease, and creatinine deiminase were entrapped in an oxazine 170 perchlorate (O17)-ethylcellulose (EC) membrane, a ratiometric fluorescent biosensor can be used to measure the levels of creatine and creatinine dissolved in the urine [32]. In this case, the enzymatic reactions between the sensing probes and target molecules produce ammonia, which reacts with the O17-EC membrane and induces a change of fluorescent signals. In another study, enzymatic reactions were combined with Raman probes, and SERS-based enzymatic assays were proposed to detect thrombin [33]. A photoelectrochemical glucose biosensor was also developed using an enzymatic reaction and photoreactive titanium dioxide (TiO_2) nanowires [34]. As these examples demonstrate, functional materials can be incorporated into enzymatic reactions to design a variety of enzymatic biosensors using proven techniques.

Although enzymes are commonly used in biosensors, enzymatic reactions tend to produce unstable and low signals. Because of this intrinsic limitation, enzyme-based nanobiohybrid materials are being studied to develop improved biosensors.

2.2 Antibody

Antibodies, also known as immunoglobulin, are Y-shaped proteins produced by a plasma B cell which is activated in an acquired immune system. Antibodies recognize and bind to specific parts of antigens, such as bacteria and viruses, in order to neutralize them [35]. The paratope, the antigen-binding site on an antibody, uniquely combines with the specific epitope present in the antigen, enabling the immune system to operate quickly and precisely. Because of these highly specific and accurate binding properties, antibodies have been used as sensing probes in biosensors for biomarkers related to various diseases [36, 37].

The enzyme-linked immunosorbent assay (ELISA) is one of the most widely used antibody-based biosensing techniques. In the case of direct ELISAs, antigens are attached to the ELISA plate, and enzyme-conjugated antibodies which can specifically bind with antigen are added to the ELISA plate. Enzymes such as alkaline phosphatase or horse radish peroxidase (HRP) are most commonly used with this technique, and they are chemically conjugated with antibodies using periodate oxidation for signal generation. The ELISA plate is then washed to eliminate any unbound antibodies, and substrates of the enzymes are added to the plate. In the presence of antibodies bound to the antigens, the enzymes conjugated with the antibodies degrade the substrate and produce detectable signals. This most commonly involves a change in color to verify detection of the target antigen [38, 39]. To improve sensitivity and amplify the signal, various types of ELISA techniques have been reported, such as indirect, sandwich, and competitive ELISA [40]. For example, in the case of sandwich ELISA, unlike the direct ELISA method, the antibody is firstly fixed to the plate for target antigen detection, and a target antigen is bound to an antibody on the plate. Then, the secondary antibodies conjugated with enzymes such as the alkaline phosphatase and HRP are combined with antigens for target antigen detection by formation of sandwich structures through the colorimetric detecting methods [41].

Another example of antibodies used in biosensing is lateral flow immunoassay (LFA). LFA detects target molecules using a similar principle as ELISA. In LFA, when liquid samples are spilled onto the LFA electrode (usually nitrocellulose film), the samples flow and the target antigens in the samples bind to antibodies conjugated with certain colorimetric labels. The sample then flows continuously and binds to the other antibody in the test line capable of binding to another site on the target antigens. Detection of the target antigens is verified by the appearance of the colorimetric signals in the test line. LFA-based biosensors are widely used in personal diagnostic devices, such as pregnancy test kits, because they are easy to use and the results can be determined visually and without any instruments [42, 43]. However, since both ELISA and LFA-based biosensors use colorimetric assays, they have limited ability to detect analytes at very low concentrations through the colorimetric change.

To overcome this limitation, antibody-based biosensors with electrochemical and SERS techniques have been developed [44]. For example, an electrochemical fibrinogen biosensor was developed using antibody-modified graphene. The

fibrinogen is measured by a varying resistance value that depends on the amounts of antigens that bind to immobilized antibodies. In addition, a SERS immunoassay-based biosensor was developed to detect chloramphenicol (Chl), which can cause aplastic anemia [45]. In this study, chloramphenicol antibody immobilized magnetic beads (MB-ChlAb) and chloramphenicol conjugated GNP as a Raman dye (GNP-Rd-Chl) were prepared. When an unknown concentration of target Chl was combined with a certain amount of GNP-Rd-Chl and MB-ChlAb in a solution, target Chl and GNP-Rd-Chl competitively bound to MB-ChlAb (Fig. 2b). After this reaction, MB-ChlA that bound to target Chl and GNP-Rd-Chl were removed by a magnet, and the supernatant was analyzed using SERS. A SERS signal which was proportional to the target Chl was generated by GNP-Rd-Chl that could not bind to MB-ChlAb due to competitive binding with Chl. As these examples demonstrate, the specific binding affinity of antibodies can be used to develop biosensors for target molecules such as antigens, proteins, and aptamers. In addition, functional groups on antibodies, such as -NH_2, -SH, and -COOH, can be used to combine antibodies with nanomaterials to develop novel nanobiohybrid materials for biosensing.

2.3 Nucleic Acids

The Nucleic acid is a macromolecule composed of nucleotides that contains a ribose, nitrogen base, and phosphate group. Deoxyribonucleic acid (DNA) is structurally distinct from ribonucleic acid (RNA), which contains one more hydroxyl group in its ribose compared to the deoxyribose in DNA. Nucleic acids carry genetic information, including cells and viruses, and have been studied in biological fields [46–48]. Because specific sequences of nucleic acid are associated with the onset of diseases, the detection of specific sequences is a key area of research in medical fields [49–51].

In addition to abovementioned nucleic acids, microRNAs (miRNAs) have been broadly studied as one of the key biomolecules for early diagnosis of diseases [52–55]. The miRNA is a small RNA which controls the levels of gene expression in functional interacting pathways in various pathogenesis [56]. Since the levels of several miRNAs show stability in various biofluids and normal tissues, profiling and detection of miRNA are also critical issues in the medical field [57]. Consequently, the detection of the target miRNA is important in the accurate and early diagnosis of disease. Also, the peptide nucleic acids (PNAs), an artificially synthesized nucleic acid which have both properties of peptides and nucleic acids, have also been studied a lot [58]. Briefly, PNAs have 4 nucleic sequences of DNA (A, T, G, and C) and a backbone which connects each nucleic acid. When the hybridization between nucleic acids, more stable duplexes are formed by PNAs with other target nucleic acids than the other homoduplexes of nucleic acids due to the peptidic structure of backbone. Therefore, PNAs have been employed as sensing probe for nucleic acid-based biosensors.

To detect particular nucleic acids, complementary nucleic acids capable of hybridizing with the target nucleic acids are used as sensing probe molecules, utilizing their highly specific binding properties. This strategy of using nucleic acids as a biosensor is called a genosensor. However, nucleic acids alone do not produce any type of signal after hybridization such as the electrochemical, optical, and Raman signals produced by other biological components. Accordingly, nucleic acid biosensors require additional molecules that generate a change in signal which is analyzed after the hybridization of the target nucleic acids with the sensing probe sequences [59–61].

For example, one study developed a label-free DNA biosensing system using gold electrodes [62]. Based on the target DNA sequence, complementary-structured sensing probe DNA was synthesized and modified on the gold film substrate. After hybridization of target DNA and probe DNA, the impedance value of the electrodes increased because the hybridized DNA on the electrodes acted as an insulator, obstructing the redox characteristics of the ferri/ferrocyanide solution. By comparing the impedances before and after the hybridization reaction, non-labeled target DNA was successfully detected.

An aptasensor, one of the nucleic acid biosensors, uses aptamers as a recognition component [63]. An aptamer is a single-stranded sequence that binds to target molecules such as proteins, pollutants, or drugs [64–66]. The binding properties of nucleic acids with specific chemical compounds have also been utilized for toxicity screening tests. One example of this is a steganographic aptasensor for detection of mycotoxins [67]. The developed fluorescence resonance energy transfer (FRET) aptasensor was composed of fluorescence-modified aptamers and graphene oxide (GO), which was employed as a quencher because of its excellent quenching property compared to other quenchers. In this study, two types of mycotoxins (zearalenone and ochratoxin) were detected simultaneously by introducing two different kinds of specific aptamers. Because GO has a high absorption capacity for nucleic acids, the two fluorescence-labeled aptamers were absorbed onto the surface of the GO. Additionally, fluorescent signals of the aptamers were quenched by FRET. When mycotoxins existed, the aptamers were released from the GO and the prominent fluorescence of the aptamers was recovered (Fig. 2c). As this example illustrates, nucleic acids are used as sensing probes for detection of toxic molecules in addition to specific nucleic acid sequences. When nucleic acids are effectively combined with other molecules capable of generating signals, they play important roles to detect various biomolecules as sensing probes. The types and properties of the representative biomaterials discussed in Sect. 2 are summarized in Table 1.

3 Nanomaterials for Nanobiohybrid Materials

Nanomaterials are being utilized and studied in scientific fields because of their merits like unique physical properties at the nanometer scale which do not appear at the bulk scale. In biological fields, nanomaterials have been applied in drug delivery systems and to monitor biological mechanisms including cellular states [68, 69]. In

Table 1 Representative biomaterials used to develop nanobiohybrid materials

Types	Property	Target	Reference
Enzyme	– Biocatalytic – Substrate specificity	– Chemical and biological substrates (e.g., H_2O_2, NO, glucose, and lactate)	[29, 31, 34]
Antibody	– Y-shaped glycoproteins – Antigen specificity	– Antigens (e.g., viruses, bacteria, proteins, polysaccharides)	[35, 40, 41]
Nucleic acid	– Complementary nucleotide base pairing	– Complementary single-stranded DNA/RNA/PNA/probe – Proteins (aptameric) (e.g., mucin, thrombin) – Small molecules (e.g., antibiotics, toxins, heavy metals, pesticides)	[62, 64–66]

the biosensor field, nanomaterials have the potential for providing a large activated surface, support for biomaterials immobilization, and the reinforcement of inherent properties of biomaterials [70, 71]. Because of these qualities, nanomaterials are being used to develop nanobiohybrid materials for improved biosensors. In this section, the representative types of nanomaterials are categorized as metal, carbon-based, TMD, and other functional nanomaterials.

3.1 Metal Nanomaterials

Metal nanomaterials have been studied in different fields due to their excellent physical, electrical, and optical properties [72, 73]. Metal nanoparticles less than 100 nm have completely different properties from their bulk forms. They have been applied in electronics, used as catalysts, and utilized in biological applications [74–76]. In addition, some nanoparticles are commercially available, making them easily accessible for scientific study. These metal nanomaterials include gold nanoparticles (GNP), silver nanoparticles (SNP), and platinum nanoparticles (PtNP). They are easily synthesized and can be used for surface modification, making them suitable to be applied in biomedical fields including clinical diagnosis [77–79].

In addition, properties of metal nanomaterials are modulated by altering their shape and size. For example, optical characteristics of metal nanomaterials are decided on the size of metal nanomaterials that can be used in the surface plasmon resonance (SPR) or localized surface plasmon resonance (LSPR) [80]. Figure 3a illustrates how these characteristics of metal nanomaterials are employed in optical biosensors by conjugation with biomolecules.

Furthermore, bi- or multi-metal nanocomposite such as alloys and core-shell structure nanomaterials combine different metallic elements to improve the function and properties of nanomaterials compared to metal nanomaterials composed of individual components [81, 82]. Alloy nanomaterials consist of a homogenously mixed structure at the atomic scale, and due to the well-mixed structure, both of the metallic elements can exist on the surface of the nanomaterial simultaneously. In

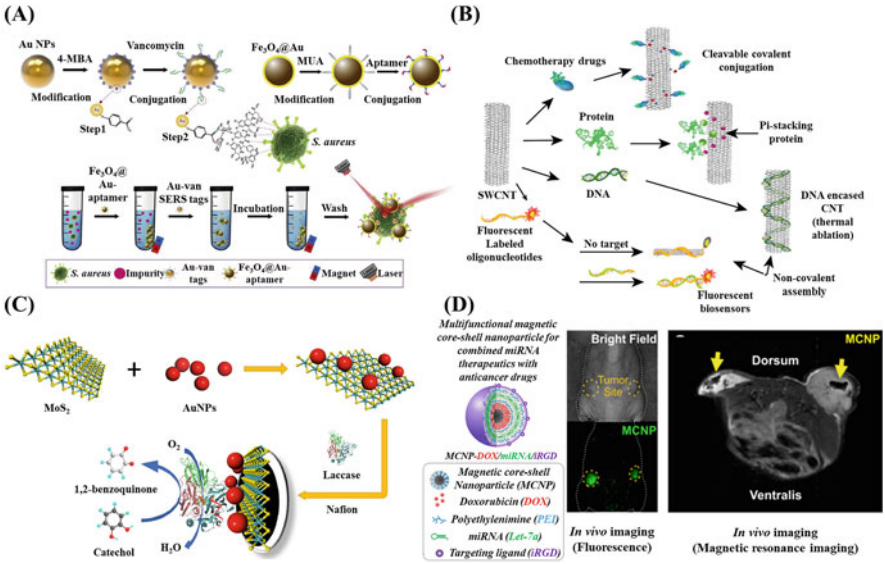

Fig. 3 (**a**) Optical biosensor composed of metal nanoparticles conjugated with biomaterials (From Pang et al. [81], with permission, copyright (2019) Elsevier), (**b**) functionalization of CNT and their biomedical applications (From Vardharajula et al. [89], with permission, copyright (2012) Dove Press Ltd), (**c**) electrochemical biosensor based on GNP-functionalized MoS_2 nanosheets for catechol detection (From Zhang et al. [105], with permission, copyright (2019) Elsevier), (**d**) MCNP construct for simultaneous delivery of miRNA and anticancer drugs and in vivo fluorescence and MRI imaging confirming the tumor-targeting capability of the MCNP constructs 24 h after intravenous injection. (From Yin et al. [118], with permission, copyright (2017) ACS)

metal nanocomposite with a core-shell structure, one metal nanomaterial is fully encased by another metal nanomaterial so unlike the alloy structure, the surface has the characteristics of one metal nanomaterial, but the inner and outer parts have distinct characteristics.

Metal nanomaterials with nanorods (NRs) structure, spherical-shaped structure and quantum dot (QD) have also been recently studied [83–85]. The NRs have some exceptional properties including efficient surface plasmon effects due to the aspect ratio of the NRs. The efficient surface plasmon effect can enhance the electric field generated by the NRs. In addition, the QD also has attractive properties such as size-tunable fluorescence and biocompatibility for biological application. The superior fluorescence properties of QD include broad absorption and narrow emission spectra, photostability, and high quantum yield. The various advantages derived from the unique structure or composition of nanomaterials can be particularly useful in the development of biosensors. New biosensors are being developed by combining biomolecules and metal nanomaterials to achieve better performance and more accurate target detection.

3.2 Carbon-Based Nanomaterials

Because of unique properties like excellent conductivity and biocompatibility, carbon-based nanomaterials have been an important area of study in biological fields [86–88]. Carbon-based nanomaterials also facilitate surface modification of biomaterials through the pi-pi interactions in the carbon nanostructure, as shown in Fig. 3b [89]. Carbon-based nanomaterials can take on various structures with multiple dimensions depending on the synthesis method. For example, a carbon nanotube (CNT) is a three-dimensional nanomaterial formed by rolled-up sheets of single-layer carbon atoms. CNTs can exhibit remarkable electrical conductivity, which is suitable for applications in electrochemical or electrical biosensors [90, 91].

Graphene, another carbon-based nanomaterial, has a two-dimensional structure and also exhibits excellent conductivity and biocompatibility [92]. GO is the oxidized form of graphene, which is a nonconductive material due to the absence of percolating pathways between sp_2 carbon clusters [93]. However, the surface of GO can be easily modified through electrostatic bonding with other amine-modified materials [94]. In addition, because of unique 2D sheet GO, the thickness of graphene-based materials can be controlled at a nanometer scale [95]. Because of its advantage of having easily controlled mechanical and chemical properties, GO has been used for electrochemical biosensors and bioelectronic devices [96]. Graphene and GO can also be used in fluorescent biosensors because of their efficient quenching properties from the resonant energy transfer via excitation of electron-hole pairs [97].

A carbon dot (CD) is a one-dimensional carbon nanoparticle that is often applied in fluorescent biosensors. CDs can be used as biolabeling reagents due to their strong photoluminescence effects, similar to a quantum dot (QD). While QDs are limited in biological applications due to the presence of heavy metals, CDs are a promising candidate for biosensors with advantages such as easy preparation, ease of surface modification, low toxicity, and excellent biocompatibility [98, 99]. Furthermore, a CD can be utilized for in vitro cell monitoring because it is not only biocompatible but also physiochemically and photochemically stable [100].

Carbon-based nanomaterials are suitable in electrochemical and optical biosensors since they have biocompatibility, high conductivity, and superior optical properties. These materials can function as a key component of nanobiohybrid materials through conjugation with biomaterials to enhance the sensing properties of biosensors with their exceptional properties.

3.3 TMD Nanomaterials

Two-dimensional (2D) nanomaterials like TMD have unique mechanical, electrical, and thermal properties [101]. Examples of these TMD nanomaterials include Molybdenum disulfide (MoS_2), Tungsten disulfide (WS_2), Titanium disulfide (TiS_2), Molybdenum diselenide ($MoSe_2$), and Tungsten diselenide (WSe_2) which have an excellent ion transport efficiency, large surfaces, and moderate toxicity

[102, 103]. In addition, TMD nanomaterials possess distinct properties depending on their crystal structures, such as 2H-phases and 1 T-phases. The 2H-phase configuration of TMD is widely used for energy applications such as batteries, catalysts, and super-capacitors because of its excellent catalytic performance and semiconducting property. The 1 T-phase in TMD exhibits enhanced electrochemical performance and charge transfer efficiency due to its metallic properties [104].

Among various TMD nanomaterials, MoS_2 nanosheets (MoS_2 NSs) and WS_2 nanosheets (WS_2 NSs) have been broadly researched for energy storage, electronics, and biological applications. The sulfur atoms on the surface of the MoS_2 NSs and WS_2 NSs can be easily modified on gold nanomaterials through the formation of strong Gold-S bonding (Fig. 3c). This Gold-S bonding between the gold and MoS_2/WS_2 NSs produces efficient charge transfer between gold and TMD [105].

TMD nanosheets can also be easily conjugated with biomolecules through simple surface modification. Large-scale liquid-phase exfoliation synthesis can be used to fabricate water-dispersible TMD nanosheets with low cytotoxicity for biological applications. These advantages make TMD nanosheets suitable for electrochemical biosensors to detect biomolecules such as proteins [106]. TMD nanomaterials, such as surface defective TMD nanosheets, flower-shaped TMD nanoparticles, and GO-modified TMD nanodomes, have been synthesized to develop highly sensitive electrochemical biosensors [107]. Some TMD nanomaterials also have unique structures with optical properties such as fluorescence quenching or emission that can be used in optical biosensing systems. For example, the QD structure of TMD (TMD QD), which is about 10 nm in diameter, has excellent optical properties and quantum confinement effects as a fluorescent probe [108]. TMD QD has been used for bioimaging because of its low cytotoxicity, excellent fluorescence properties, and small size which is suitable for recollection from cells after bioimaging, [109]. In addition to these applications, TMDs are being used to develop high-sensitivity SERS substrates. For example, the heterostructure of the GNP/WS_2nanodome/graphene system and the microsphere structure of the MoS_2 with 3D GNP array were fabricated for amplification of SERS signals [110, 111].

TMD is an example of a nanomaterial that can be combined with biomaterials to develop novel nanobiohybrid biosensors. Its primary advantages in this application include biocompatibility, excellent electrical properties, and easy surface modification.

3.4 Other Functional Nanomaterials

Magnetic nanoparticles offer unique merits including low cost for synthesis, chemical and physical stability, and biocompatibility [112–114]. In addition, the ferromagnetic property of magnetic nanoparticles can be used to efficiently collect functionalized magnetic nanoparticles by applying magnetic fields. This characteristic can be used to improve the sensitivity of biosensors through collection of magnetic nanoparticle-based sensing probes bound to target molecules. Moreover, the magnetic properties provide noninvasive detection methods such as using the

magnetic permeability [115], frequency-dependent magnetometer [116], or magnetic relaxation switches [117] to collect the magnetic, nanoparticle-based sensing probe after treatment to cells or tissues.

Among the biological applications of magnetic nanoparticles, magnetic core-shell structural nanoparticles (MCNP) prepared by surface modification have been a particular area of interest. For example, multifunctional MCNP modified with anticancer drugs and miRNA has been proposed to sensitize cancer cells and for drug delivery. In this study, the MCNP was composed of a biocompatible mesoporous silica shell modified with anticancer drugs and miRNA, as well as a highly magnetic core to significantly increase saturation magnetization to improve MRI contrast (Fig. 3d). As shown in this example, MCNP is a versatile platform for conducting multiple functions such as drug delivery and effective biological monitoring [118]. Likewise, the superparamagnetic iron oxide nanoparticles (SPOINs), one of the various types of MCNPs that consist of iron oxide core possess unique properties such as superparamagnetism and high field irreversibility, and have been studied recently for biosensing application [119]. As the promised candidate for the bioimaging and drug delivery system with biocompatibility, the SPIONs have been utilized in biosensors for a decade [120–122].

The UCNP is another promising material in the bioimaging field due to its fluorescent emission properties such as the upconversion of low-energy, near-infrared light (NIR) to high-energy ultraviolet or visible light [123–125]. In addition, UCNP has an excellent penetration depth and low toxicity for long-term tracking of biomolecules and real-time monitoring [126]. In one example, polyethylene glycol (PEG)-functionalized UCNP was used as both the nanocarrier for drug delivery and the fluorescent nanomaterial for cell imaging [127]. In this study, HeLa cells were treated with PEGylated UCNP with doxorubicin, one of the commonly used chemotherapy molecules. By introducing the multifunctional UCNP, targeted cell imaging and drug delivery were achieved simultaneously. This demonstrates the potential for UCNP in therapeutics to both deliver drugs and monitor target molecules in a nondestructive manner.

Other types of nanomaterials include organic nanomaterials such as micelles and liposomes [128, 129], as well as conductive hydrogels [130]. The advantages of these nanomaterials in biosensors include their unique characteristics of bi-solubility in both water and oil and excellent biocompatibility. The types of the representative nanomaterials with their constituting materials, structure, and properties discussed in Sect. 3 are summarized in Table 2.

4 Nanobiohybrid Materials

In general, a nanobiohybrid material is defined as a hybrid composite at nanometer scale made through the combination of biomaterials and nanomaterials. Through the synergistic properties derived from unique properties of biomaterials and nanomaterials, nanobiohybrid materials are being utilized with great potential in lots of scientific fields, especially in the field of biosensor by imparting the high

Table 2 Representative nanomaterials used in nanobiohybrid materials

Types	Materials	Structure	Property	Reference
Metal nanomaterials	Gold, silver, platinum	Nanoparticle	– Easy for surface modification – Suitable for biomedical applications	[74–76]
		Alloy and Core-shell	– Improved electronic properties – Excellent catalytic effect – Enhanced local electric field	[81, 82]
		Nanorod	– Polarized and directional emission – Efficient surface plasmon effects	[83, 84]
Carbon-based nanomaterials	Carbon	Nanotube (CNT)	– Three-dimensional – High electrical conductivity – Excellent biocompatibility	[90, 91]
		Nanosheet (graphene)	– Two-dimensional – High electrical conductivity – Excellent biocompatibility	[92]
		Nanosheet (go)	– Oxidized form of graphene – Electrostatic bonding with amine-modified materials	[94–96]
		Nanoparticle (CD)	– One-dimensional – Strong photoluminescence – Excellent biocompatibility	[98, 99]
TMD nanomaterials	MoS_2, WS_2, $MoSe_2$, WSe_2	Nanosheet	– Easy modification with gold nanomaterials – Low cytotoxicity	[105, 106]
		Nanoparticle	– Increase of the surface area	[18, 107]
		Quantum dot	– Excellent quantum confinement effects – Low cytotoxicity and good dispensability	[108, 109]
Other nanomaterials	$NiCo_2O_4$, Fe_3O_4	Nanoparticle (MNP)	– Ferromagnetic – Excellent biocompatibility	[112–114]
	$NaYF_4$:Yb^{3+}/Tm^{3+}, $NaYF_4$:Yb^{3+}/Er^{3+}	Nanoparticle (UCNP)	– Bright emissions – Suitable for long-term observation	[126, 127]

sensitivity and selectivity to the biosensors. Here, we discuss nanobiohybrid materials developed by combining nanomaterials and biomaterials, which are utilized as sensing probe molecules, to be applied for the development of biosensors.

4.1 Enzyme-Based Nanobiohybrid Materials

As discussed in Sect. 2.1, numerous enzymes, including the widely used GOx, have been researched for use as sensing probes in enzymatic biosensors. Enzymes have also been utilized in applications ranging from biofuel cells to biobatteries [131, 132]. One study suggested that an enzymatic biofuel cell composed of GOx and bilirubin oxidase (BOx) could be used to generate electricity via enzymatic reactions [133]. This biofuel cell was used to demonstrate a self-powered drug-release system. Another study developed a three-dimensional enzymatic biohydrogel electrode composed of GOx, bovine serum albumin (BSA), and arginine (Arg) entrapped in carbon fiber networks to achieve enhanced bioelectrocatalytic electron transfer reactions [134]. However, despite the broad applications of enzymes in wide scientific fields, their limitations include low stability and difficulty in measuring accurate signals from enzymatic reactions [135]. These limitations are critical obstacles for developing highly effective enzymatic biosensors and other enzymatic biodevices.

To overcome these intrinsic limitations, researchers have investigated nanomaterials that can be combined with enzymes to develop enzyme-based nanobiohybrid materials. These nanomaterials provide a large activated surface, effective support for enzyme immobilization, and reinforcement of inherent characteristics of enzymes for reinforcing the characteristics of biomaterials. For example, Choi et al. employed GNP on a metalloprotein to enhance the electrochemical signal derived from redox properties of metalloproteins. This indicated that the excellent conductive property of metal nanoparticles could be utilized to develop bioelectronic devices [136]. Graphene has also been coupled with enzymes to improve their stability, activity, and loading efficiency [137]. In addition to these examples, many other nanomaterials are being combined with enzymes to enhance their properties.

Going beyond this bulk combination of each material by self-assembly method, the conjugation of enzymes and nanomaterials at the nanometer level could maximize the effectiveness of enzymes and nanomaterials. To achieve this, many enzyme-based nanobiohybrid materials have been produced through the complex conjugation of enzymes and nanomaterials. In one study, nanoparticles such as GNP and QD were combined with complementary DNA and added to a conjugated metalloprotein-DNA platform. The resulting bioprocessing device demonstrated processing functions such as signal reinforcement, regulation, and amplification which was impossible to be achieved without delicate conjugation of biomaterials and nanomaterials [138]. Through the sophisticated conjugation of protein and nanomaterials, redox characteristics of biomaterials were applied to demonstrate specific electronic functions. In another study, a novel biofuel cell was developed using enzyme-based nanobiohybrid materials [139, 140].

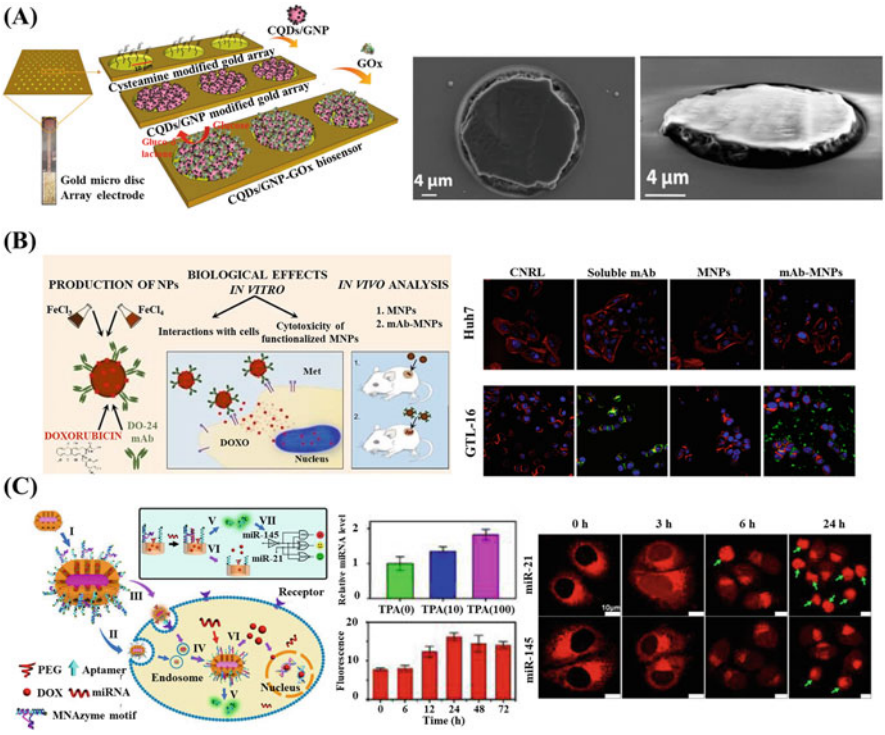

Fig. 4 (**a**) Enzyme-based nanobiohybrid material composed of GOx, GNP, and CQD on the gold micro disk arrays and its scanning electron microscopy images (From Buk et al. [141], with permission, copyright (2018) Elsevier), (**b**) scheme of the MNP-mediated drug delivery and microscope images of GTL-16 cells and Huh7 cells after incubation with nanobiohybrid nanoparticle composed of monoclonal antibody, magnetic nanoparticle, and monoclonal antibody (From Oltolina et al. [157], with permission, copyright (2019) MDPI), (**c**) MNAzyme-based nanodevices for controlled drug-release and intracellular imaging, target miRNA expression levels in cells determined by qRT-PCR, evaluation of expressed target miRNA levels after drug treatment by time variation, and microscope images of cells that received nanocarrier treatment. (From Zhang et al. [170], with permission, copyright (2015) ACS)

Above all, these enzyme-based nanobiohybrid materials have the huge potential to develop superior biosensors through the maximization of biosensing properties from biomaterials used as sensing probes. For instance, the nanobiohybrid materials composed of GOx, GNP, and carbon quantum dot (CQD) were developed to demonstrate the electrochemical sensing of glucose [141]. Here, the nanocomposite composed of GNP and CQD was conjugated with GOx to provide effective support for GOx immobilization and facilitation of the redox reaction between GOx and glucose. As a result, developed enzyme-based nanobiohybrid material could provide a powerful sensing probe for the detection of glucose electrochemically. Moreover, the gold micro disk arrays were employed as the electrode to modify the sensing probes regularly and uniformly for improving the sensitivity (Fig. 4a). In another

research, the GOx was conjugated with tobacco mosaic virus (TMV) nanotube-based nanocarrier to develop the excellent nanobiohybrid material for electrochemical biosensing application [142]. In that study, the TMV nanotube was employed as the support that provided a large activated surface area for efficient sensing probe modification [142]. In addition, several enzyme-based nanobiohybrid materials were proposed to develop fluorescent biosensors [143, 144]. In one study, the alkaline phosphatase was hybridized with lipid vesicle-based fluorescent nanoparticles to develop the enzyme-based nanobiohybrid material for fluorescent phosphate determination using the strong fluorescence emitting property [145]. Besides, various enzyme-based nanobiohybrid materials have been studied to be applied for SERS-based biosensing applications using their exceptional plasmonic properties of them [145, 146]. In addition to enabling the production of superior enzymatic biosensors, these nanobiohybrid materials could potentially be used to augment the strength of biorobots [147]. As these examples demonstrate, enzyme-based nanobiohybrid materials have huge potential across diverse scientific fields, especially for biosensors.

4.2 Antibody-Based Nanobiohybrid Materials

Antibodies are a promising component for nanobiohybrid materials because of their exceptional and accurate binding properties which can be leveraged for specific disease diagnosis and treatment [148–151]. Antibody-based nanobiohybrid materials are core components used in drug delivery systems. Nanobiohybrid materials can be utilized for effective drug delivery through the efficient binding of drug- and antibody-loaded nanomaterials to disease cells with overexpressed antigens [152, 153]. The antibody-based nanobiohybrid materials have several advantages like excellent stability in the biological condition, sufficient functional groups on the surface, and easy conjugation with target materials. These properties make them extremely suitable for biological applications [154, 155].

One example of this is a research that developed a biodegradable silica nanoparticle for mitochondria-targeting intracellular delivery of antibodies [156]. In this study, the biodegradable silica nanoparticle was fabricated using disulfide monomer Bis[3(triethoxysilyl)propyl]-disulfide, which showed a slow biodegradation profile. The slow endogenous biodegradable property led to effective mitochondria localization, as well as the efficient release of the encapsulated antibody. Moreover, the surface of the silica nanoparticle was capsulated with cell-penetrating poly (disulfides) and triphenylphosphonium for achieving fast cell uptake and mitochondria targeting. After incubation of the fabricated biodegradable silica nanoparticle with cells, the proposed biodegradable silica nanoparticle exhibited rapid cellular uptake with specific localization, biodegradation, and effective release of the antibody inside the organelles.

In another study, a magnetic nanoparticle was functionalized with a monoclonal antibody for tumor targeting [157]. The magnetic nanoparticle was a potential nanocarrier to deliver doxorubicin to tumor sites. The monoclonal antibody

specifically interacted with the surface of GTL-16 cells (Fig. 4b). To confirm the accurate delivery of doxorubicin, Huh7 cells were applied as a control. Huh7 cells were incubated with fabricated magnetic nanoparticles functionalized with monoclonal antibodies. The results showed that the fabricated nanoparticles had specific interaction with GTL-16 cells, while there was no detectable interaction from nanoparticle-treated Huh7 cells.

Furthermore, antibody-based nanobiohybrid materials can be applied to develop various biosensors including electrochemical-, optical- and SERS-based biosensors because of the synergistic effects by hybridization of antibody and nanomaterials. For example, the nanobiohybrid material consists of the GNP, graphene quantum dots (GQD), and poly-amidoamine (PAMAM) was fabricated to detect the cardiac troponin I (cTnI) antigen by electrochemical method [158]. Because of the branched tree-like structure of PAMAM, the cTnI antibody was effectively modified on a gold surface of a biosensor. In addition, excellent properties of GQD, including easy functional group modification and large surface area, helped to improve the sensitivity of a biosensor by nanohybrid materials. In another study, the MoS_2 nanosheet and gold nanorod (GNR) were conjugated with the microcystin-LR (MC-LR) antibody to detect the MC-LR electrochemically [159]. The GNR was an effective nanomaterial for electrochemical biosensors because of its attractive properties such as biocompatibility and high surface area. Moreover, the GNR could prevent the MoS_2 nanosheet aggregation for enhancing the electrochemical characteristics. Besides, various types of antibody-based nanobiohybrid materials were reported to be applied for fluorescent and SERS biosensors [160, 161]. For instance, the core-shell structural nanoparticle composed of cadmium telluride (CdTe) QDs and SiO_2 were synthesized as a fluorescence probe and conjugated with PSA antibody [162], or the GNP-modified Fe_3O_4 nanomaterials and avian influenza virus antibodies were conjugated and combined with SERs tags to be applied as SERs biosensing probe to detect target molecules [163]. As this section demonstrates, antibody-based nanobiohybrid materials have been widely used as targeting ligands because of their specific interaction through the antigen-antibody binding affinity and various functions derived from nanomaterials such as slow biodegradation. The next section will cover biosensor applications using antibody-based nanobiohybrid materials.

4.3 Nucleic Acid-Based Nanobiohybrid Materials

Nucleic acids have been researched for applications including biosensors and drug delivery systems [164, 165] because of their inherent characteristics. The highly specific binding property of nucleic acids with their complementary sequences offers a simple method for precisely immobilizing target molecules on substrates [166, 167]. However, a biosensor system composed of only nucleic acids tends to produce low signals, limiting its ability to achieve highly sensitive detection of target biomolecules in electronic devices, as well as excellent efficiency of drug delivery [168, 169]. This limitation can be overcome by combining nanomaterials with nucleic acids to improve the properties required for effective biosensing.

For example, one research developed a multiplex nanodevice by assembling multicomponent nucleic acid enzymes (MNAzymes) on a surface of mesoporous silica-coated gold nanorods (MSGRs) [170]. As shown in Fig. 4c, the fabricated nanodevices performed multiple functions: (1) a logic gate for cancer risk assessment, (2) intracellular imaging of miRNAs, and (3) controllable release of doxorubicin. When the target miRNA (miRNA-21 and miRNA-145) existed with a nanodevice, fluorescently labeled nucleic acid strands in MNAzymes were cleaved, and the intensity of the fluorescent signal emitted from the fluorescently labeled nucleic acid was monitored to detect miRNA levels. Furthermore, by using these fluorescent signals that were responsive to miRNA-21 and miRNA-145, a biologic gate was developed. This nanodevice also achieved accurately controlled drug release.

In another example, the aggregation of GNP induced by DNA hybridization can be utilized in a colorimetric biosensor because of the remarkable optical property of GNP [171]. The optical property derived from these aggregated GNP was utilized to develop a fast and simple colorimetric protocol for polynucleotide detection, [172].

In one study, single-walled CNTs (SWNTs)-modified DNA was employed as a bridge to develop electronically connected gold electrodes [173]. The single-stranded DNA was self-assembled on each of the gold electrodes by binding affinity between the gold and thiol-terminated DNA. Subsequently, the SWNTs-modified complementary DNA was hybridized with self-assembled DNA on gold electrodes and I-V measurements were performed to confirm electrical connection between the electrodes. From the results, 11.7% of the overall electrodes were electrically connected by the addition of SWNTs-modified complementary DNA on the single-stranded DNA-immobilized gold electrode through specific DNA binding. However, only 0.7% of the electrodes were connected when using SWNTs-modified non-complementary DNA due to nonspecific interactions. Based on these results, the proposed nucleic acid-based nanobiohybrid material composed of SWNTs and DNA could provide a precise method to connect or conjugate materials at the nanoscale.

The nucleic acid-based nanobiohybrid materials have significant potential for diverse biological applications including biochips, cell imaging, and drug delivery. These materials also may provide a particularly versatile platform for developing excellent biosensors. One research employed the SNP, one of the noble metal nanoparticles, as an electrochemical signal amplifier for highly sensitive and selective target DNA detection [174]. Here, the PNA was immobilized on the gold electrode and a target DNA was captured on the electrode selectively by hybridization of PNA and the target DNA. After atom transfer radical polymerization (ATRP) initiator was attached to PNA/DNA hybrid structure, the polyaldehyde was polymerized from polysaccharides by ATRP on the hybrid structure. Next, the SNP was deposited on the polymer by silver mirror reaction for achieving the high sensitivity because of electrochemical signal amplification by deposited SNP on the electrode. In addition, an electrochemical biosensor composed of carbon nanotube-gold nanoparticle (CNT-GNP) nanoclusters and the dual-DNA (reporter and linker) was developed to detect a target DNA ultrasensitively using properties of nanoclusters such as the large surface of 3D nanostructure and excellent electronic

conductivity [175]. The nucleic acid-based nanobiohybrid materials were also employed as core components on fluorescence biosensors. For instance, the nanobiohybrid material consists of fluorescence dye-tagged DNA probe and silicon nanodots, which quench signal from the fluorescence dye, and was developed to detect a target sequence DNA [176]. Also, a biosensor composed of spiky gold nanoshell-coated magnetic nanoparticles and Raman-labeled probe DNA was used for SERS-based detection of target DNA [177]. Summarizing, various types of nucleic acid-based nanobiohybrid materials have been reported by conjugation of unique properties of nucleic acids and nanomaterials that promise effective performance in biomedical and bioelectronics fields compared with the conventional platforms.

5 Electrochemical/Fluorescent/SERS Biosensors Using Enzyme-Based Nanobiohybrid Materials

Novel biosensors are being produced with enhanced sensing capabilities due to the use of enzyme-based nanobiohybrid materials. The following sections describe different types of biosensors using these materials.

5.1 Electrochemical Biosensors Using Enzyme-Based Nanobiohybrid Materials

Enzyme-based nanobiohybrid materials have been used to produce electrochemical biosensors that utilize the redox properties of metalloenzymes. Nanomaterials combined with metalloenzymes exhibit excellent conductivity and facilitate electron transfer reactions between metalloenzymes and target molecules [178]. As a result, conductive metal nanomaterials, carbon-based nanomaterials, and TMD nanomaterials are frequently utilized for this application. In one study, an enzymatic H_2O_2 electrochemical biosensor was developed by a combination of HRP and the GNP-embedded nanofiber synthesized through an electrospinning technique [179]. In another study, SNP was conjugated with HRP-loaded colloidal nanoparticles to electrochemically detect H_2O_2 with high sensitivity and stability [180].

Carbon-based nanomaterials, including CNT and graphene, are utilized to effectively immobilize enzymes and enhance electron transfer reactions. A hierarchical nanobiohybrid material composed of HRP and graphene sheets was proposed as an easy and effective platform to detect H_2O_2 [181]. In the other study, the electrochemical glucose biosensor was developed by the employment of CNT for effective GOx immobilization, which was used as the sensing probe [182]. The authors used an enzyme precipitate coating (EPC) technique for aggregative adhesion of GOx onto the CNT to efficiently prepare the nanobiohybrid material (Fig. 5a). The aggregated GOx on the CNT showed excellent redox characteristics (Fig. 5a) and exhibited superior sensing performance for glucose detection. Furthermore, by

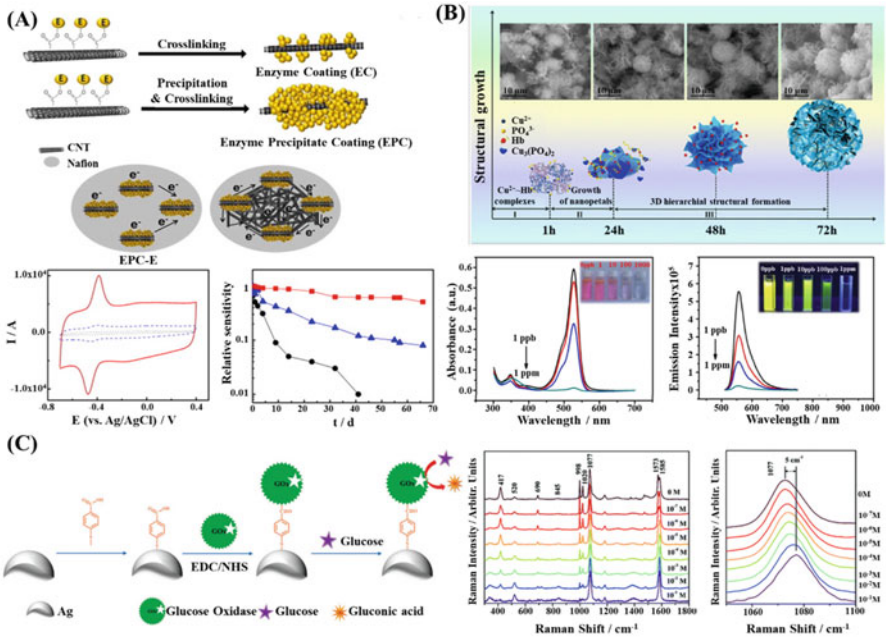

Fig. 5 (**a**) Development of enzyme-based nanobiohybrid material composed of GOx on the CNT using the EPC technique and results showing its excellent redox properties and glucose sensing performance (From Kim et al. [182], with permission, copyright (2014) Elsevier), (**b**) synthesis of Hb-modified hybrid nanoflower composed of copper phosphate ($Cu_3(PO_4)_2$ and H_2O_2 detection through fluorescent analysis (From Gao et al. [144], with permission, copyright (2018) ACS), (**c**) enzyme-based nanobiohybrid material composed of GOx-conjugated with 4-MBA assembled on SNP and its glucose sensing property measured by SERS technique. (From Yu et al. [189], with permission, copyright (2016) RSC)

employing the aggregated form of GOx in the nanobiohybrid material, the developed electrochemical biosensor retained its sensing capability for over 2 months thereby overcoming an intrinsic limitation of biomolecules (Fig. 5a).

In addition, TMD nanomaterials have also been used in nanobiohybrid materials for biosensors. TMD nanomaterials such as MoS_2 and WS_2 have been applied in enzymatic electrochemical biosensors that utilize their unique characteristics such as a rapid heterogeneous electron transfer rate [183]. To maximize the effects of TMD nanomaterials and provide a greater surface area to immobilize the enzyme, the nanosheet, nanoparticle, and other nanostructures of TMD nanomaterials have been utilized in biosensors. Among the various TMD nanomaterials, MoS_2 has been widely studied because of its excellent conductivity and biocompatibility. For example, a nanosheet of MoS_2 was combined with LOx to develop an enzyme-based nanobiohybrid material on a glassy carbon electrode to detect lactate [184]. The resulting biosensor exhibited excellent lactate detection capability with a decreased electron transfer resistance and enhanced electrochemical response.

Different structures of MoS_2, such as the microflower shape, have been researched to further increase the advantages derived from MoS_2 [185].

The conjugation of two or more different nanomaterials with biomaterials has been conducted to induce synergetic effects from each introduced nanomaterial. Choi et al. reported several biosensors using the nanohybrid material composed of MoS_2 and GO to achieve high sensitivity [27]. By conjugating MoS_2 and GO, the developed nanomaterial (GO@MoS_2) improved the charge transfer reaction by preservation of the carrier mobility of GO and facilitation of electron transfer occurred on the interface between them. To further enhance this effect, MoS_2 with a nanoparticle structure was synthesized and introduced in this study to extend the activated surface area. To encapsulate MoS_2 nanoparticles by GO, a specific chemical linker (L-Homocysteine thiolactone hydrochloride, L-hth) was used to create an amine group on MoS_2 nanoparticles, which would be enclosed by GO through the electrostatic bond. Then, Mb capable of detecting H_2O_2 was immobilized on the GO@MoS_2 to make the enzyme-based nanobiohybrid material. This biosensor exhibited excellent H_2O_2 sensing capability (concentrations: 10 nM) and selectivity. Due to the presence of biocompatible GO, the Mb retained its redox property for detecting H_2O_2 for over 9 days. To improve the conjugation efficiency of MoS_2 and GO without using intermediate chemical linkers, they synthesized surface-modified MoS_2 with an amine group to develop a nitric oxide (NO) biosensor [18]. Other nanohybrid materials composed of more than two different nanomaterials, such as platinum-decorated magnetic nanoparticles, are being researched for hybridization with enzymes to develop highly sensitive biosensors [186].

5.2 Fluorescent Biosensors Using Enzyme-Based Nanobiohybrid Materials

The advantages of fluorescent biosensors include rapid response, easy operation, and simple result confirmation by optical methods [187]. One study proposed a Hb-modified hybrid nanoflower composed of copper phosphate ($Cu_3(PO_4)_2$) to detect H_2O_2 by fluorescent and colorimetric methods [144]. In this study, the $Cu_3(PO_4)_2$ was used to improve the bioactivity and stability of Hb and accelerate the detection of H_2O_2 through the conversion of H_2O_2 into a hydroxyl radical by hybridization with the Hb (Fig. 5b). Rhodamine 6G (Rh6G) was used as the fluorescent probe, which could be combined with a hydroxyl radical and induce fluorescent quenching and hypochromatic color effects. To develop this nanobiohybrid material, Hb was incubated with copper sulfate ($CuSO_4$), and four copper ions interacted with one Hb through the conformational change of the Hb to form the nucleation growth site of the $Cu_3(PO_4)_2$ nanoflower. The conformational change also exposed the active center of Hb to react with H_2O_2 more effectively. This fluorescent biosensor demonstrated excellent fluorescent and colorimetric H_2O_2 detection dissolved in real samples (rainwater and wastewater). These samples were analyzed by colorimetric methods through fluorescence changes from yellow-like color to colorless and from pink to colorless, respectively (Fig. 5b).

In a different study, a fluorescent, microarray-based enzymatic biosensor was developed using nanobiohybrid materials composed of acetylcholinesterase (AChE), QD, and silica-coated silver nanoparticles (Silver@Silica). This biosensor was used to detect paraoxon, a well-known neurotoxin compound [188]. To develop this biosensor, AChE was used as the sensing probe capable of hydrolyzing the paraoxon into p-nitrophenol, and the QD on the surface of Silver@Silica was used as the fluorescent reporter which could be quenched by p-nitrophenol. Furthermore, the Silver@Silica could induce a metal-enhanced fluorescence (MEF) effect through control of the distance between QD and silver to achieve higher sensitivity. Lastly, all of the components, including the AChE and QD-decorated Silver@Silica were entrapped in a PEG hydrogel microarray to detect paraoxon via fluorescence. Results showed that the enzyme-based nanobiohybrid material prepared in a PEG microarray exhibited highly sensitive paraoxon detection capability due to the MEF effect.

5.3 SERS-Based Biosensors Using Enzyme-Based Nanobiohybrid Materials

Although SERS requires expensive equipment and expert skills for operation, it is an excellent sensing technique because of its exceptional sensitivity at picomolar or attomolar levels, which is difficult to achieve with other techniques. To operate a SERS-based biosensor, a Raman-active probe such as cyanine 3 and 4-mercaptobenzoic acid (4-MBA) is required. Nevertheless, by introducing Raman-active probes into enzyme-based nanobiohybrid materials, excellent SERS-based enzymatic biosensors have been reported. For example, a research conjugated GOx with 4-MBA on SNP to develop a SERS-based glucose biosensor using regular changes of the vibrational frequency and intensity from the reaction between glucose and GOx [189]. To develop this nanobiohybrid material, the 4-MBA was first coated on SNP via Silver-thiol bonding. Then, the GOx was attached to the 4-MBA-coated SNP through covalent bonding by EDC and NHS reactions (Fig. 5c). When different amounts of the glucose were added to prepared nanobiohybrid material, Raman peaks derived from the nanobiohybrid material shifted and exhibited a change in intensity, especially at the 1077 cm^{-1} band. With an increased concentration of glucose, the band of 1077 cm^{-1} shifted to higher frequency range and the intensity decreased (Fig. 5c). This phenomenon was produced by the polarizability between the SNP and 4-MBA through the change in charge transfer caused by reaction between glucose and GOx. This study suggested a new potential approach to develop SERS-based biosensors using enzymatic reactions. In addition to this study, another study developed a SERS-based biosensor using peptide-based nanobiohybrid materials [146].

5.4 Flexible Biosensors Using Enzyme-Based Nanobiohybrid Materials

Flexible biosensors are a popular area of research with the potential application of combining personalized diagnosis with devices such as smartphones and smartwatches [190]. Electrochemical biosensors are most suitable for this application of flexible biosensors [191]. To develop this type of biosensor, flexible substrates are required, and most studies utilize various polymers. However, the nonconductivity of most polymers hinders the development of excellent electrochemical flexible biosensors. To overcome this limitation, enzyme-based nanobiohybrid materials may be a potential solution. Furthermore, enzymatic reactions are suitable for targeting substances that are frequently monitored for healthcare. In one research, a nanofilm structure composed of GOx on a gold/MoS_2/gold sandwiched structure was employed on a flexible polymer substrate to achieve high conductivity for an electrochemical glucose biosensor [192]. The nanofilm produced excellent conductivity on the nonconductive polymer substrate and exhibited highly sensitive glucose detection via the enzymatic reaction. In another study, LOx was combined with zinc oxide nanoflakes on a gold-coated polymer substrate to develop a flexible lactate biosensor [193].

As shown by these examples, enzyme-based nanobiohybrid materials have significant potential to advance the sensing capability of biosensors. To achieve this goal, numerous studies are underway to fabricate the delicate, enzyme-based nanobiohybrid materials that are required to develop superior wearable biosensors that can be commercialized.

6 Electrochemical/Fluorescent/SERS Biosensors Using Antibody-Based Nanobiohybrid Materials

As previously introduced, antibody-based nanobiohybrid materials have been widely utilized for biological applications. In particular, sensing probes fabricated by conjugating nanomaterials and antibodies can be used to create ultrasensitive biosensors by achieving enhanced sensing signals.

6.1 Electrochemical Biosensors Using Antibody-Based Nanobiohybrid Materials

To detect electrically inactive target analytes such as antigens, antibody-based nanobiohybrid materials are employed to generate signals for electrochemical biosensors [194, 195]. Particularly, nanomaterials such as GNP, CNT, and GO are introduced to promote electron transfer reactions [196–198]. Sandwich-structured immunosensors have been reported by conjugating these nanomaterials with antibodies [199, 200]. For example, a human chorionic gonadotropin (hCG) antibody-modified single-walled carbon nanotube (SWCNT) was modified on the

screen-printed carbon electrode (SPCE) and coated with a blocking agent (mixture of succinimidyl ester, 1-pyrenebutanoic acid, and ethanolamine) for prevention of nonspecific binding. After the binding of hCG with the hCG antibody on the SWCNT, the hCG was detected by a gold-linked second hCG antibody with 2.5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ as the redox probe [201]. In a similar study, the GNP/gold/spiky gold/silver was conjugated with the anti-prostate specific antigen (PSA) antibody for the detection of PSA with high sensitivity. Because of the enhanced properties from the spiky structured GNP and Silver, the fabricated electrochemical immunosensor exhibited a detection limit three orders higher compared to immunosensors using GNP (26 nm diameter) as the signal probe in 1.0 M KCl solution [202].

In a different study, a sandwich-type electrochemical PSA immunosensor composed of delaminated MXene and GNP was proposed [203]. Here, an immunosensor was developed by modification of GO with GNP and p-aminothiophenol (ATP) (GNP-ATPGO). The GNP-ATPGO was immobilized on a glassy carbon electrode (GCE), and anti-PSA antibody1 (Ab_1) was immobilized on the GNP of GNP-ATPGO (Ab_1/GNP-ATPGO) through the amine-gold affinity. The GNP-coated MXene (MXene@GNP) was introduced to amplify the electrochemical signal. This MXene@GNP was conjugated with anti-PSA antibody2 (Ab_2) (Ab_2/MXene@GNP) through amine-gold binding between the amine group of Ab_2 and the gold atom of MXene@GNP. To measure the electrochemical signal for PSA detection, H_2O_2 was used as the redox probe. As shown in Fig. 6a, the fabricated immunosensor showed excellent electrochemical properties due to the edge-plane-like defective site of GO for effective electron transfer. The synergetic effect of metal nanoparticles and GO also caused enhanced surface conductivity. After the binding of PSA with Ab_1/GNP-ATPGO through the antigen-antibody interaction, Ab_2/MXene@GNP was used for amplifying the signal. The fabricated sensor showed a highly sensitive limit of detection (3.0 fg/mL) and excellent repeatability for 60 days. The developed immunosensor indicated selective sensing performance in the presence of six different analytes, such as carcinoembryonic antigen (CEA), human immunoglobulin (IgG), and bovine serum albumin (BSA).

Another study used an electrochemical immunosensor composed of AuPt hetero nanoparticles and vertical graphene (VG) to detect alpha-fetoprotein (AFP) using sandwich-type strategies [204]. The vertically oriented graphene nanosheet was synthesized on a GCE (VG/GCE) using chemical vapor deposition (CVD), and AuPt hybrid nanoparticles were prepared on VG layer (AuPt-VG/GCE). In addition, AFP antibody (Ab_1) was modified on AuPt-VG/GCE and incubated with a BSA solution to prevent nonspecific binding. Then, to demonstrate sandwich-type electrochemical immunosensing, CNT modified with gold was fabricated and conjugated with methyl orange (MO), which was used as the redox probe (MO/CNT-gold). Next, the fabricated MO/CNT-gold was combined with second AFP antibody (Ab_2) and filtered through suction filtration to collect the antibody-based nanobiohybrid material (MO/CNT-gold/Ab_2). Detection of AFP was conducted by sandwich-type and label-free strategies. In the sandwich-type strategy, after the binding of AFP with Ab_1-VG/GCE through the antigen-antibody

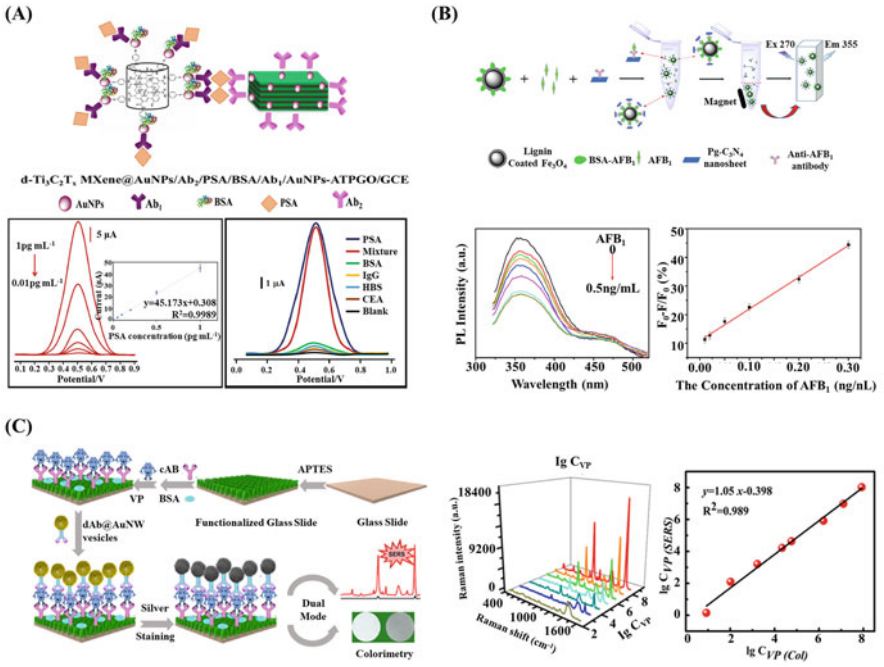

Fig. 6 (**a**) Immune reaction between Ab_2/MXene@GNP and Ab_1/GNP-ATPGO and DPV analysis of fabricated immunosensor by adding different concentrations of PSA antigen and different biomolecules (From Medetalibeyoglu et al. [203], with permission, copyright (2020) Elsevier), (**b**) fluorescent immunosensor using MNP for AFB1 detection and photoluminescence intensity and a calibration curve of the pg-CNNSs-MNPs complexes with different concentrations of AFB1 (From Xie et al. [210], with permission, copyright (2018) Elsevier), (**c**) schematic image of dAb-MBA/GNW vesicles-based immunosensor and its *Vibrio parahemolyricus* sensing property measured by SERS technique. (From Guo et al. [214], with permission, copyright (2018) ACS)

interaction, the MO/CNT-gold/Ab_2 was bound to the AFP on the Ab_1-VG/GCE. This formed a sandwich structure that exhibited an amplified electrochemical signal. Due to the redox characteristics of MO/CNT-gold/Ab_2, when the amounts of AFP increased, peak current values greatly increased. In the label-free strategy, detection of AFP was conducted by binding AFP with Ab_1-VG/GCE with MO presence, a redox probe. The peak current value decreased when the electrode resistance increased due to the increase in AFP concentration. The fabricated immunosensors using these two strategies demonstrated a similar limit of detection of 0.7 fg/mL and a linear concentration range of 1 fg/mL to 100 ng/mL due to the use of the same VG/GCE as a working electrode.

6.2 Fluorescent Biosensors Using Antibody-Based Nanobiohybrid Materials

Fluorescent immunosensors commonly incorporate antibody-based nanobiohybrid materials [205, 206]. Among the various fluorescence-based biosensing methods, FRET is a commonly used energy transfer mechanism resulting from distance-dependent interactions between donor and acceptor chromophores [207, 208]. In one example of this technique, a research proposed using UCNP and CdTeQD for the detection of procalcitonin (PCT) by the sandwich method through the FRET effect [209]. To achieve this, the UCNP (donor) was coated with poly(acrylic acid) (PAA) for enhanced solubility in water and then conjugated with 3D3 antibody (3D3 mAb-labeled UCNP) for PCT detection. The CdTe QD (acceptor) was conjugated with 2E6 antibody (2E6 mAb-labeled QD). For effective FRET between the 3D3 mAb-labeled UCNP and 2E6 mAb-labeled QD, the ratio of 3D3 mAb-labeled UCNP to 2E6 mAb-labeled QD was optimized. At the optimized concentrations of 800 μg/mL QD and 0.7 mg/mL UCNP, the immunosensor demonstrated a highly effective FRET reaction without free nanoparticles. The fluorescence signal of 540 nm was measured to determine the PCT antigen concentration in the presence of 3D3 mAb-labeled UCNP and 2E6 mAb-labeled QD mixture. As a PCT antigen concentration increased, the fluorescence signal of the mixture gradually decreased because of a sandwich structure formation. The sandwich method-based FRET immunosensor showed a highly sensitive limit of detection of 0.25 ng/mL ranging from 0.1 ng/mL to 10 ng/mL.

In a different type of fluorescent biosensor, a magnetic nanoparticle was used to detect aflatoxin B1 (AFB1) [210]. This biosensor used graphitic-phase carbon nitride (g-C_3N_4), one of the carbon-based materials, as the fluorescent probe. However, the emission peak of g-C_3N_4 overlaps with the emission peak of biomolecules such as AFB1 and BSA. To avoid this problem, the authors fabricated porous g-C_3N_4 nanosheets (pg-CNNSs) and an acidic etching solution to shift the emission peak to 355 nm. The surface of the pg-CNNSs was functionalized with the anti-AFB1 antibody. In addition, Fe_3O_4 magnetic nanoparticles (MNPs) were synthesized and modified with lignin to provide rich amine groups for AFB1 conjugation (AFB1@MNPs) via amine-amine reactions to capture the pg-CNNSs fluorescent probe. AFB1@MNPs were mixed with various concentrations of AFB1, and then the pg-CNNSs functionalized with the anti-AFB1 antibody were added to form magnetic-based complexes (pg-CNNSs-MNPs complexes) to detect AFB1. Next, the magnetic-based complexes were collected using an external magnetic field to achieve highly sensitive fluorescence measurement. Due to the fact that an affinity of the antibody with pg-CNNSs for the antigen was stronger in AFB1 than AFB1@MNPs, the concentration of the pg-CNNSs-MNPs complexes decreased as the concentration of AFB1 increased (Fig. 6b). The fluorescence intensity also decreased as the concentration of AFB1 increased.

6.3 SERS-Based Biosensors Using Antibody-Based Nanobiohybrid Materials

Among the various techniques for immunosensors, SERS is one of the most suitable methods to confirm highly specific antigen-antibody binding because of its exceptional sensitivity [211, 212]. In one research, the SERS-based immunosensor based on the nitrogen/silver-codoped CD ($CD_{N/Ag}$) was developed to detect clenbuterol (Clen) [213]. To develop this SERS-based biosensor, the $CD_{N/Ag}$ was synthesized using fructose, urea, and $AgNO_3$ via the microwave method. The synthesized $CD_{N/Ag}$ demonstrated excellent catalytic activity because of the synergistic effects of Ag and N. To detect Clen, the Clen antibody was attached to the surface of the $CD_{N/Ag}$ using electrostatic bonding. $HAuCl_4$ and trisodium citrate (TC) were added to the $CD_{N/Ag}$ to synthesize the nanogold. Because of the catalytic activity of $CD_{N/Ag}$, the synthesis of the nanogold was completed rapidly compared to the synthesis rate of nanogold without $CD_{N/Ag}$. When the synthesized nanogold was combined with the Raman probe, Victoria Blue B (VBB), the Raman intensity at 1615 cm^{-1} indicated which intensity could be used to detect Clen. When the Clen antibody was attached to the surface of the $CD_{N/Ag}$, the catalytic activity decreased, but in presence of Clen, the Clen antibody detached from the $CD_{N/Ag}$ surface due to the antibody-antigen affinity, and the catalytic activity was restored. As a result, when the quantity of Clen increased, the Raman intensity also increased due to the increase in synthesized nanogold caused by the catalytic activity of $CD_{N/Ag}$. The SERS-based immunosensor showed an ultrasensitive limit of detection of 0.68 pg/mL in the linear range from 0.0033 ng/mL to 0.067 ng/mL.

In addition to this study, a SERS and colorimetric dual-mode immunosensor were designed using gold nanowire vesicles (GNW vesicles) and silver to detect *Vibrio parahemolyricus* (VP) [214]. For this biosensor, a polystyrene (PS) template was used to synthesize the MBA-labeled GNW vesicles (MBA/GNW vesicles). $HAuCl_4$ and MBA were added to the PS template to locate the gold seed on the PS template using the reduction of gold ions in $HAuCl_4$. Then, the PS template was removed after synthesis of the MBA/GNW vesicles on the PS template, and the MBA/GNW vesicles were conjugated with the detection antibody (dAb) (dAb-MBA/GNW vesicles) as the detection probe for Raman intensity (Fig. 6c). The glass substrate was modified with capturing antibody (cAb), and the BSA was immobilized to avoid nonspecific binding. Next, different amounts of VP were added, and dAb-MBA/GNW vesicles were incubated with VP to form the sandwich structure of the detection probe. The silver enhancer solution was then introduced to VP-immobilized dAb-MBA/GNW vesicles. The silver ions of this solution were added to the surface of GNW vesicles to induce rapid catalytic reduction by GNW vesicles. The immunosensor demonstrated a highly sensitive Raman intensity and a linear relationship with VP concentration. For the colorimetric detection of VP, the silver enhancer solution was inserted into dAb-MBA/GNW vesicles. Due to the sharp-tip structure with a large surface area, the silver ion was deposited on the surface of the dAb-MBA/GNW vesicles through rapid catalytic reduction. When the VP quantity increased, the dAb-MBA/GNW vesicles increased due to

antibody-antigen binding, and the reduction of silver also increased. This immunosensor demonstrated a detection limit for VP of 10 CFU/mL using the colorimetric change through the extraction of silver ions.

As illustrated in this section, the specific antibody-antigen binding affinity of antibody-based nanobiohybrid materials provides a significant advantage in the development of excellent antibody-based immunosensors.

7 Electrochemical/Fluorescent/SERS Biosensors Using Nucleic Acid-Based Nanobiohybrid Materials

Nucleic acid-based nanobiohybrid materials are an ideal candidate for use in biosensors because of their excellent selectivity via DNA hybridization and the enhancement of sensing signals using novel nanomaterials.

7.1 Electrochemical Biosensors Using Nucleic Acid-Based Nanobiohybrid Materials

Certain metal ions, like Ag^+ and Cu^+ can be inserted specifically into mismatched sequences of double-stranded DNA to efficiently stabilize the mismatched duplex structures [215, 216]. This unique interaction between the mismatched region of the double-stranded DNA with the metal ion can be applied in novel electrochemical biosensors [217–219]. For example, an electrochemical H_2O_2 biosensor based on metallic DNA and a topological insulator was fabricated by Choi et al. [220]. In this study, eight silver ions were inserted in the cytosine-cytosine mismatches of designed double-stranded DNA, and these inserted silver ions with the double-stranded DNA could be used to detect H_2O_2.

In another example, the bismuth selenide nanoparticle (Bi_2Se_3 NP)-sandwiched gold film was utilized as a topological insulator to enhance an electrochemical signal. This biosensor showed a highly sensitive 10 x 10^{-7} M detection limit and a rapid current response (1.6 s) for H_2O_2 detection. Furthermore, the biosensor composed of DNA and metal ions detected H_2O_2 released from two cancer cells and could distinguish the two different cell lines by differences in their H_2O_2 generation. This nanobiohybrid material composed of DNA and metal ions could also provide a novel biosensing platform to electrochemically detect specific DNA or RNA using the redox signals from the inserted metal ions directly.

Similarly, the novel structure based on parallel double-stranded DNA and silver ions was utilized in the biosensor for precise nucleic acid detection [221]. As shown in Fig. 7a, a sensing probe to detect target nucleic acids consisted of recombinant azurin, which provided a stable anchoring site, as well as the imperfect double-stranded DNA. The silver ions were inserted at the mismatched portion of the top of the double-stranded DNA to generate an electrochemical signal by charge transfer from the redox of the silver ions. The imperfect DNA strand is capable of forming a double-stranded DNA segment at the mismatched portion after hybridization with

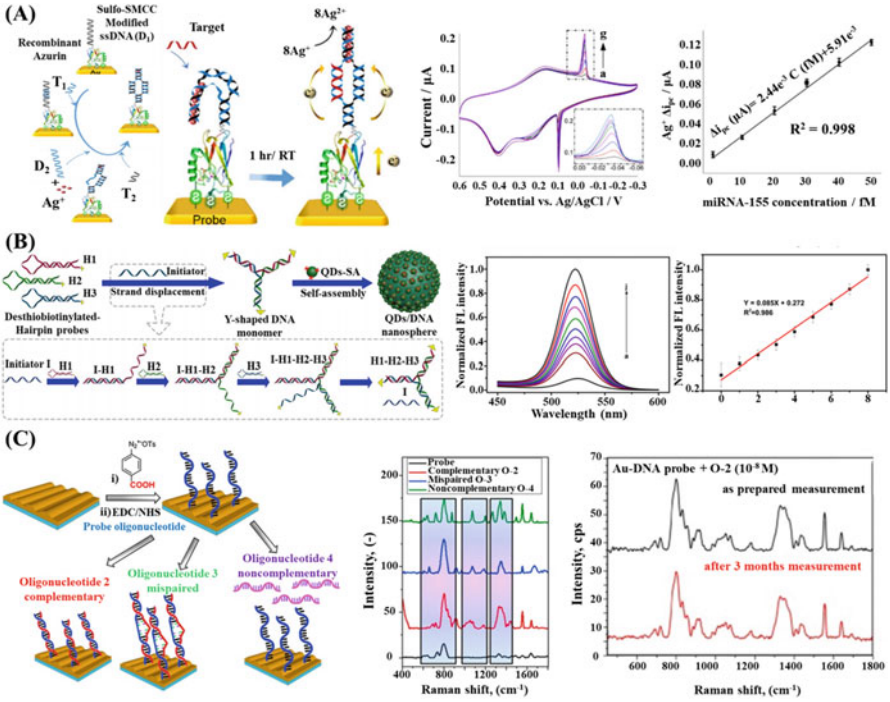

Fig. 7 (**a**) Immobilization process and detecting function of the biosensor, cyclic voltammograms for 6 different concentrations of the target RNA, and calibration linear curve (From Mohammadniaei et al. [221], with permission, copyright (2017) Elsevier), (**b**) self-assembled QD/DNA nanosphere with Y-shaped DNA monomers assembled from three hairpin probes (H1, H2, and H3) via conjugation with QD-Streptavidin (From Wen et al. [230], with permission, copyright (2017) Elsevier), (**c**) schematic demonstration of DNA detection using gold grating patterned-structure, Raman spectra of captured three types of DNA on the gold grating patterned-structure, and Raman spectra of captured complementary DNA on fabricated SERS sensor over 3 months of the storage period. (From Guselnikova et al. [234], with permission, copyright (2018) Elsevier)

the target RNA. However, because the DNA was not in an upright state and the silver ions were close to the electrodes, this resulted in a high electrochemical signal of silver ions due to the high-efficiency charge transfer. When the target single-stranded RNA existed, an imperfect part of the complementary DNA hybridized with the target and formed upright double-stranded DNA. Therefore, the electrochemical signal from the silver ions decreased as the distance between the silver ions and the electrodes increased.

This proposed biosensor based on silver-intercalated double-stranded DNA demonstrated high selectivity for target RNA detection and an excellent limit of detection (~0.5 fM), measured by cyclic voltammetry. Based on these results, incorporating novel nucleic acid-based nanobiohybrid materials into electrochemical biosensors shows the potential to significantly enhance detection capability for target nucleic acids.

7.2 Fluorescent Biosensors Using Nucleic Acid-Based Nanobiohybrid Materials

Fluorescent biosensors to detect nucleic acids have also incorporated nucleic acid-based nanobiohybrid materials and utilized the unique optical characteristics of nanomaterials [222–224]. Among the nanomaterials used for nucleic acid detection by fluorescent techniques, high-efficiency quenching materials such as gold nanoparticles and graphene oxide are widely accepted as ideal materials for the sensing probe [225, 226].

For example, a DNA biosensor based on FRET utilized UCNP as a fluorophore and GO as the quencher [227]. First, the surface of synthesized UCNP was covered by a carboxyl group and functionalized with single-strand DNA by a covalent bond. To verify the quenching effect of GO, the fluorescence signal of DNA-functionalized UCNP was measured after incubation with different GO concentrations. The measured fluorescence intensity showed a more than 95% quenching effect when the concentration of GO reached ~0.3 mg/mL with 0.4 mg/mL of DNA-functionalized UCNP. In addition, the fluorescence intensity of the DNA-functionalized UCNP gradually recovered by an increase of the complementary DNA concentration. This occurred because GO was released from the UCNP through hybridization of the complementary DNA and the DNA on the surface of UCNP. This led to the cleavage of pi-pi interactions between GO and the DNA on the surface of UCNP. These results demonstrated that the biosensor composed of GO and DNA-functionalized UCNP detected the target DNA in the picomolar range with excellent selectivity.

Similarly, a carboxylic carbon quantum dot, functioning as a nanoquencher, was utilized in a fluorescence biosensor to detect nucleic acids [228]. A fluorescence biosensor was also proposed to detect the neurotoxin acrylamide using GNP as the nanoquencher [229]. When the acrylamide existed, the single-stranded DNA formed the complex with the acrylamide, which inhibited the hybridization between the single-stranded DNA and the fluorescence-labeled complementary DNA. Upon the addition of GNP, the non-hybridized fluorescence-labeled complementary DNA was absorbed onto the surface of the GNP by an electrostatic interaction. The absorption of the non-hybridized fluorescence-labeled complementary DNA onto GNP led to the quenching of the fluorescent signal by GNP. Conversely, strong fluorescence emission was detected in the non-presence of acrylamide because a fluorescence-labeled complementary DNA hybridized with the single-stranded DNA instead of the GNP. This biosensor detected acrylamide with high sensitivity, a low detection limit of 1×10^{-8} M, and a wide linear range of 0.05×10^{-7} M. Additionally, acrylamide in a real sample (potato fries) was precisely detected.

In another study, the biosensor was proposed to detect extracellular respiring bacteria using a QD and a DNA nanosphere [230]. The QD/DNA nanosphere was self-assembled using QD-streptavidin conjugation and a Y-shaped DNA monomer (Fig. 7b). The Y-shaped DNA monomer was composed of three different hairpin structures modified with desthiobiotin, which specifically binds with streptavidin. Once a single-stranded DNA opened the hairpin structure, the three different hairpin

structures combined with each other until most of the hairpin probes were exhausted. Finally, the Y-shaped DNA monomers were self-assembled on the QD-streptavidin. The concept of this proposed method is illustrated in Fig. 6c.

To detect the target bacteria selectively, the anti-bacterial antibody-coated magnetic beads were used to strongly bind with the bacteria and isolate the bacteria by an immunomagnetic effect. Next, the isolated bacteria hybridized with the desthiobiotin-modified anti-bacteria antibody. Then, it formed a sandwich structure after a reaction with the QD/DNA nanosphere. This reaction occurred through interaction between streptavidin on the QD/DNA and the desthiobiotin on the bacteria-captured desthiobiotin-modified anti-bacteria antibody. For the next step, the sandwich complex composed of the bacteria-antibody-QD/DNA nanosphere was magnetically separated and released the QD-streptavidin via a competitive affinity reaction between desthibiotin-streptavidin and biotin-streptavidin after introducing biotin into the solution. Finally, the fluorescence of QD-streptavidin was measured by spectrophotometry to evaluate the detection efficiency for target bacteria. The proposed biosensor had a wide detection range of 1.0 cfu/mL to 1.0 x 10^{-8} cfu/mL and a detection limit of 1.37 cfu/mL.

7.3 SERS-Based Biosensors Using Nucleic Acid-Based Nanobiohybrid Materials

The SERS technique is a detection method advantageous in biosensors due to its high efficiency, rapid response, and excellent sensitivity [231–233]. Because of these characteristics, SERS-based biosensors with nucleic acid-based nanobiohybrid materials are a promising bioanalytical platform.

In one study, a SERS-based biosensor based on a functional gold grating-patterned structure was reported to detect the DNA rapidly and portably [234]. The grating-patterned structure was fabricated using a large-area excimer laser on a polymer surface followed by sputtering of the gold layer. The gold grating-patterned structure supported the surface plasmon polariton and produced a homogeneous distribution of the plasmon intensity. Then, as a sensing probe, the single-stranded DNA was modified on the carboxylated arenediazoniumtosylates (ADT-COOH)-modified gold grating-patterned structure by EDC/NHS (Fig. 7c). To estimate the sensing performance of this biosensor by Raman technique, three different DNAs were employed that had a complementary, mismatched, and non-complementary structure. Based on the measurements, each of the three types of DNA showed different SERS spectra. Visible changes in the SERS spectra between the complementary and mismatched DNA were observed because the vibration bands from each added DNA appeared, and the peak position of the previously grafted DNA chain shifted. Also, there was a slight shift in the SERS spectrum for the non-complementary DNA due to molecular rearrangement during the experimental procedure. By utilizing principal component analysis (PCA) to examine the SERS spectra, the three types of DNA were distinct and showed the 10^{-14} M detection limit in the case of complementary DNA. This fabricated biosensor

had high stability since there was less than a 10% reduction in response 3 months after fabrication. Based on the results, the biosensor demonstrated highly sensitive and stable detection of three different types of DNA through SERS spectra and PCA analysis.

This section highlights some of the advanced biosensors developed with nucleic acid-based nanobiohybrid materials. Due to the diverse advantages of nucleic acid-based nanobiohybrid, these novel biosensors exhibited enhanced sensitivity, stability, and selectivity compared to conventional biosensors. Moreover, these materials could be used to develop biosensors for early diagnosis and management of nucleic acid-based epidemics such as COVID-19. Table 3 provides an overview of the various nanobiohybrid materials utilized in the development of biosensors discussed in Sects. 5-7.

8 Conclusion and Future Perspectives

In biological and medical fields, highly sensitive detection and monitoring of harmful or important molecules is one of the most important capabilities to promote human health and welfare. To meet this need, numerous biosensors have been developed to detect chemical and biological molecules associated with fatal diseases. However, due to the intrinsic properties of biomaterials used as sensing probes, it is difficult to develop biosensors with exceptionally high selectivity and sensitivity. To overcome this limitation, researchers have developed nanobiohybrid materials that are composed of biomaterials and nanomaterials. By utilizing the synergistic effect derived from the properties of these components and the advantages gained from each of them, nanobiohybrid materials provide a powerful platform to develop excellent biosensors that use electrochemical, fluorescent, and SERS techniques.

In this chapter, the overview of nanobiohybrid materials utilized in biosensors and the techniques employed in these applications are provided. The biomaterials that are commonly used as sensing probes in biosensors include enzymes, antibodies, and nucleic acids. The nanomaterials that are frequently utilized in nanobiohybrid materials are categorized as metal, carbon-based, TMD, and other functional nanomaterials. The nanobiohybrid materials are categorized according to the type of biomaterial it includes, such as the enzyme-, antibody-, and nucleic acid-based nanobiohybrid materials. These nanobiohybrid materials are being utilized in the diverse range of biosensors that have achieved high sensitivity, high selectivity, large sensing ranges, good linearity, and stability over time.

However, there are still barriers to commercializing and implementing practical applications of nanobiohybrid material-based biosensors. These challenges include achieving more efficient conjugation of biomaterials and nanomaterials, achieving high reproducibility, and mass production of nanobiohybrid materials. Although continued research is required to overcome these obstacles and advance the use of nanobiohybrid material-based biosensors, this chapter describes some of the innovative approaches underway for developing improved biosensors for biological and medical applications.

Table 3 Summarized table of research on nanobiohybrid materials-based biosensors

Nanobiohybrid materials usedin biosensors					
Type of nanobiohybrid material	Composition	Target	Sensing probe	Measuring technique	Reference
Enzyme-based	Aggregated GOx/CNT	Glucose	Gox	Electrochemical	[182]
	Hb/$Cu_3(PO_4)_2$/Rh6G	H_2O_2	Hb	Fluorescent	[144]
	GOx/4-MBA/SNP	Glucose	Gox	SERS	[189]
Antibody-based	Ab_1/GNP-ATPGO	PSA	Ab_2/ MXene@GNP	Electrochemical	203
	pg-CNNSs- MNPs complexes	AFB1	pg-CNNSs	Fluorescent	210
	dAb-MBA/GNW vesicles	VP	MBA/GNW vesicles	SERS	214
Nucleic acid-based	Gold/Bi_2Se_3 NP/gold/silver ions	H_2O_2	Silver ions	Electrochemical	220
	QD/Y-shaped DNA/desthiobiotin-antibody	Extracellular respiring bacteria	Desthiobiotin-antibody	Fluorescent	230
	Gold grating pattern/single-stranded DNA	Complementary, mismatched, non-complementary DNA	Single-stranded DNA	SERS	234

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Conflicts of Interest The authors declare no conflict of interest.

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Biosynthesis, Biofunctionalization, and Bioapplications of Manganese Nanomaterials: An Overview

Bandita Panda, Archita Lenka, Prasanna Kumar Dixit, and Sandip Kumar Dash

Abstract

Nanotechnology primarily deals with materials of nano size and having customized shape, size, composition, or properties. Recently, these materials have attracted researchers across the globe by their properties and applicability. Manganese (Mn) has curved out a niche for themselves among the different types of nanomaterials (NMs) available at present. Mn NMs exists mostly in form of their oxides or in form of composites with other metals (bimetallic or polymetallic). Composite NMs exhibit synergistic as well as superior properties than their monometallic counterparts and preferred when it comes to the synthesis of NMs in vitro. Synthesis of NMs either follow a physicochemical or biological route proceeding through top-down or bottom-up approaches. Although, physicochemical route-based NM synthesis is quite common, but require extreme parameters and may either utilize or may produce harmful chemicals. As a result, biosynthesis, being comparatively simpler and eco-friendly, is preferred over the other. The NMs after their synthesis, often get agglomerated, mostly because of their surface energy and/or van der Walls force. In order to overcome this, the NMs are stabilized by using a suitable stabilizing agent. Apart from this, surface functionalization with chemical or biological molecules is another post-synthetic change carried out to the NMs in order to allow them for super-conjugation with more molecules and broaden their applicability. However, among the different conjugants used for functionalization, biconjugants are preferred the most, because they are biocompatible, exhibit binding specificity for complementary analyte, and can be modified according to requirement. Further, looking into the applicative aspects of different Mn NMs, bioapplication is the most prevalent and explored area as it directly impacts to the human life. Different bioapplications of

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the Mn NMs include drug targeting, in vivo tracking the path of molecules, bioimaging of tissue, implementation as antimicrobial agents and so on. In this chapter, we have discussed in detail about different biosynthesis approaches for the synthesizing Mn NMs as well as bioconjugants used for their functionalizing and finally their biological applications.

Keywords

Biofunctionalization · Biosensors · Biosynthesis · Manganese nanomaterials · Nanomaterials

1 Manganese Nanomaterials

Nanomaterials (NMs) basically include materials in the range of 1–100 nm, such as nanoparticles (NPs), quantum dots, quantum wires, ultra-thin films, fullerenes, dendrimers, nanotubes, and so on [1]. For decades, NMs have been used in a variety of fields including nanocomposite synthesis, nanocoating, solar cell design, producing supercapacitors, drug targeting, and/or bioimaging. These NMs have succeeded to attract the attention of researchers worldwide because of their unique characteristics such as surface-to-volume ratio, energy, charge, solubility, and other parameters [2]. These properties can be customized further just by changing their surface topography and size. Looking into the orientation and shape of these NMs, they can be one-dimensional (nanowires, nanotubes, and nanorods), two-dimensional (nanofilms and nanoplates), three-dimensional (nanocomposites and nanoclusters), or even zero-dimensional (quantum dots and NPs) [3], On the same time, NMs can also be classified according to their compositions and particularly metallic NMs have been explored most because of their dynamic properties and diversified applicability.

Metallic NMs can either be in form of singlets, i.e., monometallic (MM) or in conjugation with other metals in form of cluster, alloy, or core-shells (spherical, polyhedral, rod, hollow, movable-core, porous, or irregular), producing bimetallic (BM) or polymetallic (PM) NMs (Fig. 1) [4]. Especially the BM and PMs have fascinated the scientists more as they are known to exhibit synergistic as well as superior properties than their counterparts [5]. Furthermore, Mn stands out among other metallic NMs, mainly because of their multi-dynamic applicability and abundance [6]. Furthermore, these NMs are either in form of Mn oxides (MnO, Mn_5O_8, Mn_2O_3, MnO_2, and Mn_3O_4) or their composites with other elements [7, 8].

2 Synthesis of Mn NMs

Researchers have tried to synthesize these NMs, following different physicochemical and biological approaches through top-down (milling, etching, sputtering, and explosion, etc.) or bottom-up approach (sol-gel, spinning, flame spraying, laser or

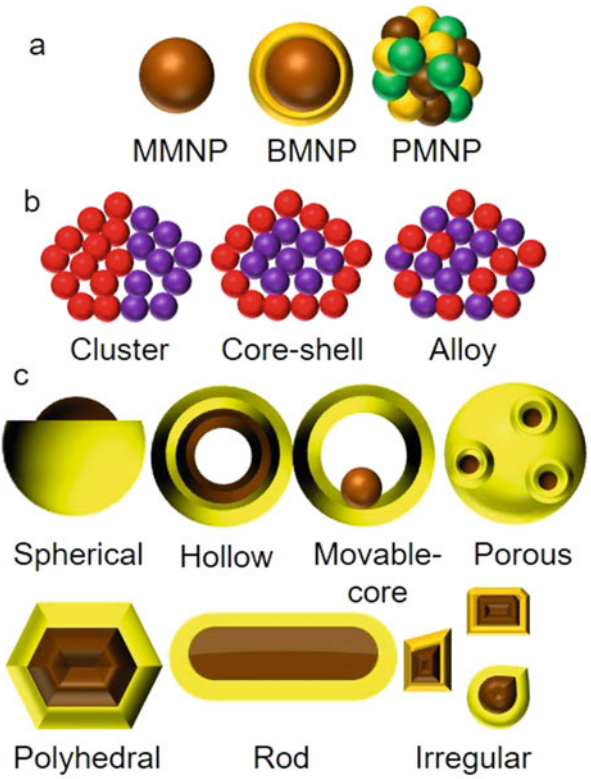

Fig. 1 (**a**) MM, BM, and PM NMs, (**b**) cluster, core-shell, and alloy, and (**c**) different forms of core-shell NMs

vapor deposition, etc.) (Fig. 2). A top-down strategy entails, first break down of bulky materials into powders and then into particles with nano size, whereas a bottom-up approach involves self-assembling of atom or sub-atomic particles into clusters, and then into NMs. However, in this chapter, we have mainly focused on the biogenic approaches, because this method is simple, eco-friendly, and in demand of the time. The Mn NMs thus synthesized in vitro, can be in singlet, fused, or agglomerated form having high (nanowire, tubes, helices, and belts) or low aspect ratio (spheres, pyramidal, and cubes) [9]. Moreover, for controlling the shape, size, and properties of the NMs, different physicochemical parameters such as temperature, pH, and pressure are monitored while synthesizing them.

2.1 Physicochemical Synthesis

Physicochemical approaches are used mainly to synthesize either pure NMs or NMs of customized shape, size, properties, and functionalization. Some of the common physical methods used for the synthesis of Mn NMs are mechanical milling [10], melt mixing, physical vapor deposition [11, 12], laser ablatio [13, 14], and electric arc deposition [15], whereas the chemical methods include colloidal precipitation,

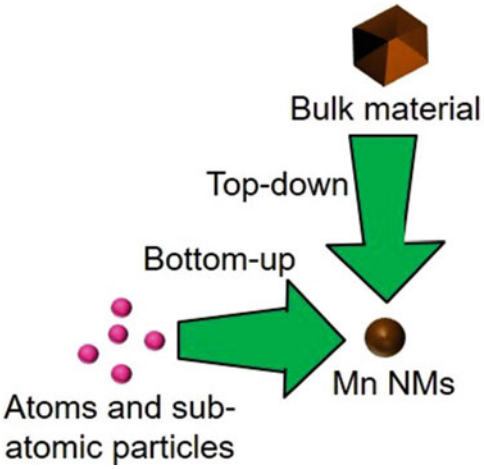

Fig. 2 Schematic presentation for bottom-up and top-down approaches of NM synthesis

co-Precipitation [16] polyol [17], sol-gel [18], micro-emulsion [19], sonochemical [20], hydrothermal [21], chemical vapor deposition [22], solvothermal [23], and others. Chemical procedures are easier to use, comparatively cost-effective, and utilize less heat than that of physical methods. However, most of the physicochemical methods require high temperature, pressure, energy, and either they produce or utilize harmful chemicals. To overcome these issues, green synthesis is favored, particularly for in vitro synthesis of NMs.

2.2 Green Synthesis

Synthesis of a NM using green route is quite simple, economic, and eco-friendly; therefore, several groups have tried this approach for the synthesis of Mn NMs. Furthermore, synthesis of a NM through green route primarily requires three components, those are source, reducing agent, and capping agent. For the synthesis of NMs of Mn, research groups have utilized different bioreducing agents such as bacteria, fungi, yeast, plant, and plant products. However, among all those agents, plant and plant products are preferred, as they can produce NMs on large scale, at a faster rate without the need for any culturing of cells [24]. This in return not only just saves time and energy but also reduces the cost of synthesis.

2.2.1 Bacteria-Based Synthesis

Bacteria are natural reducing agents, available almost in all parts of the earth, and can be cultured easily. Therefore, these are preferred highly as a bioreducing agent for the synthesis of NMs [25, 26]. A todorokite-like porous MnO_2 of disordered fibril array was produced by Kim and group [27] using *Leptothrix discophora* SP-6. A few years later, Sinha et al. [28] added sterilized $MnCl_2{\cdot}4H_2O$ into a *Bacillus sp.* and allowed it to grow. As a result, very small, uniform particles appeared around the

cytoplasm after 24 h. On continuing the culture till 48–72 h, spherical, homogeneous, and non-agglomerated NPs were formed in the cytoplasm. Also, the group has succeeded in synthesizing the NPs ex situ, simply by using the bacterial lysates instead of the bacteria itself. *Shewanella sp.* being a well-known oxidizer, different strains of the bacteria such as *S. oneidensis* MR-1, *S. putrefaciens* CN-32, *S. putrefaciens* 200, *S. loihica* PV-4, and *S. denitrificans* OS217 were used by wright et al. [29] for the synthesis of Mn NMs. In their process, each of the strains was cultured to mid-log phase and then inoculated into PYE media containing $MnCl_2{\cdot}4H_2O$ and grown for 20 days. Although, most of the strains oxidized successfully the Mn^{2+} into Mn^{4+}, the rate of oxidation varied from one another of the order *S. loihica, S. putrefaciens, S. putrefaciens, S. denitrificans, and S. oneidensis*. Similarly, the appropriate concentration of the $MnCl_2{\cdot}4H_2O$ required for the synthesis was found to be 1–10, 3–5, 2–4, and < 1 mM for *S. loihica, S. putrefaciens, S. denitrificans*, and *S. oneidensis*, respectively. In the case of *S. loihica* and *S. putrefaciens* CN-32, oxidation of the precursor started after 2–3 days and continued till stationary phase, exhibiting the highest rate of oxidation at lag phase of bacteria. Another group Jiang et al. [30] also used the same *S. loihica* PV-4 for reducing $KMnO_4$ into Mn_2O_3 nanostructure which was then calcinated at 500 and 700 °C for 5 h to produce NPs.

In another study, conducted by Hosseinkhani and Emtiazi [31], *Acinetobacter sp.*, isolated from Persian Gulf water and identified through 16 s rRNA sequencing was inoculated into $MnCl_2$ containing K medium and then allowed to grow for 14 days at 28 °C and 180 revolutions per minute (RPM). It was observed that oxidation of the Mn started after the day of attaining stationary phase (3 days) and reached its maximum level after 14 days. The method produced small and agglomerated bixbyite-like Mn_2O_3 NPs. The effect of the concentration of the $MnCl_2$ at 0.1, 1, 10, and 100 mM was evaluated on the synthesis of NP and found that the growth decreased at 100 mM. Furthermore, synthesis of the NMs was inhibited by sodium azide, which may be due to the inhibition of enzymes. *Pseudomonas putida*, another bacterium used by Feng et al. [32] for the synthesis of MnO NPs from $MnSO_4{\cdot}H_2O$. In this process, 19 h cultured bacterial cells were exposed to $MnSO_4$ and incubated for 48 h at 200 RPM to yield MnO NPs. MnS NPs synthesis was carried out by using *Streptomyces* sp., the largest Gram-positive bacterial genus of the class Actinobacteria [25].

2.2.2 Yeast-Based Synthesis

Salunke et al. in 2015 [33] used $KMnO_4$ to synthesize MnO_2 NPs using a bacterium (*Saccharophagus degradans*) and yeast (*Saccharomyces cerevisiae*) separately. They observed that the supernatant of both microbial cultures could do it successfully. When they tried with just *S. degradans* cells, they found no NMs, but the culture media and its constituents did. They concluded that *S. degradans* is unable to synthesize MnO_2 NPs. In contrast to this result, *S. cerevisiae* synthesized NMs both with or without the culture media. They arrived at the conclusion that not all bacteria are capable of synthesizing NMs. Table 1 lists other Mn NM production methods based on bacteria and fungi.

2.2.3 Fungi-Based Synthesis

In a study carried by Ogunyemi and group [34], the fungus *Paenibacillus polymyxa* strain Sx3 was used to synthesize MnO_2, ZnO, and MgO NPs by following the same procedure which was followed by Rajabairavi et al. 2017 [35] with certain modifications. In this process, the bacteria were grown at 30 °C for 24 h under 180 RPM followed by mixing the supernatant with corresponding metal oxides through stirring for 24 h at RT. The MnO_2 NPs were produced in this method and were of irregularly spherical shape. In another experiment, Uddin and coworkers [36] used *Fusarium oxysporum* to produce antiferromagnetic Mn_5O_8 NPs. In their experiment, mycelium was extracted by culturing the fungus at 25–27 °C for 96 h with 200 RPM which was mixed with $MnAc_2{\cdot}4H_2O$ and incubated. The procedure yielded stable, dispersed, and quasi-spherical Mn_5O_8 NPs. Surprisingly, the NPs got enlarged by ~4 nm up on calcinating at 400 °C for 3 h may be due to the removal of the upper fungal protein coat. On increasing the temperature to 700 °C, the weight of particles was lost by 64%.

2.2.4 Plant-Based Synthesis

In case of plant-based biosynthesis, the plant proteins or other metabolites, act as both reducing as well as a capping agent in addition to controlling the NM morphology, enhancement of antimicrobial or anti-inflammatory properties, and increasing biocompatibility. Majority of the research on plant or plant product-based Mn NM synthesis found focused on the synthesizing MnO_2 NMs from $KMnO_4$ [37–39], $C_4H_6MnO_4$ [40], $MnCl_2$ [41], and $MnAc_2.6H_2O$ [42, 43]. Abuzeid et al. [37] synthesized fine interconnected wire-shaped α-MnO_2 NPs from $KMnO_4$, using both juice and peels from orange. Although, both reactions produced NMs, the rate of production and size of the NMs were different. Those NPs were then dried through heating at 90 °C overnight, followed by 300 °C for 5 h. In a similar study, *Datura stramonium* leaf extract (LE) was used to reduce $KMnO_4$ into polymorphic MnO_2 NPs. The leaves were collected, washed, dried, ground, boiled, and then filtered before adding them into $KMnO_4$ at pH 6.0. The solution was stirred for 3–4 h till brown NPs were precipitated, which was then homogenized through sonication and washed before drying [38]. Ullah et al. in 2020 [39] also synthesized α-MnO_2 NPs from $KMnO_4$ but using the LE of *Bryophyllum pinnatum*. The LE was prepared the same as Joshi et al. (2020) [38] and added to $KMnO_4$ dropwise in 1:1 ratio at 90 °C, which resulted in spherical, ferromagnetic, partially agglomerated NPs. Recently, Dewi and Yulizarin [41], used *Euphorbia heterophylla* LE for the synthesis of the same MnO_2 NPs. They macerated the leaves in methanol and stirred for 1 week, then resuspended in methanol: hexane (1:1, v/v). The LE was added to $KMnO_4$ at 80 °C along with constant stirring followed by calcination at 500 °C for 2 h. This process yielded irregular NPs of 56.68 nm. In another experiment, *Yucca gloriosa* was used to produce spherical MnO_2 NP through time, pH, and LE optimization. The extract was prepared by boiling the powdered leaves followed by cooling and centrifugation. For the synthesis of the NPs, 10, 20, and 50% of the extract was added to $MnAc_2{\cdot}6H_2O$ at pH 4, 6, and 8 for 40, 80, and 120 min at RT [42]. Recently a group headed by Al-Tamimi et al. [43] synthesized rod-shaped MnO_2 NP by incubating a

Table 1 Different green approaches for the synthesis of Mn NMs by using bacteria, fungi, yeast, plant, or plant products as reducing and capping agents

Sl. No.	Mn NMs	Plants/parts	Source	Shape	Size	Refs.
Bacteria-based						
1	MnO_2	*L. discophora*	Mn(II) metal	–	L: 20–100, W: 1.5–4.0 nm	[27]
2	MnO	*P. putida*	$MnSO_4 \cdot H_2O$	Rope	L: ~100, W: ~10 nm	[32]
3	Mn_2O_3	*Acinetobacter sp.*	$MnCl_2 \cdot 4H_2O$	–	<500 nm	[31]
4	MnO_2	*Bacillus sp.*		Spherical	~ 4.6 ± 0.14 nm	[28]
5	MnS	*Streptomyces sp.*	$MnSO_4 \cdot H_2O$	Polymorphic	10–20 nm	[25]
6	Mn_2O_3	*S. loihica*	$KMnO_4$	Cube	Edge length: 1.8 μm	[30]
8	MnO_2	*Shewanella sp.*	$MnCl_2 \cdot 4H_2O$	Spherical	–	[29]
9	MnO_2	*P. polymyxa* strain Sx3	MnO_2 solution	Spherical	19.8–63.9 nm	[34]
Fungal-based						
10	MnO_x	Acremonium KR21–2	$MnSO_4 \cdot H_2O$	–	–	[53]
11	Mn_5O_8	*F. oxysporum*	$MnAc_2 \cdot 4H_2O$	Quasi-spherical	10–11 nm	[36]
12	MnO_2	*S. cerevisiae*	$KMnO_4$	Hexagonal, spherical	~34.4 nm	[33]
13	Mn	*F. nygamai*	Mn^{+2}	Spherical	11.90–43.29 nm	[54]
Plants or plant product-based						
14	Mn_3O_4	*Musa sp.*	$KMnO_4$	Spherical	20–50 nm	[45]
15	MnO_2	*C. limon*	$MnAc_2 \cdot 4H_2O$	Polymorphic	50 nm	[55]
16	MnO_2	*K. pictus*	$KMnO_4$	Spherical	~ 9.2 nm	[56]
17	MnO	*A. Malabarica*	MnO solution	–	0.5–2.0 μm	[57]
18	Mn_3O_4	*A. comosus*	$KMnO_4$	Spherical	10–34 nm	[46]
19	MnO_2 Mn_2O_3 Mn_3O_4	*S. mukorossi*		Needle Spherical Cubic	< 10 nm 28.7–63.1 nm 21.6–65.4 nm	[50]
20	Mn_3O_4	*A. indica*	$MnAc_2 \cdot 4H_2O$	Spherical	20–30 nm	[47]
21	MnO_2	*B. oleracea*	$KMnO_4$	Tetragonal	10–20 nm	[58]
22	MnO_2	*C. limon*	$MnAc_2 \cdot 4H_2O$	Spherical	50 nm	[44]
23	Mn_3O_4	*Chaenomeles sp.*	$Mn(NO_3)_2 \cdot 6H_2O$	Spherical	40–131 nm	[59]
24	MnO	*S. aromaticum*	$C_4H_{14}MnO_8$	–	2.5 ± 0.88 nm	[60]
25	Mn_3O_4	*J. adhatoda*	$MnSO_4 \cdot H_2O$	Tetragonal	~ 44 nm	[7]
26	MnO_2	*P. amarus*	$MnAc_2 \cdot 4H_2O$	Rod	D: 100–200 nm	[61]
27	α-MnO_2	*V. vinifera* *M. domestica*	$KMnO_4$	Rod Rod	D: 40–80, L: 150–800 nm	[62]

(continued)

Table 1 (continued)

Sl. No.	Mn NMs	Plants/parts	Source	Shape	Size	Refs.
					D: 28–70, L: 85–180 nm	
28	MnO_2	*A. marmelos*		Tetragonal	27.3 nm	[63]
29	MnO_2	*E. heterophylla*	$MnCl_2{\cdot}4H_2O$, $KMnO_4$	Irregular	~56.68 nm	[41]
30	α-MnO_2	*C. limon*	$KMnO_4$	Irregular Needle Rod	< 10 nm L: ~50 nm D: 17, L: 150 nm	[64]
31	MnO_2	*Y. gloriosa*	$MnAc_2{\cdot}6H_2O$	Spherical	~80 nm	[65]
32	MnO	*D. graveolens*		Spherical	~38 nm	[8]
33	α-MnO_2	*C. sinensis*	$KMnO_4$	Wire	–	[37]
34	MnO	*A. calamus*	H_8MnO_8S	–	–	[48]
35	MnO	*C. verum*	$C_4H_{14}MnO_8$	Spherical	50–100 nm	[49]
36	MnO_2	*M. chamomilla*	MnO_2	Irregularly spherical	16.5 nm	[66]
37	MnO_2	*Y. gloriosa*	$MnAc_2{\cdot}4H_2O$	Spherical	~32 nm	[42]
38	MnO_2	*G. resinifera*	$C_4H_6MnO_4$	Spherical	~17–35 nm	[40]
39	MnO_2	*C. zuluensis*	$KMnO_4$	Flake	11–29 nm	[67]
40	α-MnO_2	*B. pinnatum*		Spherical	4–19 nm	[39]
41	MnO_2	*A. sativa*	$MnAc_2{\cdot}4H_2O$	Rod	50–100 nm	[43]
42	MnO_2	*D. stramonium*	$KMnO_4$	Polymorphic	–	[38]
43	Ag-MnO_2	*C. pepo*		Spherical	15–70 nm	[51]
44	Ag-MnO_2	*C. majus* *V. minor*		Polygonal Oval and Spherical	32.47 ± 0.73 nm 10.09 ± 0.14 nm 9.36 ± 0.19 nm	[52]
45	Ag-Mn	*A. pintoi*		Spherical	3.3 nm	[24]

mixture of boiled oat powder and $MnAc_2{\cdot}6H_2O$ for 1 h at RT and pH 8. Using the same Mn precursor, another group also synthesized MnO_2 NPs but at a temperature of 50–60 °C and using lemon and curcumin extracts. NP production was confirmed through the yellow coloration of the pale green suspension which turned reddish brown on treating with curcumin extract for 2 h [44]. The phenols of *Gardenia resinifera* were targeted by Manjula et al. for reducing $C_4H_6MnO_4$ into MnO_2 NPs [40].

Other commonly biosynthesized Mn oxides (Mn_xO_y) include Mn_3O_4, MnO, Mn_2O_5, and so on. Yan and coworkers [45] used extracts from the peels of bananas to synthesize crystalline, spherical, tetragonal Mn_3O_4 NPs of 20–50 nm. In another

experiment also peel extract was used but from *Ananas comosus* [46]. The extract was prepared by stirring fine pieces of peels at RT for 4–5 h followed by filtration and then added into $KMnO_4$. The mixture when incubated at RT for 5 h produced spherical NPs (40–50 nm). Sharma et al. [47] used boiled LE of *Azadirachta Indica* for the synthesis of the same NPs from Mn(Ac)2·4H2O. Similarly, *Justicia adhatoda* LE was also tried for the synthesis of protein-coated Mn_3O_4 NPs but from $MnSO_4{\cdot}H_2O$ solution and with continuous heating at 90 °C in presence of NaOH as catalyst. Addition of NaOH led to an increase in the pH and the particle formation started at pH > 6 in which, the NPs were then washed and dried at 110 °C. Nano powders were collected after crushing. Those NPs changed into Mn_5O_8 on heating at 498.3 °C and then to Mn_2O_3 at 581.3 °C [7].

Polyphenols secreted by *Acorus calamus* rhizome were utilized by Arasu and group in 2019 [48] for the synthesis of MnO NP. The rhizome was cleaned, dried, crushed, and heated in a microwave for 3 min before adding into $MnSO_4{\cdot}4H_2O$ at 32 °C to produce MnO NPs. Another group attempted to synthesize the same NPs by using the bark of *Cinnamomum verum*. In their experiment, they sun dried the bark and then ground it into powder followed by boiling at 70 °C for 2 h and filtering. For the preparation of NP, a homogenized mixture of $MnAc_2$ and extract was sonicated at 60 °C for 7 h in the presence of sodium alginate. The suspension on heat-drying at 70 °C for 3 h followed by 550 °C for 7 h, resulted in spherically agglomerated NPs (50–100 nm) [49].

Some of the reports published earlier have also claimed the synthesis of multiple types of Mn_xO_y NPs. One such study conducted by Jassal et al. in 2016 [50] reported three distinct oxides: MnO_2, Mn_2O_3, and Mn_3O_4 of needle, spherical, and cubic shape, respectively. The *S mukorossi* fruit was dried at 50–60 °C for 48 h followed by grinding and suspension. Different mixtures were incubated for different time periods for the synthesis of different NPs. For MnO_2 NP, a mixture of KMnO4, $MnCl_2$ was mixed with fruit extract at 2:3 ratio along with constant stirring at RT. Similarly, for Mn_2O_3 NPs, fruit extract was added to $MnCl_2$ in methanol, with continuous stirring followed by the addition of ammonia. However, for Mn_3O_4, fruit extract was added to $MnCl_2$ solution in the presence of NaOH and then stirred at RT for 24 h.

Scientists have also tried to synthesize metal-doped Mn_xO_y NPs or metal composites. A study carried out by Krishnaraj et al. [51] states that *Cucurbita pepo* LE can act as an efficient reducing agent for the synthesis of MnO_2 as well as Ag-doped MnO_2 NPs. The LE was prepared just like any of the previously described methods through microwave-based boiling and added to $KMnO_4$ for the synthesis of MnO_2 NPs (15–70 nm) while a mixture of $KMnO_4$ and $AgNO_3$ at pH 7 for the synthesis of Ag-doped NPs (5–40 nm). The concentration of LE, $KMnO_4$, $AgNO_3$, and pH were optimized. In a similar type of study, Ciorita and group [52] used *Chelidonium majus* and *Vinca minor* to produce Ag-doped MnO_2 NPs in a two-step process. In the first step, the plant extract was added to $KMnO_4$ and sonicated for 1 h for the synthesis of MnO_2 NPs. In the next step, those NPs were added to $AgNO_3$-containing plant extract and stirred at 1000 RPM for 6 h at RT. NPs having different morphologies such as, polygonal, oval, and spherical shapes were

synthesized from *C. majus* (32.47 nm ±0.73 nm) and *V. minor* (10.09 nm ±0.14 nm) while at 1:1 ratio (9.36 nm ± 0.19 nm), all the NPs produced were of core-shells with Ag being outer cover. Tien and coworkers [24] used *Arachis pintoia* leaf and stem extract to prepare uniformly distributed spherical Ag-Mn BM NPs of 3.3 nm along with Ag NPs. In their experiment, they heated the cleaned and ground material (10%) for 2 h at 70 °C. The filtrate at RT was added to either $AgNO_3$ or a mixture of $AgNO_3$ and $KMnO_4$ (1:4) and stirred at 300 RPM for 1.5 h.

3 Functionalization

NMs after their synthesis, get agglomerated, mostly because of their surface energy and *van der* Waals forces [68]. To prevent this, the NMs are functionalized using different functionalizing agents such as CA, OA, polyethyleneimine (PEI), PEG, oleyilamine, poly (ethylene-co-vinyl acetate), mercaptopropionic acid (MPA), and PVP (Fig. 3) [69, 70]. Functionalizing agents like PEI and PEG are used even as linkers for allowing conjugation with other molecules such as antibodies, folic acid (FA), hyaluronic acid (HA), and so on. These composite NMs are used in positron emission tomography (PET), magnetic resonance imaging (MRI), or drug targeting [71, 72]. Furthermore, adding proteins, dendrimers, gelatins, dextrans, and chitosans to these NMs can enhance their antimicrobial characteristics, while fluorophores can help in tracking the NMs both in vivo and in vitro. Different strategies employed for the functionalization of Mn NMs and their implementation has been listed in Table 2.

3.1 Biofunctionalization

Biofunctionalization mainly involves the conjugation of biomolecules onto NMs to enhance their applicability and biocompatibility. Tripathy and group [71] employed single-stranded DNA (ssDNA) probe-immobilized Mn_2O_3 nanofibers to detect dengue fever. A cobalt tetracarboxyl phthalocyanine and amino-functionalized octahedral Mn molecular sieve composite was used for the degradation of diclofenac at pH 5–9 [73]. Functionalization of the CA onto $MNFe_2O_4$, not only improved its biocompatibility [74, 75], rather enable it for hypothermia treatment, contrast enhancement in MRI [76], and antimicrobial application. However, encapsulation of NMs with CM-P5 peptide has considerably increased their antifungal efficiency [77]. Chen et al. [78] synthesized MnO NPs in the presence of OA and then conjugated them with PEG-FA for application in brain glioma diagnosis. Yang et al. [79] employed the same functionalization onto $SiO_2@Mn_3O_4$ NPs for MR-based tumor imaging in the same year. Haneefa et al. [44] produced MnO_2 NPs with curcumin as a stabilizer then functionalized them with salicylalchitosan and found better antimicrobial activities, while Anwar [80] in 2018 used chitosan on MnO_2 NPs for Pb^{2+} adsorption as well as antibacterial property enhancement.

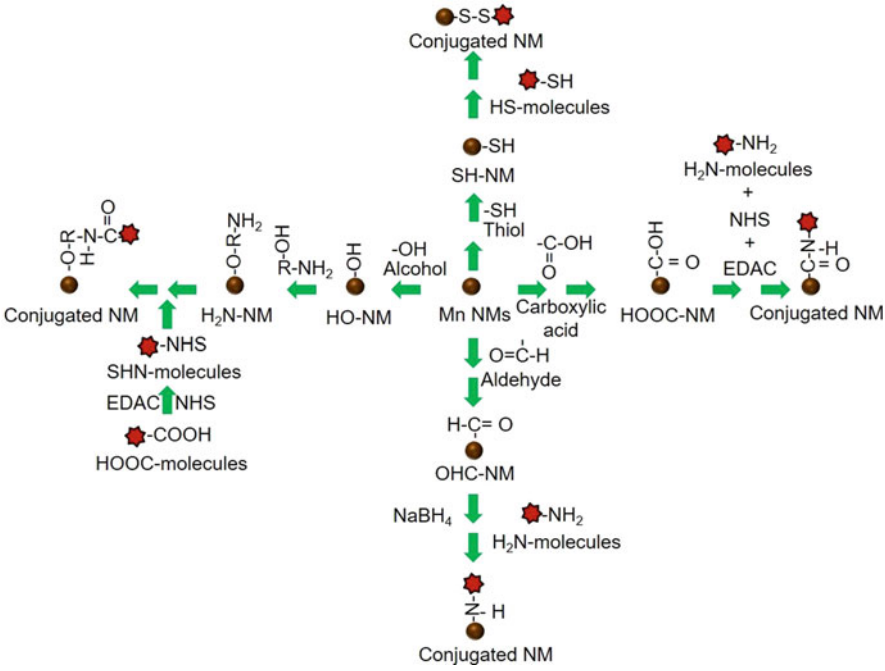

Fig. 3 Schematic presentation of different functionalizing agents and their functionalization onto Mn NMs

3.2 Polyfunctionalization

Polyfunctionalization refers to multilayer conjugation of more than one bio or chemical molecules in repeated layers, enabling them to execute multiple functions (Fig. 4). Shukoor and co-group [81] used OA as a stabilizer to prepare Mn_3O_4 NPs, which then anchored by dopamine linker to a pentafluorophenyl acrylate polymer. This nanocomposite was then immobilized using ssDNA probes, to imitate a cancer cell-specific pathogen. The NPs were also labeled with rhodamines to facilitate in vitro tracking. The authors also suggested for potential application of such NPs in contrast enhancement in MRI or cancer diagnosis. In another piece of work, Luo and coworkers [82] noticed an improved colloidal stability as well as hydrophilicity of PEI@Mn_3O_4 NPs, and on further encapsulation the NPs with fluorescein isothiocyanate (FI) and PEG-FA, they could use the NPs for diagnosis and tumor-targeting, respectively. In their subsequent work, they functionalized those NPs with L-cysteine [83].

In another study, PEG-anti-CD105 Ab TRC105 was conjugated to Mn_3O_4 NP for PET and MRI applications, whereas ^{64}Cu-2,2′-(7-(2-((2,5-dioxopyrrolidin-1-yl) oxy)-2-oxoethyl)-1,4,7-triazonane-1,4 diyl) diacetic acid (NOTA) and FI were functionalized onto the NPs for tracking them [94]. In a similar experiment, PEG-FA was conjugated onto PEI@Mn_3O_4 NP for PET and MRI, while the same

Table 2 Different linkers and functional groups used for the functionalization of Mn NMs

S. No.	Mn NMs	Functionalization method	Linker	Functionalizing agent	Application	Ref
01	MnO	Phosphoramidite chemistry	Dopamine, rhodamine	ssDNA	Activation of TLR 9 pathway bioimaging in tumor cell	[81]
02		Ligand exchange	Dopamine, PEG	Protoporphyrin IX	MRI and cytoyoxicity study of Caki-1 cells	[84]
03		EDC/NHS chemistry	DOPA, PEI	Herceptin	Cancer-targeted siRNA delivery and MRI	[85]
04			TETT silane	FA	MRI of cancer cells	[78]
05			TETT, PEG	Cy5.5	MRI of brain cancer cells	[86]
06		Covalent coupling reaction	PEG	AS1411 aptamer	MRI of renal carcinoma	[87]
07	MnO_2	Carbodimide reaction	CA	Anti-Sticholysin II mAb	Magnetic separation of sticholysins	[88]
08		Ligand exchange	Gallol group	PEG-OH	MRI	[89]
09		Carbodimide reaction	CA	CM-P5 peptide	Antifungal activity	[77]
10		Biofunctionalization	–	Salicylalchitosan	Antimicrobial activity	[90]
11		Michael addition amidation reaction	APTES	PAMAM	–	[91]
12		Ligand exchange	PE-HBPG	FA	MRI in aqueous media	[72]
13		EDC/NHS chemistry	ODA, PEG	ASA, MEF, and NAP	Anti-inflammatory activity	[92]
14		Silanization	–	APTES	Magnetic hyperthermia therapy	[69]
15	Mn_3O_4	Silanization	NH_2	Folate RBITC	MRI and cytotoxicity study with HeLa cell	[93]
16		–	PEG	FA	MRI of HeLa cells	[79]
17			PEI, mPEG, and FI	PEG-FA	MRI of tumor cell	[82]
18		Thiol-maleimide-coupling	PEG, sodium citrate PEG	L-cysteine	MRI of tumor cell	[83]
19		–	PEG	TRC105, ^{64}Cu-NOTA	PET and MRI of cancer cells	[94]

20			CF	PEG	MRI	[95]
21		NHS/EDC chemistry	FA, FI, PEI, and PEG	^{64}Cu-NOTA	MRI of cancer cells	[96]
22	Mn_2O_3		MPA	Carboxyl	Detection of dengue	[71]

DOPA 3,4-dihydroxy-L-phenylalanine; *TETT* triethylenediaminetriacetic acid; *ODA* octadecylamine; *ASA* acetylsalicylic acid; *MEF* mefenamic acid; *NAP* naproxen; *RBITC* rhodamine *B* isothiocyanate; CF carbon framework; *mPEG* PEG monomethyl ether

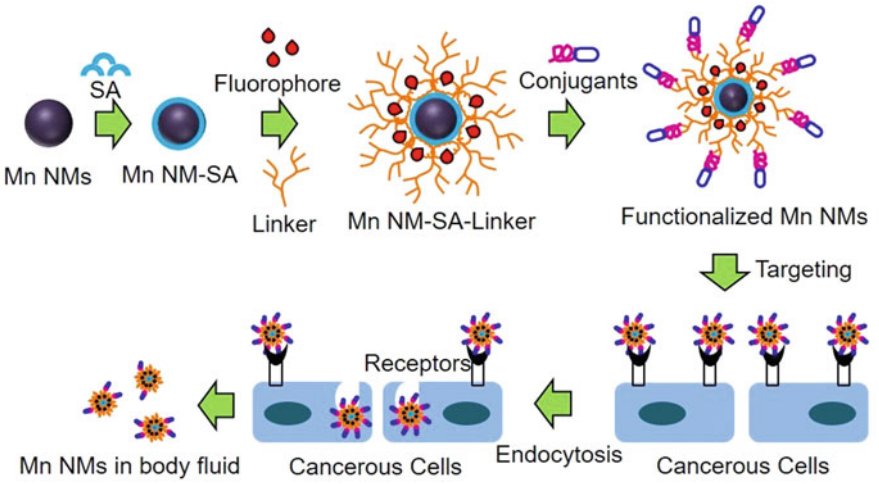

Fig. 4 Schematic presentation for polyfunctionalization of a Mn NM and conjugation with drugs for targeting cancerous cells

conjugants were used for diagnosis and tracking [96]. Ertürk and Elmac [91] fabricated a poly(amidoamine) dendrimer@$MNfe_2O_4$ composite while Augustine and group [72] fabricated FA-functionalized L-α-phosphatidylethanolamine-hyperbranched polyglycidol(PE-HBPG)@$MNfe_2O_4$ composites for multiple applications.

4 Bioapplications

Mn_xO_y NMs are well-known for their dynamic properties and applications, which are broadened further by synthesizing their clusters, composites, or alloys. The application of Mn NMs includes improvement of capacitance, acting as a contrast enhancer in MRI, carrying and targeting drug, diagnosing cancer, treating hypothermia, antibacterial activities, catalysis, and scavenging.

4.1 Theranostic Applications

Despite significant advancements in the fields of medicine and therapeutics, certain diseases continue to fetch challenges for researchers. Cancer is one of those diseases, known for its rapid pathophysiological progression, deadly consequences, and dreadful secondary sequels. Furthermore, chemotherapeutic drugs used for treating cancer are known to have a variety of acute or long-term side effects [97]. Aside from that, malignant tissue's diverse blood arteries and dense stroma act as a self-barrier to identification and treatment [98]. Therefore, designing an effective and more importantly specific drug to combat various types of cancer is critical.

Similarly, clinicians are quite concerned about the diagnosis of damage or abnormalities in a tissue, and MRI can be a reliable and effective tool in this regard. However, this technique from its inception has been associated with issues with regard to sensitivity and clarity. In form of theranostic materials, scientist have found a solution to both diagnosis as well as treatment problems [99–101]. These materials are generally Fe, Gd, Dy, and Mn containing paramagnetic, superparamagnetic, or ultrasmall superparamagnetic NPs such as superparamagnetic iron oxides (SPIONs) [102, 103]. The unique features about these oxides are their magnetic behavior, stability, reactivity, and target-specificity [104, 105]. In order to improve their properties, these NMs are further customized, functionalized, and/or doped [106–108]. These oxides, once their task is over, are engulfed and degraded by macrophages to excrete out [109].

Kanagesan et al. [110] conducted a dose-to-time cytotoxic study of $MnFe_2O_4$ NPs on breast cancer cells (4 T1) and found that cell viability reduced as the NPs concentration and exposure were increased. Mary Jacintha and coworkers [111] reported an antiproliferative effect of $MnFe_2O_4$ NMs on both human lung cancer (A549) and breast cancer cells (MCF-7). Similarly, hollow-spherical $MnFe_2O_4$ and $ZnFe_2O_4$ NPs were synthesized, separately by using ethylene glycol and sodium acetate trihydrate and observed anticancer properties of both the NMs against D407, MW35, B16F10, and A549 cell lines. However, the second one was found to be more effective [112]. In another study, Akhtar and group [113] assessed the cytotoxicity of $Mn_{0.5}Zn_{0.5}Sm_xEu_XFe_{1.8\text{-}2x}O_4$ NPs against cancer (HCT-116), embryonic kidney (HEK-293), and normal cells, and found that NPs are completely selective to the first. Rehman et al. [114] reported a similar property of these NPs in separate research.

4.2 Antimicrobial Agent

Mn_xO_y NMs, particularly MnO_2, display antimicrobial properties against a wide spectrum of bacteria such as *Staphylococcus aureus, Klebsiella pneumonia, Escherichia coli, Bacillus subtilis, and Pseudomonas aeruginosa* [115]. In another study, MnO_2 was found inhibiting *Salmonella typhi* including the other two bacteria *P. aeruginosa* and *E. coli,* reported in the earlier study [116]. Because of the synergistic effect of the reducing or capping agent used and the NP itself, biogenic NMs exhibit broader antimicrobial properties than their physiochemically synthesized counterparts [66]. Biogenic MnO_2 NPs produced by using *Paenibacillus polymyxa* strain Sx3 were found inhibiting *Xanthomonas oryzae* pv. oryzae [34] whereas, *Citrus limon*-based synthesized NPs showed antimicrobial activities against *S. aureus*, *E. coli*, *Cochliobolus lunata,* and *Trichophyton simii* [55]. The same NPs produced using *Matricaria chamomilla* flower extract could inhibit *Acidovorax oryzae* strain RS-2 [66]. Joshi et al. [38] used *Datura stramonium* as a reducing agent to produce similar NPs with antimicrobial property against a broad range of bacteria including *S. aureus, Proteus vulgaris, S. typhi, Streptococcus mutan*, and *E. coli*. Other biogenic Mn_xO_y NPs such as Mn_3O_4 NPs have

demonstrated antibacterial activity against *P. aeurogenosa* and *S. pyogenes* [59] while MnO NP was found inhibiting *E. coli* and *S. aureus* [49].

Tailoring, doping, and functionalization of the NMs have shown to improve their antimicrobial activities further. Krishnaraj et al. [51] examined the antibacterial properties of MnO_2, Ag, and Ag-doped MnO_2 NPs against *B. cereus, S. aureus, L. monocytogenes, E. coli, S. typhi, and Salmonella enterica*. Similarly, composite NMs such as $MnFe_2O_4$ NPs showed antimicrobial effect against *S. aureus*, *Streptococcus pneumoniae*, *P. aeruginosa*, *S. paratyphi,* and *Candida albicans* [111] while, Ag-Mn bimetallic NPs found inhibiting *E. coli*, *salmonella*, *P. aeruginosa*, *S. aureus*, and *B. cereus* [24]. In another work by Akhtar and coworkers [113], hydrothermally produced $Mn_{0.5}Zn_{0.5}Sm_xEu_XFe_{1.8-2x}O_4$ NPs demonstrated substantial antibacterial characteristics against *S. aureus* and *E. coli*. On further studies, the authors found an increase in the antimicrobial property for increased Fe content of the NM. Studies have reported that smaller the size of a NM, better is its antimicrobial effacacy [117].

4.3 Biosensor Fabrication

Mn NPs have long been a choice among researchers when it comes to the fabrication of nanocomposites [47]. In return, these composites are used to design solar cells [118], supercapacitors [70, 119, 120, and], and electrochemical sensors [121, 122]. Eremko et al. used screen-printed graphite electrode to devise three electrochemical sensors, using amorphous, β-, and γ- forms of MnO_2 for sensing thiocholine, glutathione, and cysteine, respectively. Among the three NPs, the last one showed highest sensitivity [122]. For designing an amperometric H_2O_2 sensor, Dontsova et al. [123] used MnO_2 deposited graphite electrode, while Wang et al. [124] used β-MnO_2 nanorod fabricated glassy carbon electrode (GCE). In a separate experiment, Ahn et al. [125] used the same principles of Wang et al. [124] for K^+ detection while used the principle of Ahmad et al. [118] to sense para-nitrotoluene, 2,4-dinitrotoluene, and 2,4,6-trinitrophenol. Others have reported the detection of L-Cysteine [126], FA [127], and glucose [128] by using GO/CNTs/AuNPs@MnO_2, CNT-decorated MnO_2, and Pd-Mn alloy-GO composites, respectively. In another study, δ-MnO_2 nanoflower (NF)/polyaniline complex was coated through naflon (Naf) onto GCE for electrochemical detection of G, A, T, and C from calf thymus DNA with a sensitivity 1.6, 1.9, 1.5, and 2.4 μA/cm^2 μM while limit of detection (LOD) 4.8, 2.9, 1.3 and 1.3 μM, respectively [129].

4.3.1 Enzymatic Sensors

In an enzymatic sensor, enzyme immobilized transducers are used to catalyze a substrate into its product for detection. A β-MnO_2 nanowire/Naf/glucose oxidase (GOx)-modified GCE-based enzymatic glucose sensor was developed by Zhang et al [130], for the detection of glucose from human urine sample with a sensitivity of 38.2 μAmM^{-1} cm^{-2}. GOx-catalyzed oxidation of glucose into gluconolactone and H_2O_2 produced current which was measured through cyclic voltammetry (CV). The

sensor retained 94% of its activity after 10 days. Almost a similar sensor was fabricated by Han et al. [131] for the detection of glucose at pH 7.4 and RT with a response time of 5 sec. LOD of the sensor was found to be 1.8 μM with 97–104% recovery. A 10% loss in the response was observed after 15 days of storage. Another electrochemical glucose sensor was developed by using a composite electrode made up of Gox-impregnated poly-L-lysine decorated onto a MnO_2 NP and chemically reduced GO (CRGO) coated GCE. The sensor was able to detect glucose in a range of 0.04–10 mM with LOD 0.02 mM at pH 7. The response of the sensor was reduced 4.02% after 20 days [132]. A disposable enzymatic sensor was developed by Vukojevic's group [133] by using MnO_2-Graphene nanoribbon (GNR) for the detection of glucose. MnO_2-GNR composite was prepared by mixing GNR solution to $Mn(NO_3)_2{\cdot}4H_2O$ along with sonication for 2 h, followed by $KMnO_4$ for 6 h. The composite was coated onto screen-printed carbon electrode and dried at 4 °C. Finally, the electrode was immobilized with GOx. Sensitivity and LOD of the sensor were found as 56.32 μA/mMol cm^2 and 0.05 mmol/l, respectively at pH 7.4. The stability of the sensor was found to be 08 days.

4.3.2 Nonenzymatic Sensors

Electrochemical

Although enzymatic sensors do have high sensitivity and specificity but low stability, researchers have tried to develop nonenzymatic sensors. Ultra-thin MnO_2 nanosheet coated GCE electrode-based electrochemical sensor was developed by Shu et al., for detecting H_2O_2 released from mouse myeloma cell SP2/0. The sensitivity and LOD of the sensor were found to be 3261 $mA.M^{-1}$ cm^{-2} and 5 nM, respectively. The response time was noted as 3 sec, with 7% loss in response after 2 weeks [134]. In 2020, Waqas et al. [128] fabricated a nonenzymatic glucose sensor by using Pd-Mn/RGO/GCE composite in which, Pd-Mn alloy NPs (3:1) deposited RGO was coated onto GCE. The sensitivity of the sensor was found to be 1.25 μM with a stability of 20 days. For the detection of L-cystine from water samples, a GO/CNTs/AuNPs@MnO_2-modified GCE was developed. The sensor, at 4.0 μL of GO/CNTs/AuNPs@MnO_2 was able to sense L-Cys in 7 min at pH 7 with a LOD of 3.4×10^{-9} mol L^{-1} [126]. Another such GCE/MWCNT/MnO_2-based electrochemical sensor was developed but for the detection of ferulic acid [127]. Gurban et al. in 2015, deposited MnO_2 nanolayer onto screen-printed carbon electrode through Naf for sensing phenols such as 4-t-butylphenol, 4-t-octylphenol, and 4-n-nonylphenol. The sensitivity of the method was found inversely proportional to the phenol length [135]. A year later, Wang and group [136], used MnO_2 nanoflower (NF) to fabricate a sensor for the detection of β-agonists such as ractopamine and salbutamol. During the fabrication of the sensor, GO was deposited onto Ni foam electrode through spraying, followed by reduction of GO and electrodeposition of MnO_2. The MnO2/RGO@ MnO_2 electrode showed highest performance at 0.5 mg/ML of GO and pH 6.0. The current output was reduced by 3.4% after 7 days while 8.9% after 1 month. A Mn_2O_3/NiO alloy NP-coated GCE was used for the detection of choline by Rahman et al. in 2019 [137]. The principle

behind the detection is the reaction of choline with O_2, which releases betaine and H_2O_2, which undergo further breakdown into H_2O and e^-s. The current produced by the e^-s is detected. The sensitivity and LOD of the sensor were found to be 16.4557 mA mM^{-1} cm^{-2} and 5.77 ± 0.29 pM, respectively.

Optical

A carbon-dot-MnO_2 composite was used for the fluorescent-based detection of glutathione from human serum sample [138]. The method is based on the principle that MnO_2 acts as a quencher to absorb the fluorescence emitted by C-dot but glutathione reduces MnO_2, disabling its quench ability leading to the emission of fluorescence. The intensity of fluorescence emission will be directly proportional to the concentration of glutathione. The assay was highly sensitive and specific. An ammonium metavanadate incorporated $MnCaO_2$ nanolayer was used in another typical nonenzymatic optical sensor, to provide the same function as GOx in glucose sensors. The H_2O_2 produced herein converted ammonium metavanadate to a colored molecule that was detected spectrophotometrically. The intensity of the color increased with increasing the glucose concentration in human serum samples. LOD of the sensor was found to be 6.12×10^{-6} M with a recovery rate of 95.4–108.9% [139].

4.3.3 Electrochemical Immunosensors

Electrochemical immunosensors mainly involve electrochemical detection of antigen-Ab interaction. Supraja and group [140] fabricated an electrochemical immunosensor using Mn_2O_3 nanofiber and mercaptopropionic acid coated GCE (GCE/MNF/MEP) for label-free detection of atrazine from bottled and tap water. In this sensor, anti-atrazine antibodies (Abs) were immobilized onto the composite electrode through N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide-N-hydroxysuccinimide (EDC-NHS) chemistry. The sensitivity and LOD were found to be 52.54 (kΩ/μgmL^{-1})/cm^2 and 0.22 mg/ml, respectively. The sensor has lost around 6.75% of its initial response when examined after 28 days of storage. Using the nanocubes of the same Mn_xO_y, another electrochemical immunosensor was reported by Singh et al. [141] for the detection of aflatoxin-B1. Anti-aflatoxin-B1 Abs were immobilized through the same EDC-NHS chemistry onto Mn_xO_y NM-coated indium tin oxide surface. The sensitivity and LOD were found to be 2.04 μA mL ng^{-1} cm^{-2} and 0.54 pg mL^{-1} for non-spiked sample and 1.484 μA mL ng^{-1} cm^{-2} and 0.54 pg mL^{-1} for spiked samples. The authors have also reported good reproducibility, repeatability, and specificity of the sensor.

4.3.4 Aptasensors

Mn_3O_4 nanoflower (NF) was used for the construction of a dual-aptasensor for the detection of breast cancer biomarker, human epidermal growth factor receptor 2 (HER 2). In this sensor, two nanoprobes were constructed, one by coating Pd@Pt NFs onto Mn_3O_4 NFs followed by horse reddish peroxidase (HRP) and single-stranded DNA probe (nanoprobe 1) and the other by coating HRP and complementary DNA oligomer onto Pd@Pt NFs (nanoprobe 2). For constructing

the sensor, an activated gold electrode was immobilized with tetrahedral DNA nanostructure through Au-SH chemistry along with blocking of the free space with 6-mercaptohexanol. The DNA immobilized electrode was then soaked with HER 2 followed by nanoprobe 2. The composite was hybridized with nanoprobe 1 to further increase the immobilized HRP and hence H_2O_2 reduction. HRP then reduced H_2O_2 into H_2O and O_2 which in turn oxidized hydroquinone to benzoquinone. The current produced by the e^-s released from the oxidoreduction couple was measured through differential pulse voltammetry for detection of the biomarker (0.1–100.0 ng/mL) with LOD 0.08 ng/mL [142].

4.3.5 Pressure Sensor

Recently, a pressure sensor was developed using Pt NP-packed Mn NF (Pt@MNO_2 NFs) for the detection of *salmonella sp.* In this sensor, an anti-salmonella polyclonal capture Ab-coated magnetic nanobead was used to isolate salmonella from water samples. This complex was labeled with Pt@MnO_2-detection Ab complex through Ab-salmonella agglomeration. The whole complex when enclosed in a tube containing H_2O_2, the NFs reduced the H_2O_2 into O_2, causing an increase in the pressure of the tube. The change in the pressure was detected by a piezoresistor-based pressure detector. The sensor could detect 15–15,000 CFU/ml of salmonella in 90 min with LOD, 13 CFU/ml [143].

5 Future Prospects

Physicochemical approaches mostly require extreme conditions such as temperature, pH, and pressure therefore focus must be shifted towards the biosynthesis of these materials. Further, since, plants or plant components are easily available and can facilitate the large-scale synthesis of the NMs, they may be preferred over other bioreducing agents. However, emphasis should be given for the synthesizing composite, cluster, or alloy NMs along with metallic doping in order to improve the properties and broaden their applicability. Although several functionalization processes have been already reported, biofunctionalization of these NMs must be preferred. Cationic or anionic antimicrobial peptides (AMPs) such as dermicidin, cecropin A, moricin, indolicidin, defensin, and drosomycin exhibit wide antipathogenic role being a part of almost every innate immune system [144]. These molecules must be used in future to replace the present-day bioconjugants [145]. These molecules can lyse the cells by interacting with their complementary charged surface molecules (Fig. 5). Mn_xO_y NMs may in future be blended with other heavy metals or their oxides for utilization as thermostable materials, fuel cells, and dielectric materials, and so on.

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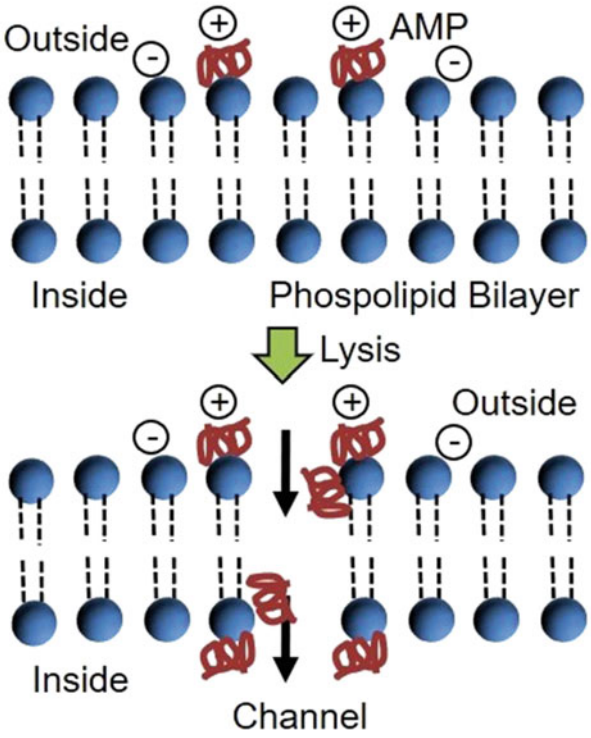

Fig. 5 Schematic presentation for microbial lysis by an AMP

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Transducers in Biosensors

Sandeep Yadav, Ashok Saini, Rooma Devi, and Suman Lata

Abstract

Biosensors are analytical tools comprising of a substrate or an analyte and a specific interface in close proximity or incorporated with a transducer to permit the quantitative development of some complex biochemical parameters. Thus, both the substrate/analyte and transducers are significant parts of these insightful devices which consist of an immobilized biorecognition component (or bioreceptor) like proteins (e.g., cell receptors, catalysts, and antibodies), nucleic acids, microorganisms, or even entire tissues that interact with particular types of analyte. The signals produced due to the reactions between a biorecognition component and its specific target analyte/s are converted into a detectable electrical or other signals by transducers through a process known as signalization. The various signals produced (electrical or optical, etc.) are usually proportionate to the quantity of analyte–bioreceptor interactions whose intensity may be directly or inversely proportional to the concentration of analyte. This chapter covers the various sorts of transducers which are utilized in various

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biosensors, their characteristics, a couple of important applications also as new trends of transducers in biosensors.

Keywords

Transducers · Electrochemical · Optical · Calorimetric · Piezoelectric · Surface plasmon resonance · Magnetic

1 Introduction

Leland C. Clark gave the concept of biosensors in 1962 and first demonstrated the feasibility of biosensing process and described the process of making intelligent electrochemical sensors using glucose oxidase enzyme immobilized in an enclosed membrane [1]. Biosensors are analytical tools comprising a substrate or an analyte and a specific interface in close proximity or incorporated with a transducer to permit the quantitative development of some complex biochemical parameters. Thus, both the substrate/analyte and transducers are significant parts of these insightful devices which consist of an immobilized biorecognition component (or bioreceptor) like proteins (e.g., cell receptors, catalysts, and antibodies), nucleic acids, microorganisms, or even entire tissues that interact with particular types of analyte. The reaction between the bioreceptors and their specific analytes produces a signal in various forms like electrons, ions heat, light, or sound which are then sensed by different types of transducers such as electrochemical, thermistor, counter, semiconductor, or sound detector which convert these signals into usable data [2–5]. A biosensor has three components—the sensitive biological elements (enzymes, antibodies, nucleic acids, cell receptors, organelles, tissues or cells), the transducer (electrochemical, optical, piezoelectric, thermometric, etc.), and the associated electronics or signal processing to display the results (Fig. 1) [6–13]. In fact, it is a probe which integrate a biosensing component (e.g., nucleic acids/entire cells/enzymes/

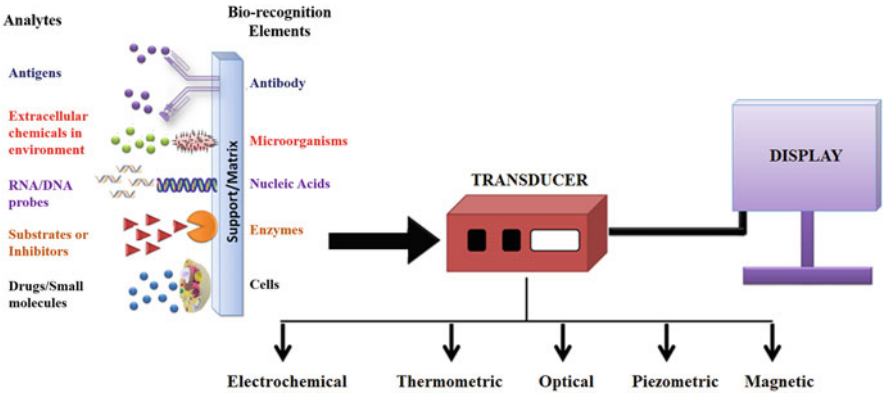

Fig. 1 Structure and components of biosensors

antibodies) creating a layer for recognition which is incorporated with the second significant part of biosensors that is a transducer by means of various method of immobilization so that both the components came in close proximity. The sensitivity and reliability of biosensor signal is determined by the stability of immobilization strategies. The biological/biologically derived sensing element are capable to recognize their specific analytes and to regulate overall functioning of the biosensors. These are important so the transducers can quickly and effectively create the particular signals in response to the going biochemical reactions, besides the conveyance ought to be corresponding to the response rate of the biocatalyst with the target analyte for a high range of linearity. The transducer fundamentally behaves like an interpreter which recognizes the interaction of various biochemical events/reactions and changes it into another signal for analysis by the processor that changes over it into a coherent/quantifiable yield. Biosensing technology offers significant advantages over other analytical techniques, such as optical, biochemical, and biophysical analysis, in terms of high sensitivity and varied sensing mechanisms, localized detection with high spatial resolution, real-time detection in a non-destructive manner, and simplified integration with standard semiconductor processing. Selection of suitable transducers and bioreceptors is fundamental for the development of an effective and sensitive biosensor. Moreover, the process used for the immobilization of a bioreceptor also plays a major role in the efficiency of the biosensors.

For research and commercial applications, the key requirements for developing a biosensor are—(1) the identification of a target analyte (or molecule), (2) availability of a suitable biorecognition element, and (3) the prospective for disposable manageable detection systems [14, 15]. Most of the transducers used in biosensors produce either electrical or optical signals that are usually proportional to the amount of interactions between bioreceptor and its analyte. Regardless of their advantages, biosensors have certain disadvantages such as low sensitivity and selectivity, complexities in immobilization methods used, long incubation time, inadequate electron communication and removal of interferences, high operational potential, and low sensor stability which are still to be improved or solved. Various analytic characteristics of a biosensor like the operational stability, selectivity, signal stability, limit of detection, and reproducibility determine the efficiency of transducing process. A bio-transducer is the recognition-transduction component of a biosensor system. The transducers used in biosensors work in a physicochemical way and have a role in conversion of the signal produced by the interaction between biorecognition and analyte into another quantifiable signal and this conversion process is known as signalization. The detector is not selective and it makes use of a physical change associated with the reaction (Fig. 2) which are as follows:

- **Amperometric** measured the movement of electrons produced in an oxidation-reduction reaction [16, 17].
- **Potentiometric** measured the change in electric potential caused due to the distribution of charges [18, 19].
- **Thermometric/Calorimetric** measured the heat output by the reaction [20, 21].

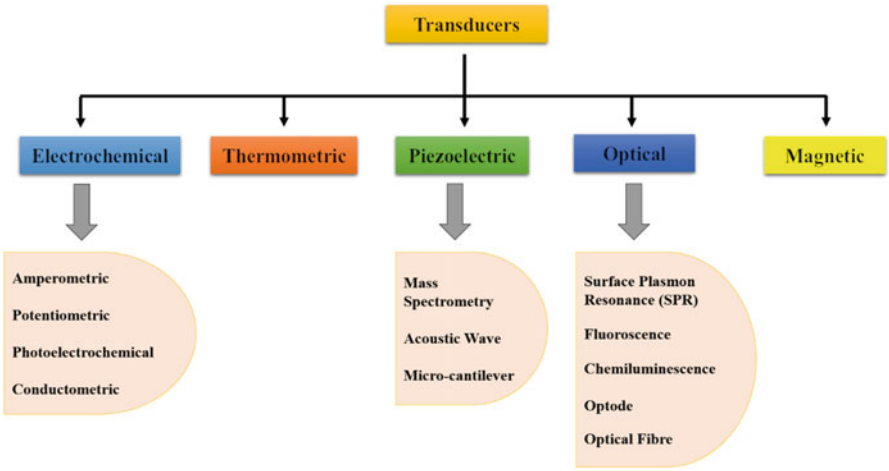

Fig. 2 Various kinds of transducers/detectors used in biosensors

- **Optical** measured the light produced during the reaction or difference between the light absorbed by the reactants and products [22, 23].
- **Piezoelectric** measured the changes due to the mass of reactants and products [24, 25].
- **Magnetic** measured the variations in the magnetic properties or effects induced magnetically [26, 27].

This chapter surveys the different types of transducers used in biosensor, their principles and applications, current trends, and future prospects of transducers.

2 Transducers Used in Biosensors

After the development of the Ist biosensor by Clark and Lyons in 1962 [1], several classes of biosensors have been developed. The presence of a bio-response element (enzyme, microorganism, nucleic acid, whole cell, antibody, or tissue) in the biorecognition layer is the most typical part in the fabrication of biosensors to provide electroactive materials for recognition by the physicochemical transducer by providing the quantifiable signal. The bio-response elements are immobilized in various ways in the fabrication of biosensors. Biosensors are classified into various forms, based on the type of bio-response, immobilization method, and transducer used. Various types of biosensors are summarized in Table 1 based on different types of immobilization methods used for the development of biosensors.

To develop the biosensors, selection of suitable transducers is required because transducer converts the signal produced (Biochemical) into a measurable signal that plays an important role in the detection process of the biosensor. On the basis of

Table 1 Different types of bio-response elements and immobilization methods used in the development of biosensors

Transducer	Bioreceptors	Method of immobilization used	References
Electrochemical	Enzymes Nucleic acids Antigens Microorganisms Whole cells	Adsorption	[28, 29]
		Covalent binding	[30, 31]
		Cross-linking	[32, 33]
		Entrapment	[34, 35]
Optical	Enzymes Nucleic acids Antigens Microorganisms Whole cells	Adsorption	[36, 37]
		Covalent binding	[38, 39]
		Cross-linking	[40, 41]
		Entrapment	[42, 43]
Calorimetric	Enzymes Whole cells	Covalent/cross-linking	[44]
		Adsorption	[45]
		Entrapment	[46]
Piezoelectric	Nucleic acids Antigens Proteins Whole cell	Covalent	[47, 48]
		Cross-linking	[49]
Magnetic	Protein Nucleic acids Antigen Bacteria	Chemical adsorption Cross-linking	[26, 27]

transducing mechanism used in the biosensors, they can be classified into the following types:

1. Electrochemical transducers.
2. Optical transducers.
3. Calorimetric (thermometric) transducers.
4. Piezoelectric transducers.
5. Magnetic transducers.

Some of these transducers can be further divided into various types which will be discussed below.

2.1 Electrochemical

Transducers depending on electrochemical detection mechanisms are the most commonly used transducers in the development of biosensors. The first electrochemical-based biosensor was developed by using glucose oxidase (GOx) for the detection of glucose [1]. After that many improvements and types have been developed.

Electrochemical transducers detect the various kinds of electrochemical species either generated or consumed during the interaction of biosensing element (enzyme/

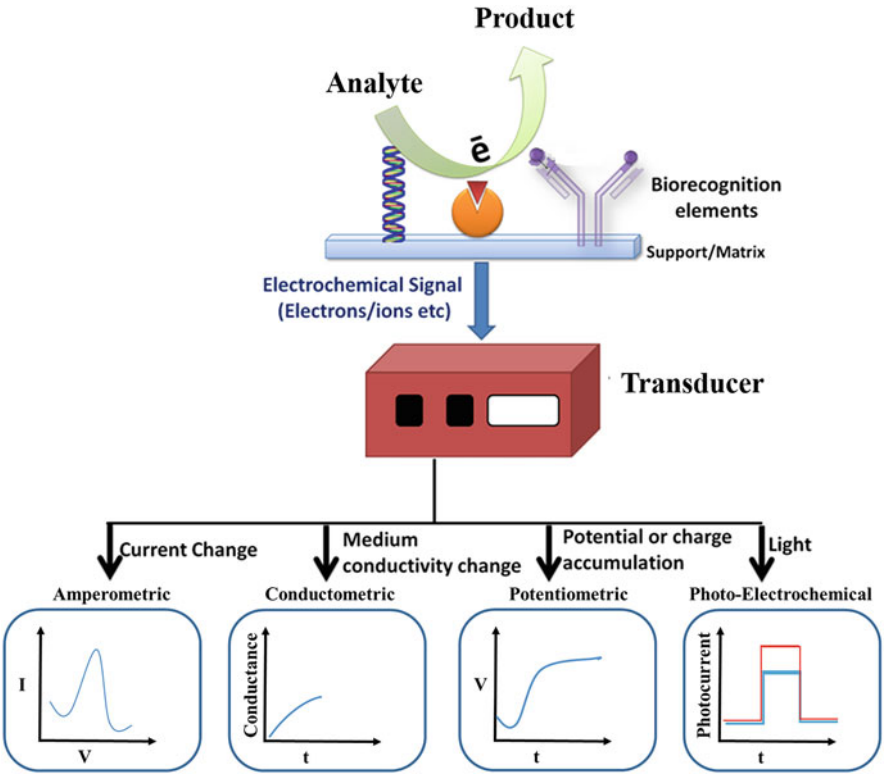

Fig. 3 Graphic representation of biosensor construction based on different types of electrochemical transducers

antibody) with the analyte (or substrate) [17, 18]. Based on the detection mechanism used for the detection of electrochemical species, EC transducers used in biosensors are mainly classified as—(1) Amperometric-based detection, (2) Potentiometric-based detection, (3) Conductometric-based detection, and (4) Photoelectrochemical (PEC) (Fig. 3).

2.1.1 Amperometric

These transducers measure the electric current produced by various electroactive species generated during a biochemical reaction. The concentration of analyte to be analyzed is linearly dependent upon the amount of current produced usually in redox reactions [50–52]. The amount of current during the reaction changes due to the increase or decrease in the thickness of the diffusion layer at the electrode.

Nernst suggested that the electrode surface is typically contacted by an immobile thin layer of solution and the local analyte concentration reaches zero at the electrode surface. The target analyte moves from the solution of higher concentration (bulk solution) to the electrode surface and is controlled by a diffusion mechanism. Thus, a

concentration gradient is developed away from the electrode surface. In the bulk solution, the amount of analyte is retained at a value of c^0 by convective transfer [53].

The current produced during the reaction can be either measured at constant potential or at variable potential. The former is known as amperometry whereas the latter is known as voltammetry. The simplest form of amperometric transducer-based biosensor was designed by Clark in which the current produced is relative to the amount of oxygen reduced at working electrode made up of platinum in reference to a reference electrode (Ag/AgCl electrode) maintains at a constant potential [54].

Voltammetry-based devices can be classified into different categories such as polarography (DC Voltage), differential staircase, linear sweep, reverse pulse, normal pulse, and differential pulse on the basis of ways to vary the potential [55, 56]. The most widely used form is Cyclic voltammetry in which the voltage is drift between two values at a static rate; however, when the voltage reaches V_2 the scan is reversed and the voltage is swept back to V_1. The critical factor is the scan rate $(V_2 - V_1)/(t_2 - t_1)$ which produces varied results as the duration of a scan must provide enough time to go through a chemical reaction [57, 58]. The voltage difference is measured between working and reference electrodes, while the current determined between the working electrode and the reference electrode and the output values are plotted as voltage vs. current in the form of a voltammogram. The current increases with an increase in the voltage approaching the electrochemical reduction potential of the target analyte and when V_2 passes the reduction potential, the current start decreasing forming a peak as the target analyte amount near the electrode surface fall off, since the oxidation potential has been exceeded. The reaction will begin to reoxidize the product from the initial reaction when the voltage is reversed to complete the scan toward V_1 with an increase in current of opposite polarity as compared to the forward scan but again decreases, having formed a second peak as the voltage scan continue toward V_1. The reverse scan also provides information about the reversibility of a reaction at a given scan rate. The shape of the voltammogram for any analyte mainly depends on the scan rate, the electrode surface which has changed after each adsorption step, and the concentration of a catalyst. It has been observed that at a given scan rate the addition of a specific catalyst (such as specific enzyme) in a reaction results in higher current output as compared with a non-catalysed reaction [58–60].

Next-generation amperometric-based biosensors use various mediators to generate current in case the analytes are incapable to work as redox partners [52, 54]. Chronoamperometry is another type of amperometric-based detection used in biosensors. In this technique, a steady state current is measured as a function of time applying a square-wave potential to the working electrode. The Cottrell equation is more related to chronoamperometry which describes the current-time dependency for linear diffusion control at a planar electrode [61]. In this equation, the current (I) is dependent on various factors—number of transferred electrons per molecule, electrode surface area (A), concentration of analyte (c^0), diffusion coefficient (D), time (t), and Faraday's constant (F). As per the equation, the changes in

current mainly depend on the diffusion rate of the target analytes at the surface of the electrode.

2.1.2 Potentiometric

These transducers usually measure the potential difference between the two electrodes—reference electrode and working electrode generated due to the redox reactions occurring between the bioreceptors and analytes at their surface. Any change in the concentration or activity of a specific analyte present in the solution leads to a change in potential difference [62, 63]. The relationship between the concentration of the analyte and the potential difference generated is governed by the Nernst equation

$$\text{EMF} \quad \text{or} \quad E_{\text{cell}} = E^0_{\text{cell}} - \frac{RT}{nF} \ln Q \tag{1}$$

where,

E_{cell} is the observed electrode potential at zero current which is usually referred as the electromotive force or EMF.

E^0_{cell} is a constant potential contribution to the electrode,

R the universal gas constant,

T the absolute temperature in degrees Kelvin,

n is the charge number of the electrode reaction,

F is the Faraday constant, and

Q is the ratio of ion concentration at the anode to ion concentration at the cathode (Buerk, D. 1993).

Many potentiometric detection-based transducers are also depend on different types of field-effect transistor (FET) devices which measure the concentrations of specific ions, changes in pH, and the kinetics of enzyme reactions [64]. The Field-Effect Transistor (FET) mainly controls the conductivity of a channel (i.e., an area used up of charge carriers) among two electrodes (known as source and drain electrodes) present in a semiconducting material using an electric field. The electric field potential is varied at an electrode known as gate relative to the source and drain electrode to control the conductivity. Depending on the configuration and composition of the semiconducting material used, the potential (negative or positive) present at the gate electrode would either attract or repel various charge carriers (e.g., electrons) in the conduction channel which either fill or empty the depletion region with charge carriers respectively. The formation or deformation of the operative electrical dimensions of the conducting channel can control the conductance between drain and source electrodes. When FET operates in a linear mode where the drain-to-source voltage is much less than the gate-to-source voltage which acts as a variable switchable resistor between two states (Conductive and non-conductive), whereas in saturation mode, FET is used as a voltage amplifier which usually operates as a steady current source. These devices are ideal for weak-signal and have high impedance applications therefore these are now extensively used in the field of electrochemical biosensing [52, 65].

Potentiometric measurement can be of two types—Direct potentiometric and potentiometric titration.

Direct Potentiometry In this type the concentration of the analyte can be measured directly as per Nernst equation. Ion-specific electrodes (ISE) have been used in these potentiometric devices which can detect only specific ions produced or consumed during the reaction even in very small amounts. The biosensors based on potentiometry have detection limit in ranges from 10^{-8} to 10^{-11} M.

Potentiometric titration It is another method used to electrically detect the end point in a biochemical reaction at which equal amounts of different solutions reach a state of equilibrium (e.g., 0.1 M HCl and 0.1 M NaOH). In this type of measurement, titration has been performed and the end point is determined by the variations in electrode potential which occurs during the changes in the concentration of the particular ion/s present in the solution at constant or zero current.

2.1.3 Conductometric

Conductometric based analysis is widely used for chemical systems in which many chemical reactions have occurred to produce or consume ionic species which changes overall electrical conductivity of the solution. These transducers measure the change in electrical conductivity which occurs in the solution between a pair of metal electrodes during the reaction between the analyte and biosensing element [66]. These transducers are frequently used to study enzyme reactions where reaction between the analytes and immobilized enzymes producing or consuming charged species leads to a change in ionic strength of the medium which affects the electrical conductivity between two electrodes [67]. The main drawback of these transducers is that they cannot measure small conductivity changes in the clinical sample having a variable ionic background in the medium [68] and also they may measure the nonspecific ions as conductivity or resistance of the solution is determined by movement of all the ions present in it.

2.1.4 Photoelectrochemical (PEC)

Photoelectrochemical enzyme-based biosensors are a new subclass of biosensors which combine the selectivity of enzymes and inherent sensitivities of PEC bioanalysis. In typical PEC enzymatic biosensors, the enzymatic system upon irradiation transforms the specific biocatalytic events into electrical signals through the interactions between the reaction chain catalyzed by enzymes and the semiconductor species. Many PEC enzymatic biosensors have now been developed and advancement is going on rapidly in this field [51, 69–73].

2.2 Optical

These biosensors (or transducers) are used for detecting an optical signal generated during a biological reaction and/or any other chemical reaction. There are two key areas of development in optical-based biosensors and these measure the change in light absorption between reactants and products of a reaction or the light produced

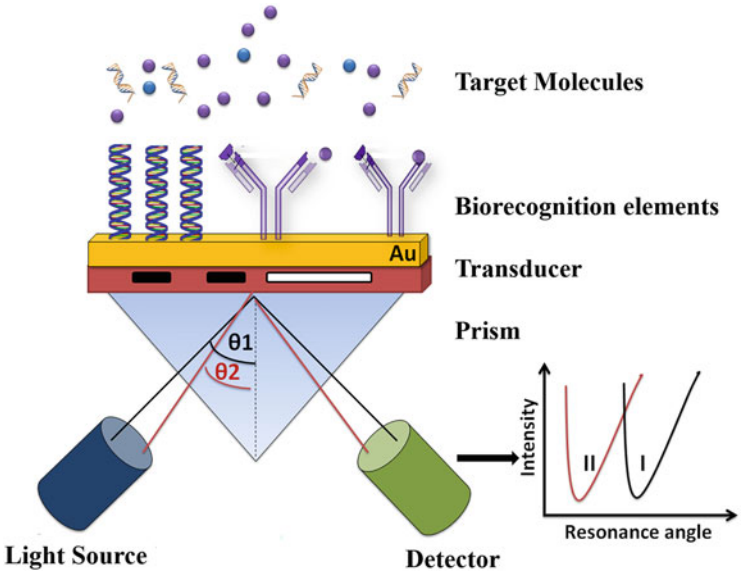

Fig. 4 Schematic drawing of biosensor construction based on optical transducer

by a luminescent process as an optical signal by using various spectroscopic detectors to quantify the signal and further correlate with the concentration of target compound/s. The optical signal can be measured directly or sometimes is further amplified to improve sensitivity (Fig. 4). Optical signals have many characteristics which can be measured but amplitude is measured commonly [74]. Various kinds of optical methods which have been used in optical detection-based biosensors are discussed below.

2.2.1 Fluorescence

Fluorescence is a phenomenon in which a molecule absorbs light at a shorter wavelength (or longer frequency) and emits the light in a longer wavelength (or shorter frequency) in visible regions of the electromagnetic spectrum. It is a broadly used optical detection-based method used for sensing the analytes which can show fluorescence. Optical detectors in fluorescence-based biosensors detect the change in the frequency of electromagnetic radiation between the excited and emitted radiation during the reaction analyte interaction with the biorecognition element [75]. Fluorescence-based biosensors are very useful for biosensing in many analytes due to its selectivity and sensitivity. These methods can be further divided into three types—direct sensing, indirect sensing, and FRET.

In direct sensing, fluorescence is measured before and after a reaction takes place by stimulating a specific molecule to be analyzed whereas in indirect sensing method target molecule is not stimulated directly rather than a dye (e.g., green fluorescent protein) is used which will optically transduce the presence or absence of a particular

target analyte. The third type of fluorescence-based sensing method known as fluorescence energy transfer (FRET) is used to generate a unique optical signal during the reaction of analyte and biomolecules. In FRET, two molecules are coupled in such a way that if one molecule is stimulated with a particular wavelength producing light of a particular wavelength which will further be absorbed by the other molecule (if present) and emits light of more higher wavelength which can be further detected.

2.2.2 Chemiluminescence

During some chemical reactions different reactants react with each other and undergo an excited state. While producing the product they again came back to the ground state along with the emission of light (luminescence). This process is known as Chemiluminescence (also chemiluminescence) as shown in the reaction given below. In this reaction, luminol reacts with hydrogen peroxide and produces 3-aminophthalate (3-APA) as a product along with the emission of light [76, 77].

$$[\text{Luminol}] + [H_2O_2] \rightarrow [3 - \text{APA}] + \text{light}$$

Some of the biosensors that exploit this phenomenon are known as Chemiluminescence biosensors which detect the light produced due to the reaction between target analyte and its respective biorecognition element using a photomultiplier tube [78]. This transducer-based platform has been widely used to identify specific biochemical reactions and their property. The chemiluminescence-based biosensor has been extensively used for immunosensing and NA hybridization studies as it provides higher sensitivity, simple instrument requirement, fast dynamic response properties, and a broader calibration range [79].

2.2.3 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) is a label-free detection method which is a suitable and trustworthy platform mainly used in the clinical analysis for measuring biomolecular interactions in a real-time manner with high sensitivity [80]. Now SPR is becoming one of the optical detection technique used in biosensors to study the interactions between bioreceptors and their analytes. SPR-based biosensors are fabricated by using a sensor chip which consists of a plastic cassette supportive glass plate whose one side is coated with a microscopic film of gold which contact with the optical correction device of the instrument whereas the other side is contacted with a microfluidic flow system. One side of the glass sensor chip is modified to allow the attachment of the biomolecules and when a photon of incident light strikes the metal surface (Gold) generates a plasmon resonance. As the light energy strikes at a particular angle at the metal surface, the electrons on the metal surface layer become excited. Whenever any analyte interacts with the attached bioreceptor, then the refractive index in the proximity of thin metal layers made up of gold, silver, aluminum, etc., changes and is thus measured by the detector. In SPR, sample solution flows across the surface and the changes in the SPR angle (angle of minimum reflectivity) are measured by changing the incidence angle and the

reflected light intensity is recorded which changed due to the binding reactions between various biomolecules. Various SPR-based biosensors have been reviewed in many papers [81].

2.3 Calorimetric (Thermometric)

The calorimetric biosensors were designed by attaching the biological component of a heat-sensing transducer known as *thermistor*. During a biochemical reaction, heat is either absorbed or produced which induces a change in the temperature of the solution/medium. The construction of these biosensors consists of miniaturized thin film thermistors and immobilized biological elements. Calorimetric-based transducers detect the changes in temperature occurring during the reaction between the biological element and its target analyte (Fig. 5). The change in temperature is usually linked with the number of reactants consumed or products formed or analyte concentration and the change observed in the form of heat is mainly measured by a thermistor (usually a metal oxide) or by thermopile (usually ceramic semiconductor) [83, 84]. Thermistors determine the temperature change at the entrance and exit of small, packed bed columns containing immobilized bioreceptors (like enzymes) within constant temperature environment and the solution is passed to waste. Various external electronics detectors determine the difference in the resistance and hence temperature, between the thermistors. These biosensors have a large number of applications in the measurement of various clinical analyte, e.g., cholesterol, glucose, ATP, urea, triglycerides, and ascorbic acid in which most of the enzyme catalyzed reactions produces heat in the range of 25–100 kJ/mol.

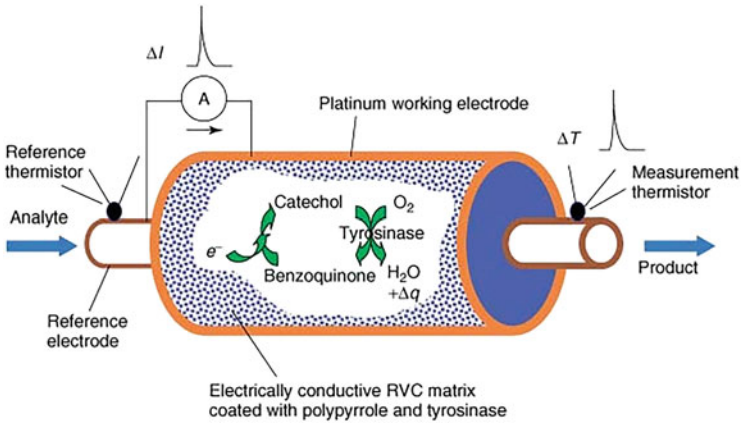

Fig. 5 Schematic drawing of biosensor construction based on calorimetric transducer [82]

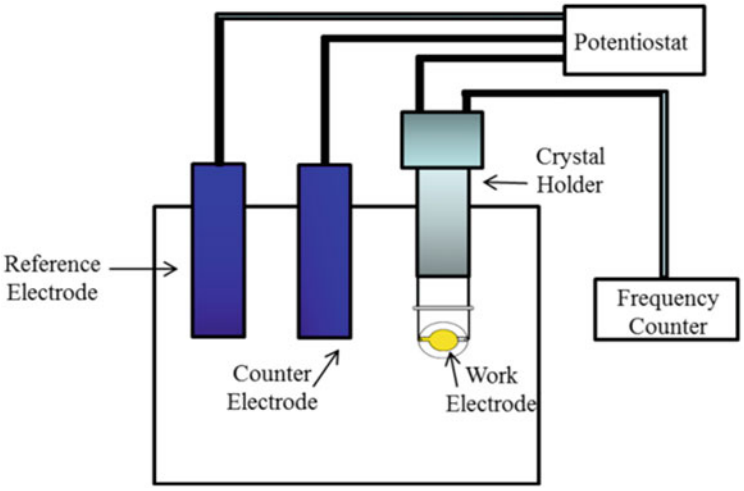

Fig. 6 Schematic drawing of biosensor construction based on piezoelectric transducer [85]

2.4 Piezoelectric

Piezoelectric transducer-based biosensors are analytical devices which senses the affinity interaction between analyte and biorecognition element and they utilize the crystals which undergo elastic deformation when an electric potential is applied to them. It consists of a piezoelectric platform or crystal which detects the change in oscillations due to change in mass, pressure, temperature density, or viscosity of the samples applied on its active surface and converting it into electrical signal (Fig. 6). These changes occurring on the surface of the piezoelectric platform transduces into a change in piezoelectric effect, i.e., changes in the frequency of oscillation or surface acoustic waves which are detected by the detector [24, 25] and this frequency is mainly dependent on elastic properties of the crystal. This piezoelectric transduction provides mechanical and electrical forces to a biological medium in the form of different types of waves like progressive or standing acoustic waves in an analyte-specific manner [86].

Piezoelectric-based sensors can be further divided into two types on the basis of frequency detections: i) Bulk Wave and ii) Surface Acoustic Wave (SAW) [87]. The sensors based on bulk waves are capable of detecting fundamental oscillations at higher frequencies whereas SAW-based devices are capable of lower detection limits [88].

Microcantilever-based sensors are another type of a label-free mass-sensitive biosensors which helps in the direct detection of various biomolecular interactions with high accuracy. These biosensors (physical, chemical, or biological) are based on the transduction of molecular adsorption and particular molecular interactions occurring at the cantilever surface. The output is measured in change in viscosity, density, and flow rate resulting from change in mechanical response at cantilever

surface. In the fabrication of cantilevers with well-controlled mechanical properties, low-cost and reliable standard silicon technologies are widely used [89, 90].

2.5 Magnetic

Due to unique properties of magnetic materials, these are being now used in the development of biosensors to monitor biological interactions for quick detection at the point of test in different fields. In the past few years, magnetic nanoparticles (MNPs) have been developed as labels for biosensing technology for the detection, identification, localization, and manipulation of an extensive range of biological, physical, and chemical agents [26, 27, 91, 92].

Magnetic biosensors are employed with paramagnetic or super-paramagnetic particles, or crystals which mainly detect the changes in the magnetic properties or magnetically generated effects such as changes in coil inductance, resistance, or magneto-optical properties happen during the interactions between biomolecules. These particles are coated with a biorecognition element such as antibodies or nucleic acid strand of sizes ranging from nanometres to microns in diameter. In the presence of target analyte, bioreceptor interacts with it causing a change in the physical properties of the particles which may be associated with mobility or size. Magnetic biosensors provide a noninvasive method to detect the activity of living systems by measuring the bio-magnetic fields produced by the biological tissues or organs with high sensitivity. Colloidal particles are manipulated by mismatches in their magnetization and are based on the exposure of the magnetic field of a magnetically labeled biomolecule interacting with a complementary biomolecule bound to a magnetic field sensor in such type of biosensors (Fig. 7).

Also in MNPs, specific particles can be separated or detected using the magnetic force based on different nonmagnetic properties such as shape, size, density, or the amount of molecules which are attached to their surface. So, due to the unique properties of MNPs they are now being used in magnetic biosensing technology for the development of novel biosensor systems. One of the key advantage of magnetic biosensors is that the magnetic field can be manipulated by using paramagnetic particles in a magnetic field which accelerate the binding interactions and permitting the movement of particles to the sensor surface where biochemical reactions are going which helps in fast detection of a specific analyte [94].

Classically magnetic biosensors are divided into two different types: substrate-based and substrate-free biosensors. In substrate-based biosensors the target analyte is present, the probe-functionalized MNPs directly bind to the sensor's surface whereas substrate-free biosensors use the resonance behavior of nanoparticles, where the probe hybridizes with its target generating a change in the resonance behavior. The magnetic biosensors detect the MNPs (Labels) in various ways based on their signal-to-noise ratio, sensor dimensions, types of particles used for the detection, experimental conditions, and the amplification technique used [95, 96]. First magnetic biosensor was developed in 1998 by Baselt [91]. He used a magnetoresistive sensor to detect the presence of micron-sized magnetic particles

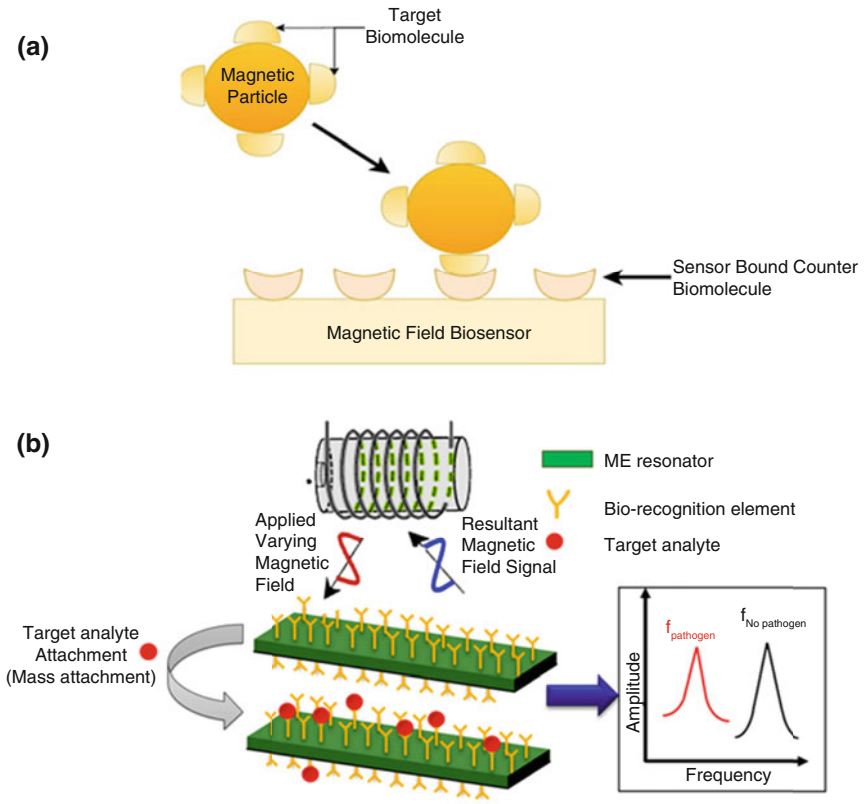

Fig. 7 (**a**) Schematic drawing of biosensor construction using magnetics utilize the magnetic field created by magnetic particles that bind to target molecules in a biological assay. (**b**) Operating principles of a magnetoelastic (ME) biosensor. In an alternating magnetic field, the ME biosensor oscillates [93]

which are used as bimolecular labels. After that many magnetic biosensors have been developed based on magnetoresistive (MR) and non-magnetoresistive (non-MR) for various biosensing applications [27, 97] which includes Anisotropic magnetoresistance biosensor (AMR) [98], Giant magnetoresistive (GMR) biosensors [99], Giant magnetoimpedance biosensor (GMI) [100], Tunnel magnetoresistive biosensors (TMR) [101], Fluxgate biosensors [102], Hall biosensors [103], and Optomagnetic biosensor [104].

3 Applications of Transducers

There are a variety of transducers available to detect diverse types of analytes such as electrochemical, optical, electrical, and thermal transducers. Transducers used in different types of biosensors open the pave to the ways not for human health, but also

in food processing/monitoring, fermentation methods, biodefense in the armed, and many more. Currently, the detection of common illnesses ranging from viral infections to serious tumors is possible due to the availability of a varied range of biorecognition elements and their measurement techniques as discussed above. The fundamental objective of biosensors is to be carried out in the clinical field. Different transducers used in biosensors can resolve the problem of finding pathogens at very low concentration and even at very early stages of infection [105]. Electrochemical detection is a very common and popular platform employed in biosensors for ultrasensitive detection of analytes. The electroactive species could exist either as a labeling agent of an antibody/antigen or working solution or the generated electroactive species products. Hence, the electrochemical-based transducers are the most beneficial mainly for on-site diagnostic tests as POC devices which are accessible to doctors and patients. Moreover, biosensors based on these transducers have many benefits like they are usually portable, easy to handle, simple instrumentation, and user friendly [105–108]. Other advantages include high specificity and sensitivity with simultaneous analysis, cost-effectiveness, and easy to be miniaturized to hand-size devices [109]. The glucose sensor is one of the most widespread instances of enzyme-based biosensors that could save a huge number of lives from diabetes.

The colorimetric transducers are very particularly attractive, they can achieve the rapid detection of a disease, as naked-eye observation can only interpret a screening result. These kinds of transducers are very useful for qualitative detection of pathogens. The colorimetric transducers examine fundamentally dependent on the accumulation of nanoparticles, like silver (AgNPs) and gold nanoparticles (AuNPs) improving the signal, have gotten impressive interest in medical diagnosis [110].

The direct colorimetric assay presented the simplest detection of biological molecules. As the simplest demonstration, the determination of the albumin (AL) to creatinine (CR) ratio has been employed for the screening of diabetes [111]. Other monitoring methods such as chemiluminescence have been used for the detection of nitrogen and sulfur in gas chromatography and capillary electrophoresis techniques. Detectors based on chemiluminescence are usually compact, affordable, and sensitive for real-time sample analysis [5].

Different transducers like piezoelectric-based transducers are utilized in different ultrasonic filtering devices in hospitals where the measure of medicine given to the patients can be checked by health professionals by the drop-counting technique utilizing a piezoelectric crystal. In this method, a lattice is connected to the piezoelectric transducer and when a drop of medicine falls on this cross-section, the piezoelectric transducer delivers a pulse which can be identified by detectors. Currently, biosensors are extensively used as tools to accurately identify a disease and these are the variety of transducers being used in biosensors that play a vital role in various areas of life science, especially in medical diagnosis. Merits and demerits of different transducers used in the construction of biosensors along with their application in different fields are summarized in Table 2.

Table 2 Advantages and disadvantages along with applications of various transducers used in biosensors

S. No.	Transducer type	Advantages	Disadvantages	Applications	References
1	Electrochemical	• Easily miniaturized • Inherently inexpensive • Lack of the high complexity of the sensor setup • Require simple electronics for molding and read-out, making them ideal for point-of-care applications • Easy to use interface with conventional electronic handling and results • Broad identification limits in any event, when just little volumes of analyte are available. Can be utilized to investigate biofluids with high turbidity and optically engrossing and fluorescing compounds • Results are highly reproducible • Satisfactory storage stabilization	• Limited shelf life • Low selectivity • Highly buffered solution may interfere • Sensitive to surrounding environment • pH and ionic strength of biofluids can shift significantly and along these lines impact the conduct of essential biosensor classes, for example, immunosensors • Narrow or limited temperature range	• Clinical diagnostics • Food production and quality control • Environmental monitoring • Detect waterborne and food-borne pathogens	[8, 17, 112–115]
2	Optical	• Highly sensitive • Rapid • Reproducible • Real-time detection • Simple-to-operate • Chemically inert • Small and light weight • Potential to be incorporated on a single chip • Equipped for observing a wide scope of synthetic and actual boundaries	• Costly equipment • Not portable • Pretreatment of sample may be required • Attaching a fluorescent tag to the analyte can interfere with the binding reaction that occurs during specific sensing • Quantitative fluorescence measurements can be inaccurate in the limits of both high and low concentrations of analyte • Presence of biomaterial in the biosensor (immobilization of biomolecules on transducers, soundness of	• Clinical diagnostics • Drug discovery • Food process control • Detection of food-borne pathogens • Environmental monitoring	[23, 116–122]

(continued)

Table 2 (continued)

S. No.	Transducer type	Advantages	Disadvantages	Applications	References
			catalysts, and antibodies) • Mass production of biosensors has been the cost factor • Susceptible to physical damage • Susceptible to interference of environmental factors		
3	Calorimetric (thermometric)	• Easy operation • Rapid detection • Highly stable	• pH-sensitive	• Enzyme activity and determination • Clinical diagnosis • Progression monitoring • Multianalyte detection • Hybrid biosensing • Environmental monitoring • Non-aqueous determinations	[20]
4	Piezoelectric	• Fast • Highly sensitive • Potable • Rapid • Simple • Stable output • Real-time and label-free detection	• Low sensitivity with fluid samples • Nonspecific binding-induced interference	• Cellular studies • Nucleic acid sensing • Biosensing of microbes • HIV in biological fluids • Cellular biology • Food safety • Pesticide detection	[24, 85, 123–126]

5	Magnetic	• Rapid detection of target • Provide precise diagnosis for multiple marker detection • Affordable due to their prevalence in magnetic data storage technologies • Also be incorporated into microfluidic systems with relative ease, making them useful devices for a limited range of measurements	• Require labeled analyte to be present in the sample • Nonspecific interactions that can occur between magnetic nanoparticles or between these nanoparticles and other materials	• Protein • DNA assays • Antibody-antigen • Bacteria • Virus	[27, 94, 97, 126]

4 Conclusion and Future Prospects

This chapter describes and characterizes different types of transducers used in construction of biosensors. A biosensor device consists of a biosensing element intimately coupled or integrated within a transducer. Biosensors offer an exciting alternative to traditional analytical methods which can be applied in various areas such as clinical diagnosis, environment monitoring, and food industry to allow rapid, real-time, and multiple analyses simultaneously. Presently the research on biosensor technology mainly emphasizes on the generation of more sensing biorecognition elements and transducers. Thus, biosensor technology offers an opportunity for the development of robust, low-cost, more sensitive, and accurate sensors for the determination of specific analytes. The next generation of biosensors dependent on nanostructures could prompt the development of devices ready to extraordinarily rival other scientific strategies utilized today The future depends on the development of new sensing elements and transducers.

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Self-Assembly and Fabrication of Biomaterials onto Transducers and Their Characterization

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Abstract

Biosensor applies the immobilized biomaterials as the sensitive components, and the performances of the biosensor are greatly defined by the activity and properties of the immobilized biomaterials, which could be affected by the fabrication and immobilization method. Proper immobilization and fabrication technologies could keep the structure and the activity of biomaterials to the full, or even make certain promotion. So the exploration of scientific and reasonable self-assembly methods and surface immobilization technologies has become an important issue to develop biosensors with high performance. This chapter mainly introduces the typical and emerging assembly methods of biomaterials onto the transducer from the following aspects: embedding method, adsorption method, chemical reaction method, electrochemistry method, spinning method and deposition method. Finally, the important characterization technologies of biosensors, including Fourier transform infrared spectroscopy (FT-IR), Raman spectroscopy, ultraviolet-visible absorption spectroscopy (UV-Vis), circular dichroism spectroscopy (CD), atomic force microscope (AFM), scanning electron microscope (SEM), and transmission electron microscope (TEM) will be briefly introduced.

Keywords

Biosensor · Characterization · Immobilization · Self-assembly · Transducers

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1 Introduction

In multiple research branches, including the field of biosensors, the current fusion of science and technology has been proven as a successful method to develop new tools with better performance and more reliability. The effectiveness of the biosensor depends on its high sensitivity and long-term stability for detecting phenolic compounds in the actual matrix, and it can be widely used in food, environment, and medical fields. In particular, the innovative explorations allow us to improve the sensitivity and robustness of biosensor through ingenious fabrication of biomaterials onto the transducers, which is urgently concerned in the world. The use of biomaterials as bioreceptors has appeared in the literature but is now revisited with modern technology to achieve smarter analysis functions. In order to show the exploration results and to achieve new breakthroughs, a case study representing the latest development of biosensors assembly has been presented in this chapter.

We summarized 88 references on the latest assembly trends in electrochemical biosensors and emphasized how cutting-edge technologies have promoted the development of biosensors and promoted their applications in the fields of agriculture, environment, and biomedicine. This review introduces the types and functions of biomaterials and focuses on the electrochemical properties of biomaterials as bioreceptors of electrochemical devices. Then, we mainly introduced the typical and emerging assembly methods of biomaterials on the transducer from the aspects of embedding method, adsorption method, chemical reaction method, electrochemical method, spinning method, and deposition method, and described this fixation strategy which can help improve the analytical performance of biosensors.

2 Embedding

The embedding method involves embedding biomaterials into organic polymer networks, inorganic materials, and hybrid materials, such as sol–gels, polymer membranes, and microencapsulated. This method is generally achieved through the co-precipitation of carrier materials and biomaterials which is relatively simple and convenient. The pore size and shape of the embedded hosts are generally controllable, allowing various immobilization demands of different biomaterials. Benefiting from the embedding effects, biomaterials could be able to resist extreme experiment conditions and keep most of their activity. Here the typical sol–gel embedding and the emerging technology based on metal-organic framework (MOF) will be explained in detail [1].

Silica sol–gel system is an excellent physical embedding agent for its biocompatibility. The inherent 3D net structure could ensure enough space for biomaterials and prevent them escaping from the system as well. Di et al. applied silica sol–gel method as self-assembly technology to propose a one-step method for constructing the hydrogen peroxide biosensor. In this work, horseradish peroxidase (HRP), together with gold nanoparticles, was mixed with the silica sol–gel and then successfully introduced onto the surface of the gold electrode by simple dipping. The

sensor could realize the direct electrochemical detection of hydrogen peroxide. The reduction of hydrogen peroxide by immobilized HRP did not require any mediator and exhibited an excellent heterogeneous electron transfer rate. The biosensor still could maintain 92% of the original cyclic voltammetry (CV) peak currents after 100 days' storage at 4 °C, while the high stability should be ascribed to embedding effects of silica sol–gel network, which could supply strong affinity and mild microenvironments for HRP [2].

In addition, biomaterials immobilized in sol–gel are usually subject to problems like mass transfer limitation or difficulty in active site exposure. In recent years, organic-inorganic hybrid materials, such as MOFs. MOFs possess large aperture and specific surface area, designable structures and function groups, making themselves the most promising support materials for biomaterials' immobilization [3]. Biomaterials can be immobilized into MOFs by adsorption, covalent attachment, and in situ embedding. MOFs can improve the stability and recyclability of bioactive components by increasing the rigidity of the biocomponents structure, changing the microenvironment for enzyme-catalyzed reactions, or forming a protective shell [4]. The self-assembly mechanism based on the MOF carrier used in the in situ embedding method has been shown in Fig. 1. The biomaterial molecule attracts metal cations and organic ligands to nucleate and crystallize on the surface, and the formed nano-shell can protect biomaterials from the external environment. Similar to the biological mineralization process in nature, this method is also called biomimetic mineralization [5].

As one of the zeolitic imidazolate framework series (ZIFs), ZIF-8 aroused great attention in the development of biomaterial immobilization platform, for its large pore size, high stability, and facile synthesis method. Lyu et al. coupled ZIF-8 and cytochrome C (Cyt *c*) by in situ embedding for the first time, preparing Cyt *c*/ZIF-8 sensor with methanol as reaction medium and polyvinylpyrrolidone (PVP) as cofactor. ZIF-8 improved the affinity of Cyt *c* to the substrate H_2O_2 [6]. Knedel et al. used the in situ embedding method to prepare Laccase@ZIF-8, by stirring laccase, Zn^{2+}, and 2-methylimidazole in an aqueous solution for 5 mins. The obtained laccase @ZIF-8 composite material showed enhanced thermal stability (up to 70 °C) and high chemical stability [7]. Wang and his workers applied the in situ co-precipitation experiment to embedding theGOx together with NiPd hollow nanoparticles into ZIF-8 (Fig. 2) thus developing an efficient colorimetric electrochemical glucose sensor GOx@ZIF-8 (NiPd). The GOx@ZIF-8 (NiPd) modified electrode showed good GOx bioactivity and high electrocatalytic activity for the oxygen reduction reaction (ORR). The relative standard deviation (RSD) of the sensor for 16 consecutive glucose measurements was 0.8%, indicating good stability and reproducibility. A clearly defined linear relationship between the current and glucose concentration was observed between 0.1 mM and 1.7 mM, indicating that the glucose of the new electrochemical glucose biosensor has a wider detection range, which can expand the application range of bioanalysis [8].

Embedding method is an increasingly perfect immobilization technology for biomaterials. The stability, biocompatibility, and cage effect of the embedding agent give the biosensor good detection performance and stability. Future research

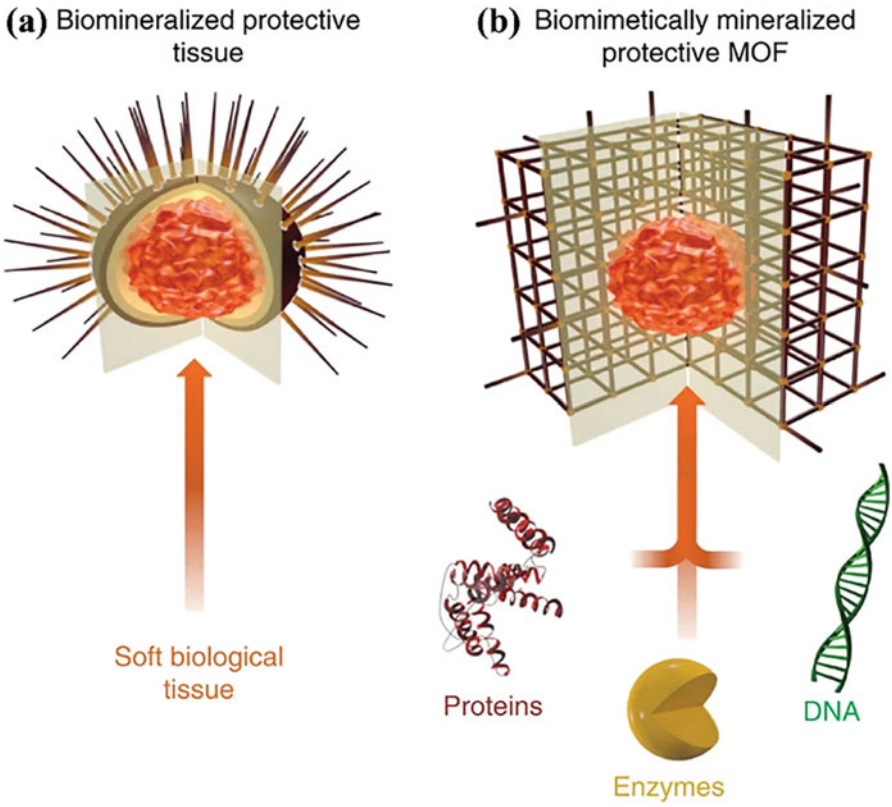


Fig. 1 (**a**) Schematic diagram of sea urchin; (**b**) Schematic diagram of MOF biocomposite, showing biological macromolecules (such as proteins, enzymes, or DNA) encased in a porous crystal shell [5]

of embedding method may focus on the development of new embedding agent or the modification of the existing embedding agent. And the biosensor with high activity and stability can be reasonably designed by suitable embedding method according to the specific application requirements. However, in the embedding method, enzymes buried in a thicker polymer film may extend the response time of the biosensor.

3 Adsorption

Adsorption method is an approach to fix biomaterials by physical adsorption or electrostatic adsorption onto the insoluble carrier. These interaction forces for adsorption may be hydrogen bonds, ionic bonds, or the corporation of the multiple forces, etc. The adsorption method possesses the advantages of simple operation, less activation and cleaning steps, little effect on biological activity, and a wide range

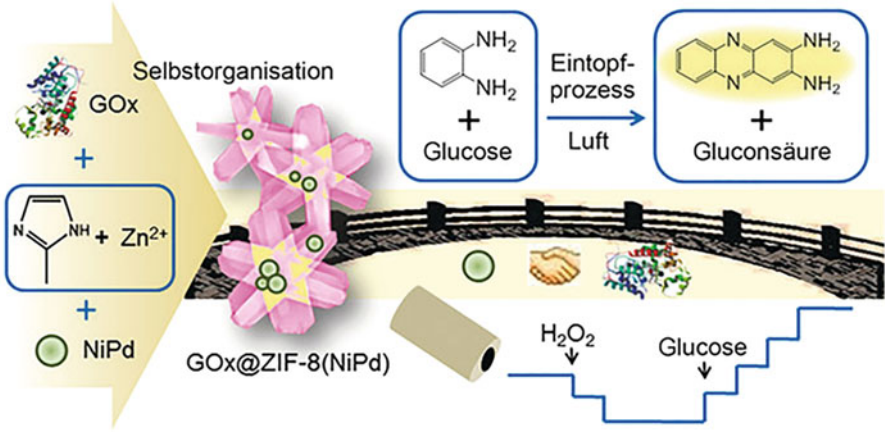


Fig. 2 NiPd hollow nanoparticles and glucose oxidase (GOx) were simultaneously immobilized on ZIF-8 by co-precipitation [8]

of host selection [9]. According to the different carriers, this method mainly can be divided into adsorption by (1) carbon-based materials like graphite, graphene, and carbon nanotube, (2) natural products, like fiber, montmorillonite, and attapulgite, (3) nanomaterials, and (4) synthetic polymer. Each of these approaches will be described in sequence.

3.1 Physical Adsorption on Carbon-Based Materials

To enhance the stability and sensitivity of the biosensors, graphite and graphene-based nanomaterials have been introduced into the self-assembly and fabrication of biosensors for their extraordinary thermal and electrical properties [10]. In fact, graphite and graphene-based materials can increase the signal response of electrochemical sensors by promoting the electron transfer between biomaterials and transducers, and provide a high surface area for loading biomaterials.

Patricia and coworkers successfully constructed a platform for laccase (LAC) immobilization by mixing graphite, gold nanoparticles (AuNP-CD), and β-cyclodextrin (Fig. 3a). Zeta potential measurement result showed interesting laccase successfully immobilized on the sensor by a strong electrostatic attraction. Due to laccase's highly efficient oxidation of rutin (Fig. 3b), the biosensor AuNP-CD-LAC could accurately detect rutin in the drug samples by square-wave voltammetry, and displayed good repeatability, repeatability, and good stability. Under optimized conditions, the linear range of rutin was 0.30~2.97 mmol L^{-1}. The detection limit could reach 0.17 mmol L^{-1} [11].

Ioana and his coworkers construct a sensitive and reusable laccase biosensors with graphene quantum dots (GQDs) and MoS_2 as electrode modifiers, and successfully applied the determination of polyphenol. Figure 4 depicts the composition and

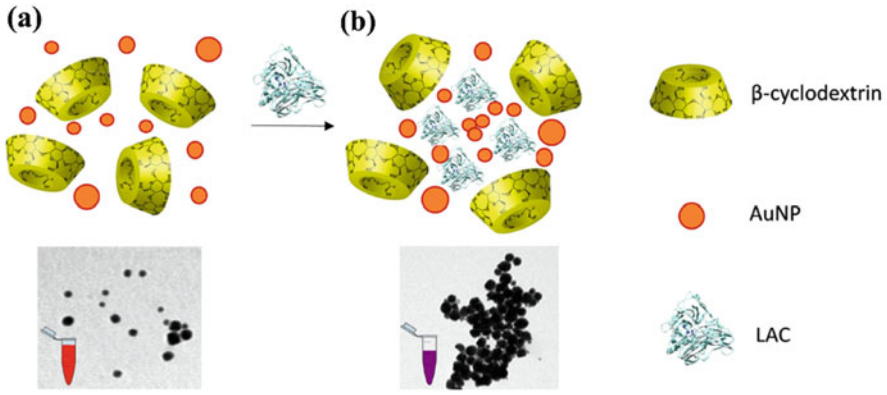


Fig. 3 (**a**) Shows the components used to build the biosensor; (**b**) LAC catalyzes the oxidation of rutin and electrochemically reduces the formation of o-quinone f on the surface of the biosensor [11]

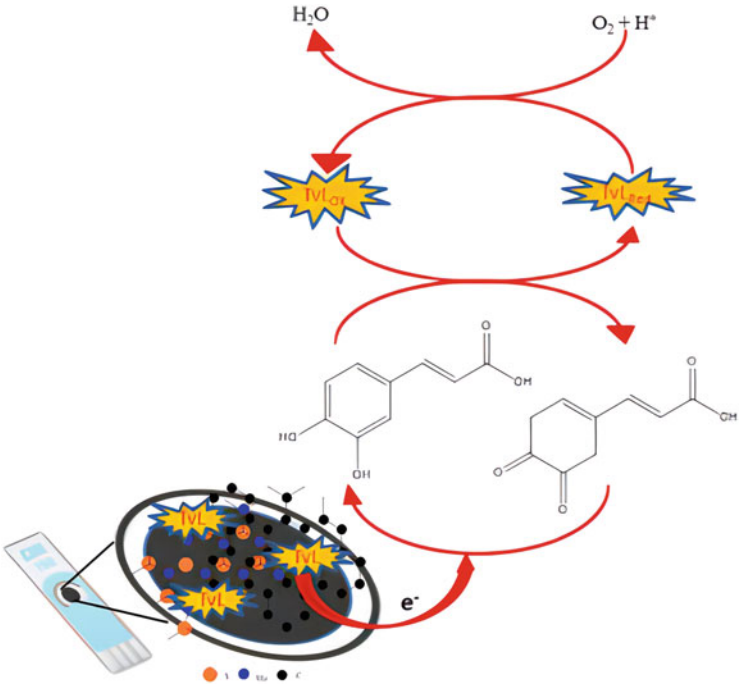


Fig. 4 The composition and detection schematic diagram of CSPE-MoS_2-GQDs-TvL biosensor [12]

detection schematic diagram of CSPE-MoS_2-GQDs-TvL biosensor. GQDs and MoS_2 nanocomposite formed with laccase provide a suitable environment immobilized electrode surface. Moreover, the electrostatic interaction between

GQDs and laccase enhanced the physical adsorption and the immobilization of laccase. The laccase biosensor had a good response to caffeic acid with a sensitivity of 17.92 nA μ M^{-1} [12].

Carbon nanotubes (CNTS) consists of sp^2 hybridized carbon atoms, which are cylinders with nanometer diameter and micro length [13]. The nanometer size of carbon nanotubes and their surface chemical properties can provide very effective adsorption for biomaterials, this makes them for fixing the biological material and the design of new biosensors attractive [14].

Sidika et al. constructed a novel ethanol biosensor by involving MWCNTs and polyfluoren-*g*-ploy (PF-*g*-PEG) for ethanol content analysis in alcoholic beverages. Firstly, poly (ethylene glycol) (PEG-FL) with fluorene functional group was synthesized and characterized by the one-step method, and then the working electrode was modified by nanotube. Finally, fix the alcohol oxidase on the modified surface. The sensor showed good detection of ethanol and displayed potential practical application for the analysis of ethanol contents in alcoholic beverages [15].

3.2 Adsorption by Natural Products

Natural fibers are important biomaterial carriers in construction of biosensors, for the economic price, easy accessibility, strong adsorption, and biocompatibility. Chen and coworkers used zein as the main carrier and obtained corn protein superfine fibers through electrostatic spinning technology. After being modified by gold nanoparticles, the zein ultrafine fibers could efficiently load active laccase and functioned as a new laccase biosensor for the measurement of catechol in actual solution specimens. The zein superfine fiber membrane prepared by electrostatic spinning can be attached to the GCE surface to achieve the firm fixation of laccase for the excellent biocompatibility and adsorption abilities of zein. The test results show that the biosensor displayed high detection sensitivity for catechol, good reproducibility, stability, and selectivity. Figure 5 describes the mechanism of A-CZNF formation (A) and the reaction principle and formation mechanism of a-CZUF biosensor (B) [16].

Bacterial cellulose (BC) is another potential biomaterial carrier for its biocompatibility and mechanical strength. BC is the natural product of gluconacetobacter xylinum and possesses inherent three-dimensional structure and excellent adsorption property. Li et al. modified the bacterial cellulose with gold nanoparticles to form the modified hybrid nanofibers (BC-AuNPs). Then the BC-AuNPs hybrid nanofibers were adsorbed on the electrode surface, and laccase and Nafion solution were added to form the biosensor of hydroquinone compounds. Since BC is conducive to the full dispersion of the active biomaterials, this sensor had a good electrocatalysis effect on dopamine [17].

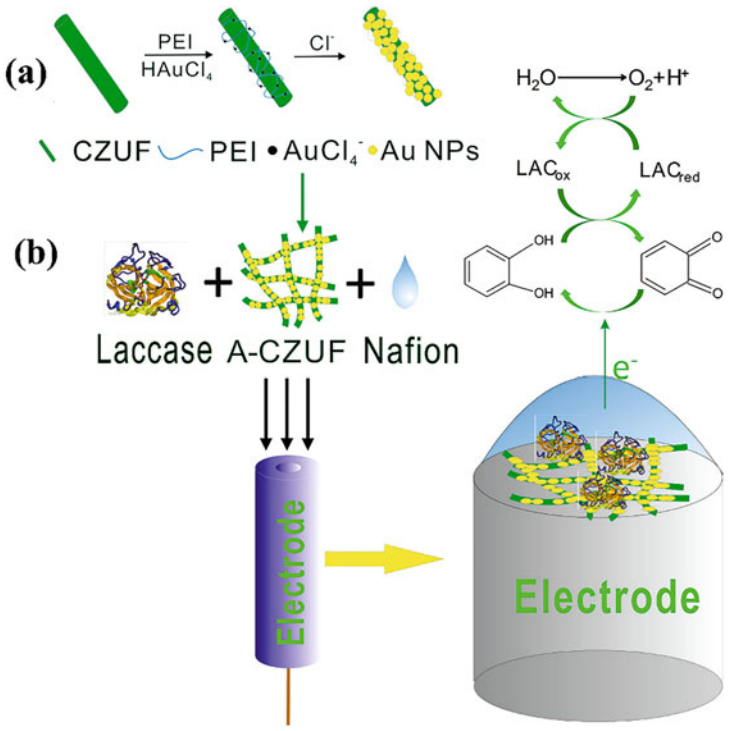


Fig. 5 (**a**) The formation mechanism of A-CZNF and (**b**) the reaction principle and formation mechanism of a-CZUF biosensor [16]

3.3 Adsorption by Nanomaterials

Nanomaterials take on nanometer size (0.1–100 nm) at least in one-dimension, and they display special physical and chemical properties such as optics, magnetism, and electricity. Compared with traditional large-size materials, nanomaterials also have a large specific surface area, easy to modify, and similar to the size of enzyme and other biomaterial molecules. As a new enzyme immobilization, they have been widely concerned in the field of biosensor technology [18].

Metal nanoparticles (MNPs), represented by Au and Pt [19], have attracted great attention in the field of biosensors because of its large immobilized surface area of bioreceptors, good biocompatibility, and electron transfer ability. In particular, the excellent conductivity can provide significant signal amplification, promote sensitivity, and greatly improves the electrochemical detection of target molecules. Further, Because of their high specific surface area, the loading capacity of biological receptors is very high, and the protein life and natural structure have been strongly preserved [20]. To take advantage of the polymer, the metal nanoparticles have been integrated into fiber membranes in order to highlight their

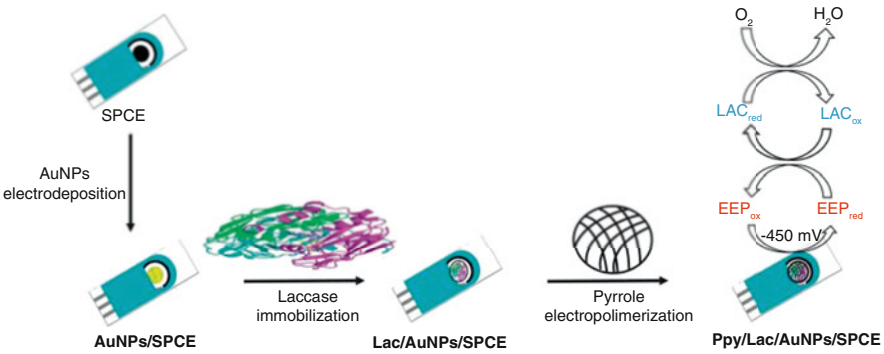


Fig. 6 Schematic diagram of SPCE modification using nanocomposite membranes (Ppy/Lac/AuNPs) to quantify polyphenols in propolis samples [22]

synergistic contribution to improving the analysis performance in terms of sensitivity and stability [21].

Mohtar et al. modified the graphite screen-printed electrode with AuNPs and then dropped laccase on the surface of the modified electrode. After continuous immersion in pyrrole solution and electropolymerization at 700 mV, the nanocomposite film consisted of laccase, AuNPs, and polypyrrole (Ppy) was obtained on the electrode surface (Fig. 6). The authors proved that the AuNPs on electrode could enhance the electron transfer and adsorption of laccase thus improved the response to polyphenols in propolis [22].

Apart from polymer, some inorganic materials with large specific surface areas have also been selected to work in collaboration with MNPs to achieve the immobilization of biomaterial. Zhang et al. used AuNPs and MoS_2 nanosheets as carriers to immobilize laccase to prepare a new type of laccase biosensor for the detection of catechol. Figure 7 is the schematic diagram of the production of AuNPs-MoS_2-Lac/GCE and the catalytic oxidation sensing process. MoS_2 had a large specific surface area, providing abundant space for enzyme immobilization; while AuNPs displayed good biocompatibility and electron conduction, which could enhance the activity of laccase and conductivity of MoS_2 thus improving the detection sensitivity [23].

After decades of development, the technology of immobilization by adsorption has made great progress, but there are still some problems to be solved. The adsorption method relies on the physical adsorption or electrostatic adsorption between biomolecules and carriers, which are weak forces. The combination of biomaterials and carriers is not firm, and it is easy to fall off, and the activity loss of biomaterials is large. How to solve the above problems is the direction to be improved for the adsorption method.

3.4 Adsorption by Synthetic Polymer

So far, various methods have been developed to assemble biosensors. Among various measurement means, electrochemical sensors show a strong, selective, and

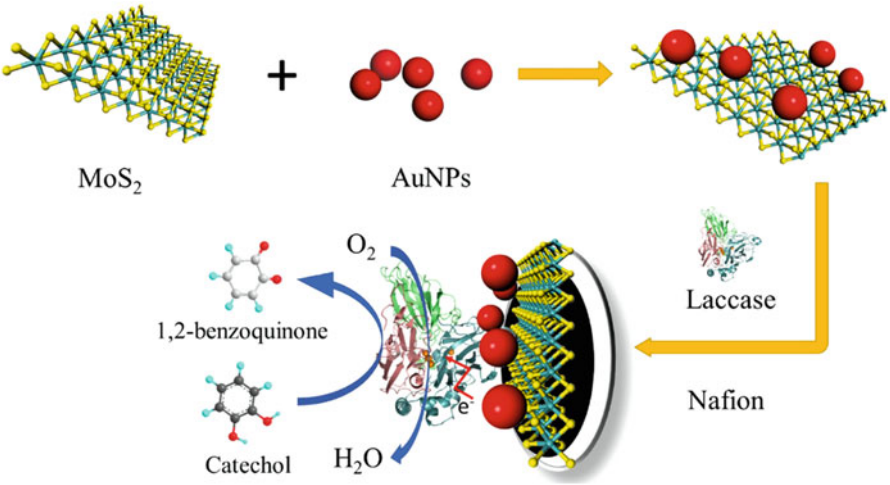


Fig. 7 Schematic diagram of the production of AuNPs-MoS_2-Lac/GCE and the catalytic oxidation of the surface of the catechol electrode [23]

sensitive real-time measurement of the potential [24]. In recent years, conductive polymers and their composite materials are widely used in various electrochemical biosensors prepared. Surface modification based on polymers can smooth the interface surface, reduce the roughness, and the introduction of extra functional groups, for instance, amines, is an indispensable condition for immobilizing biological receptors [25].

Jun Ren and his coworkers [26] discovered the polymer platform of the minicircle resonance type biosensor by fixing the receptor protein on the polymer-based optical device by direct physical adsorption, as shown in Fig. 8, to stabilize the biosensor surface. The test conclusions show that the polymer film surface is glazed, the hydrophobicity is tempered, and the touching angle with water is 97°. This degree of hydrophobicity provides the necessary binding strength to stabilize the proteins on the surface of the material under a variety of sensing conditions. Through the particular discern of human immune globulin G, validated the biological activity and biosensing performance of immobilized Staphylococcus protein A. This experiment proved the feasibility of creating a serried, uniform, particular and steady biosensing surface.

Hurija and his coworkers [27] tried a sensor design combining conjugated polymer and magnetic nanoparticles. Through a two steps method (Fig. 9). Firstly, magnetic nanoparticles were disposed with SiO_2 and amended with a carboxy group to construct a novel stable surface, that is, a conjugated polymer with suspended carboxyl group of magnetic nanoparticles, so that biomolecules can be covalently bound. This method improves the characteristics of biosensor and the stability of matrix. The experimental result indicated that the biosensor has a rapid response speed, short detection limit, and strong sensitivity.

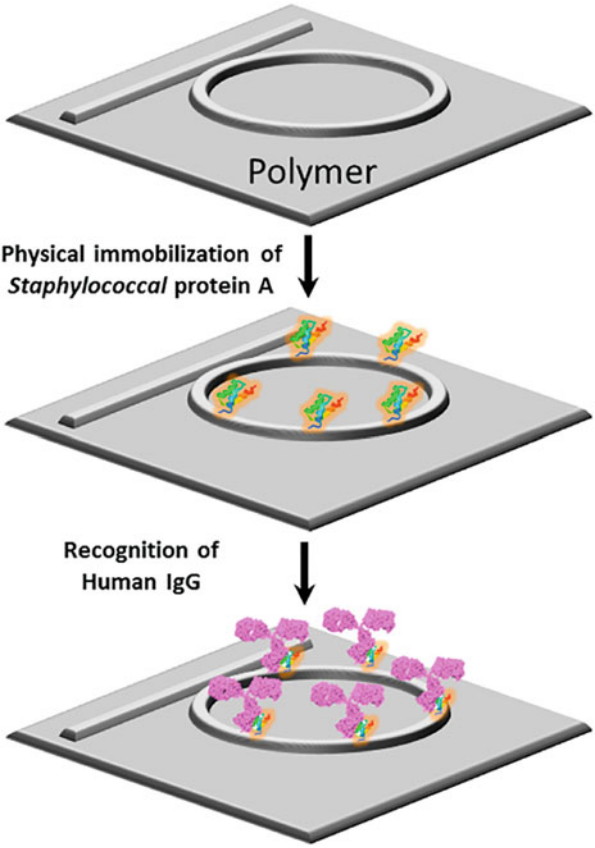


Fig. 8 Schematic diagram of PA/IgG affinity coupling biosensor process [26]

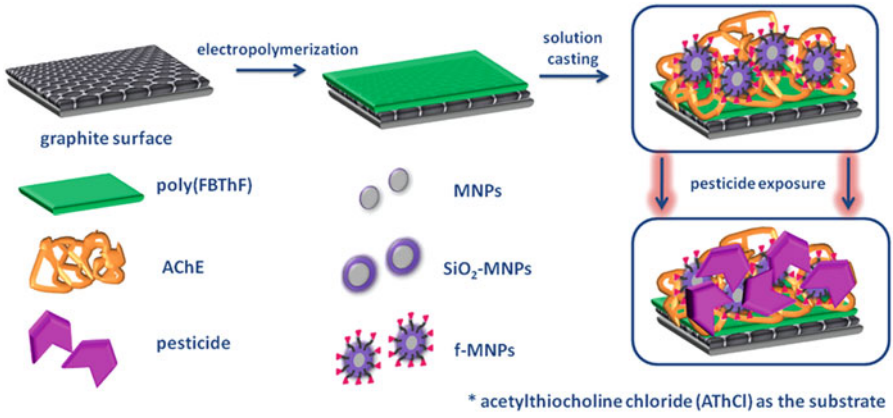


Fig. 9 Biosensor assembly diagram based on FBThF and SiO_2 [27]

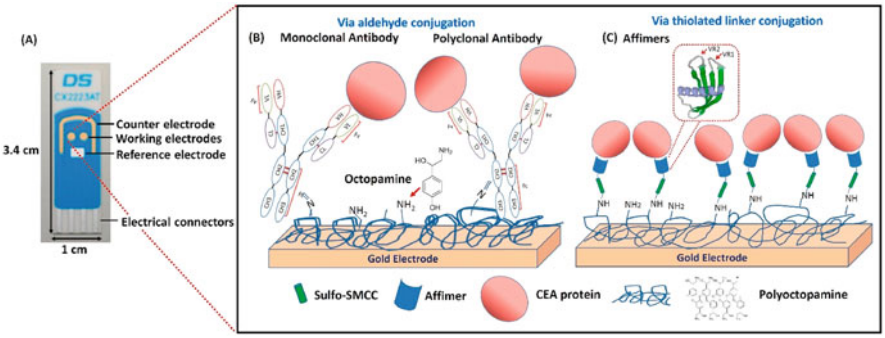


Fig. 10 Schematic diagram of CEA biosensor based on polyoctopamine [28]

Millner et al. [28] used Polyoctopamine (POct) as the transducer layer of the electrochemical biosensor and developed a label-free electrochemical impedance biosensor (as shown in Fig. 10) on the screen-printed gold electrode for the detection of carcinoembryonic antigen (CEA). The majorized POct-based biosensor was inspected in addition to anti-human serum. As a result, the electropolymerization of octopamine on the silk-screen printing gold electrode produced a low-resistance polymeric membrane, immobilized biological receptor close contact with the transducer layer greatly improving the detection sensitivity. Therefore, octopamine is a simple barrier biosensor using amine functionalization technology, which has the characteristics of providing fast responsive time, highly sensitive, and non-marker detection.

4 Chemical Immobilizations

The chemical reaction immobilization method uses direct chemical reactions to generate simple covalent bonds or cross-linked covalent bonds between the carrier and the biomolecules to realize the loading and immobilization of the biomaterial. According to the different reaction types, it mainly includes the following methods: simple covalent bonding, chemical cross-linking reaction, specific recognition between proteins, and click chemistry.

4.1 Covalent and Non-covalent Linking

In the covalent bonding method, biomaterials can form covalent bonds with the carrier through their functional groups on the surface, so that the biomolecules can be strongly fixed on the carrier. Generally, there are two methods to form covalent bonds between the biomaterials and the carrier: (1) The surface of the carrier is activated and directly covalently bonded to the biomaterials; (2) The surface of the

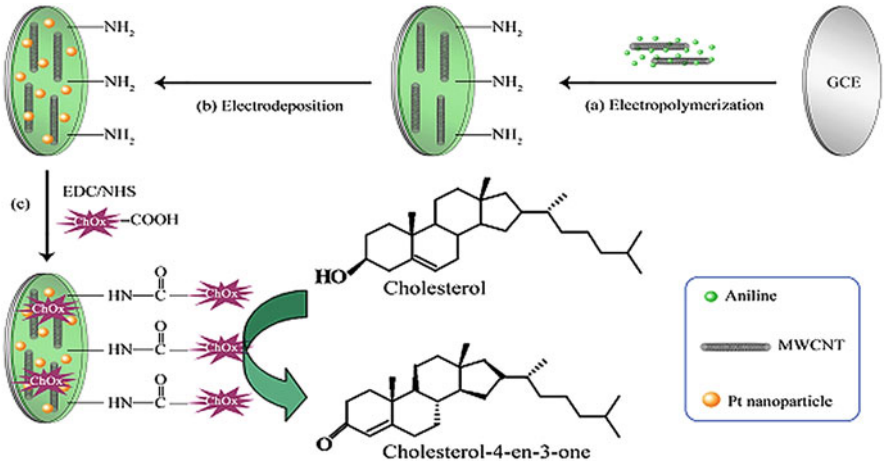


Fig. 11 The preparation process of cholesterol biosensor and the reaction on the surface of the ChOx-modified electrode during cholesterol determination [30]

carrier will be grafted with bifunctional reagents first, then the reagents form covalent bonds with the biomaterial. The covalent bonding method, on the one hand, improves the rigidity of the structure of the biomaterial, and reduces the damage to its conformation by the external environment; on the other hand, covalent attachment fixation helps to strengthen the orientation of biomaterial active sites towards the substrate and increase the chance of contact with the substrate [29].

Organics, like polymers, with free functional groups, which are very extremely suitable for the reactivity and biocompatibility of covalent bond formation with biological materials. Xu and his colleagues modified the surface of GCE with MWCNT-PANI (polyaniline) nanocomposites so that the surface of the electrode was grafted with active $–NH_2$. Cholesterol oxidase (ChOx) could form covalent bonds through its free carboxyl group –COOH and $–NH_2$ of PANI on the GCE surface, so as to be covalently fixed on the transducer to form the high-sensitivity cholesterol oxidase current biosensor. Figure 11 shows the preparation process of the enzyme electrode. The cholesterol biosensor had good performance, with a good linear range and low detection limit. After 45 days, the sensor could still maintain 87% of the initial current. Reproducibility for 5 electrodes gave a relative standard deviation (RSD) of 3.9%. The excellent stability and reproducibility are perhaps ascribed to the strong covalent fixation of ChOx, which also creates the ideal biocompatible environment [30].

In addition to the function of embedding, MOFs are also newly developed materials for covalent immobilization of biomaterials. Enzyme covalently fixed on the MOF is realized by the formation of amide bond between the biomaterials and the MOF free $-NH_2$ and $-$COOH groups on the surface, which is obviously different from the embedding method of MOF. Toromote the formation of amide

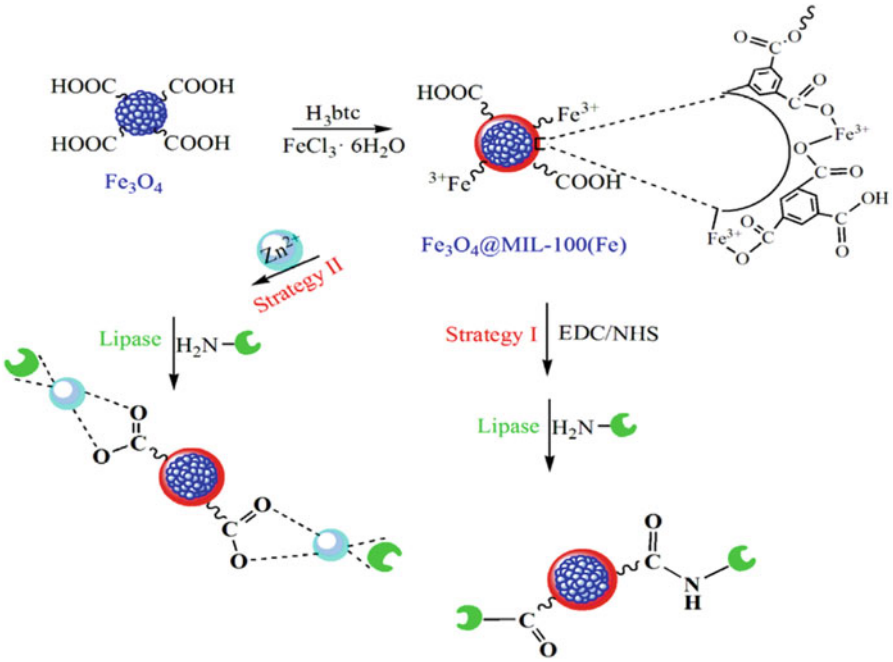


Fig. 12 Synthesis of Fe_3O_4@MIL-100 (Fe) microspheres and immobilization of lipase [33]

bonds between biomolecule and MOF, carboxylic acid activation molecules, such as N,N′-dicyclohexylcarbodiimide (DCC), are always required to activate −COOH and accelerate the reaction process [31].

Mehta et al. prepared UiO-66-NH_2 with amino-terephthalic acid (NH_2-BDC) and Zirconium chloride ($ZrCl_4$) by solvothermal method, and used DCC to activate the carboxyl group. The activated carboxyl group of UiO-66 formed amide bonds with the amino group of the organophosphate hydrolase, realizing the covalent connection and fixation of the organophosphate hydrolase. Immobilized enzyme activity of the free enzyme is higher than nearly 40%. Also, significantly increased storage stability. After 60 days of storage under 4 °C, 80% of the relative activity was still maintained, while almost all the free enzymes were inactivated [32].

Covalent bonding is also beneficial to keep the activity of biomaterials. Wang et al. compared the effect of covalent bonding (strategy I) and affinity interaction of metal ions (strategy II) on the retention of activity of fixed lipases (Fig. 12). Though the loading amount of lipases by covalent bonding method (83.48%) is lower than that of the metal ion affinity (87.20%), after 10 cycles' working, the residual activity of lipases immobilized by the covalent bond is much higher (74.04%) than that of metal ion affinity (61.16%). The results further verified that covalent bonding immobilization could greatly keep the activity and promote the stability of biomaterials [33].

Compared with physical adsorption, the covalent bonding force is stronger and the stability of biomaterials is higher. However, the covalent bonding process is complex and costly, and some functional groups on the surface of biomaterials may be destroyed during the fixation process, affecting their final activity and sensing ability. Therefore, the design of covalent bonds should avoid using functional groups in biomaterials.

Chemical cross-linking method conducts certain cross-linking reactions between biomaterials and carriers by the use of bifunctional or multifunctional reagents, leading to the formation of functional networks on the transducers. Functional groups such as $-NH_2$, $-COOH$, $-SH$, and $-OH$ in biomaterials often participate in cross-linking. As with the covalent binding method, cross-linking method also applied chemical binding to immobilize the biomaterials, while the difference is that the cross-linking method requires suitable cross-linking agents, like glutaraldehyde (GLT), hexamethylene diamine, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, and so on. The biomaterial can be firmly fixed onto the transducers by chemical cross-linking and could not fall off easily. While there are also some disadvantages that the degree of cross-linking reaction is difficult to control, the diffusion resistance is relatively large, and the required amount of biomaterial samples is large [34].

Glutaraldehyde (GLT) is the most common cross-linking agent for the high activity of aldehyde groups on both ends. Ceren et al. constructed a new and sensitive L-lysine amperometric biosensor based on Lysine oxidase (LyOx) with GLT as cross-linking agent. LyOx was cross-linked by the GLT/bovine serum albumin (BSA) and then immobilized on the modified GCE, which had been modified by graphene (GR) and redox polymer poly(vinylferrocene) (PVF) in advance. Figure 13 shows the preparation process of the biosensor. Chemical cross-linking treatment enables the biosensor to make full use of the advantages of LyOx, GR, and PVF, resulting in short response time (<5 s), high sensitivity, low detection limit, and good reproducibility [35].

In some cases, multiple cross-linking agents may be used together. In the preparation of a hormone-based parathyroid hormone (PTH) detection biosensor, Hakkı applied poly amidoamine dendrimer (PAMAM) as an active end increasing agent to link the carrier and anti-parathyroid hormone molecule. 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide/N-hydroxysuccinimide (EDC/NHS) and GLT functioned together as the cross-linking agents. Use EDC/NHS solution to connect PAMAM to the modified Au electrode surface, and GLT activates the amino end of PAMAM so that the anti-parathyroid hormone molecule could be covalently fixed onto the modified Au electrode. Figure 14 is the assembly process of the PTH biosensor. And various characterization results show that the biosensor has good reproducibility [36].

4.2 Protein-Molecular Interactions

Specific recognition is one of the most important layer-by-layer (LBL) self-assembly reaction for proteins and other bio-macromolecules, which is driven by the specific

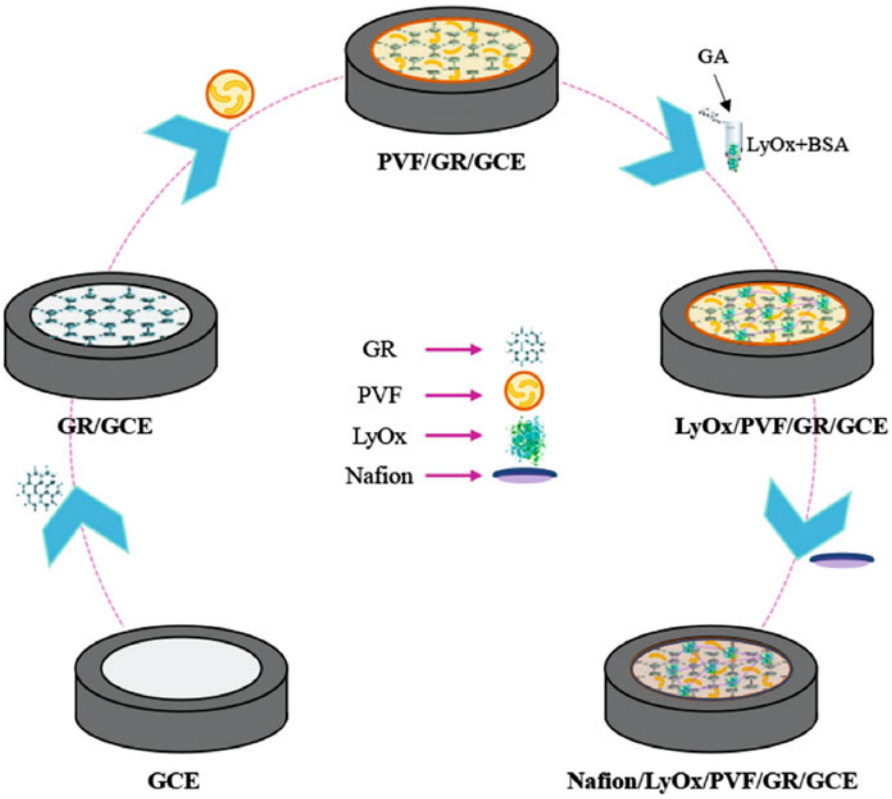


Fig. 13 Scheme of L-lysine biosensor procedure [35]

recognition between specific biomolecules. Presently, the common types of specific recognition between biomolecules include enzyme-substrate, antigen-antibody, aptamer-thrombin, antibiotic protein (avidin)-biotin, Concanavalin A (Con A)-Glycoproteins, Con A-HRP, etc., among which the last two types have been frequently used in the construction of biosensors. Bio-molecular layers self-assembly by specific recognition possess high ordering, stability, and controllability, which could enhance their advantages in the construction of biosensors [37].

Lin group constructed a multilayer film of HRP on the surface of the modified Au electrode by the alternative deposition of Con A and natural HRP under the drive of the recognition between Con A and HRP. One Con A molecule can be combined with four HRP molecules to detect thiols through the inhibitory of enzymes. The biosensor showed high selectivity and stability. Under these conditions, the linear response range of the biosensor to cysteine is 0.1–23.5 μM, and the detection limit was 0.02 μM [38].

Anzai et al. further developed the HRP/GOD (glucose oxidase) dual-enzyme biosensor through the recognition of Con A and glycoprotein, which expanded the

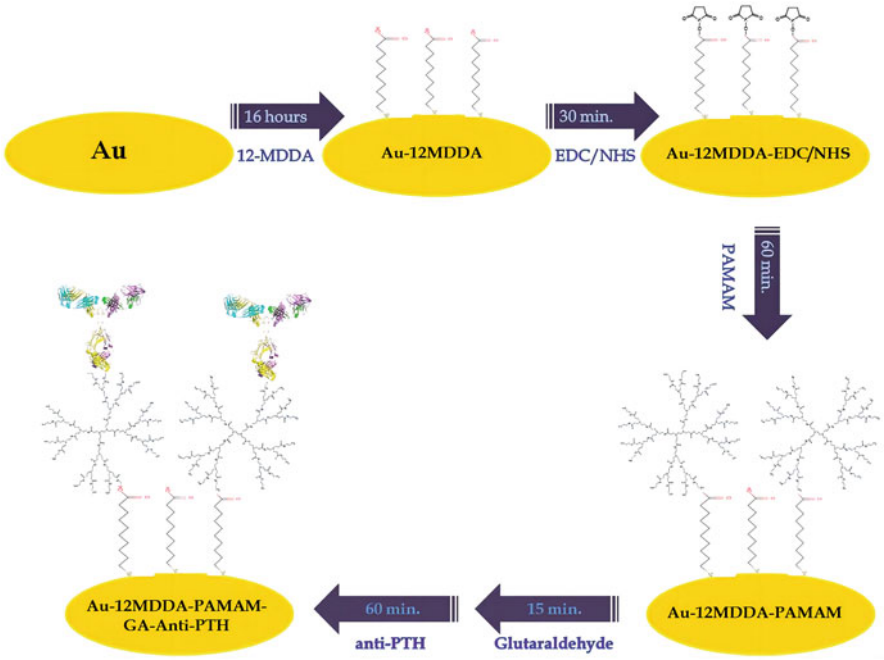


Fig. 14 The manufacturing process of biosensor based on PAMAM [36]

application of this immobilization method. Figure 15 shows the deposition of Con A and glycolase on the solid external. The study found that in order to obtain a high-performance sensor, HRP layer should be first coated on the electrode surface, then fix the GOx layer on the outer layer. By changing the amount of layer and immobilization order of the enzyme, the load and geometric configuration of the enzyme in the multilayer film were optimized. The response intensity of glucose sensor enhanced with the increase of GOx layer, while the HRP bilayer was sufficient to achieve the maximum response [39].

4.3 Click Chemistry

Click chemistry is a new synthesis concept introduced by professor Sharpless from the famous American Scripps Research Institute in 2001. This method is based on the formation of C-X-C bonds to complete the chemical synthesis of various molecules through the splicing of small group units [40]. One of the most representative "Click" chemical reaction is the 1,3-dipole ring addition reaction of azide and endyl acetylene catalyzed by Cu^{+}. This reaction not only has a high degree of independence, integrity, and specificity but also has good biological compatibility. In addition, the reaction product is extremely stable under physiological conditions. Consequently, many biosensors have been constructed with Click chemistry.

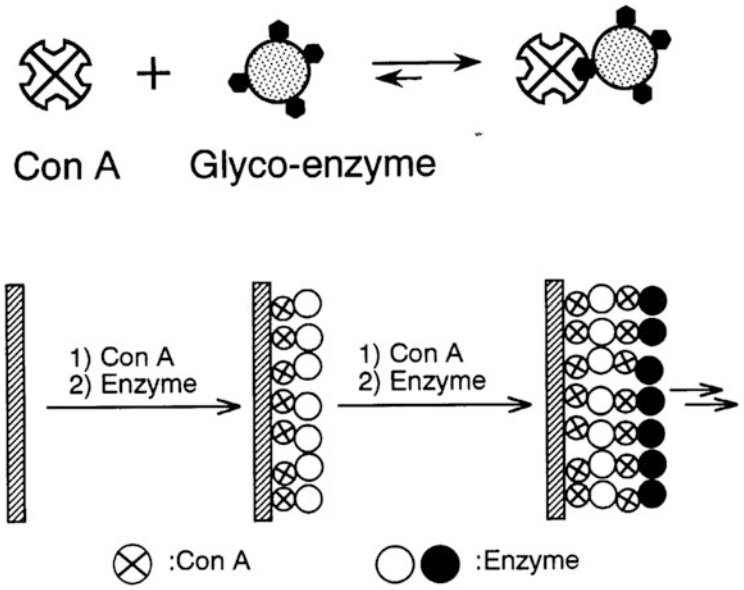


Fig. 15 The deposition of Con A and glycol-enzymes layer-by-layer on the solid surface [39]

Brennan et al. conducted the biological coupling reaction between lipases and nano-gold through click chemistry. Firstly, gold nanoparticles were azidation and lipase was alkynylated at the same time, and then the "click" reaction took place under the catalysis of Cu (I). Figure 16 illustrates the process of functionalizing gold nanoparticles and attaching lipase to nanoparticles with "click" chemistry. Studied by capillary electrophoresis and fluorescence gave the results that seven active lipase protein molecules were efficiently linked to 1 Au nanoparticle [41].

Jin's team conducted an extended study on the "click" chemistry, where the alkynyl-terminated monolayers were prepared by self-assembly technology, and then the azido-HRP were covalently fixed on the monolayer through the "click" reaction (Fig. 17). The assembly process of the electrode was studied in detail by infrared spectroscopy and surface-enhanced Raman scattering spectroscopy. The results showed that the surface of the fixed electrode horseradish peroxidase (HRP) molecules maintain a high biological activity in the case of invariance enzyme structures. The electrocatalytic reduction of H_2O_2 by immobilized horseradish peroxidase could be evaluated linearly in the range of 5.0–700 μM. The heterogeneous electron transport constant between the HRP and the electrode was 1.11 s^{-1}, and the apparent Michaelis-Menten constant was 0.196 mmol L^{-1}, indicating a high affinity between HRP and the substrate [42].

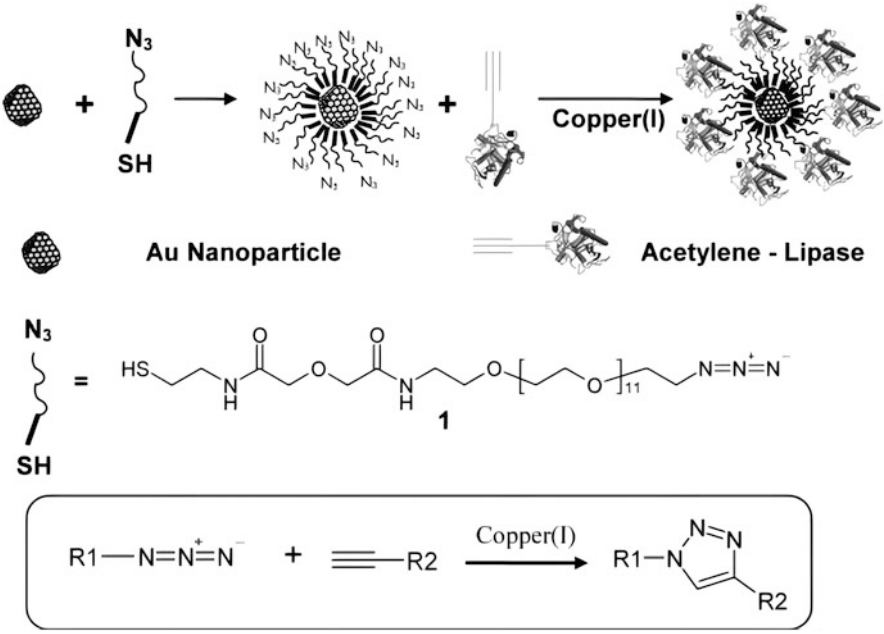


Fig. 16 The process of functionalizing gold nanoparticles with "click" and attaching lipase to nanoparticles [41]

5 Electrochemical Polymerization

Electrochemical polymerization is an efficient fixation method of biomaterial developed in recent years, which involves mixing biomaterial molecules with polymer monomers in an electrolyte. The monomers could be oxidized or reduced by potentiostatic scanning or cyclic voltammetry scanning, and then polymer films were formed on the electrode surface. Biomaterial molecules are embedded in the polymer membrane during the electrochemical polymerization, so that can be fixed on the electrode surface. The electrochemical polymerization method has the following advantages: (1) strong anti-interference; (2) The fixation and electrochemical polymerization of biomaterial molecules can be completed in one step, which simplifies the experimental steps; and (3) The composition of the biomaterial and the thickness of the polymer layer can be easily controlled, so the electrochemical biosensor has a good reproducibility [43, 44].

Samuel et al. prepared an amperometric nitrate biosensor by embedding the Aspergillus niger nitrate reductase (NAR) in the Ppy membrane during the galvanostatic polymerization (GALP) of pyrrole. This amperometric nitrate biosensor can be used to detect nitrate at relatively low concentrations, with a sensitivity of

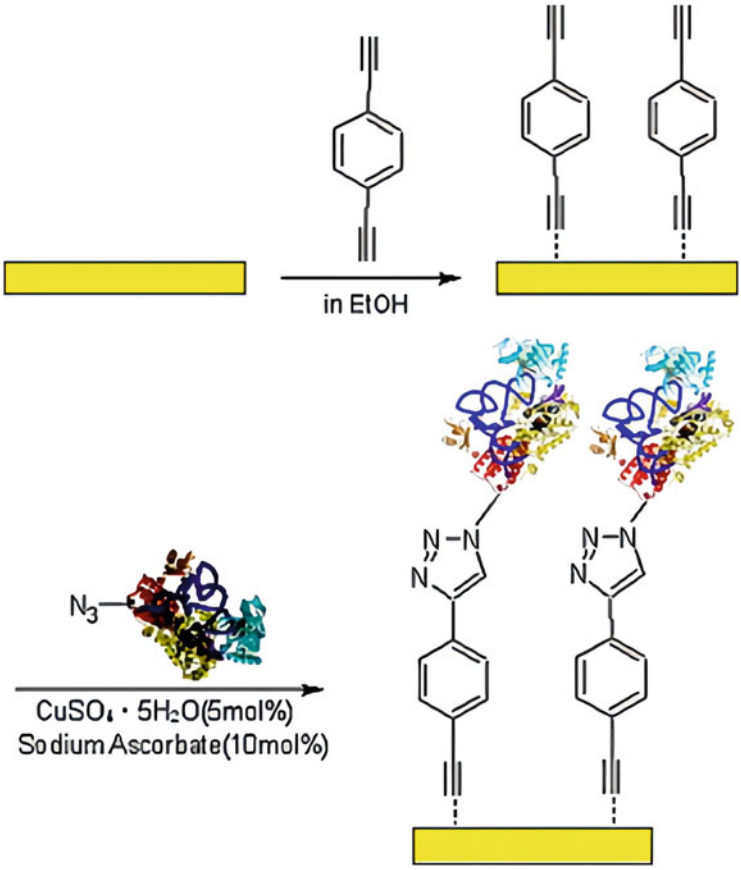


Fig. 17 Fabrication strategy of HRP/DEB-modified electrode [42]

40.8 nA μM^{-1} cm^{-2}. This method allows the enzyme to expose more active sites thereby providing effective electron transfer [45].

Krishnendu et al. prepared a novel cholesterol biocomposite electrode using a simple method. The biocomposites were prepared from Ppy, green reduced graphene oxide (gRGO), and ChOx. In the process of electrodeposition of pyrrole by cyclic voltammetry, gRGO improved its adhesion on the electrode surface through co-deposition, and the ChOx enzyme was immobilized through this co-deposition process thus completing the one-step fabrication of the biosensor. Figure 18 is a schematic diagram of ChOx-gRGO-PPy/GCE. The optimized enzyme biosensor showed excellent performance, such as high sensitivity (1095.3 μAmM^{-1} cm^{-2}), wide low detection limit (3.78 μM), and linear range (0.01~6 mM) [46].

Chen and his coworkers designed a new biosensor for the detection of Brucella based on the combination of the directed immobilization technology of biomolecules and signal amplification technology. It has been modified to graphene oxide/

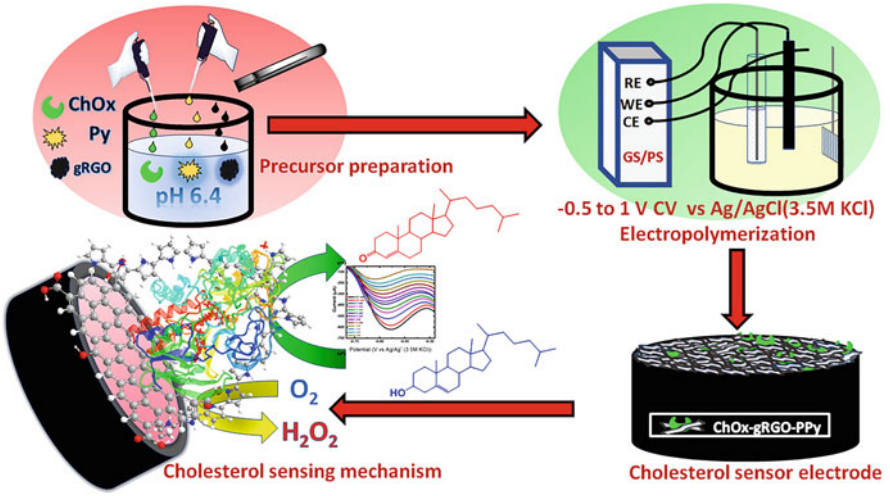


Fig. 18 Schematic diagram of ChOx-gRGO-PPy/GCE [46]

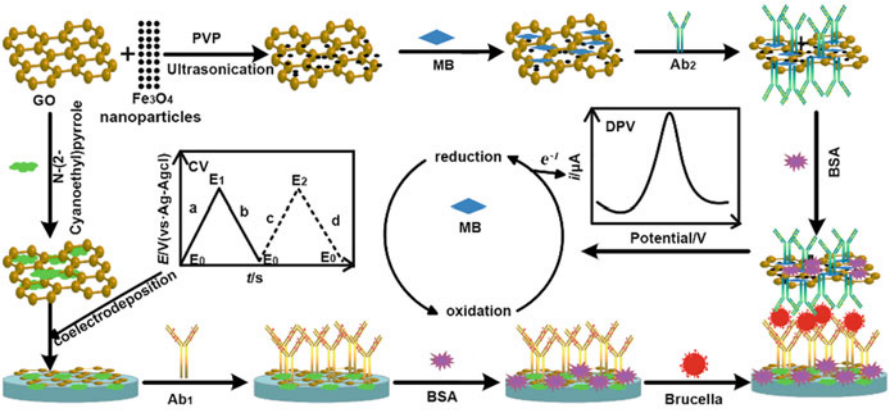


Fig. 19 Step-by-step assembly process of biosensors [23]

polypyrrile co-electrodeposited by screen-printed gold electrode, successfully immobilized antibody by cyclic voltammetry (Ab1), to improve the binding efficiency of the antibody to the antigen. The signal amplification of the second antibody nanoprobe GO/Fe_3O_4/MB/Ab_2 (graphene oxide/nanometer iron oxide/methylene blue /Ab_2) was completed by physical and chemical methods. Figure 19 shows a step-by-step assembly of the biosensor. The results showed that the logarithm of *Brucellosis* concentration was positively correlated with the corresponding current, the detection limit is 2.2×10^2 CFU/mL, and the linear range is 1.6×10^2 to

1.6×10^8 CFU/mL. In addition, the biosensor demonstrated good specificity, reproducibility, and stability [47].

6 Spinning and Deposition

In addition to the former traditional fixation methods, some new immobilization techniques beneficial from the development of high and new technologies, such as laser and electric field, have been applied to construct biofilms onto the sensor. Specifically, spinning techniques (such as electrospinning) and laser deposition techniques (such as laser-induced forward transmission) have been applied to the fabrication of biosensors. In this part, electrospinning technology, electrostatic spray deposition, plasma deposition, and laser deposition will be discussed.

6.1 Electrospinning

Electrospinning technology is a fiber manufacturing process in which polymer melt or solution is atomized in a strong electric field and solidified into filaments through a small jet [48]. In order to prepare biosensors by electrospinning, biomaterials and polymer precursors are usually mixed as raw materials, which is similar to the treatment in electrochemical polymerization. The mixed raw solution of biomaterial and polymer is placed in a high-voltage electrostatic field, and the solution droplet is accelerated at the Taylor cone of capillary under electric field. The droplet overcomes the surface tension and forms a jet trickle. In the process of trickle injection, the solvent evaporates or solidifies, and finally the mixtures of polymer and biomaterials fall on the receiving device, the formation of bio-fibers of different diameters. Electrospinning nanofiber membranes have high porosity, diversified components, and uniform diameter distribution, widely used in biosensors and biomedical. The increased surface area can improve the sensitivity of the conductivity sensor. In addition, the electrospinning technology is simple to operate, low in cost, and has strong practical value. Biosensors based on electrospinning membranes have been widely used to analyze various substances, for example, glucose and uric acid [49].

Lee et al. Prepared PVA/laccase-Au-NPs/Pt electrode by electrospinning the mixture of PVA-laccase-Au NPs onto the surface of Pt electrode. Figure 20 is the structure diagram of the modified PVA/laccase-Au-NPs/Pt electrode. This sensor showed good selectivity to ascorbic acid and stability in several interference reagents. The standard deviation of 76 days of continuous testing was 4.3%, indicating good durability [50].

6.2 Electrostatic Spray Deposition

With the same principle of electrospinning, electrostatic spray deposition (ESD) also produces aerosol by applying the electric field on the biomaterials droplets, which

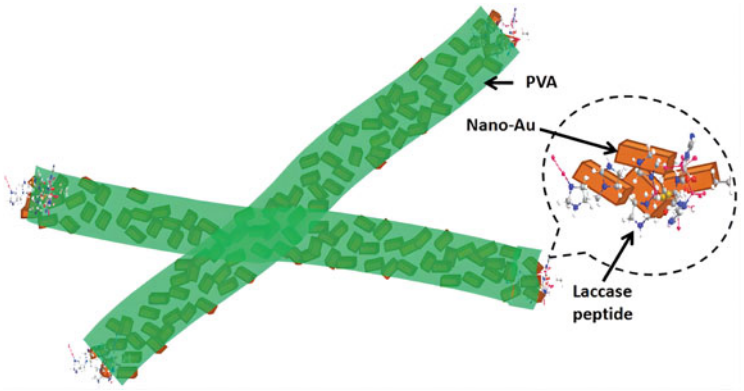


Fig. 20 The hierarchical structure diagram of modified PVA/laccase-Au-NPs/Pt electrode [50]

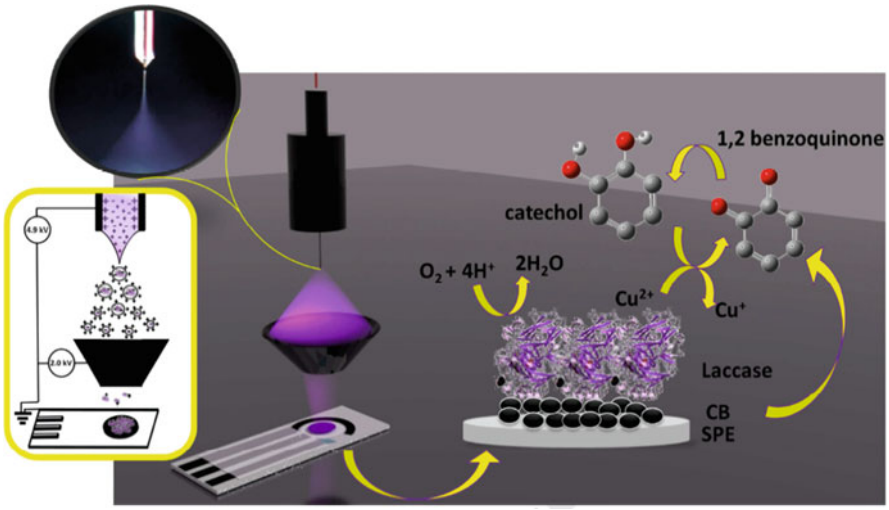


Fig. 21 The left picture is a schematic diagram of an electrostatic discharge device used for immobilization of CB-SPE laccase; the inset is a photograph of the Taylor cone during the deposition process; the arrow in the right figure highlights the catalytic effect of laccase on catechol oxidation and oxygen reduction [51]

will fly to the substrate and be immobilized as a bio-membrane. Unlike electrospinning, ESD usually can directly form the membrane without the spinning of fiber. Therefore, it is not necessary to emphasize the addition of polymer in the preparation of precursor solution. According to the needle direction, ESD can be divided into vertical ESD devices and horizontal ESD devices. Mattea et al. used ESD to successfully immobilize laccase on electrode screen-printed of the modified carbon black and fabricated an amperometric biosensor for the detection of phenolic compounds. The left picture in Fig. 21 is a schematic diagram of an electrostatic

discharge device used for immobilization of CB-SPE laccase, which is the usual vertical ESD device. The arrow in the right figure highlights the catalytic effect of laccase on catechol oxidation and oxygen reduction. The results showed that the laccase still could maintain its activity after electrospray ionization treatment and deposition. The prepared laccase biosensor applied catechol as the analyte, the linear range is 2.5~50 μM, and the detection limit is 2.0 μM, together with good working stability and repeatability at room temperature [51].

7 Plasma Deposition

In recent years, plasma deposition technology, especially soft plasma polymerization (SPP) at ambient pressure and room temperature, has been very popular as a powerful tool for immobilization of biomaterial and surface engineering. In SPP experiment for biosensors, the original monomers and biomolecules will be directly cross-linked into a thin film coating, which is deposited on the substrate without significant degradation or denaturation so that it will not lose important functional groups from the biomolecules [52]. SPP technology can deposit the biocoating without using toxic solvents, so it is more suitable for the fixation of biomaterials [53]. Compared with traditional methods (such as wet chemistry), the biomaterials on the transducer formed by SPP always possesses a higher cross-linking degree and adhesion [54].

With the help of low energy density Helium Corona plasma jet, Szymon et al. applied the SPP experiment directly polymerized laccase molecules onto the surface of a clean glass to obtain laccase coating (Fig. 22). The bioactivity of the coating by SPP was only 59% of the original activity, which might be due to the structure damage of laccase molecules under the action of high energy materials in plasma. However, after being washed by water, the activity of laccase coating deposited by SPP was seven times higher than that of non-SPP method. It is suggested that the SPP method has a good immobilization effect on laccase due to the higher cross-linking degree brought by the plasma method. In the study, the authors also found that, as expected, bioactivity declined with the increase in the severity of plasma conditions, i.e., the increase in power density and exposure time [55].

Therefore, in order to avoid this shortcoming, a new “cold” technology is considered to be an innovative construction method for laccase voltammetric biosensors. Malinowski and his group used a new SPP method with cold plasma to polymerize and bond the layer of laccase molecules onto the surface of GCE under atmospheric pressure. The results GCE/Laccase sensor for the determination of Rutin in actual drug samples showed that the analytical performance parameters of laccase biometric recognition layer deposited by corona plasma jet were close to or better than those prepared by traditional methods. The biosensor had the best analytical signal stability and a wide analytical response linear range. The new Corona SPP method proposed in this work is a one-step, environment-friendly, allowing the deposition of biometric layer, without using any additional chemicals (except water and alcohol) [56].

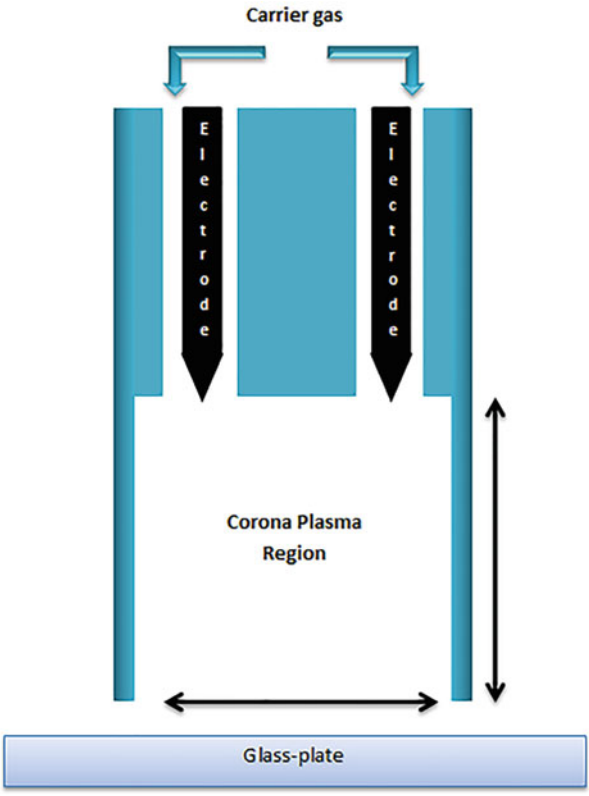


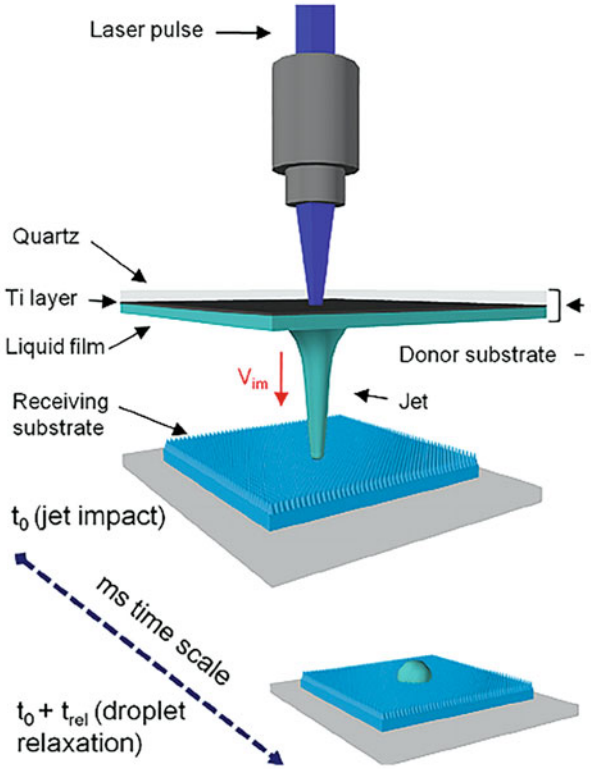
Fig. 22 Schematic diagram of corona plasma discharge device for laccase coating deposition [55]

7.1 Laser Deposition

In addition to the abovementioned advanced technologies, new immobilization technologies based on laser also be used in the progress of biosensors [57], such as Laser Induced Forward Transfer (LIFT) technology [58] and Matrix Assisted Pulsed Laser Evaporation (MAPLE) technology [59].

In the LIFT technology, the efficient immobilization of biomaterials mainly depends on the high-speed transport of droplets in laser pulses. Figure 23 is a sketch of the main part of LIFT setup. Biomaterials, prepared as liquid or solution in usual, are firstly coated on the surface of certain transparent substrate, and another receptor is placed close to this surface (about a few hundred microns). The pulsed laser passes through the transparent substrate and focuses on the biomaterials. Under laser irradiation, the biomaterials will produce a high-speed jet, which will impact the receiving substrate, resulting in the adsorption and fixation of biomaterials on the substrate (Fig. 24). LIFT technology has been used to deposit and immobilize laccase [60].

Touloupakis et al. used LIFT technology to successfully immobilize laccase on a graphite SPE. Compared with the one prepared through pipetting method, the



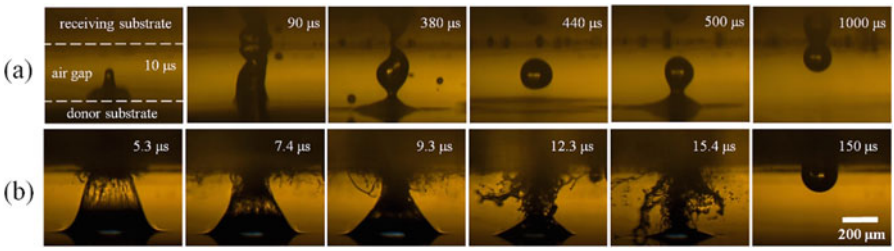


Fig. 23 Sketch of the main parts of the LIFT setup

Fig. 24 Side view imaging of the evolution of LIFT printing on the superhydrophobic substrate (**a**) for low (300 mJ/cm^2) and (**b**) for high (930 mJ/cm^2) laser fluence [60]

contact angle between the laccase droplets and the graphite SPE became smaller. With the increase of the laser ernegy from 125 to 300 mJ/cm^2, the contact angles would decrease from 89.1° to 18.5°, changing from a partially wetted state to a fully wetted state (Fig. 25). This should be ascribed to the efficient immobilization of laccase of high energy laser. The biosensor can detect catechol in an aqueous solution within the nanomolar concentration range. The sensitivity of the biosensor

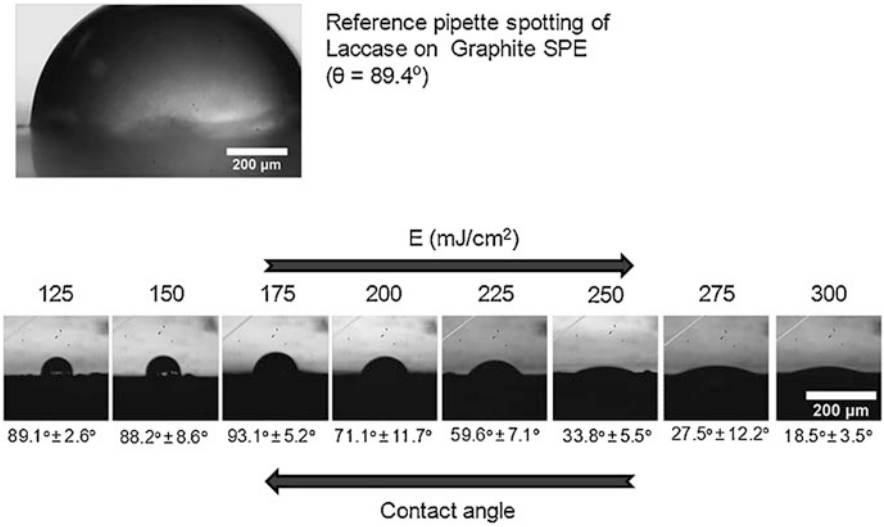


Fig. 25 Laccase optical microscopy image of the droplet, respectively reference pipette and the LIFT method pipette deposited on the graphite SPE [61]

was 0.43 nA ± 0.04 nA/μM. It retained 90% of its activity for up to 35 days at 4 °C [61].

In MAPLE, pulsed laser beam is focused in a vacuum chamber, the target hits the rotating surface [62]. The biomaterials will be dissolved in the appropriate solvent and frozen on the target. Under laser impacts, the frozen solvents absorb the laser energy and evaporate together with the biomolecules, and finally deposited on the substrate, forming uniform films. Due to the usage of frozen solvents, the structure and activities of biomaterials can be preserved to the largest extent. As a result, MAPLE has been described as the most appropriate technology to construct biofilms.

Maria and his coworkers created a laccase-based biosensor for the determination of phenols by using MAPLE deposition technology. Figure 26 is the MAPLE experimental device diagram. This method can not only fix laccase firmly on the surface but also control the thickness of laccase film by controlling laser pulse parameters. The sensor has good film-forming performance, wide linear range (1–60 μm), high sensitivity, and response stability, which is suitable for the evaluation of TPC in plant extracts [63].

8 Characterization of Biomaterials on Transducers

Good biosensors not only need to load enough amount of biomaterials, but also should guarantee the immobilized biomaterials, the reasonable distribution on the surface of the transducer, the inherent stable structure and activity, and effective

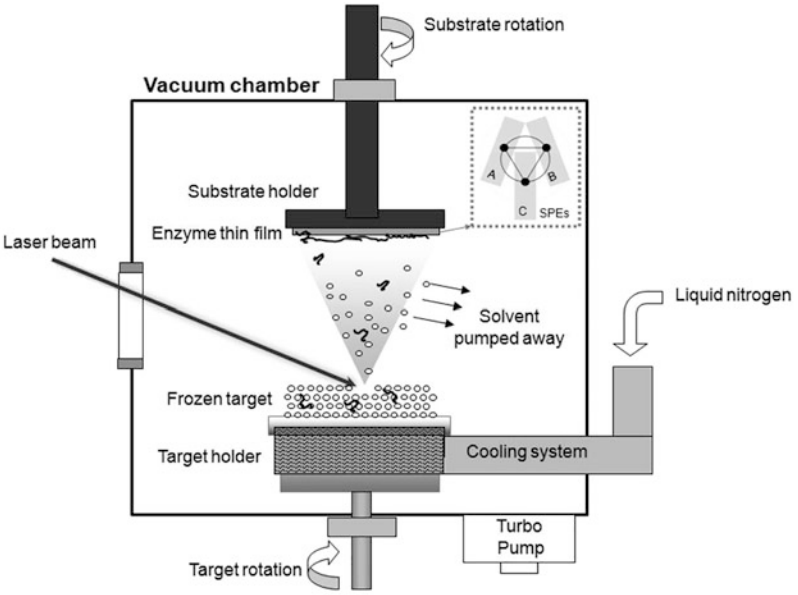


Fig. 26 The MAPLE experimental device diagram [61]

binding force with the transducer, so as to ensure the specificity and stability of the sensor. Different characterization methods on the surface of carriers and biological materials can understand the above properties and characteristics. In this part, the approaches usually used to test the characteristics will be reviewed, including FT-IR and Raman, UV-VIS, CD, AFM, SEM, TEM, etc.

8.1 FT-IR and Raman Spectroscopy

Organic functional groups are animportant basis to confirm the existence and activity of biomaterials. FT-IR technology can recognize the biomolecules based on the detection of the vibrational and rotational energy of functional groups in the infrared range of 400 cm^{-1}–4000 cm^{-1} and can be applied to analyze the composition, the loading, conformation, and activity of biomolecules fixed on the substrate surface [64] according to the wavenumber and intensity of the characteristic infrared absorption peaks of functional groups. FT-IR technique avoids the structure damage of the biomolecules and greatly simplifies the test of some special samples. It has the characteristics of in situ, real time, non-radioactive, and non-destructive. It is suitable for the nonuniform and concave convex surface and can obtain the infrared spectrum image of the spatial distribution of the functional groups on the surface of the micro area [65].

Jaffrezic-Renault applied FT-IR and proved the immobilization of laccase onto the laccase/chitosan (GHIT)-lambda-carrageenan (CAR)-based voltammetric

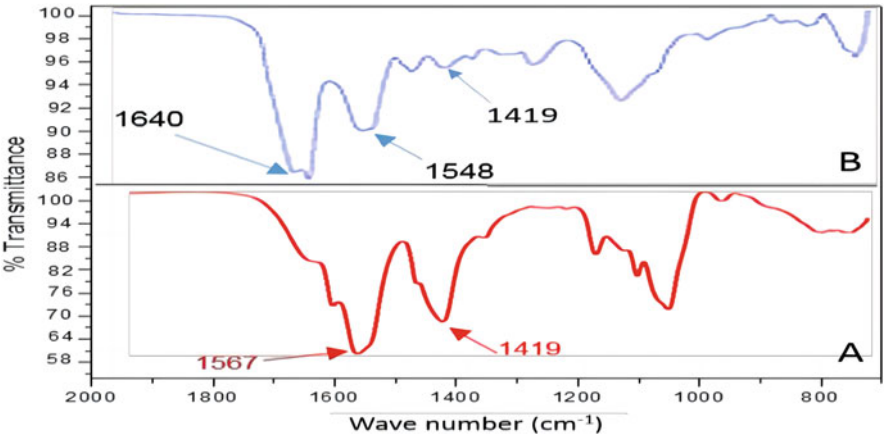


Fig. 27 (**a**) FT-IR spectra of the (CHIT+CAR) and (**b**) of the LAC/CHIT+CAR film [66]

biosensor. Before the introduction of laccase, IR spectrum of CHIT-CAR clearly gave the adsorption peak at 1562 cm^{-1}, corresponding to the $–NH^{3+}$ group in CAT. The band at 1443 cm^{-1} shifts to 1419 cm^{-1} compared with the free CAT, which could be assigned to the formation of polyelectrolyte by CHIT and CAR. On the further modification of laccase, the new adsorption in the peak at 1640 cm^{-1} and 1548 cm^{-1} showed up (Fig. 27), indicating the existence of Amide I and Amide II bands of laccase. In addition, FT-IR had been used for the calculation of the degree of deacetylation (DD) of chitosan by applying the ration of adsorption intensity at 1313 $cm^{-1}$and 1415 cm^{-1}, resulting in the molecule weight of 42.7 kDa and DD as 80% [66].

Zhang's groups found that the laccase adsorbed on Black Pearl 2000 (BP2000)-modified GCE would result in structural change for the strong interaction between laccase and BP 2000. After the loading of laccase, the original adsorption peaks of BP2000 at 1584 cm^{-1} and peaks at 1546 cm^{-1} of laccase disappeared, and the characteristic adsorption of laccase at 1630 cm^{-1} shifted to the long wavelength along with a decrease in intensity (Fig. 28). The change of typical adsorption peaks might be aroused by the intense interaction of laccase and BP2000 [67].

Raman spectroscopy is another important way to characterize the structure and biomolecule activity besides FT-IR. Like FT-IR, Raman spectrum also gives the structural information of molecules based on the detection of the vibrational and rotational energy. Differently, Raman spectrum can be analyzed without special sample preparation, which is more convenient. Raman spectrum could apply water as the solvent, which is forbidden in FT-IR. Consequently, Raman is friendly to the biosamples which contain plenty of water or preserved in water [42, 68].

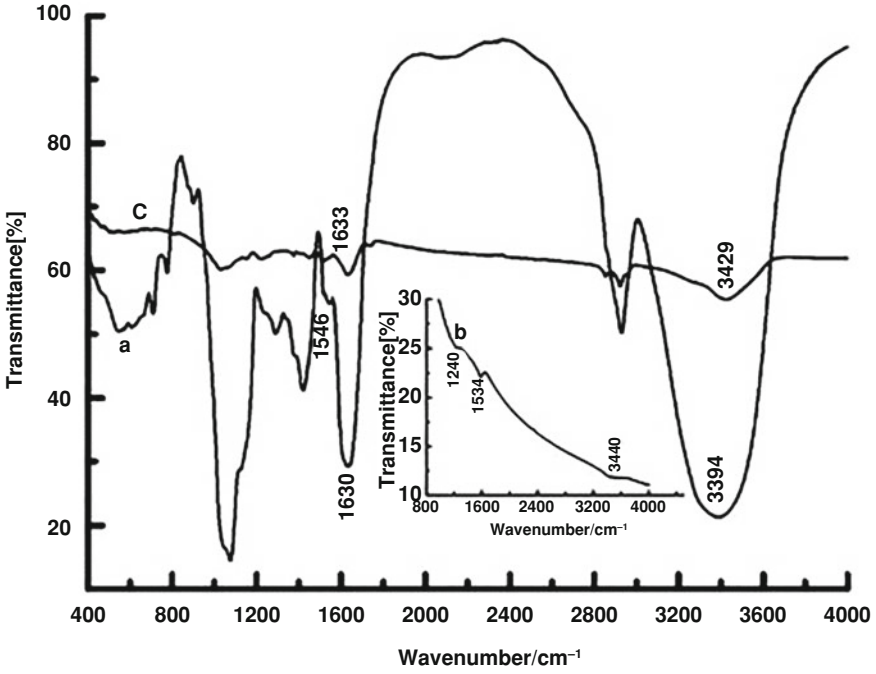


Fig. 28 FT-IR spectra of (**a**) pure Lac, (**b**) pure BP2000, and (**c**) Lac immobilized on BP2000 matrix [67]

8.2 UV-vis Spectroscopy

UV-Vis adsorption spectrum can be used to analyze and determine the biomaterials according to the spectrum generated by the adsorption of ultraviolet and visible light at 200~800 nm by the biomolecules. The functional groups of biomolecules could be concluded through the characteristic UV absorption band, and the quantitative analysis could be carried out by Lambert Beer's law and the UV absorption intensity. Since most biomaterials display UV-Vis adsorption, UV-Vis spectrum is an important technology to analyze the structure, composition, and content of biosamples on the sensor [69].

Sundramoorthy applied the CV method to construct the biosensor based on Flavin adenine dinucleotide (FAD). UV-Vis had been adopted to confirm the immobilization of FAD. As shown in Fig. 29 (iv and v), the typical bands of FAD at 220 nm, 270 nm, and 450 nm showed up in the UV-Vis spectrum of the final electrode composites, indicating that the FAD had been successfully immobilized into the composites and could keep its stability well.

UV-Vis can also be used to confirm the multilayer structure and figure out the number of layers. Dai et al. confirmed the multilayer structures of avidin labeled by FITC on quartz slides through the UV-Vis spectrum [70]. The experimental absorbance of the composites at 500 nm was 0.0044/deposition (Fig. 30) while

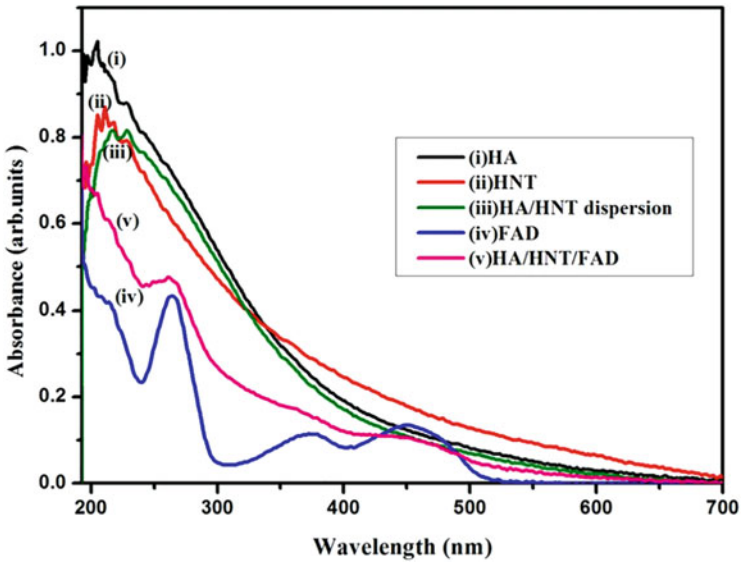


Fig. 29 UV–Vis spectra of (i) HA, (ii) HNT/H_2O, (iii) HA/HNT, (iv) FAD solution, and (v) HA/HNT/FAD dispersions

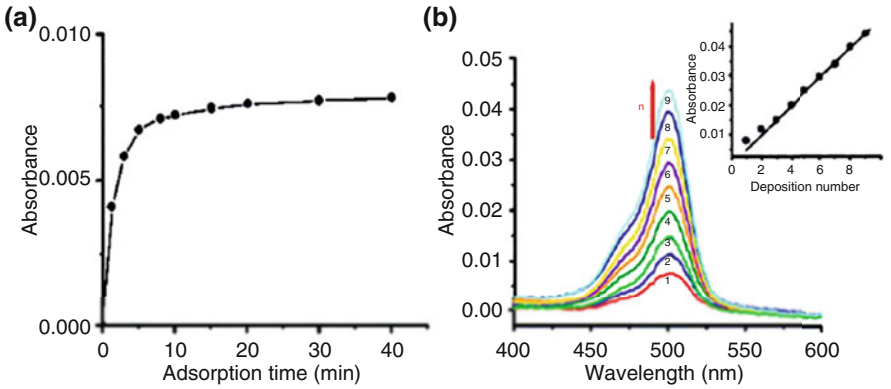


Fig. 30 (**a**) The time course of AF adsorption on the surface of lead-plated quartz glass slide. (**b**) The absorption spectrum of the (PB/AF)n ($n = 1, 2, 3, 4, 5, 6, 7, 8, 9$) multilayer film formed on a quartz glass slide as a function of the number of depositions [71]

considering the absorbance of monolayer of AF as 0.0022, there should be two layers of the FITC–avidin immobilized on the quartz slide after each deposition [71, 72].

8.2.1 CD Spectroscopy

Bio-macromolecules often exhibit optical anisotropy. Circular dichroism (CD) has been widely applied to characterize the three-dimensional structure of molecules

through the different absorption of R and L circularly polarized light. It is mainly applied to measure the secondary structure of bio-macromolecules, such as proteins, nucleic acids, and polysaccharides. CD can reflect the biological activity, chemical environment, and secondary structure changes of biomolecules [73, 74].

Zhou et al. constructed the colorimetric aptamer biosensor for As^{3+}, based on Ars-3 aptamer and hexadecyl-trimethyl-ammonium bromide (CTAB). CD had been applied to evaluate the interaction among the aptamer, CTAB and As^{3+}. As3 added in Ars-3+ aptamer solution only results in a reduction of negative and positive peaks of intensity at 240–260 nm at 270–290 nm, and the peak position without displacement (Fig. 31). While the addition of CTAB, the positive peak could shift to the long wavelength by 5 nm, without the change of peak area. The CD experiment proved that CTAB had made a great function in the interaction with the DNA base [75].

Puri et al. immobilized the lipase onto the MWCNT and analyzed the secondary structural change of lipase after covalent attachments with the help of CD spectroscopy. Once immobilized, the amount of α-helix in lipase would decrease by about 20% as calculated by the residue ellipticity at 222 nm (Fig. 32). Though the adsorption of MWCNT would make an effect on the active structure, while considering the increase in stability after immobilization, the preserved secondary structure could still display high activity [76].

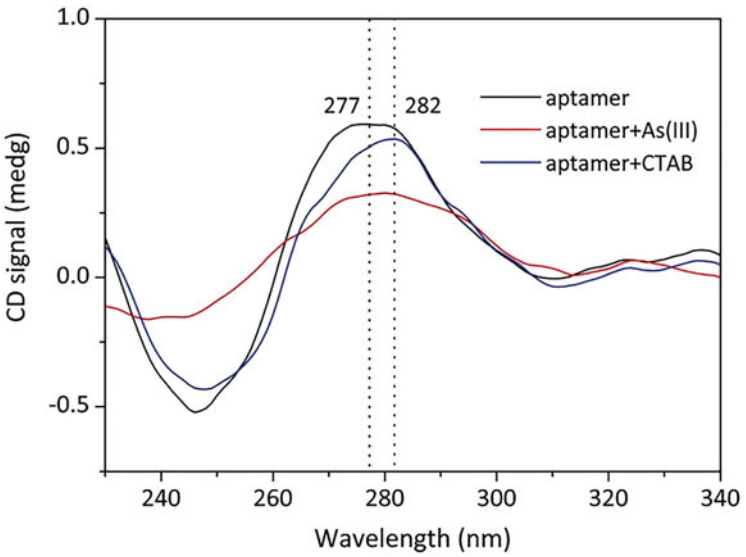


Fig. 31 CD spectra treatment Ars-3 aptamer by CTAB solution and As (III) [75]

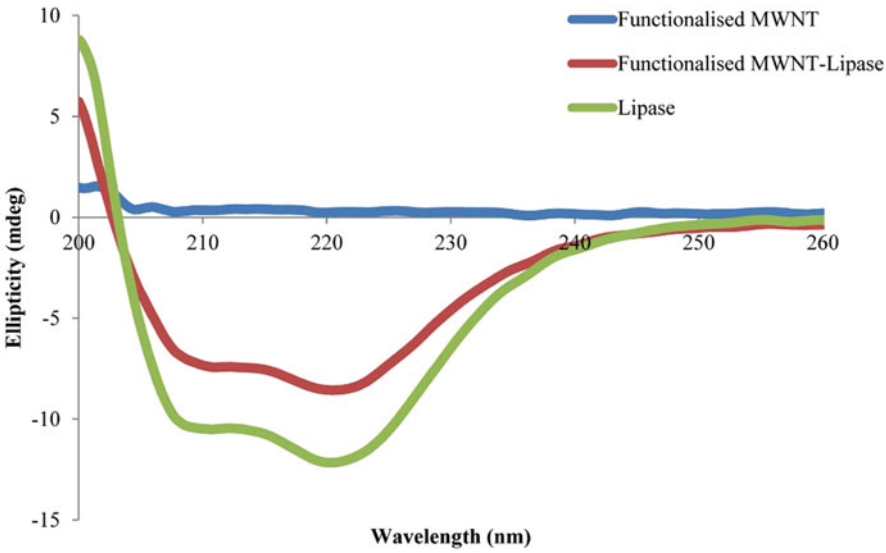


Fig. 32 CD spectrum of free and MWNT bound enzyme [76]

8.3 Atomic Force Microscopy

The performances of biosensors largely depend on the immobilization state of biomaterials on the transducer, which is generally determined by the interface interaction between biomolecules and carriers. As a member of scanning probe microscopies (SFM), atomic force microscopy (AFM) can effectively demonstrate the interaction between biomaterials and carriers, as well as the thickness and surface roughness of biomaterials layers on three-dimension at nano-scale. AFM presents the characteristics of the sample surface by the change of interaction force between the probe and the sample, and mainly consists of contact, tapping, and non-contact mode, according to the different interaction types.

Bulut et al. synthesized a new testosterone (TES) biosensor based on antibody. Testosterone antibody (AbTES) was immobilized on the surface of the poly (benzenediamine-Bis[(2-ethylhexyl)oxy] benzodithiophene) (pBDBT)-coated screen-printed carbon electrode (SPCE). They used the non-contact AFM to characterize surface morphology of the bare SPCE, SPCE/pBDBT, SPCE/pBDBT/AbTES, and SPCE/pBDBT/AbTES/TES (Fig. 33). The changes of surface roughness confirmed the successful immobilization and modification of polymers pBDBT and AbTES on the electrode surface. Moreover, the contact angle increased with the modification of hydrophobic polymer pBDBT and decreased with the fixation of hydrophilic antibody and adsorption of testosterone. The change of contact angle also indirectly confirmed the loading process of the material [77].

The Brucella sensor constructed by Chen showed excellent performance. Through AFM images, they intuitively reflect the effect of diverse fixation methods

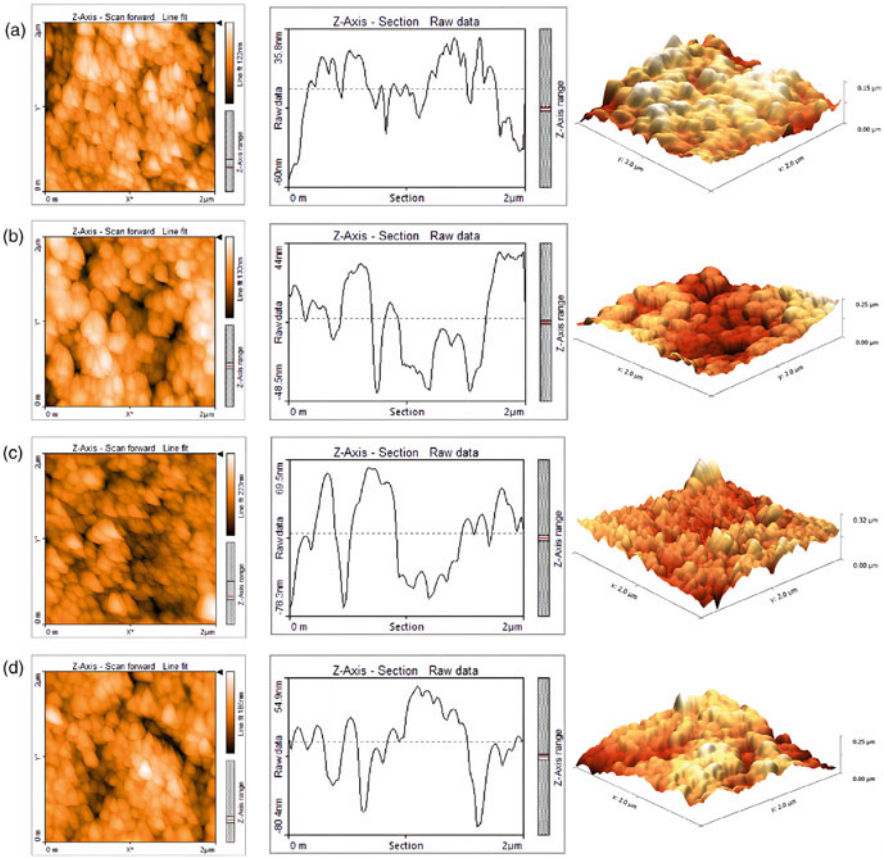


Fig. 33 3D AFM topographic images and section analysis with height images of (**a**) bare SPCE, (**b**) SPCE/pBDBT, (**c**) SPCE/pBDBT/AbTES, and (**d**) SPCE/pBDBT/AbTES/TES [77]

on the sensor surface. After graphene modification, the surface of Au electrode changed from rough to smooth (Rq changing from 58.4 nm to 46.4 nm, Rq representing the Root mean square roughness) (Fig. 34). The surface would become further smoother after the modification of Ab1. Noteworthy, the electrode modified with Ab1 by the CV method possessed a smaller Rq than that prepared by physical adsorption. This AFM experiment not only successfully proved the fixation of biomaterials but also illustrated the different fixation characteristics that the CV method could form ordered film while physical adsorption tended to produce irregular surface [47].

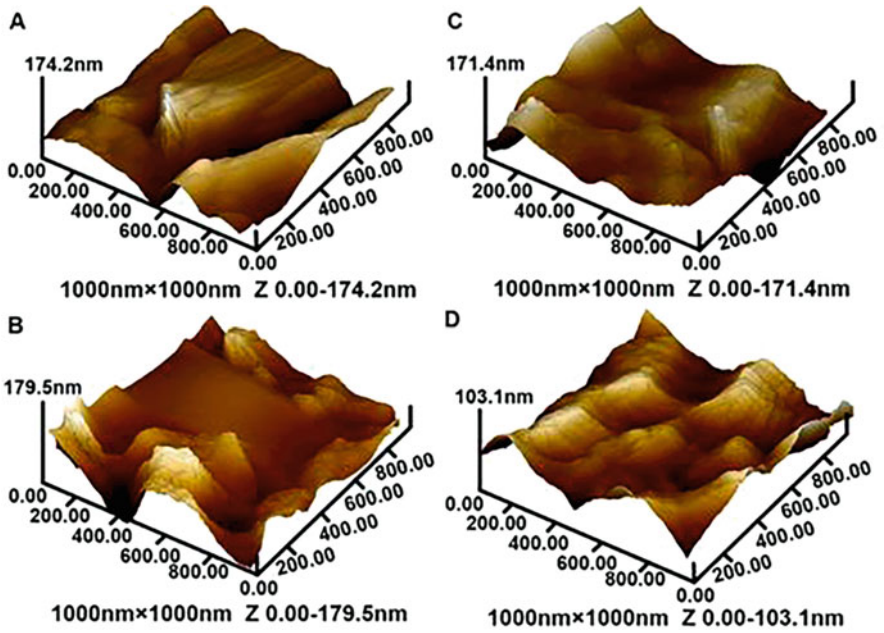


Fig. 34 AFM image of (**a**) Bare SPGE; (**b**) GO/Ppy/SPGE; (**c**) Ab1 modified GO/Ppy/SPGE by physical adsorption; (**d**) Ab1-modified GO/Ppy/SPGE by CV [47]

8.4 Scanning Electron Microscopy

Scanning electron microscopy (SEM) is a vital means to characterize the structure of biosensors. The microstructure, size, composition, and distribution of elements, SEM can even clearly observe the loading of biomaterials on the sensor surface. Due to the lack of conductivity of most biomaterials, gold spraying is often used before SEM experiment. In addition, SEM works through the interaction between high-power electron beams and the sample surface, so the electron impact may have some influence on the sample. However, the electron probe current used in SEM observation for biomaterials and biosensors is relatively small (generally about 10^{-10} to 10^{-12}a), so as the beam spot size of the electron probe and the energy of the electron probe (the acceleration voltage can be as small as 2 kV). Moreover, the bio-sample is irradiated by grating scanning instead of fixed point scanning. Therefore, SEM is an important approach to observe biomaterials on the surface of sensors, for small damage and intuitive images [78].

Wei and his coworkers developed a novel biosensor based on polydopamine (PDA)-laccase (Lac)-nickel nanoparticle loaded carbon nanofibers (NiCNFs), denoted as PDA-Lac-NiCNFs/MGCE. SEM image showed that the short rod-like NiCNFs were fully wrapped in the PDA-Lac-NiCNFs hybrid after the mixture and polymerization of Lac and PDA (Fig. 35). Apart from providing the substrate for

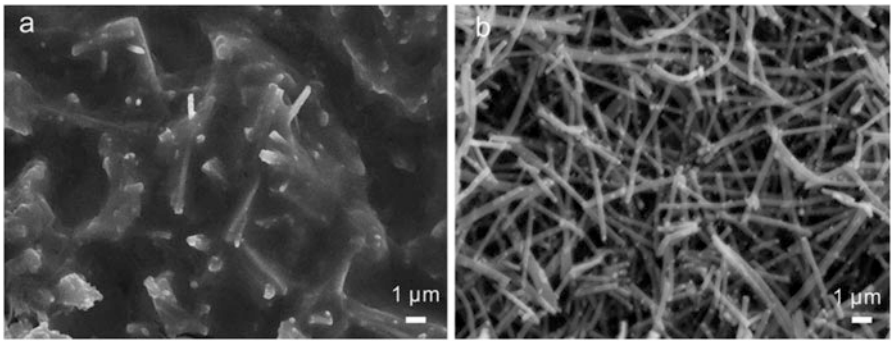


Fig. 35 SEM images of the surface of PDA-Lac-NiCNFs/MGCE (**a**) and NiCNFs (**b**) [79]

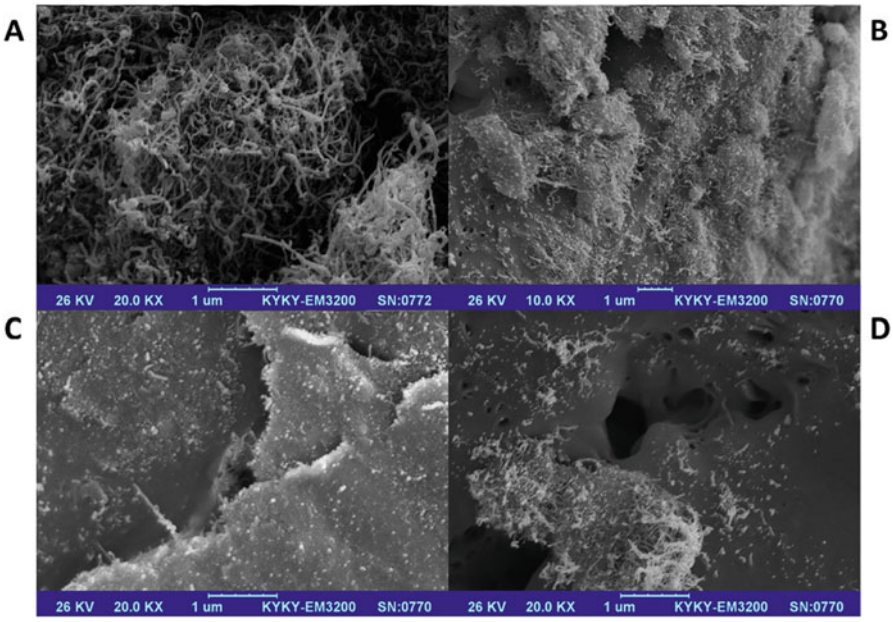


Fig. 36 SEM images of (**a**) MWCNTs/GCE, (**b**) Ch/MWCNTs/GCE, (**c**) CPRCP450/Ch/MWCNTs/GCE and (**d**) DNA/CPRCP450/Ch/MWCNTs/GCE [80]

Lac, the wrapped NiCNFs could also bridge the active sites and the MGCE to provide the conductivity [79].

Zangeneh designed an impedimetric biosensor by immobilized Cytochrome P450 reductase (CPR) and cytochrome P450 (CP450) onto the GCE modified by MWCNT and chitosan (Ch) by cross-linking method. SEM showed that the MWCNT was curling at free state (Fig. 36a), while a new layer formed after the addition of Ch (Fig. 36b). With the cross-linking of CPR and CP450 onto the composites, another new layer came into existence on the former layer (Fig. 36c).

After the detection of DNA, a darker layer consisted of DNA finally formed on the top of the sensor (Fig. 36d). SEM images of different stages clearly showed how the sensor had been constructed and its working ways [80].

8.4.1 Transmission Electron Microscope

The transmission electron microscope (TEM) is a technical method for further structural analysis and testing of materials for its high resolution, which can further study the microstructure of the biomaterial and analyze the element composition and distribution at nanometer level. However, there are still several problems in the analysis of biomaterials [81]. First, since biomaterials are mainly composed of light elements, it is difficult to obtain lattice fringe. Additionally, the biomaterials can be easily damaged by the electron beam of TEM. Usually, negative staining will be used to prevent damage to the biomaterials [82].

Koh got the TEM images of streptavidin-gold nanoparticles before and after the conjugation to biotinylated antibodies in the bright field (BF). The streptavidin appeared as light halos with the width of about 5 nm surrounding the Au particles, which indicated the successful immobilization of streptavidin (Fig. 37) [83].

Verma ML et al. also applied TEM to investigate the morphology of the composites of Au nanoparticle immobilized lipase (Fig. 38). The Au nanoparticles appeared in aggregation with the sizes of 20–50 nm, while after the coating of lipase, the nanoparticles seemed to be dispersed uniformly, and surrounded by light halos of lipase [84].

TEM images could provide evidence of the combination of biomaterials and carriers, and an estimate of the loading thickness of biomaterials and the loading modes or morphology.

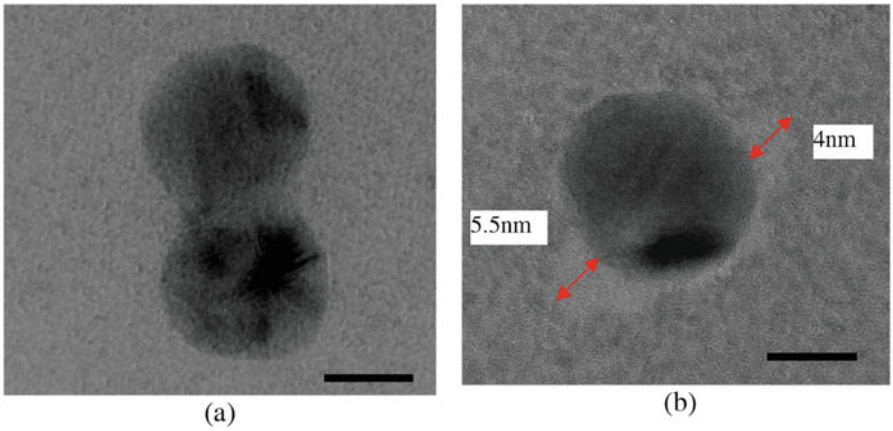


Fig. 37 TEM BF images of (**a**) 15 nm plain gold and (**b**) 15 nm gold functionalized with streptavidin [83]

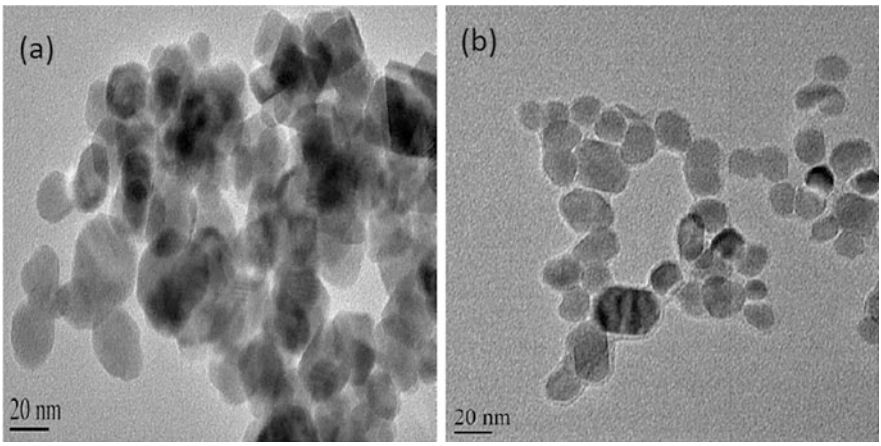


Fig. 38 TEM images of functionalized magnetic nanoparticle (**a**) and nanoparticle immobilized lipase. (**b**) Nanoparticle immobilized lipase [84]

8.5 Electrochemical Impedance Spectroscopy

Electrochemical impedance spectroscopy is one important electrochemical measurement based on the small amplitude sine wave voltage (or current) as the disturbance signal, which can provide information on film capacitor and resistor [85], to characterize biosensors during the manufacturing process.

Cui et al. [86] prepared a biosensor for the detection of organophosphorus pesticides (OPs) by sorption of acetylcholinesterase (AChE) based on chitosan (CS), titanium dioxide sol–gel, and decreased rGO. The preparation process was explored and confirmed by electrochemical technique. As shown in Fig. 39, the R_{ct} value of the rGO/GC electrode is about 43 Ω. The big specific surface acreage and interlamellar barrier of rGO films lead to the increase of charge shift resistance. With the introduction of TiO_2-CS nanocomposite CS membrane and AChE, the R_{ct} value first increased, then decreased, and finally increased sharply, it is important to note that the use of CS membrane significantly reduced the R_{ct} value, which was due to the sorption and amassing of negatively charged probe molecules on positively charged CS membrane.

M. Alagappan et al. [87] discovered an electrochemistry cholesterol biosensor that immobilized ChOx with gold nanoparticle-functionalized-multiwalled carbon nanotubes (MWCNT)-polypyrrole (PPy) nanocomposite modified electrode. PPY serves as the supporting substrate to support ChOx, and AU NPs-*f*-MWCNT presence of increased conductivity. CV showed due to the competitive effect of Triton X100, the current decreases with increasing cholesterol concentration, a surfactant used to prepare cholesterol solution. In addition, an impedance spectrometric method was also demonstrated for the determination of cholesterol. The interface properties of different electrodes were studied by EIS. Figure 40 shows Nyquist plots, the frequencies from 10^3 Hz to 1 Hz. The results showed that the R_{ct} value of AU-*f*-

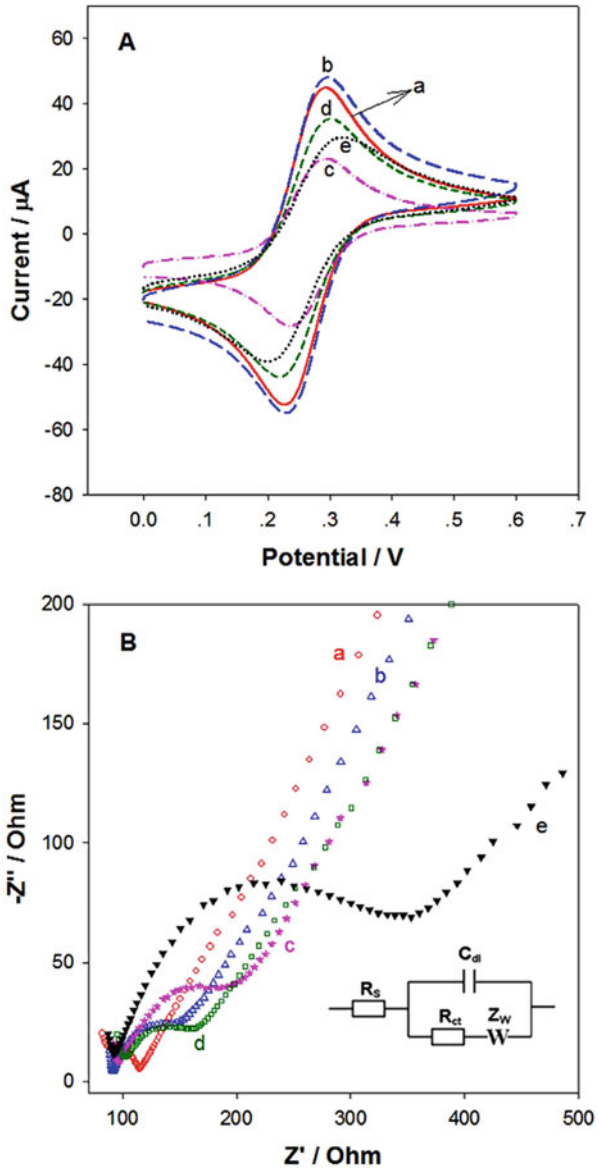


Fig. 39 (**a**) The CVs; (**b**) the EIS of (a) GC, (b) rGO/GC, (c) TiO_2^m-CS^m/rGO/GC; (d)CS@ TiO_2^m-CS^m /rGO/GC; (e) AChE/CS@ TiO_2^m-CS^m/rGO/GC electrodes [86]

MWCNT-PPy/GCE was lower, indicating that the electrical conductivity of the prepared sensor was improved.

Millner et al. [28] used Polyoctopamine (POct) as the transducer layer of the electrochemical biosensor and developed a label-free electrochemical impedance biosensor (as shown in Fig. 41) on the screen-printed gold electrode for the detection of carcinoembryonic antigen (CEA). The performance of the optimized POct-based

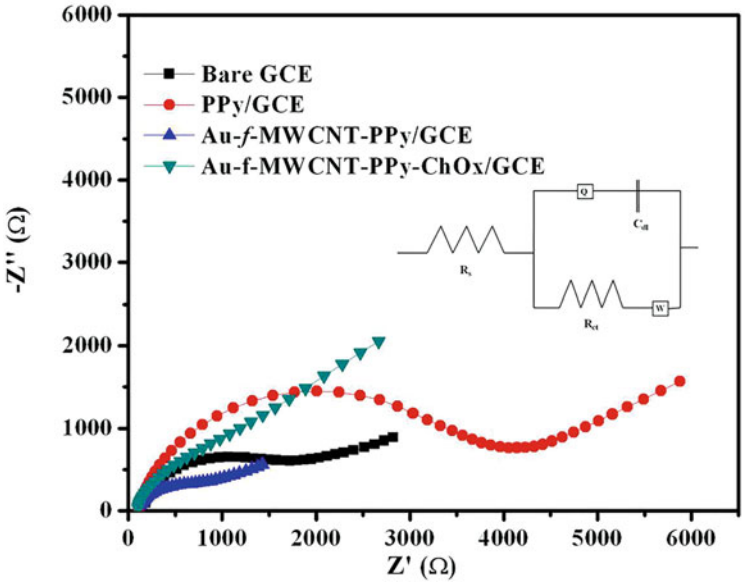


Fig. 40 Nyquist diagrams of different modified electrodes [87]

biosensor was tested in the addition of human serum. The impedance data from the figure (Fig. 41b) shows that when the scanning period exceeds two cycles, the sensor becomes highly capacitive and resistive. Compared with the bare gold electrode in Fig. 41c, no redox waveform is observed on the POct-modified electrode. These data show that the thin POct polymer film successfully passivated the electrode surface, and its resistance was low (137.5 kΩ). As a result, the electropolymerization of octopamine on the silk-screen printing gold electrode produced a low-resistance polymeric membrane, immobilized biological receptor, and transducer layer close contact greatly improving the detection sensitivity.

9 Summary

This chapter mainly introduced the common self-assembly methods and characterization methods. The self-assembly methods have been listed in Table 1, including the embedding, adsorption, chemical immobilizations, electrochemical polymerization, and spinning and deposition methods. The characterization methods have been listed in Table 2, including FT-IR and Raman, UV-vis spectroscopy, CD spectroscopy, AFM, SEM, TEM, and EIS.

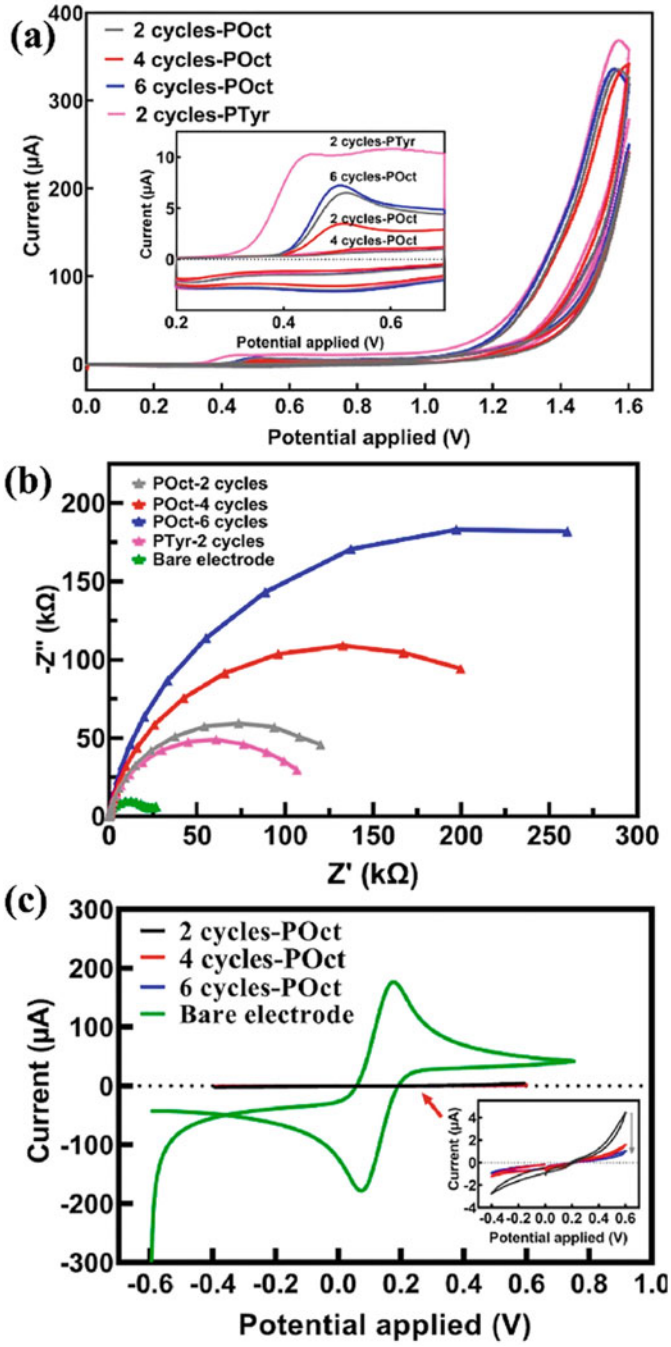


Fig. 41. (**a**) shows the variation of CV deposited at a constant scanning rate of 100 mV/s, PTyr was deposited twice using the same scanning settings as the comparison model; (**b**) Comparative impedance; (**c**) CV diagram of electrodeposited polymer [28]

Table 1 Self-assembly and fabrication of biomaterials onto transducers

Method	Immobilization technique	Biosensor configuration	Ref.
Embedding	Embedding	The glucose oxidase (GOx) together with NiPd hollow nanoparticles into ZIF-8	[8]
Adsorption	Physical adsorption on carbon-based materials	Laccase (LAC) immobilization by mixing graphite, gold nanoparticles (AuNP-CD) and β-cyclodextrin	[11]
		Disulfide (MoS_2) and graphene quantum dots (GQDs) was used for enzyme immobilization	[12]
		MWCNTs and polyfluoren-g-ploy (PF-g-PEG) for alcohol oxidase immobilization	[15]
	Adsorption by natural products	After being modified by gold nanoparticles, the zein ultrafine fibers could load laccase	[16]
	Adsorption by nanomaterials	Laccase immobilized on modified the graphite screen-printed electrode with AuNPs	[22]
		AuNPs and MoS_2 nanosheets as carriers to immobilize laccase	[23]
	Adsorption by synthetic polymer	Receptor proteins were immobilized on polymer-based optical devices	[26]
Chemical immobilizations	Covalent and non-covalent linking	Cholesterol oxidase (ChOx) immobilized on MWCNT-PANI (polyaniline) nanocomposites	[30]
		Lysine oxidase immobilized on the modified GCE which had been modified by graphene (GR) and redox polymer poly (vinylferrocene) (PVF) in advance	[35]
	Specific recognition between proteins	Layer-by-layer deposition of Con A and glycol-enzymes on the solid surface	[39]
	Click chemistry	Attach lipase to nanoparticles	[41]
		Azido-HRP were covalently fixed on the monolayer	[42]
Electrochemical polymerization	Electrochemical polymerization	ChOx enzyme immobilized on the biocomposite which is prepared by polypyrrole, green reduced graphene oxide (gRGO)	[46]
Spinning and deposition	Electrospinning	PVA-laccase-Au NPs immobilized on Pt electrode by electrospinning	[50]
	Electrostatic spray deposition	Laccase enzyme immobilized on carbon black modified screen-printed electrodes	[51]
	Plasma deposition	Polymerized laccase molecules onto the surface of a clean glass to obtain laccase coating	[55]
	Laser deposition	Laccase immobilized on the surface	[63]

Table 2 Characterization of biomaterials on transducers

Characterization methods	Characterization objects	Characterization results	Ref.
FT-IR and Raman spectroscopy	Laccase/chitosan (GHIT)-lambda-carrageenan (CAR)	FT-IR proved the immobilization of laccase onto the laccase/chitosan (GHIT)-lambda-carrageenan (CAR)-based voltammetric biosensor	[66]
	Laccase	The laccase adsorbed on Black Pearl 2000 (BP2000) modified GCE would result in structural change for the strong interaction between laccase and BP 2000.	[67]
UV-vis spectroscopy	Flavin adenine dinucleotide	UV-vis had been adopted to confirm the immobilization of FAD	[69]
CD spectroscopy	As(III)	CD had been applied to evaluate the interaction among the aptamer, CTAB, and As^{3+}	[75]
	Lipase	CD spectroscopy analyzed the secondary structural change of lipase after covalent attachments	[76]
Atomic force microscopy	Testosterone antibody	The changes in surface roughness confirmed the successful immobilization and modification of polymers pBDBT and AbTES on the electrode surface	[77]
	Brucella	AFM images intuitively reflect the influence of different fixation methods on the surface of the sensor	[47]
Scanning electron microscopy	Laccase	SEM image showed that the short rod-like NiCNFs were fully wrapped in the PDA-Lac-NiCNFs hybrid after the mixture and polymerization of Lac and PDA	[79]
	Chitosan	SEM showed that the MWCNT was curling at free state, while a new layer formed after the addition of chitosan	[80]
Transmission electron microscope	Streptavidin	Streptavidin immobilized successfully	[83]
	Lipase	Investigate the morphology of the composites of Au nanoparticle immobilized lipase	[84]
Electrochemical impedance spectroscopy	Chitosan	The introduction of CS membrane significantly reduced the R_{ct} value	[86]
	Cholesterol	The interface properties of different electrodes were studied by EIS, indicating that the electrical conductivity of the prepared sensor was improved	[87]

10 Conclusion and Prospect

This chapter mainly summarizes the self-assembly and immobilization methods of biomaterials on the transducer surface, the common use characterization methods, and the examples of the preparation and characterization of biosensors. Actually, it is clear that, whether the preparation or the characterization of biosensors cannot rely on a single technical method, but calls for the combination of a variety of technical means, to meet the improved demand for biosensors and the progress of science and technology. In the future construction of new biosensors, the combined use of multi-technologies should be explored to improve the load effect and develop a new way for further optimization of sensor performance and accurate characterization.

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Biocatalytic Sensors: Potentials, Maxims and Mechanisms for Optimal Performance

Samuel Eshorame Sanni, Emmanuel Rotimi Sadiku, Emeka Emmanuel Okoro, Babalola Aisosa Oni, and Amanda Onyinye Ndubuisi

Abstract

Biocatalytic sensors are devices which consist of bioactive functionally grafted layers of catalysts or analytical pieces which are in contact with transducers that help to convert biological signals into electrical pulses. They are essentially distinct materials whose design, application, immobilization, and transducing capacities induce/infuse distinct properties that offer several advantages in science, engineering, and medicine. The essentiality of biocatalytic sensors cannot be overemphasized; however, for successful application, it is necessary to understand their origins, nature, mechanism of operation, as well as their behavioral activities in different media within favorable conditions. Hence, three categories of biosensors, whose mechanisms of operation would be discussed include the biocatalytic, bioaffinity, and microbial groups. In addition, the synthesis and mechanisms of immune, DNA, thermal, and piezoelectric biosensors, will be discussed in relation to their indispensable functionalities in multitudinous facets, such as the food industry, where quality checks are conducted to detect poisonous substances and glucose levels, in metabolic engineering, where in vivo

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assessments and monitoring of cell responses to metabolism are carried out and in medicine, where drugs, heart diseases, and the human papilloma virus can be X-rayed; biosensors also find application in defense/military technology and marine science, just to mention a few. In today's world, a myriad of biosensors, assume the form of membrane-bound microorganisms/enzymes, antibodies, receptors, or multilayered (matrix-enzyme) nanocomposites, all geared towards the maximization of the synergistic effect which these combinations offer in order to advance humanity. With the advent of newly discovered hyperthermophiles, it would be an interesting thing to consider their usage in biosensing especially at temperatures that can sometimes be twice above 50 °C, which may be unfavorable for most enzymes. However, the potentials of these biosensors are yet to be exploited maximally owing to the dearth in the understanding of the basic principles underlying the conditions within which they work best. To effectively optimize the potentials/performances of biosensors, a good understanding of the nature/characteristics of such systems, the principle on which they operate alongside the system's pH, temperature, and type of medium, which either favor or mare their activities are required. Hence, this chapter's discourse will essentially focus on the mechanisms and modes of operation of existing biosensors as well as recent/futuristic applications of potential bioactive materials, anchored on graphene and other potential substrates.

Keywords

Biofabrication · Biorecognition · Biocatalytic sensors · Electrochemical sensors · Optical sensors · Piezoelectric/magnetic biosensors · Mechanical biosensors · Thermal biosensors

1 Introduction

Biosensors are coordinated systems which provide quantitative/semi-quantitative data responses by means of a spatially arranged biological receptor/identifier/recognition element in connection with a transducer [1–3]. They are compact analytical systems/devices having biological detectors in sync with a physiochemical transducer which converts the bio-interactive operation into measurable responses/signals that can be picked up by a signal processing system/processor for processing the signals from machine format into readable data [4]. Biosensing or bioelectronics is an operation that combines biochemical engineering with computer science/engineering, all centered on integrating/combining the properties of biological systems with electrical/automated systems. The advent of digital biosensing using biological catalysts is made possible due to the outstanding potentials that are inherent in the advantages offered by combining the synergistic effect of enzyme sensitivity with smart-responsive electrodes. Advances in research have opened up greater opportunities that underscore the application of biological systems/enzymes as replacements for their chemical counterparts. Evidence also has it that these enzymes or biological catalysts can be cascaded to improve their transmissivities (i.e.,

biochemical signal reception from biological systems) when integrated into transducers that help to process bio-responses into electrical signals. These digitalized bio-signals emanating from enzymatic mediators are logically processed via Boolean logic networks characterized by biomolecular reactions before being converted to electrical pulses (i.e., Yes/No signals) [5]. Another major potential exhibited by biosensors is bioaffinity. Bioaffinity sensors adopt hormone receptors as means of detecting hormones or antigens [6, 7]. Progressive advances in enzymology, molecular biology, and metabolic engineering have resulted in the emergence of hybrid biomaterials and nanoparticle assemblies which assume the form of enzyme-isolates or whole cells with outstanding properties/advantages including stereo-/region-selectivity, adaptability to mild conditions, and biodegradability over chemical catalysts. The application of nanoparticles in bioelectronics for the detection of molecular/biomolecular analytes has helped in the development of synthetic functionalized nanoparticles fused with biomaterials and polymers which in turn serve as effective tags for amplifying bioaffinity assays. The constituent properties of analytes which constitute enzymes, antibodies, proteins, nucleic acids, and glycans ought to be given utmost consideration in the fabrication-stages of biosensors [8–11]. The literature has it that biosensors have been employed in disease-diagnosis, environment-monitoring, drug-screening/delivery/discovery as well as therapeutics, [12–16]. A highly effective biosensor requires the synergistic effect of apt biomarkers for effective disease-diagnosis [17–19]. Reports from the literature have confirmed the high efficacy of pathogenic disease diagnosis of some biosensors [20–26], which include electrochemical biosensors [27], acoustic biosensors [28], plasmon resonance biosensors [16], and the waveguide biosensors [15]. Modern developments in these areas birthed highly sensitive nanostructures/nano-mediated enhancers including nanoflowers [26], nanowires [29], nanocapsules [30], and nanoparticles [31] onto the surfaces of these sensors for high performance. According to some studies, it is pertinent to be able to garner quick information about analytes which will aid their easy/simultaneous quantification, reporting, and detection for environmental, diagnostic, and clinical applications [32, 33]. Biosensors have also recorded high-throughput protein patterning for bacterial and toxin detection [34]), as well as measurement of protein interactions [35]. In the following sections, the types (varieties), potentials (viability), maxims (working principles), and mechanisms (functionalities) of biocatalytic sensors are discussed.

2 Biofabrication Techniques for Detecting Cell Behaviors in Analytes

In bioprinting, rather than flow-based delivery of molecules, systematic patterning makes for the direct delivery of cells/molecules or analytes at intended locations which in turn improve and favor increased molecular binding of molecules to substrates over random-molecular interactions with resultant deposition of proteins, antibodies, and molecules on target substrates while measuring the degree of molecule/cell-substrate binding [36–38]. Biosensor fabrication techniques include:

2.1 Contact-Based Fabrication Techniques

Efficient patterning is usually enhanced by soft lithography-based techniques aided by stamped elastomers characterized by relief-features made from silicon wafer master-chips [39, 40]. Although chemical reactions occurring between patterned polymers and the substrate tend to limit the types of polymers and substrates used, a photomask is usually employed in stimulating selective photoresistant polymerization of self-assembled monolayers onto a silicon surface (this together makes up the master-chip) which is used in the fabrication of polydimethylsiloxane (PDMS) into stamped elastomers/molds. Fabrication techniques, such as lithographic-based techniques, are contact-based thus they depend on high stress/pressure application on the stamped elastomers in order to transfer signals from a donor substrate to the receptor.

2.2 Non-Contact-Based Fabrication Techniques

Another method by which patterning can be achieved is the non-contact deposition technology (as evidenced in inkjet printing) as well as direct laser writing (DLW). Non-contact fabrication approaches allow for direct patterning of materials/cells, in which the binding chemistry of cells to the substrate is not crucial [41].

Non-contact deposition technique offers some merits over contact-based patterning which is characterized by the prevalence of a chemical reaction or physical comingling of reactant-molecules and cell development is usually confined to a domain. However, in order to encourage routine cell functions, unrestricted growing of cells is required to stimulate high biosensitivity. With uncharacterized or unrecognized cell functions, production of cells from analytes may become altered, thus transmitting error-signals from the biosensor. In order to abate substrate modification and its influence on cell capacities, non-contact-based bioprinting such as DLW and inkjet printing techniques are more suitable for use relative to contact-based methods as they are capable of delivering higher throughputs, although both deposition-techniques are distinct in performance with peculiar trade-offs between the expected throughput and biosensor precision, i.e., LDW gives higher accuracy and precision, while inkjet printing gives higher throughput.

Other fabrication techniques for detecting changes in cell behavior include Electric Cell-substrate Impedance Sensing (ECIS) which houses a transducer which measures the impedance on a membrane surface relative to that which results from changes in cell behavior as influenced by the presence of a toxin [42–46]. Miniaturized/scalable ECIS can be fabricated into biochips [34]. Surface Plasmon Resonance (SPR) signals help in transducing/transmitting cellular responses to a reference-stimulus [47]. The ECIS and SPR, help in the identification of single-analytes; however, for hybrid sensors or the biorecognition of several target molecules, some sophisticated fabrication modules which combine the aforementioned technologies may then become a necessity.

2.3 Cell Encapsulation

In order to achieve optimum performance in biosensors, their biological elements need to be immobilized on active transducers. However, the immobilization of living cells is difficult because these cells may traverse/drift away from the transducer which is usually anchored on a homogenized substrate; this situation is similar to the localization/confinement of cells into a specific region while these cells proliferate and experience a 2D motion [51]. This then brings to mind the subject of cell encapsulation (a technology that thrives on microbeads, microscale spheroids, and microcapsules-use in biosensors). In comparison to the configuration of a 3D matrix, 2D systems have a spherical outer shell-constrained geometry which envelopes the environment's area within which the encapsulated cells can migrate; hence, the geometries can be sized while also accounting for the total number of encapsulated parcels/cells. Conventional micro-bead/capsule fabrication techniques cannot adequately confine the fabricated beads/structures in a specific location which is necessary for the transduction of multiple signals. To abate this challenge, one-step fabrication/patterning of the encapsulated microbeads is required [48].

The 3D matrix fabrication technique in Fig. 1a–g uses large constructs emanating as molded masses from an injection mold [53]. In this technique, a cross-linkable hydrogel suspension/hydrogel blend is transferred to a mold for subsequent cross-linking of the suspension [54]. This method aids the easy production of large/small cell capsules within a geometric control volume whose features are defined by the quality of the resulting sample mold. Modern imaging technologies also employ Magnetic Resonance Imaging (MRI) and Micro Capsulation Technologies (micro-CT) in the fabrication of highly detailed molds [53]. Injection molding is highly compatible with cross-linking polymer solutions/hydrogels such as collagen, hyaluronic acids, gelatin, alginates, and agarose. [55, 56]. Although injection molding is an inexpensive strategy for synthesizing 3D tissue cells of uniform geometry, issues related to nonspatial monitoring of cells and their compositions in the crosslinked suspension and the creation of the tissue cell's internal architecture are predominant. However, according to Chang et al. [57], one good fabrication technique for controlling this problem is the adoption of a layer-by-layer fabrication (patterning) technique for the 3D cell architectures. Inkjet and DLW users use the combined technique of multiple layer fabrication while continuously extruding the hydrogel strands onto the cross-linker.

3 Types of Biocatalytic Sensors

Biochemical reactions in biosensors are usually stimulated by confining enzymes to nano-scaled compartments for improved enzymatic activity and substrate channeling considering the enzyme's proximity [58, 59]. Protein nanoparticles (PNPs) provide a unique platform for achieving these nanoscale assemblies, such that the enzymes employed are used at high-surface concentrations or they are packaged within PNPs via encapsulation which helps in improving enzyme stability while defending these

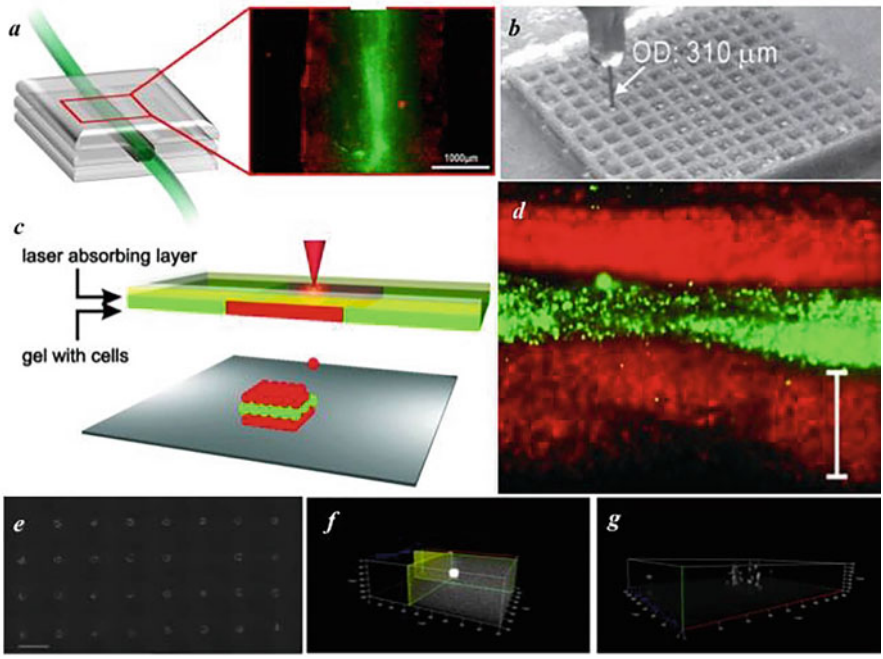

Fig. 1 3D patterned structures: (**a**) inkjet vascular graft print out [49], (**b**) printed continuous flow structural architectures [50], (**c**) laser-induced cell arrays of hydrogels patterns [52] (**d**) skin graft fabrication from random cell layers [52], (**e**) laser-induced cell patterns of microbeads [48], (**f**) 3D z-stack image of rhodamine-microbead [48], and (**g**) z-stack-3D distribution of cells in a sample microbead [48] with permissions obtained from Elsevier; John Wiley and Sons and IOP Publishing. Adopted from Dias et al. [41]

enzymes against proteases/thermal denaturation [58]. Clark and Lyons produced the first biosensor in the year 1982. They plunged a glucose active enzyme (glucose oxidase—GOx or GOD) on amphoteric oxygen electrodes for direct measurements of its glucose concentration; their effort is a good starting point for making intelligent electrochemical sensors including pH, polarographic, potentiometric, and conductive meters that are integrated into enzyme transducers having the same semblance with membrane enclosed sandwiches [60].

3.1 Electrochemical Biosensor

Electrochemical biosensors are actively enforced by biocatalytic reactions occurring in a medium between immobilized biomolecules and target analytes which are receptors or donors of ions/electrons that influence the electrical potential and current of the overall medium/electrolyte [61]. Electrochemical biosensors comprise of three electrodes (i.e., the reference, working, and counter electrodes). The

biomolecular identifier with a physiochemical transduction system makes up the working electrode (transduction system/redox/sensing electrode). A typical reference electrode comprises of Ag/AgCl; it must be kept a few distances off the area where the reaction is incipient in order to attain a poised electrode potential. The counter/auxiliary electrode helps to transmit electrical currents/pulses via the electrolyte to the working electrode. The commonly used electrodes are made of carbon, platinum, gold, and silicon owing to their high conductivities and chemical stabilities [62]. Transducer types adopted in electrochemical biosensors include conductometric, potentiometric, and amperometric transducers which transform impulses from bioreceptors into measurable signals [63].

3.1.1 Maxim and Mechanism of Operation

As discussed by Rathee et al. [6], the following reaction schemes explain the process in a sample electrochemical biosensor (Fig. 2a).

$$\text{L-Lactate} + \text{oxygen} \xrightarrow{L-\text{Lactate oxidase}} \text{pytruvate} + H_2O_2 \quad (1)$$

$$H_2O_2 \xrightarrow{\text{enzyme}} O_2 + 2H^+ + 2e^- \quad (2)$$

$$\text{Substrate} + O_2 \xrightarrow[Catalysis]{enzyme} \text{product} + H_2O/H_2O_2 \quad (3)$$

$$\text{Substrate} + NAD^+ \xrightarrow{\text{enzyme}} \text{product} + \text{NADH} + H^+ \quad (4)$$

The electrochemical biosensor shown in Fig. 2a is characterized by an interdigitated electrode (IDE) which comprises of terminal nanoscale-arrays. It consists of a silver coating (reference electrode) on the surface of platinum (working electrode) onto which the polymer (polypyrole) is attached. It has a better sensitivity relative to impedimetric biosensors. A surface area modification of 200 nm^2 is possible when a part of the biomolecule is bound to probes of the electrochemical biosensor, which is the reason for its enhanced responsiveness relative to conventional electrodes with a size range from micrometer to millimeter. In addition, this type of biosensor only requires a small portion of disease sample from affected patients for analysis on the biosensor chip; these chips attract lower costs in larger sizes. According to Lakshmipriya and Gopinath [3], some chronic pathogenic diseases and infections are usually detected via DNA sequence binding of the pathogens to the appropriate IDE probes (i.e., binding an antigen to an antibody). Lactase detection which uses the enzyme (lactase oxidase) makes use of oxygen for reaction initiation as a way of enhancing the biorecognition of lactates that may be mixed in analyte tissues, nucleic acid, antibodies, lectins, organelles, antibodies, and enzymes as shown in Fig. 2b. When a sample/analyte of a specific disease is bound to an IDE probe, the direct impulse emanating from this contact is picked up by the analyser of the dielectric impedance system. Thereafter, fluctuations in impedance may be influenced by the changes in electrical pulses arising from the levels of attachment of the antigen to the antibody. The ratio of length to the surface area of an IDE (i.e., the area of inter electrode gaps) as well as the metals used in manufacturing

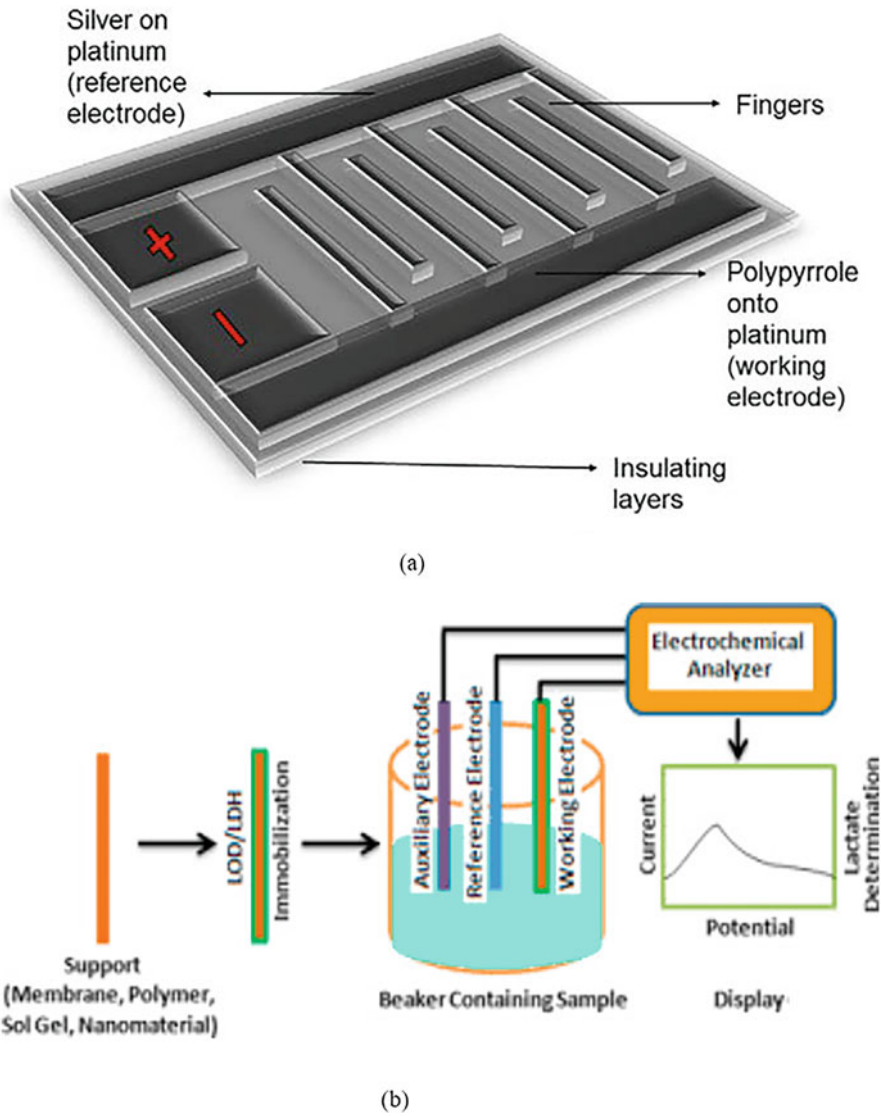

Fig. 2 (**a**) Electrochemical biosensor with nanogaps and interdigitated electrodes [3], (**b**) Lactate detection using an electrochemical sensor [6]

the IDEs can be maximized to obtain high performance. For instance, biologically compatible components such as silicon/silica and glass exist as a lone/single molecule thus very tiny components can be manipulated on a microscale by taking advantage of the flow of electric charges on the terminals/electrodes of the biosensors. Reports also have it that the shape and dispersion of nucleic acids within

the inter electrode gaps of an IDE can be adjusted due to their bipolar nature. A very significant application of Electrochemical Impedance Spectroscopy (EIS) is a technique that measures dielectric properties such as protein–molecule interactions or protein–protein interactions with antigen-antibody strength of attachment/analyte specificity. At low to moderate frequencies, these interactions generate a single layer charge (capacitance) which varies with the capacitance of the dual layer whose impedance varies inversely with its capacitance, and hence a nanotype interlocked electrode is a highly sensitive immunosensor manufactured on a miniature scale. Despite the advantages of this sensor type, its entrenched xanthine oxidase enzyme acts as a catalyst that stimulates the production of xanthine and hypoxanthine, which when produced in excess, can cause renal failure. In some applications, graphene-based carbon nanotubes are integrated into electrochemical biosensors for high analyte sensitivity and recognition as a result of their inherent superior physico-electrical characteristics including excellent carrier mobility/specific surface area, ambipolar field effect, flexural ability, and wide adaptability to microfabrication methods which makes them easily integrated into transistors/chemiresistor configurations commonly found in portable/field-deployable biosensors [64]. The four types of electrochemical biosensors include amperometric, conductometric, potentiometric, and impedimetric biosensors.

For biosensors incorporated with the lactate enzyme, membranes of varying porosities are employed as supports for the enzymes during fabrication, such that they are in close proximity to electrode surfaces. This is done by immobilizing an enzyme on a natural/artificial membrane that is compatible with the enzyme after which the membrane enzyme system is attached to a transducer for improving the biosensor's selectivity and sensitivity. This in turn provides a cheap, portable, and rapid approach for determining lactate concentration as well as maintaining the stability of the enzyme for improved service life of the biosensor. This way, certain drawbacks, such as direct transducer enzyme immobilization, are avoided which further prevents enzyme loss and allows for mass production and timely response of enzymes to stimulus or analytes thus enhancing the reproducibility of biosensor signals. Supports for enzyme immobilization for this kind of biosensor include membranes made from amucin, albumin, hydrogel, and Nafion [65], porous hydrophilic or mesoporous silica or screen-printed Prussian Blue (PB) membranes [66], membranes coated with polydivinylbenzene (PDVB), ethylvinylbenzene (EVB) [67], cellulose acetate(CA) membranes [68], carbon nanotubes/polysulfon membranes [69], polypyrrole [70], polycarbamoyl sulfonate (PCS) hydrogel on Teflon membrane/Pt-support [71]. These membranes make for sensor flexibility and mechanical durability over wide pH ranges which enhances the sensor's selectivity and rejection of interfering substances towards improved response signal amplification.

3.1.2 Amperometric Biosensors

These types are the most abundant types of biosensors which help to establish the current flow at a fixed voltage [72]. Their response rates or time measurements and dynamics have some semblance with those of potentiometric biosensors; however,

they have a higher sensitivity and are more commercially viable than their potentiometric counterparts.

3.1.2.1 Maxim and Mechanism of Operation

This type of sensor takes advantage of the current produced when a redox reaction occurs at its electrodes. The produced current responses are proportional to the specific solute concentration in a solution/sample analyte. The current flow results from the potential difference set up between its working and reference electrodes which bring about the occurrence of electrocatalytic redox reactions of the active components. The magnitude of the current generated has a strong correlation with the active concentrations of the redox active agents or products of the enzymatic reaction. Generally, enzymatic amperometric biosensors are used in the identification of L-lactate in blood or tissues, due to their simplicity in design and performance [6]. The immobilized enzyme in these biosensors must possess the ability to catalyze or stimulate the reaction of the analyte via the uptake of an electroactive species which is in consonance with the generation of an electroactive product. Typical enzymes for this kind of sensor include hydrophobic oxidases and reduced β-nicotinamide adenine dinucleotide such as dehydrogenases, which help in the catalytic decomposition/disintegration of substrates as shown in reaction schemes 6 and 7 [73].

$$\text{Lactate} + \text{NADH} \xrightarrow{\text{LDH}} \text{pyruvate} + \text{NADH} + \text{H}^+ \tag{5}$$

$$\text{NADH} \xrightarrow{\text{E}} \text{NAD}^+ + \text{H}^+ + 2\text{e}^- \tag{6}$$

The maxim of this sensor type is informed by its name "Amperometric," which is indicative of current flow. Examples of this type of biosensor include single potential amperometry and direct current amperometry. For the aforementioned sensors, a difference in potential is set up at the terminals of the sensor cell in order to induce current flow through the analyte. The working electrode in this type of biosensor consists of a noble metal which is encapsulated by a bio-indicator/biorecognition component, usually an enzyme, which helps to catalytically convert electroactive species in the analyte that is in direct contact with the enzyme layer supported by the electrode; thereafter, the current produced is then measured/estimated [74] as a result of its response to the analyte concentration at a nonvarying potential.

Figure 3a, b shows an amperometric biosensor whose function is similar to that of a Universal Sensory Board (USB) that can transmit data at high speed by drawing power from the battery of a smartphone. The resulting digital signals are then processed and uploaded onto a smartphone for processing in the smartphone app so as to show the resulting assay data for easy access by the user. Enzymes are bioreceptors/proteins/biological catalysts which help to identify and quantify the concentrations of substrates/biomolecules (alcohol, cholesterol, uric/lactic acid, glucose, etc.) undergoing biochemical reactions that are feasible within the human body temperature. One potential enzyme used as a bioreceptor in amperometric biosensors is the oxidoreductase enzyme that sticks/attaches itself onto a target

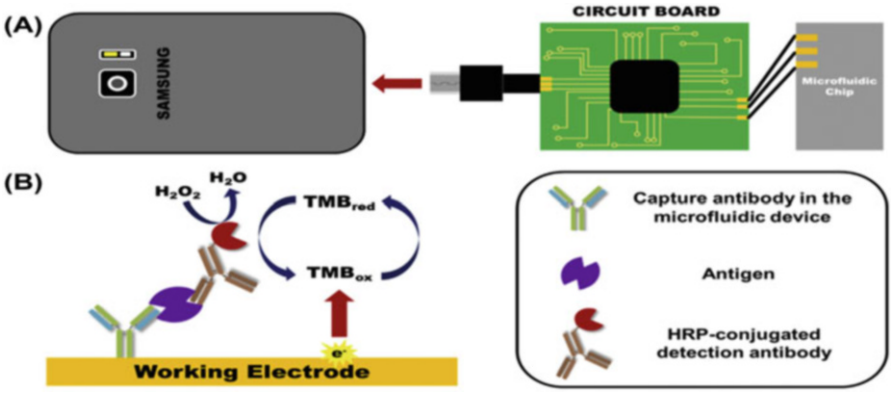

Fig. 3 (**a**) Microfluidic chip of an amperometric electrochemical immunoassay for the transmission of digitalized data from a micro-USB port to a smartphone, (**b**) working electrode. Adapted from Yoon [75]

substrate which gets oxidized while the enzyme in turn becomes reduced/inactive. This redox reaction can be made to undergo repetitions to complete a cycle by introducing an electronic mediator/transducer. Glucose as a target substrate can be detected by a biosensor with glucose oxidase (GOx) and ferricyanide ion $Fe(CN_6)_3$ as the biocatalyst and electrical pulse mediator, respectively. At first, the glucose oxidase speeds up the oxidation of glucose by taking advantage of the dissolved oxygen in the blood. The reaction is shown in Eq. 7.

$$\text{Glucose} + O_2 + \text{GOx} \rightarrow \text{Oxidized gluconic acid} + \text{Reduced GOx} + H_2O_2 \quad (7)$$

In the reverse direction, the ferricyanide converts the reduced/inactive GOx to its oxidized form, with subsequent reduction of the ferricyanide to ferrocyanide as represented in Eq. 8:

$$\text{Reduced GOx (reduced)} + Fe(CN_6)^{3-}\text{ (oxidized)} \rightarrow \text{Oxidized GOx} + Fe(CN_6)^{4-} \quad (8)$$

3.1.3 Conductometric Biosensor

This type of sensor takes advantage of a transducer, which measures the electrical conductivity of a solution as influenced by several batches of biochemical reactions [76]. As a result of the difference in the rate of flow of electrons and the rate of generation of ionic species, as well as the resulting resistivity of the solution/antigen in which the analyte is present, there is a change in the overall conductivity.

3.1.3.1 Maxim and Mechanism of Operation

During the biochemical reaction, there is an interaction between biomolecules and the analyte which subsequently alters the conductivity of the solution [61]. According to Ohm's law, when a voltage spans across the terminals/electrodes of metal conductors, it leads to a flow of current between the terminals. With the help of an ohmmeter/conductivity meter, the variation in conductance can then be measured during the biorecognition/biochemical reaction which is often stimulated by an enzyme (biorecognition element) which acts as a catalyst. This type of sensor is not readily used like other types of sensors [76].

3.1.4 Potentiometric Biosensor

This type of sensor measures the difference in electrode potential across a working and reference electrodes mediated by a semipermeable membrane when there is no flow of electric current [61, 76].

3.1.4.1 Maxim and Mechanism of Operation

Despite the zero flow of current, the measurement of the potential difference is caused by the changes in solution pH or proportional index pI or chemical potential [63] during the biochemical reaction/biorecognition step as initiated/stimulated by the enzyme/microbial–analyte interaction. According to Pohanka and Republic [77], due to ionic flow and analyte-adherence to the surface of the selectively permeable membrane, there is an output signal that responds to these changes which eventually results in an alteration in the initial solution concentration and thus gives a variation in the solution properties as well as pH. For variations in pH with analyte properties, the Nernst formula (Eq. 9) is apt for estimating the overall potential difference of the resulting solution [72].

$$E = E_0 + RT = bF \ \ln C \tag{9}$$

Where E, E_0, R, T, b, F, and C are the potential difference (Volts), standard cell potential, universal gas constant (J/mol.K), standard temperature in Kelvin, valence electron/ion, Faraday's constant (Coloumb/mol), C is the ratio of C_o to C_i as well as the individual external and internal ionic concentrations of the resulting species, respectively.

Useful hints/general working principles of electrochemical biosensors:

Considering the illustration in Fig. 4a, if the voltage across its electrodes is sourced from a DC source with a difference of +3 V, there would be a flow of electrons along the path defined by the net charge. Supposing the voltage at the anode (i.e., ground/reference electrode) and cathode are 0 and −3 V, respectively this then gives a resulting difference in electrode potential of +3 V. Figure 4b shows all the parametric profile measurements obtainable for all four types of electrochemical biosensors.

As soon as current begins to flow to/through the two resistors, where one serves as a normal resistance wire while the other is a biosensor, the voltage at the cathode must begin to drop and may eventually fall back to zero (same voltage at the anode)

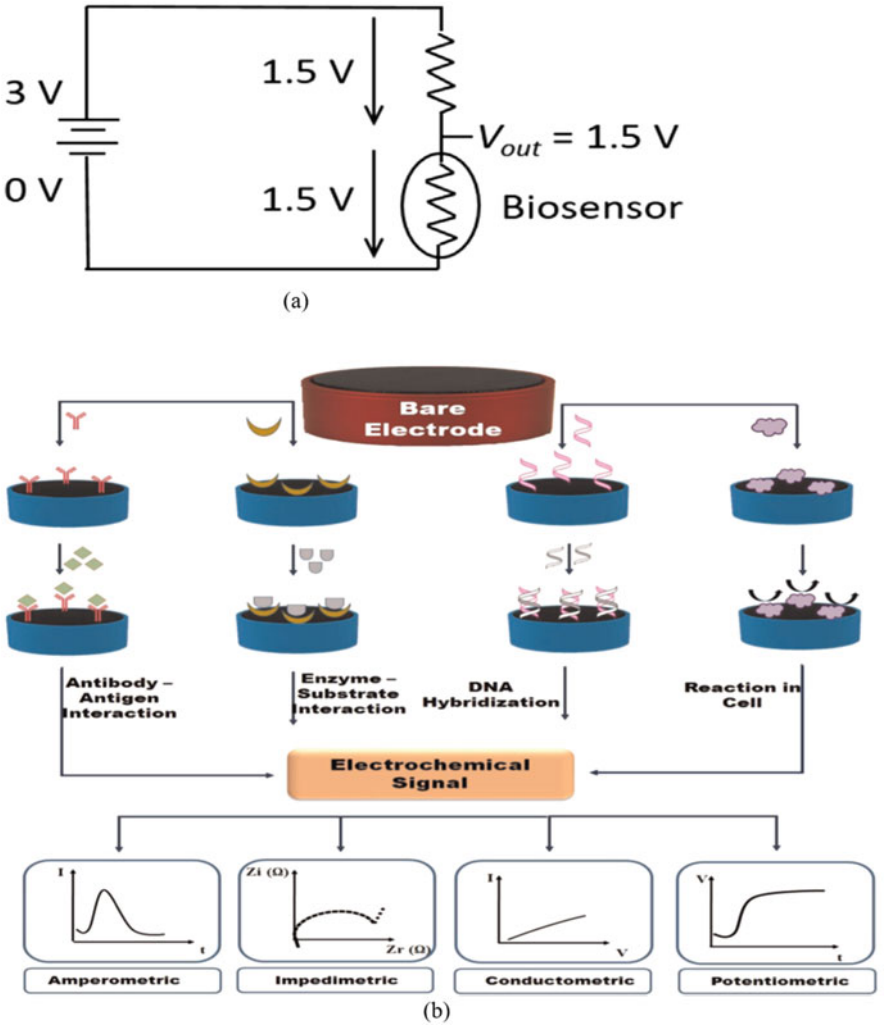

Fig. 4 (**a**) Simple circuit diagram consisting of an electrochemical sensor. Adopted from Yoon [75], (**b**) An electrochemical sensor with schematic profiles of parametric measurements for all sensor types. Adopted from Bahadır and Sezgintürk [78]

in order to nullify the constraint. For two resistors of equivalent resistance, each will have a comparative resistance of magnitude 1.5 V, and thus the drop in voltage across the terminals of each resistor equals half of 3 V (i.e., 1.5 V); this equals the measure of voltage reduction across each resistor. In the same regard, the output voltage at a point midway between the two resistors will be 1.5 V. Since one resistor is fixed and the other is the variable type resistor/biosensor which varies with the biological variable/molecule being tested, the output voltage at the point midway between the resistors (V_{out}) will then be altered. If the value of this output voltage is

detected and read off as a biosensor readout, the biosensor then functions as a potentiometric biosensor. However, when the electrochemical biosensor resistance offered by the resistor is constant despite varying current flow, such that the variation in current is detected and measured, such a sensor is the amperometric type. In principle, if the measured constraint examines the change in output resistance rather than current or voltage, this kind of electrochemical sensor is the conductometric type where the inverse of resistance is taken to account for the imposed/offset conductance [75].

3.1.5 Impedimetric/Impedance Biosensor

This type of electrochemical biosensor measures the variation in electrical impedance induced by the cell analyte which is contained within a microfluid. The overall resistance offered to the flow of transduction signals emanating from the transducer is then computed as a measure of the detection/deflection which is seen on the sensor. This type of biosensor is a functional part of the Wheatstone bridge. It consists of two electrodes with an alternating voltage of about 100 mV applied between its terminals. Impedimetric biosensors are used in the assessment of urea assays with urease as the biorecognizing component. The equation for the reaction is given in Eq. 10. Details of some electrochemical biosensors are shown in Table 1.

$$(NH_2)_2CO + 3\,H_2O \rightarrow 2NH_4 + HCO_3^- + OH^- \tag{10}$$

3.1.6 Chemiresistive, Capacitance-Based Sensors

Chemiresistive systems which exhibit changes in electrical resistance as responses to chemical stimulus have gained popular interest in biosensor application owing to their cost effectiveness, portable nature, allowance for monitoring real-time processes alongside the direct interference with electronically connected devices such as smartphones [103]. Metal-based HCHO chemiresistive sensors are available in the literature [18] although their limitation still remains that they are only suitable for temperatures above 200 °C, and hence they are energy intensive and undergo cases of low HCHO selectivity relative to its volatile counterparts. Some promising single/multi-walled functionalized carbon nanotube (S/MWCNT)-HCHO chemiresistive sensors have also been reported to have good sensitivities and selectivity at humid and ambient conditions [104–110]. Tin oxide-doped MWCNTs have shown high HCHO detection capabilities in 0.03 ppm concentrations of HCHO at 250 °C [111]. An investigation to sense the presence of HCHO in air is also available in the literature [112].

3.2 Optical Biosensor

By design, these kind of biosensors are developed for measuring the amount of dissolved oxygen, CO_2, and the degree of acidity or alkalinity of the target analyte [113]. Lightwave transmission by this device simulates the transmission of electrons

Table 1 Tips on electrochemical sensors' supports, their techniques, analytes, and fluid types

Support	Biofluid	Recognizing element/ molecule	Technique	Target/analyte	Ref.
Denture	Saliva	Glass membrane	Potentiometry	pH	Graf and Mühlemann [79]
Cobalt chrome denture	Saliva	Lanthanum fluoride	Potentiometry	Fluoride	Graf and Mühlemann [80]
Mouth guard	Saliva	Lactate oxidase	Amperometry	Lactate	Kim et al. [81]
Graphene supported on silk	Saliva	Peptides	Resistometry	Staphylococcus aureus	Mannoor et al. [82]
Polytetrafluoroethylene	Tears	Au	Conductometry	Electrolytes	Ogasawara et al. [83]
Gas perm-selective membrane	Tears	Pt	Amperometry	O_2	Iguchi et al. [84]
Polypropylene	Tears	Glucose oxidase	Amperometry	Glucose	Iguchi et al. [85]
Polyimide	Tears	Glucose oxidase	Amperometry	Norepinephrine and glucose	Kagie et al. [86]
PET contact lens	Tears	Glucose oxidase	Amperometry	Glucose	Yao et al. [87]; Liao et al. [88]; Yao et al. [89]
Polyethylene terephthalate contact lens	Tears	Lactate oxidase	Amperometry	Lactate	Thomas et al. [90]
Cotton	Sweat	Carbon	Amperometry	β-NAD and H_2O_2	Yang et al. [91]
Polyimide/Lycra blend	Sweat	Na-ionophore	Potentiometry	Na	Schazmann et al. [92]
Cotton yarn	Sweat	H_2, NH_3, and K-ionophore	Potentiometry	pH, NH_4, and K	Guinovart et al. [93]
Polyester	Sweat	Ag/AgCl	Potentiometry	Cl^-	Gonzalo-Ruiz et al. [94]
Gas permeable membrane	Sweat	Pt	Amperometry	O_2	Mitsubayashi et al. [95]
Elastomeric stamps	Sweat	Carbon	Voltammetry	Uric acid	Windmiller et al. [96]
Temporal tattoo	Sweat	Lactate oxidase	Amperometry	Lactate	Jia et al. [97]

(continued)

Table 1 (continued)

Support	Biofluid	Recognizing element/ molecule	Technique	Target/analyte	Ref.
Temporal tattoo	Sweat	Polyaniline	Potentiometry	pH	Bandodkar et al. [98]
Temporal tattoo	Sweat	NH_4-ionophore	Potentiometry	NH_4	Guinovart et al. [99]
Temporal tattoo	Sweat	Na-ionophore	Potentiometry	Na	Bandodkar et al. [100]
Parylene skin patch	Sweat	Lactate oxidase	Conductometry	Lactate	Khodagholy et al. [101]

Adapted from Bandodkar and Wang [102]

through the UV-Vis Spectrophotometer, surface plasmon resonance, X-ray fluorescence, laser dispersion/scattering, chemiluminescence/bioluminescence, and reflection spectroscopy, and it works by the same principle [76, 114]. Based on the work of O'Toole and Diamond [115], this type of sensor works basically by the principle of propagation of light where colorimetric measurements, as well as light intensities, are weighed as they traverse through reactants or products in a reactor/system where the resultant luminescence and fluorescence are produced by a high-voltage photomultiplier or a low-voltage photodiode hybrid system.

3.2.1 Types of Optical Biosensors

Optical fiber-based biosensors operate on the basic principle of propagation and total internal reflection of light in which rays of light are wholly propagated/directed through a medium and the fiber-core of the optical fiber. The fiber-core is characterized by a refractive index ($n1$) with an outer covering/layer whose refractivity is indexed ($n2$). For total internal reflection to take place, the refractive indices must be such that $n1 > n2$ [116]. A ray incident on the outer layer of the core as well as the interior part of the core will be totally internally reflected if the incident angle is higher than the critical angle depicted by Snell's law:

$$\theta_c = \mathrm{Sin}^{-1}\left(\frac{n_2}{n_1}\right) \tag{11}$$

θ_c= the critical angle and $n1$ and $n2$ are the refractive indices at the outer layer of the fiber core and the core's interior.

For totally reflected light from the interior part of the core, the intensity of the reflected light gradually dampens to zero at the outer layer of the fiber core. Thus, a portion of light sieves through the reflector at a wavelength good enough for the identification of analytes attached to the different layers of separate refractive indices [116]. An electromagnetic field is then established with an evanescent waveform that undergoes exponential decadence between the outer layer of the core and the core's interior of lower "*n*." Hence, a penetration depth (dp) is defined as the maximum distance through which the amplitude of the electric field = $1/e$ or 0.37 of the value at the outer layer which increases as a measure of the light's wavelength and the incident angle.

3.2.1.1 Attenuated Total Reflection Optical Biosensors (ATROB)

When the established waveform in Sect. 3.2.1 tangles with molecules along the "dp", it gives some net photon energy across the surrounding core/reflecting layer with refractive index $n2$ and this helps to balance light transmission in the evanescent field. The transferred photons cause attenuation in the ensuing reflectance which is the mechanism of operation of absorption sensors known as attenuated total reflection optical sensors (ATROS).

3.2.1.2 Fluorescence Total Reflection Optical Biosensors Biosensors

For detectors in which the evanescent light excites a fluorophore, the emitted fluorescence may be reverted to the fiber and subsequently transmitted into a detector. This causes the total reflection of the light's fluorescence which has helped in the design of immunosensors applied in the field of medicine.

Optical fiber biosensors can be combined with spectroscopic methods such as phosphorescence, absorption, Raman, surface plasmon resonance (SPR), and fluorescence. For absorbance measurements, the biological receptor is immobilized in proximity to the optical fiber or attached to its surface. Interactions between the recognition element and the analyte cause changes in the absorbances and transmittances of the sensing layer (Beer's law) which provide useful information about the concentrations of the analyzed species.

3.2.1.3 Surface Plasmon Resonance (SPR) Biosensors

SPR is the situation that is brought about by the transmission of light which is orchestrated by the difference in the oscillating charge densities that exist on the interface between two media characterized by oppositely charged dielectric constants (i.e., a metal and a dielectric material) [117]. The interaction between the light of apt wavelength and the dielectric material's interface occurs at an angle known as the resonance angle. The resonance emanating from the energy exhumed by the light photons has a semblance with the electrons radiating from the metal surface. Photon energy transmitted to the dielectric's surface is in form of electron packets/plasmons thus, the reflected light from the dielectric's surface is then attenuated.

According to Haake et al. [118], SPR can be radiated over fixed angles, white light, and spectral detection; however, based on the studies of Earp and Raymond [119] and Purvis et al. [120], several factors (the incident light-wavelength, the dielectric material, the nature of the medium in contact with the interface and the refractive index of the adsorbed analyte/molecules) influence the resonance angle. An alteration in "n" also has an effect on the conditions surrounding the SPR couplings with resultant shifts/variations in the resonating angle of the radiated light. Two commonly adopted configurations for coupling light rays onto surface plasmon resonators include prisms and diffraction gratings, which are quite different from other forms of optical fibers [121].

SPR has found useful application in immunosensors where the specificity and sensitivity limitations of prior techniques are evident. SPRs can be used in analyzing simple/rapid assays of analytes in different fields including biotechnology, environmental science, medicine, and proteomics where labeling is not necessary [117, 122]). The advantages of SPRs include high sensitivity for the detection of protein levels in sub-femtomole levels of complex fluids and the monitoring of the binding kinetics of the receptor and ligand interactions in the absence of fluorescence/radioisotope labeling of analytes.

Some optical fiber SPR sensors possess monomode and multimode fibers [123, 124]. In the multimode optical sensor, the interaction zone is confined to a few millimeters. Another challenge is the complexities associated with depositing a homogeneous layer with excellent chemical functionalization of the surface of the

device; the modal light dispersion is influenced by changes in mechanical/surface properties. Commercially available biosensors include the BIAcore fiberoptic SPR manufactured by a Swedish company which is compatible with the dipstick-based sampling techniques.

3.2.1.4 Surface Enhanced Raman Scattering-Biocatalytic Sensors (SERS-BS)

According to Nylander et al. [125] and Liedberg et al. [126], despite the extensive use of SPR-biosensors for sensing/biosensing, some associated challenges include low sensitivity to biomolecules of low molecular weight and adaptation to miniaturization. This has led to the localization of SPRs in arrayed nanoparticles/nanostructures in a confinement of electromagnetic field molecular identification/Raman spectroscopy which gives rise to the SER phenomena, which finds usefulness in the probing of mono-molecular substrates as discussed by Rodríguez-Lorenzo et al. [127] and biomolecules [128]. With these kind of sensors uniform/flexible/reproducible SERS substrates can be obtained at high sensitivities relative to those of single molecule ultrasensitive detectors of chemical/biological analytes.

3.2.1.5 Bioluminescent Optical Fiber/Optrode Biosensors

These types employ a recombinant array of bioluminescent cells and bioluminescent signals from an analyte. A typical example is a genetically modified Escherichia coli bacterial strain immobilized on an optical fiber (i.e., a highly dense array of microwells) for the emission of luminescent pulses in environments where genotoxic agents are present, in order to obtain optrode responses to genotoxins such as atrazine within a limit of 10 pg/L [129]. Biran et al. [130] observed that each microwell houses a genetically modified bacterium that responds to a specific analyte towards ensuring the multiple identification of genotoxins.

Other optical biosensors include:

3.2.1.6 Optical Waveguide Interferometric/Resonant Waveguide Grating (RWG) Biosensors

They combine evanescent sensing and the difference that exists between the optical phase measurements. Here, the changes in the probed-volume refractive index cause a phase shift in guided mode relative to a reference field similar to that of the waveguide structure. According to Kozma et al. [131], the interference generated by the different fields gives a superimposed wave signal that is often picked up at the sensor's output section; the output signal has a relationship with the analyte concentration. Zaytseva et al. [132] asserted that RWG sensors are useful for identifying redistributed cellular constituents as well as, monitoring cellular responses/processes, and have been applied to the detection of the avian influenza virus [133].

3.2.1.7 Ellipsometric Biosensors

These types indicate the difference in polarized light having undergone surface reflection. They have been used in the attachment of viral strains of influenza-A to several structures of a glycan panel. Zhang et al. [134] opined that microarrayed biosensors of this type exhibit reflection imaging ellipsometry for serum tumor biomarker (CA19-9)-identification within the limits of 18.2 units/mL.

3.2.1.8 Reflectometric Interference Spectroscopy Biosensors

These types employ label-free time-resolved approaches in sensing signals within a thin layer white light noncomplex optical setup. The resulting phase and amplitude differences in the plane-polarized light give hints about the thicknesses/refractive indices of any adsorbed analytes/protein layers. An example is the identification and estimation of the amount of diclofenac contained in bovine milk matrix where protein levels/concentration of 0.112 μg/L was observed [135].

Maxim and Mechanism of Operation

One area of application is in the monitoring of blood sugar levels of a diabetic patient, where cellulose-based disposable colorimetric test strips are doped with reagents. According to Galindo [113], immunoassays or biochemical tests for measuring the concentrations of macromolecules/small molecules in a solution of plasma-formulated protein cells or antibodies (immunoglobins) that are stimulated by the presence of an antigen/toxin is made possible by reflection and luminescence spectroscopy. To complement the last statement, an example of optical sensors is one that uses light emitted from the firefly (i.e., firefly lucriferase/photinusluciferin 4-monooxygenase) to identify bacteria in analytes. Figure 5 shows incident light, falling at an angle onto a prism surface which rests on a flat surface of the upper

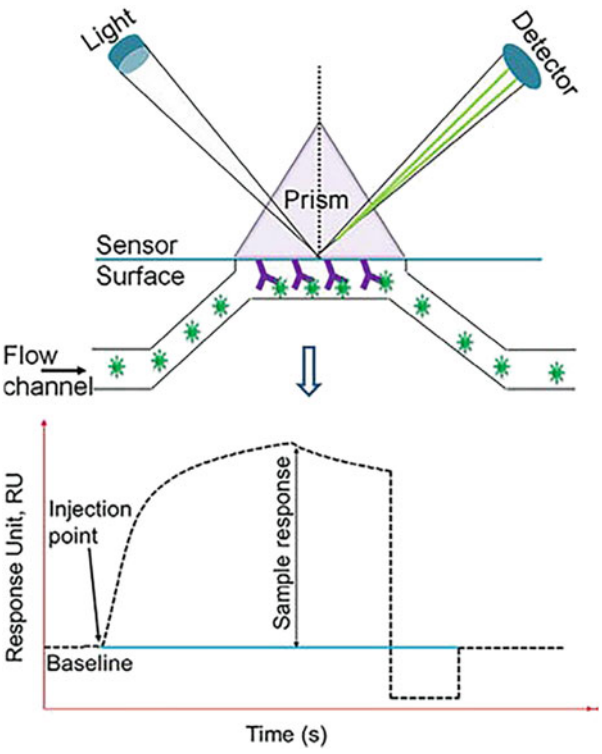

Fig. 5 Optical sensor showing two sections (an upper basic system and a basal section) with surface plasmon resonance. Adopted from Lakshmipriya and Gopinath [3]

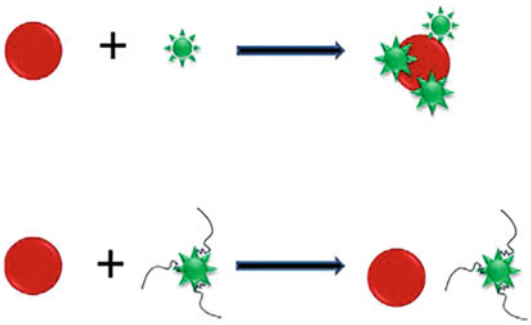

Fig. 6 Colorimetric detection of protein attachment and detachment (biofouling prevention can be aided by injection of polyethylene glycol polymers). Adopted from Lakshmipriya and Gopinath [3]

section of a sensor. The analyte sample to be tested is injected at a point along the baseline (lower section) of the response unit of the sensor. A detector at an angle of reflection then helps to see the response of the analyte that was activated by the incident light, as its molecules (Y-shaped cells) flow along the flow channel. Figure 6 is an illustration of the calorimetric measurements of the attachment and detachment of molecules initiated by the energy of photons released into the cells; the upper interaction yields an attachment while the lower interaction shows there is no form of attachment despite the inherent interaction of the protein molecules or cells.

Two merits of optical biosensors are their low transducer costs and biodegradable electrodes. In an optical sensor, a biological agent/biorecognition element is annexed onto a transducer. The sensed analyte which is bound to a complementary bio-recognizer is also immobilized on an optical substrate which produces an electronic signal whose magnitude/frequency corresponds to one or more analyte concentrations to which the biosensing device is bound [136]. Optical biosensors are named after their biorecognition elements and these include optocouple enzyme-substrate, nucleic acids-complementary sequences, and antibody-antigen systems. Most optical biosensors follow the evanescent field detection principle for biomolecular property detection [137].

3.3 Piezoelectric/Magnetic Biosensor

This type produces electrical pulses in response to some applied mechanical stress. It comprises of an elemental biorecognizing component fused with a quartz-like crystal coated with gold/tourmaline/lithium niobate or aluminum nitride electrode (piezoelectric component) which showcases the piezoelectric effect (i.e., generates electric current when mechanically stressed or stimulated by a stimulus) [61].

3.3.1 Maxim and Mechanism of Operation

As soon as there is a comingling of the coated crystal material with the sample analyte, a change in mass of the crystal ensues with resultant vibrations of the crystal in response to the imposed constraint [61]. In the study conducted by Galindo [113]), the frequencies of oscillation depend on the difference in quartz crystal mass and that of the molecular coating. Subsequently, analyte attachment to the crystal surface causes a resultant growth/reduction in mass of the crystal with an alteration in the frequency of oscillation; these oscillations are then quantified electrically with the aim of obtaining the observed changes in terms of mass/growth of the crystal [61]. Immunosensors of this kind can be used to detect gas/pesticide concentrations as well as hormones/hormonal disorders [76].

3.4 Calorimetric/Thermal Biosensor

Thermal biosensors use the synergistic combination of an enzyme and a transducer to detect the amount of thermal radiation/heat generated from a biochemical reactor [76]. The quantity of heat absorbed/generated is directly proportional to the target analyte's concentration/amount as well as its molar enthalpy [61]. Examples of thermal biosensors include enzyme thermistors.

3.4.1 Description, Maxim, and Mechanism of Operation

The thermal biosensor is one equipped with a calorimetric device for measuring the heat capacity of the solvent alongside a highly sensitive thermistor which helps to sense temperature fluctuations ranging from 0.05 to 10^{-4} °C in a biochemical reactor. It can monitor/track concentrations of target analytes of $\leq 10^{-5}M$ [113]. According to Leung et al. [63], changes in entropy and Gibb's free energy can be estimated for the biochemical reaction by first obtaining the temperature of the system, all aimed at determining the change in enthalpy of the system (heat change between products and reactants). Although this sensor type finds application in the pharmaceutical, food, and cosmetic industries, one major shortcoming of this device is that it cannot be improvised or made to function as an electrochemical or optical biosensor [3].

3.5 Mechanical Biosensors

Micro and nanofabrication methods have helped in the production of biosensors with micro- and nano-sized mechanical parts. This is made possible by the use of standardized wafer scale semiconductor processing techniques which take advantage of exquisite mass resolution, i.e., the correlation between the minimum added/detectable mass and the sensor's total mass. Arlett et al. [138] and Braun et al. [139] provided evidence of mass detection in zeptogram and nanoscales, especially when operating in a vacuum and fluid, respectively. Also, the flexibility/conformity of the device in terms of the uniformity of its trimmed dimensions makes room for easy displacement or deformation within a defined space; hence, an applied force is

measured along a suitable displacement which is analogous to the proportional gain (mechanical analog) in the electronic circuit. Mechanical biosensors have the capacity to sense biological signals within very short times, which allows for the detection of the biological/stochastic molecular interactions occurring in fluids alongside tracking these interactions within milliseconds. Chemistry-based classification of mechanical biosensors is broadly on the basis of the interactions that exist between the analyte and the recognizing biological molecule/sensor.

3.5.1 Biological Assays for Mechanical Biosensors

3.5.1.1 Bioaffinity-Based Assays

In this type of sensor, a high affinity/specificity of the identifier and the target is first ensured on the device's surface, all aimed at achieving improved selectivity and identification of target cells/analytes (antigens) by identifiers (antibodies).

3.5.1.2 Fingerprint Assays

These take advantage of the multiple effect of less-selective functionalized layers in identifying analytes via their binding affinities to an ensemble sensor.

3.5.1.3 Separation-Based Assays

These are mechanical biosensors whose activities are dependent on the chemical affinities (chemical potential) of the immobilized species and a mobile analyte which in turn enhance the spatiotemporal separation of the target analytes.

3.5.1.4 Spectrometric Assays

For these types, the weight or optical properties of the target analyte are first determined for easy identification.

Maxim

In a mechanical biosensor, the measured responsive force makes it possible to read off the miniscule forces that stimulate biological interactions. Reports have it that nanomechanical sensors can attain force resolutions of approximately 10 pN which can accurately detect rupturing hydrogen bonds. One major challenge in biosensing is to be able to develop biochemical agents of reliable suites that are capable of capturing target biomarkers (species of interest). High-affinity binding of the analyte is based on biomolecular recognition in the fluid phase where the actual detection takes place [140]. Alternatively, the detector can be detached from the fluid phase after the target has been captured, it is then dried thereafter, prior to taking measurements. In situ (in liquid) biomolecular detection is quite immediate and simple [141, 142]; however, biomechanical sensing is adversely affected by the damping forces of the viscous fluid which subsequently reduces the mass resolution from the device relative to that obtained in a vacuum or gaseous medium. A mechanical biosensor consists of a small central element/cantilever/sensor that helps to detect target biomolecules.

3.5.2 Types of Mechanical-Biocatalytic Sensors

3.5.2.1 Surface Stress Biosensors

In this type of sensor, quasistatic deflections of a cantilever are achieved by the binding of biomolecules to surface functional groups. Surface binding of the analyte results in an exerted stress which is a measure of the attractive/hydration effects, steric hindrance, and electrostatic effects which in turn induce the deflection of the cantilever. A thorough investigation on how stress influences free energy on a surface was carried out by Ibach [143] in which binding proteins, DNA, and mRNA were observed to show drug interactions [144]. The deflections from the cantilever were made possible via reflections received from the laser beam of light rays incident on the cantilever. Each microlever is prone to parasitic stimulants that engender its exposure to the aliquot sample which may cause strong repeated deflections introduced by a variation in the refractive index, temperature, and fluid composition. Batch measurements were taken in situ between the inductor/strain on the functionalized/passivated cantilevers; sensitivities of this device have been reported to lie between 100 picomolar (pM) to a few nanomolar concentrations [145]. According to Stoney [146], for devices with large aspect ratios (ARs), i.e., length to thickness ratio >10, the exerted stress on a self-assembled monolayer sensor can be estimated by inputting the surface stress measurements from the sensor in the Stoney equation; otherwise, the Sader analysis is employed in determining the exerted stress [147].

3.5.2.2 Dynamic-Mode Biosensors

As the name implies, these types are not quasistatic; however, they vibrate/resonate periodically at a specific frequency which changes as soon as molecules of the analyte hit the cantilever. The operating modes and media for these sensors include:

- Humid environments: where timely measurements of small bacterial colonies are made possible by growing these microorganisms on a sensor that is indirectly immersed in a humid fluid/gaseous medium. Hourly measurements of the growth of antibodies/E coli measured with this device have been found to compare favorably with daily growth monitoring data obtained by conventional approaches [148]. Thus, the multiplexed detection of several bacteria is feasible with an array of this device.
- Vacuum: Mechanical biosensors have proven to give an exquisite mass resolution under vacuum/air [91]. This operation involves using the device in a fluid while disengaging it as soon as the analyte becomes bound/attached to the recognizing molecule. Conscious efforts must be made to dry the analyte in a desiccator prior to mass detection. However, spurious molecular binding of molecules to the device may ensue during drying, however, this must be avoided as it can lead to errors in measurements thus truncating the continuous data monitoring/capturing process; considering the efforts made by Gupta et al. [149] and Ilic et al. [150], this approach has been tested as a potent method for the detection of particulate viruses that are massive in nature. In a study conducted by Waggoner

et al. [151], sandwiched assays helped in detecting femtomolar concentrations of prostate antigens by employing two affinity-based probes (two separate antibodies) aimed at enhancing the effective affinity of multiplex systems. A label is usually tagged on the second probe using fluorescent assays or massive nanoparticles to enhance mass detection of the analyte cells; this makes for easy readouts of the biological signal transmitted to the transducer.

Based on the findings of Hwang et al. [152] and Lee et al. [153], discrepancies were observed for adsorbed masses and frequency shifts in a liquid medium, while McFarland et al. [154] observed the same in a gas medium, where mechanical biosensors used in taking measurements, were seen to give responsive effects caused by adsorbate-induced surface stresses. There is also evidence that clamping severely affects the surface stress exhibited by short cantilevers; however, the expected frequency shifts of the induced stresses are usually lower than those recorded from experiments. Also, frequency shifts can be caused by changes in the composite elasticity of the biosensor as induced by the analyte to be measured. Such variations can overwhelm the frequency changes stimulated by the loaded masses, even when the thickness of the species adsorbed is far less than 30 nm (i.e., ≫30 nm thick) compared to that of the device which is 35 nm. Experimental and theoretical-based evidences were provided for such stiffening effects in thin-layer antibodies in microcantilevers of 30 nm thickness. Other mechanical biosensors include suspended microchannel resonators (SMRs) where the fluid is confined to channels within the resonator of the sensor [155]. According to Bryan et al. [156] and Godin et al. [157], SMRs have been employed in measuring mass/density of yeast cells; the growth rates of unicellular bacterial/mammalian cells were established by Godin et al. [157], while the antibiotic resistances offered by bacterial strains were recorded by Knudsen et al. [158].

3.5.2.3 Quartz Crystal Microbalances (QCM)

QCMs are scaled biomechanical resonators which measure the inertia-mass of analytes bound to a biosensor surface within a vacuum/gas/fluid. A shift in resonance frequency takes place during the attachment stage of the analyte prior to real-time electronic tracking of the target molecule by the biomarker. QCM-fluid concentrations are in the range of nano to femtomolar compositions. Continuous analyte monitoring is usually done in nano-concentrated media via indirect competitive assays. However, during post-capture drying, and in-vacuo measurements, femtomolar concentrations of approximately 100 fM are used to carry out end-point assay detection without any form of contact with the fluid. Femtomolar sensitivity combines end-point vacuum detection with sandwiched assay thus allowing for immunospecific target-mass enhancements [159, 160].

3.5.2.4 Whispering-Gallery Microcavity (WGM):

In this device, a toroidal resonator fiber connection is established. The surface attachment of the analyte on the resonator alters the resonance frequency of the transducer signals received from the biorecognizing molecule. Armani et al. [161]

successfully documented approximately 100 aM sensitivity within response times of about 1 s; however, the challenge still remains that their data is not reproducible due to inconsistencies in the expected resonance shifts/binding kinetics [162]. As opinionated by Armani et al. [161], several prior studies have shown that conservative and highly reproducible results for large influenza-A virion detection at picomolar concentrations have been achieved in less than 10 s.

3.5.2.5 Optical Microring Resonators (MRRs)

These types of mechanical biosensors are similar to WGMs and can be fabricated using standard techniques. They are often integrated into multiplexed detectors/systems; hence, they are characterized by low quality optical sensitivities owing to the fact that reliable measurements can only be obtained within media of nanomolar concentrations in time responses of about 1 min. MRRs can help to carry out quantitative parallel detection of cells in mixtures of several proteins [163] as well as the detection of sandwiched assays with high sensitivity of approximately 6.5 pM [164].

3.5.2.6 Nanowire Biosensors

These types are made from carbon nanotubes and semiconductor wires. They are characterized by their ability to exhibit changes in conductance when target molecules are bound to the sensor surface. There arises a process called "electrochemical gating" caused by the variations in the force of attachment inducing the surface-localized potential that exists between the target-analyte and the sensor, or pH variation. Although enzymes/microbes are most active at pHs of 4–7 however for enzyme nanowire configurations, the anchoring wires increase the tendencies of the immobilized enzymes to withstand/remain somewhat active at wider pH ranges. Furthermore, the biosensing media for these sensors have fluid concentrations within femtomolar [165] to picomolar scales [166]) for accurate frequency-dependent detections. Optimizing sensor measurements via subthreshold biasing can significantly improve the sensor's accuracy. Given the miniature area available for binding on the surface of a nanowire, the works of Squires et al. [167] and Gao et al. [168] suggested that at femtomolar concentrations, bio-capture/sensing should be done within the interval of a few days in order to ensure adequate/reliable sensing owing to the inconsistencies associated with estimating the binding kinetics. Rich and Myszka [169] noted that nanomolar sensitivity for label-free protein biosensing has been carried out using surface plasmon resonance (SPR)/photonic-bandgap (PBG) biosensors. The adjustments in the accuracies of both sensors can help take measurements on the femtomolar scale via sandwiched assay end-point detection for which label probes are coated with gold nanoparticles that enhance SPR performance [170]. The studies of Cesaro-Tadic et al. [171], Fan et al. [172], and Diercks et al. [173] show that optical fluorescence detectors can be employed as sensors within fluid media of picomolar cell concentrations; however, they require hourly incubation periods in order to obtain reliable/dependable measurements within appreciable levels of accuracy.

Maxim

The shortcomings of nonspecific binding of nanowire biosensors can be overcome with advanced procedures (sandwich assays) as a way of enhancing target capture specificity [172]. With the help of a nanoribbon sensor, a two-step method that factors in the variation of sandwich assays can be adopted as a means of detecting prostate-specific blood-antigens (PSAs) of 60 pM or 2 ng/mL concentrations in 20 min [174]. Alternatively, since protein molecules/assays cannot be detected at high resolutions, the amplification of the target analyte may suffice, which helps to raise its concentration above the required/minimum concentration. This then stimulates the chain reaction of the polymerase biomolecule/enzyme which exponentially amplifies the noise signals or pulses from the initial target species thus enabling the biological recognition and measurements of the DNA of several cells >100 μL [175]; the amplified signals of mechanical biosensors are different from those of non-biomechanical types (enzyme-linked immunosorbent assays) (ELISA) because, in the latter, enzymes are used to produce/detect linear signal increments (i.e., subpicomolar sensitivities) with time. Another strategy helps to achieve high signal amplification to machine readable form by a two-step process which includes: (1) enhancement of molecular detection specificity via sandwich assays before the actual amplification of the signaled molecule/detected molecule. SPR sensors can be used to achieve nanomolar molecular concentration sensitivities in label-free forms; however, femtomolar sensor sensitivity is achieved by the inducement of plasmonic signals caused by the immunospecific coating/doping of the target analyte with gold nanoparticles at the final step within 2 h ([160, 170]—they employed gold nanoparticle labels in seeding more gold precipitation thus enhancing the quartz crystal microbalance of the mechanical sensor adopted for detecting femtomolar concentrations of a DNA molecule. However, for optical microring resonators (OMRRs), the label-free step can be achieved within detection limits of 0.6 nM sensitivities in a few minutes [163], whereas the final labeling step can be achieved within specificities of 6.5 pM in 45 min, hence the reason for its ability to detect small protein molecules such as those of cytokine [164]. Biobarcode sensors take advantage of the synergistic effects of signal amplification and nanoparticulate labeling for sensitivities within 500 aM concentrations of the analyte [176]. Generally, scaling of labeling and amplification/modification of non-polymerase chain reaction techniques to advanced multiplexed assays is herculean owing to the fact that they are one-shot detection methods which makes them unsuitable for real-time continuous monitoring of analytes. Although it is often difficult to develop a highly robust and effective immunoassay for biosensor applications, this further situates the idea that nearly all selective sandwich assays are predicated on two bioaffinities (capture agents and antibodies) for high sensitivities [140].

Mechanisms: Diffusion, Convection, and Biochemical Kinetics

Diffusion, convection, and biochemical capture kinetics play significant roles in the overall performance of a mechanical biosensor. In most cases, high flow rates are necessary for the optimal performance of microfluidic biosensors, however, this may reduce the total percentae of target molecules captured and in turn increase the

number of actual molecules captured per time. These types of sensors use small aliquots (in micro liters or less) which makes the biosensing process time-intensive. Based on the study of Diercks et al. [173], for small analyte-volumes (in μL), one may consider the depletion of the bulk solutions at steady concentrations of about 90% to be somewhat significant. Signal sensitivities of about 2 zeptomoles (i.e., 10^{-20} mole or approximately 1000 cells) were obtained via the confinement of several cells within a 5 nanoliter (nL) section containing a bead-type-immunofluorescence assay (BT-IFA). For reaction-controlled systems, time-dependent tissue/cell captures can be expressed as:

$$\delta C_A/\delta t = k_A(b_m - b(t))(c_0 - b(t))/(V^* N_A) \tag{12}$$

$b(t)$ = no. of target molecules attached to a surface at time (t), V = volume of sample, and N_A = Avogadro number.

Note: At minimal concentrations, higher order terms such as $(b(t)/bm)^2$ can be ignored.

Some challenges:

One major limitation of nanoelectromechanical biosensors (NEMS) is the attainment of efficient actuation and transduction [177]. In optical detection, the use of the atomic force electron microscope (AFM) is not feasible due to the small dimensional scaling of the device to the tune of very small wavelengths. Alternatively, dimensions in the range of 50 nm width have been measured optically using optical interferometric devices which do not require the integration of a biochip as light source (single types) or any form of grating [178]; however, those that employ non-interferometric transducers for an array of cantilevers [179], do not need a coherent light source, in lieu of the fact that the latter holds prospects for co-integration with optical chips. There is also an evidence of evanescent coupling with propagating light field substrates as drive mechanisms for NEMS [180]. Generally, microelectromechanical systems (MEMS) used with electrostatic detectors and actuators lose their efficiencies for measurements in nanoscale. Although the measured capacitances are proportional to the areas of separation between the capacitors, the limitations imposed by their sizes are often imposed by their inherent drive gate gaps. NEMS operate at higher frequencies relative to MEMS because a high proportion of the electrostatic/detection-derived signals are often dissipated when these capacitors become storage points for the implied signals. However, this can be corrected by the integration of an L-C (Inductor-Capacitor) network in series for effective impedance transformation at reasonable frequencies (i.e., frequencies >100 MHz) [181].

The functionalities/performances of thermoelastic actuators in photothermal heaters/NEMs have been reported for measurements taken in air [182], liquid [183], and air-liquid systems using electrothermal heaters [184, 185]. Literature has it that, the high performance of thermoelastic actuators is due to the fact that the elastic strain which is a measure of the accumulated energy density, remains unchanged in conformance with the uniformly scaled dimensions of the device. Also, according to Piazza et al. [186], piezoelectric actuators are currently being

integrated into MEMS used in aerated environments, while Hwang et al. [152] reported their application in liquids. The recent developments of qualitative piezoelectric ultra-thin film materials of <20 nm size have broadened their application/integration in NEMS in lieu of the small exceptional rise in power consumption of NEMS void of piezoelectric films.

4 Features/Qualities of a Good Biosensor for Optimal Performance

A good biosensor must be relatively inexpensive and affordable. It must have high precision in the diagnosis and identification of analytes. It should possess improved surface modification and a biorecognition element of high sensitivity and specificity which are measures of its bioaffinity for biomolecules. It must be resistant to biofouling, especially when used for RNA, DNA, proteins, and other micromolecular detection. It must have a good connectivity with the corresponding transducer, i.e., it must be compatible with the inherent transducer that helps to convert biological signals to easily interpretable electrical pulses. It must have a good lower limit detection, i.e., it must be ideal for measuring analytes of very low concentrations. A good biosensor must have a high specific surface area to length ratio. It must be able to accommodate a flexible range of scaled multiplexed detectors and transducers in order to abate unforeseen challenges at the biofabrication stage. Since biosensors require the integration of multiple transduction elements for adequate processing of several analytes, it then suggests that high-throughput transducers and/or detectors are of great essence for optimal biosensor performance.

5 Limitations of Biosensors

Biosensors may find it difficult to detect high-affinity molecules at low concentrations owing to their high levels of signal to noise ratio. Cross reaction of biomolecules may result in biofouling and low performance of biosensors due to the undesired random binding of biomolecules onto the sensor's surface, especially highly weighty proteins, which have the tendency to increase biofouling at the surface, although, blocking agents including skimmed milk, polyethylene glycol, ethanolamine, and bovine serum albumin can help to abate this effect. Each biosensor is designed for a unique purpose, i.e., it is output-specific as no one type of biosensor can carry out all biological measurements as desired per target analyte. Some biosensors are hydrophobic by design hence, they are not water friendly owing to the fact that water can affect the sensitivity of the device. High-throughput biosensors are fabricated using sophisticated technologies that assume the form of arrayed micromechanical cantilevers for the successful detection of chemical compounds [187]. Huang et al. [188], Aristotelous and Ahn [189], Hartati et al. [190], and Lee et al. [50, 51] have reaffirmed that regular biosensors are fabricated to detect one compound or analyte with some measure of compromise for sensitivity;

however, no biosensor is said to be 100% accurate. Miniaturized biosensors are low-performing because when in use, they rely on surface capturing of molecules that are poorly detected during the transportation of target molecules towards the sensor [167]. Usually, real-time biosensors are somewhat stereotypically inclined to one analyte because of the specificity of the sensor [191]. Real-time biosensors in which cell-substrate binding is not required are usually integrated with sophisticated transducers which makes them somewhat expensive. For biological systems, an alteration in their microenvironment may result in undesirable variations in pH, absorbance, signal cells, and the complex impedance of membrane cells. Although a school of thought posits that pH/absorbance variation may not always be biologically significant in real-time biosensors, healthy looking cells thrive at well-defined pHs (say 4–7) hence, measuring the resulting transmittance/absorbance is necessary upon introducing fluorescent labels to the biological signal detectors of these sensors.

Table 2 shows several biosensing techniques, the time duration for target analyte detection, and the detection limits of some biosensors.

6 Sensors: The Future

Ultimately, since it is a known fact that mechanical biosensors combine precise microfluidic handling of samples with automated protocols, highly sensitive enzyme-element binding in an array of multiplexed systems can be assembled for mass production of signals using existing microfabrication techniques. Also, many suspended hybrid cantilever-enzyme systems can be manufactured in form of calibrated chips which will help take milli-metric measurements (i.e., measurements in millimeters). However, some basic challenges include the complexity associated with the differential functionalization of closely packed sensors as well as the nature of multiplexing electrical outputs of a dense arrangement of sensors. The advent of silicon microelectronics is one promising way out of this problem, which provides the opportunity of taking advantage of large-scale integrated microelectronic silicon chips as complementary metal oxides (MO) or semiconductors that can actively drive NEMS; several steps adopted include (1) the development of a monolith-NEM-SIO_2, as well as a multilayer and multichip component for 3D-stacking or hybridization of the NEM and MO [196]. High-multiplex microfluids are currently being exploited for integration in densely arrayed nano-scaled biosensors where their mass sensitivities determine the degree of sensitivity or biosensing obtainable from such devices. A typical example is in the area of detecting rare biomarkers in a sample fluid (serum/albumin) wherein the concentrations of highly predominant proteins far outweigh those of the target molecules. To some extent, immunoaffinity or preconcentration reduction of the serum may be useful but the precision of this approach may be compromised by competing molecules which ought to be concentrated/depleted alongside the target molecule. In addition, small or less abundant target proteins (cytokines) may be trapped/captured by more concentrated proteins in the serum (albumin). This is because evidence has shown that trapped

Table 2 Characteristics of biosensing techniques, their period of detection, and detection limits

Detector category	Description	Detection conditions	Time required for analysis	Sensor detection limits	Refs.
Optical-label-free real-time detectors					
Microring resonator (MMR)	Label-free detection via a microring resonator	5 protein mixture in BSA-PBS (0.1 mg mL^{-1} BSA)	2 min	0.6 nM	Washburn et al. [163]
Surface plasmon resonance (SPR)	Label-free SPR detection	0.1 mg mL^{-1} BSA	10 s	3 nM	Rich and Myszka [169]
End-point-labeled detectors					
Lateral flow assay (LFA)	Pregnancy test	Urine	3 min	10 μM	Lipsitz [192]
Immunofluorescent assay (IFA)	1. ELISA 2. Integrated blood barcode chip (IBBC) with DEAL 3. Microfluidic fluorescent immunoassay 4. Bead-based microfluidic immunoassay with zM sensitivity	Serum Whole blood Cell culture supernatant 4-protein mixture in PBS with 1% BSA	60 min 90 min 45 min 210 min	0.1 pM 1 pM 1 pM 0.4 pM	Lipsitz [192] Fan et al. [172] Cesaro-Tadic et al. [171] Diercks et al. [173]
Microring resonator (MRR)	Labeled detection using a microring resonator	IL-2 in BSA-PBS	45 min	6.5 pM	Luchansky and Bailey [164]
Biobarcode amplification (BBA) assay	Protein amplification through functionalized nanoparticles	Serum	45 min	0.5 fM	Goluch et al. [176]
Surface plasmon resonance (SPR)	Labeled detection via SPR (DNA detection)	TNE	2 h	1.4 fM	
Mechanical-label-free real-time detector					
Microcantilevers (MC)	1. Static mode (surface stress sensors, SSS), functionalized reference 2. SSS, unfunctionalized reference, piezoresistive detection 3. SSS, no reference cantilever 4. Dynamic-mode detection (mass sensing)	HBST buffer 0.1 mg mL^{-1} BSA 1 mg mL^{-1} HSA PBS	10 min 12 min 100 min 12 min	15 nM 300 pM 100 pM 0.3 pM	Backmann et al. [141] Wee et al. [193] Wu et al. [145] Hwang et al. [152]

(continued)

Table 2 (continued)

Detector category	Description	Detection conditions	Time required for analysis	Sensor detection limits	Refs.
Suspended microchannel resonator (SMR)	Protein detection in serum	Serum	1 min	300 pM	Von Muhlen et al. [142]
Quartz crystal monitor (QCM)	Detection of C-reactive proteins	0.1 M sodium phosphate buffer	10 min	1 nM	Kim et al. [194]
End-point-labeled detector					
Microcantilevers (MC)	Mass sensing with liquid phase capture and vapor phase detection	Serum	4 h	1.5 fM	Waggoner et al. [151]
Quartz crystal monitor (QCM)	1. Mass sensing with liquid phase capture and vapor phase detection 2. DNA detection using a sandwich assay with mass amplification	Serum 0.4 M phosphate buffer	180 min 220 min	85 fM 1 fM	Kurosawa et al. [159] Weizmann et al. [160]
Electrical label-free real-time detector					
Nanowire (NW)	1. Nanowire FET for DNA detection 2. Nanowire FET for identification of PSA in time domain 3. Nanowire FET for identification of PSA in frequency domain	Buffer Buffer Buffer	10 min 17 min 33 min	10 pM 5 pM 0.15 pM	Bunimovich et al. [166] Zheng et al. [195] Zheng et al. [195]
End-point-labeled detectors					
NW: nanowire	Nanowire FET for DNA detection	Whole blood	11 min	0.6 pM	Stern et al. [174]

Adapted from Arlett et al. [138]

biomarkers can be detected at concentrations ranging from 10 to 500 times higher than those of their unbound counterparts [197]. Also, in recent times, high-temperature hyperthermophiles have been discovered as thermally resistant microbes located in sandstone reservoirs. The enzymes of these microbes can then be exploited/extracted and immobilized on nanocomposite membranes charged with active nanoparticles not only to serve as membrane filters but to also serve as detectors of polymer saturation levels in petroleum reservoirs during enhanced oil recoveries. In essence, these filters can be designed as sensors linked with transducers that can read up saturation levels of the polymers in order to perceive polymer saturation or plugging since it is evident that these microbes have high thermal stabilities within temperatures of about 100–110 °C. This then suggests that the enzyme extracts of such microorganisms can be immobilized on active supports that will help retain their bioactivities/bioaffinities such that they are able to exhibit high sensitivities within temperatures in the range of 100–110 °C, which is quite unusual of enzymes because enzymes are known to be thermally resistant between 0 and 50 °C. In the area of computer biosensing, unlike the common biosensors which require single input (analyte), biochemical logic systems may be fundamentally designed with apt focus on their interfaces and transducers as well as their fluid compositions and methods of immobilization of the biocomputing surface within levels of efficient scalability for the transmittance of the required end signals. Unlike in earlier studies where multiple constituents were contained in solutions, biosensors for computer applications may assume the forms of biochemical logic-based sensors which require optimally confined surfaces of their biocomputing layers. Hence, a prudent tuning/tweaking of the fluid medium/microenvironment is necessary for high efficiencies, considering the high complexity of bio-logic gate sensors relative to their enzyme-electrode counterparts. The main objective is not just to provide a synergy between a biolayer and the transducer/electrode surface, but to also effectively synergize separate bio-logic gate elements in order to enhance the effective assembling of enzyme cascades without compromising enzyme stability in different media or buffer concentrations of the substrates. Furthermore, the selected enzymes should be void of cross-reactions with other biocatalysts/enzymes in situations where multiplexing with biosensors is feasible. In the medical arena, there are proposals for the consideration of graphene-based/nanocomposite biosensors for identifying the novel COVID-19 virus which has caused the GDPs of several nations' economies to decline owing to the recent global pandemic/high mortality caused by the virus. Graphene is a 2D monolayer SP^2-hybridized carbon-based material which has been proven to possess qualities that have led to its application in drug delivery, containment as well as the treatment/identification of cancerous cells. Graphene-based biosensors of various transduction modes are used in biosensing owing to their high surface areas, electrical conductivity, high electron transfer rate, and compatibility with prospective immobilizates. In addition, nanographenes can serve as quenchers in transducers for fluorescent biosensors and reports from literature are in support of the fact that graphene (G), and its oxides, i.e., graphene oxide (GO) and reduced graphene oxide (rGO) are highly efficient fluorescent quenchers. This is made possible because of its conjugate

structure, which facilitates increased electron transfer between bioreceptors and transducers for signal amplification and sensitivity; hence, it is highly patronized for the construction of electrochemical biosensors. In lieu of this fact, it becomes expedient/urgent to begin to look into its consideration for integration into other types of biosensors. Furthermore, the application of biosensors in engineering science, medicine, biochemistry, electrical engineering, computing, etc., holds great prospects in the near future. However, for large-scale applications, efforts should be made towards synthesizing biological catalysts at low costs or better still, have these catalysts in micro forms/nanodots such that cost implications of the fabricated biosensors are minimized. The potential of nanotechnology in biosensing is a promising technology for future biosensors as about 100% enhancement in the biosensing ability of the tyrosinase enzyme was reported when copper ions were used as cofactors for the sensitivity enhancement of a Clarkson-dissolved oxygen probe covered with Teflon membrane on which the tyrosinase was immobilized [198]. This then confirms the high prospects underlying the use of other nano-metal conductors, cofactors, or hybrid catalysts with enzymes towards maximizing/multiplexing the sensitivities/electrical readouts of biosensors.

7 Concluding Remarks

Biosensors integrated with DNA chips are majorly of high interest to biotechnologists and molecular biologists. Their principle of operation depends on the specificity of the recognition process occurring between a biomolecule and its complementary molecule. A good biosensor is expected to have high sensitivity and selectivity because it is usually difficult to artificially achieve sensitivities and selectivity that are higher than those of the specific recognition processes occurring between biomolecules such as those involving an enzyme and its substrate, hence the need for immobilization; this then implies that the onus lies on ensuring to have the precise immobilized biomolecule for effective biorecognition of the target analyte [199]. In an enzymatic biosensor, the enzyme stimulates a reaction in its substrate via attachment, which in turn produces a chemical signal that may assume the form of a generated product or coproduct. Ultimately, the bulk majority of biosensors have their bioactivities stimulated by immobilized enzymes. In some biosensors, the binding of specific antibodies to their corresponding antigens is the mechanism via which the biosensing is initiated, and hence biorecognition is then stimulated between complementary molecules. The creation of a biosensor begins with identifying a biomolecule that exhibits the desired potential for sensing analytes. This biomolecule must be surface-immobilized, such that its activity can be retained/localized in order to prevent it from being washed off by solvents containing samples to be measured. Enzyme immobilization also helps to ensure that changes in reactant/product concentrations are measured with high precision and very minimal signal losses. Oftentimes, the strength of immobilization is key to achieving high-performing biosensors; this helps to keep the immobilized enzymes fastened onto their anchors in order to prevent fluid wash-offs as well as limit the tendencies for

loss of bioactivities. Methods of enzyme immobilization include surface binding, embedment/entrapment, covalent linking/binding, cross-linking, etc. The choice of adopting a specific immobilization procedure is governed by the nature of the biomolecule to be immobilized, the measurement technique, the planned lifespan, and cost [200–204]. Furthermore, the construction of multistage/multiplex biosensors can be achieved with several biomolecules; however, efforts must be put in place to ensure compatibility between the immobilized biological agents/enzymes. To maximize the precision/accuracy of a sensor effectively, it is necessary to get acquainted with its principle of operation, the type of measurements to be taken, the environment where the sensor would be used, its working principle and accuracy, as these will help ensure that the user is well informed prior use. In theranostics (diagnostics for disease-detection), treatment regimen selection and monitoring procedures for patients' responses to therapies/molecular target drugs have been developed as a cure for several diseases, especially for patients with cell expressions similar to that of the target molecule in the drug [205]. Hence, an assessment/examination of the quantity of target molecules/biomarkers expressed by a patient's cell is essential for apt patient selection for specific drug administration. In addition, companion matching diagnostics is currently being exploited by the "US Food and Drugs Administration guidelines," as a valid decision-making tool for reviewing target drugs prior approval for patient administration.

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Part III

Potential Role and Applications of Bio-based Sensors

Polyhydroxyalkanoate-Based Sensors and Their Applications

Subhasree Ray, Archana Kumari, Madan Sonkar, and Prasun Kumar

Abstract

Polyhydroxyalkanoates (PHA) are unique polyester of microbial origin with features equivalent to synthetic plastics. Its thermoplastic, biodegradable, biocompatible, and nontoxic characteristic makes it a suitable biopolymer for various sectors of modern sciences and industry, including biomedical and related applications. The physical properties of PHA can be tuned by changing its monomeric units (hydroxyl acids) and/or modifying the flexible R-group present within the monomers. Thus, the PHA polymer can have broad crystallinity, optical, and piezoelectric activities that can be customized. Recent advancements in industrial manufacturing techniques led to the utilization of PHA polymers in sensing applications and developing microelectronic devices. Sensing devices based on PHA polymer along with other composite materials have been used to detect gases, volatile organic compounds, Urea, H_2O_2, antibiotics, body fluids, and live microorganisms. In addition, PHA-based devices also found their role as triboelectric nanogenerators, time-temperature indicators, strain sensors, chemosensors, and pressure sensors. In this chapter, these unique applications of PHA in sensing applications will be discussed, along with the recent advancements and associated challenges. The PHA polymers are great

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alternatives for miniaturized electronic devices with lower ecological impact, and with recent developments in biocomposite fabrication. Soon the PHA-based sensors will find their place in our daily lives.

Keywords

Bioplastic · Biosensors · Piezoelectric · Biomedical application · Detection · Triboelectricity · Polyhydroxyalkanoate nanocomposite

1 Introduction

Polyhydroxyalkanoates (PHAs) are a family of bacterial biopolyester synthesized by bacteria as intracellular storage of carbon and reducing energy equivalents. These polymers are accumulated by the microbes during stationary stage of growth phase on substrates such as sugars and/or volatile fatty acids [1, 2]. This microbial polyester is a polymer of saturated and/or unsaturated hydroxyalkanoic acids; therefore, PHAs are nontoxic, thermoplastic, biodegradable, and biocompatible. PHA-producing microbes are widely distributed in nature and are capable of accumulating this polymer upto 90% of their dry cell weight in the form of discrete granules. Both Gram-positive and Gram-negative bacteria (belonging to more than 400 genera) are known to synthesize PHA under nutrient imbalance conditions particularly when carbon sources are available in plenty but other nutrients (e.g., N, P, Mg, or O) are limited. Well-known PHA producers include *Ralstonia eutropha*, *Bacillus megaterium*, *Pseudomonas* spp., *Rhodobacter sphaeroides*, and engineered strains of *Escherichia coli*. More than 100 types of monomers are known to be present within the PHA polymer. On the basis of the number of carbon atoms present in the monomer, PHAs are categorized as short-chain-length PHAs (scl-PHA, containing monomers of 3–5 carbon atoms) and medium-chain-length PHAs (mcl-PHA, containing monomers of ≥6 carbon atoms). Polyhydroxybutyrate (PHB) having only 3-hydroxybutyrate monomers are the most common type of PHA polymer (brittle in nature). The intracellular accumulation of PHA polyester and its monomeric composition varies depending on the type of organism, growth substrate, and growth conditions being provided [3]. There could be homopolymer, co-polymer, or tercopolymers of PHA. Due to a broad range of monomers incorporated within the polymer by the enzyme PHA polymerase (encoded by *phaC* gene); the physical properties of the synthesized polymer vary and the polymer can also be customized for different applications [4]. It is also possible to functionalize this polymer for various applications. This fact makes PHA a potent alternative to petroleum-based plastics. Thus, PHAs and their polymeric eco-friendly blends have attracted significant attention worldwide. From a commercial viewpoint, a few important PHAs are polyhydroxybutyrate (PHB), poly(hydroxybutyrate-*co*-hydroxyvalerate; PHBV), poly(hydroxybutyrate-*co*-hydroxyhexanoate; PHBHHx), and poly(3-hydroxybutyrate-*co*-4-hydroxybutyrate; P3HB4HB) that can be produced through fermentation. There is a lot of scope and expectation from this unique biopolyester. The degradation of PHA occurs through successive exposure to

bacteria present in soil or marine sediment. It begins when microbes start colonizing the PHA surface and release the PHA depolymerase enzyme that degrades the polymer into hydroxyacid monomers. These hydroxyacids are utilized by the native microorganisms for growth and biomass production. Under aerobic conditions, the final end product of microbial PHA degradation is CO_2 and water, while CO_2 and CH_4 are the final products during anaerobic digestion [5].

Being a polyester with diverse physicochemical properties there are several applications of PHA, the most common usage of this polyester is in the packaging industry [6]. Despite all these fascinating properties, large-scale production of PHA polymers suffers from huge production costs owing to expensive raw materials and manufacturing processes. In the past two decades, several microbes and alternative substrates (including agro-industrial wastes) have been explored for cost-effective PHA production. A wide range of agro-industrial wastes, vegetable wastes, wastewater originating from industries, waste cooking oil, lignin derivatives, etc., had been exploited using natural or genetically engineered organisms [7–9]. In addition, the co-polymer content within the PHA polymer was also manipulated by changing feed composition or genetically modifying the PHA synthase or the whole operon regulating PHA biosynthesis [4, 10, 11]. Among them, a few notable advancements were those related to the integrated production of PHA and other bioproducts in a two-stage process, other researchers have also suggested producing valuable co-product by the same microbe such that the overall production cost can be minimized provided that the substrate competition during bioconversion is averted and cheap carbon sources are utilized properly [12–14]. For instance, the production of biohydrogen and PHA was found to be suitable and cost-effective as biodegradable wastes can be used in this process [15–18]. Pigment production, exopolysaccharides, and integration of bioelectrochemical systems with PHA production were also proposed to be an effective strategy in alleviating production costs [13, 19–21].

A few large-scale PHA productions had been of limited success with the commercial brands namely—Biogreen, Mirel 4000 series, Biocycle, Biomer 300, GreenBio, Kaneka PHBH, AONILEX, TephaFLEX, and ENMAT Y1000 [6]. Conventional techniques can be used to process PHAs that can be used later for packaging, disposable materials, food and medical products, and other industrial uses. Especially, pure PHA polymer can be exploited in the biomedical industry with a desirable profit. The Food and Drug Administration, USA approved the use of poly (4-hydroxybutyrate) for surgical applications. Subsequently, the interest in other biomedical applications of PHA has increased significantly [5]. Several novel applications of PHAs are known and are being developed further for the benefit of humankind (Fig. 1). PHA has been well studied for its ability to deliver drugs/antibiotics, etc. A common platform technology was designed where PHA film and a fusion partner (having PHA depolymerase) were used to immobilize proteins onto the PHA surface. Once the target protein gets immobilized on the PHA surface (can be a thin film or coated material and even microbeads) it can be used to study specific interactions between the two protein molecules or protein-drug/biomolecule interactions. In the past few years, several reports have explored PHA granules

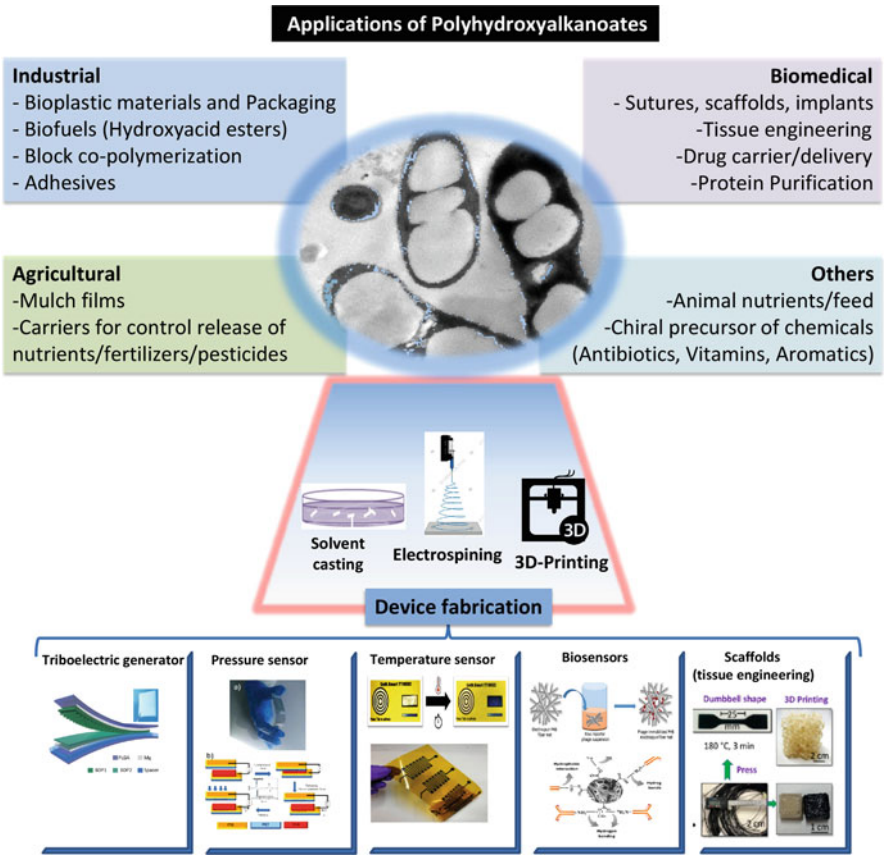

Fig. 1 Applications of PHA and PHA-based materials in various fields

and associated proteins for the development of biosensor and theranostic activities [22].

Owing to their nontoxicity, piezoelectric property, thermoplasticity, and or elastomeric properties, PHA polymers, and PHA-based composites were shown to be useful in electronics, wearable devices, drug encapsulation, tissue engineering, agriculture, etc. However, very limited studies are available on the use of pure/extracted PHA polymer for biosensor applications. Therefore, this chapter discusses the use of various PHA polymers in sensing applications and the underlying challenges as well as the scope for better utilization of these biodegradable polymers in our everyday life.

2 Use of Polyhydroxyalkanoates as Sensors

Naturally, the intracellular accumulation of PHA polyesters occurs with the polymerization of a variety of monomers providing them with unique properties (crystalline, piezoelectric, and optical active polymer). Moreover, the processability of PHA with industrial manufacturing techniques traditionally used for oil-based plastics (viz., injection molding, thermoforming, extrusion, etc.), makes it a potential candidate for applications in electronic devices [23, 24]. The development of such devices is also associated with the miniaturization of the electronic component to lower the environmental impact of the organic matrix in which they are held.

2.1 Pressure Sensor

PHB polymer can also be used for a device that uses the charging process during friction to generate electric power from mechanical energy. Such a demonstration was made recently while fabricating a triboelectric generator (TEG) by stacking PHB film between poly(ethylene terephthalate) (PET) coated with indium tin oxide and PET sheet. Charge transfer occurs between the materials as the material having a high dielectric constant becomes positively charged while the other becomes negatively charged. There will be a flow of charges across an external load if the two surfaces were rubbed with each other. The TEG produced a short-circuit current density and an open-circuit voltage [25]. Such a TEG setup can be utilized as a pressure sensor and have the potential to harness energy from mechanical vibrations, touch screen, etc. Thus, these devices may be applied as self-powered sensors for environmental monitoring and other large-scale application. It was suggested that in this case, the energy conversion involves two key steps: (1) as an external compressive force is applied to the device, the two polymer layers come in contact and electrification occurs between the two layers. In this initial step there will be no charge induction in the external load, as there is no charge separation (the two layers are strongly attached to each other); (2) when the compressive force is released, due to the elasticity of the material an air gap between the tribo-charged layers will exist. The latter introduces charge separation and a potential difference between the top and bottom electrodes. It is the driving force for the electrons flow in the external load [26]. The contact electrification between polymeric films is further enhanced due to nanoscale roughness on their surface. On a similar theme, an emerging class of advanced technology—Transient electronics has become increasingly popular. It refers to an ability of a device to physically or chemically disintegrate, dissolve, and degrade in an actively or passively controlled fashion to leave harmless by-products in the environment.

2.2 Triboelectricity Generator

The transfer of electrons as a result of two objects contacting each other and then separating is known as "triboelectric charging." In this process, one object gains electrons (becomes negatively charged) on its surface while the other loses electrons and becomes positively charged depending on the material's relative tendencies to gain or lose electrons. Some materials have a higher tendency to gain electrons than others; similarly, some others tend to easily lose electrons. Materials that have a strong triboelectrification effect act like an insulator (i.e., less conductive; transferred charges are retained for an extended period of time). Although such things are considered to be a negative effect on technology developments, it must be noted that insulating materials allow longer retention of triboelectric charges. Usually, in a physical process, energy conversion occurs in three stages: (1) charge generation, (2) charge separation, and (3) charge flow. In contrast, piezoelectric nanogenerators create the potential under mechanical strain. Flexible TEG was recently developed comprising all-polymer-based materials working either on contact mode (here, vertical out-of-plane separation of the tribo-charged layers is used to induce polarization) or sliding mode (here, the difference in potential is produced by in-plane separation of tribo-charged layers).

Transient electronics fabricated with degradable materials have shown immense potential for in vivo sensors and therapeutic devices. Most of these devices need an external source of power limiting their in vivo applications. A bio-based triboelectric nanogenerator (BD-TENG) was reported for harvesting biomechanical energy in vivo (Table 1). The advantage of this biodegradable device is that after completing its work cycle it can be resorbed in the host body without any adverse effects. Four different biodegradable polymers, viz., poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid) (PHBV), poly(caprolactone) (PCL), poly(L-lactide-*co*-glycolide) (PLGA), and poly(vinyl alcohol) (PVA) were used to fabricate BD-TENG with tunable electric outputs and biodegradation characteristics [31]. A DC pulse was generated when BD-TENG was applied to power two complementary micrograting electrodes. As described previously, the as-fabricated TENG is a multilayer structure comprising of encapsulation, friction, electrode layers, and the spacer (Fig. 2). Here, two BDP layers having nanoscale structures on the surface may be kept together as friction parts with a thin magnesium film (electrode layer) deposited on one side of each friction layer. The BDP encapsulates the whole structure to keep it contacting surroundings. This TENG setup was used to culture endotheliocytes (ECs) for 7 days. It also supported the neuron-repairing process and nerve cell growth. Biopolymers used to prepare TENG are an alternative to commercial polymers, cost-effective, and undergo facile processing methods with solvents such as casting and spin coating. The BD-TENG could be a potential power source for transient medical devices.

The output performance is considerably affected by the intrinsic electrical properties (ability to gain or lose e^-) of two friction layers used in TENG. The relative ability to gain or lose electrons can be ranked on a BDP triboelectric series where charge tendency of the biopolymers was observed as

Table 1 Application of PHA biopolyester for various sensing applications

Polymer type	Preparation condition	Application	Reference
PHA-based sensors			
Polyhydroxybutyrate	Drop cast PHB film between poly(ethylene terephthalate) sheet and indium tin oxide coated PET	Pressure sensor	Valentini et al. [25]
Polyhydroxybutyrate	Brilliant Blue FCF dye-Loaded Polyhydroxybutyrate	Time-temperature indicator	Anbukarasu et al. [27]
Polyhydroxybutyrate	PHB-rGO composite	Strain sensor	Dan et al. [28]
Polyhydroxybutyrate	Electrospun fiber of Polystyrene/ Polyhydroxybutyrate doped Graphene and Porphyrin	Chemiresistor gas sensor	Avossa et al. [29]
Polyhydroxybutyrate	Drop-coating of PHB-rGO composite onto silver electrodes (ink-jet printed)	Temperature sensors	Dan and Elias [30]
Poly(3-hydroxybutyric acid-*co*-3-hydroxyvaleric acid)	Solvent casting method (5% solution in chloroform)	Biodegradable triboelectric nanogenerator	Zheng et al. [31]
PHA-based chemosensors			
Polyhydroxybutyrate	Myoglobin-PHB/Pyrolytic graphite	H_2O_2	Ma et al. [32]
Polyhydroxybutyrate	Hemoglobin-PHB/ Pyrolytic graphite	H_2O_2	Ma et al. [33]
Polyhydroxybutyrate	Gold Interdigitated Microelectrodes Fabricated on Polyhydroxybutyrate	Urea sensor	Slaugther [34]
Polyhydroxybutyrate	Blend of polyaniline and poly-3-hydroxybutyrate (PANi/PHB) electrospun nanofibrous scaffold	Ammonia, triethylamine, and acetic acid	Macagnano et al. [35]
Polyhydroxyalkanoate	Enzyme immobilized on PHA patterned interdigitated array (IDA) gold electrode	Glucose and organophosphorus pesticide	Moore et al. [36]
PHA-based biosensors			
PHA polymer (3-hydroxyvalerate (3HV), 5-hydroxydecenoate (5HDE) and 3-hydroxyoctadecenoate (3HODE))	PHA/AuNP/HRP/ITO	Quantitative detection of artemisinin	Phukon et al. [37]
Polyhydroxyoctanoate (mcl-PHA)		Remote detection of liquid solution (e.g.,	Stojanović et al. [5]

(continued)

Table 1 (continued)

Polymer type	Preparation condition	Application	Reference
	Inductive capacitive (LC) resonant circuit structure	Saliva, simulated gastric fluid)	
Polyhydroxyalkanoate	PHA depolymerase substrate binding domain (SBD) as fusion partner to immobilize proteins on PHA film, microbeads, or printed PHA surface	Detection of hepatitis B virus and Coronavirus	Park and Lee [38]
Polyhydroxybutyrate	Electrospun PHB Fiber Coated with Polymethacrylate	Detection of Dengue enveloped virus	Hosseini et al. [39]
Polyhydroxybutyrate	Reporter nanoluciferase Bacteriophage, PP01 immobilized on electrospun polyhydroxybutyrate fiber	Detection of *Escherichia coli* O157:H7	Chen et al. [40]

PCL < PVA < PHBV < PLGA from negative to positive. This is due to the diverse molecular structure and functional groups of the biopolymers. Thus, triboelectric series could be helpful in the fabrication of TENG of different electric outputs by using selective combinations of BDPs having different triboelectric properties. For this purpose, a classical TENG system was designed using the Kapton file as a reference contact layer to demonstrate the relative ability of BDPs' electron exchange in a triboelectric process [31].

2.3 Temperature Sensor

In addition to various biomedical applications, PHA polymers have also found their usage in the food packaging industry. A successful demonstration of PHA-based time-temperature indicator (TTI) was done (Table 1). The system was based on enzyme activation that releases dye loaded on PHB film. The PHB-acetic acid solution was loaded with Brilliant Blue dye and cast as a thin film such that the dye gets uniformly distributed within the film. By the action of PHA depolymerase enzyme from *Comamonas testosteroni*, degradation of the polymer starts releasing the dye in solution. Consequently, the optical transition of the film from clear to colored occurs as a function of time [27]. The dye release kinetics due to the depolymerase enzyme was also tested at a broad temperature range of 4–37 °C. Kinetic analysis revealed that the dye release process has an activation energy of 74 kJ/mol, while the dye gets released completely within 6 h in contrast to ~7 days when incubated at 4 °C. These kinetic parameters suggest the suitability of Enzyme assisted dye-loaded PHB TTI for cold packaging applications, particularly for fresh and frozen food products. The PHB film thickness also governs the response time of

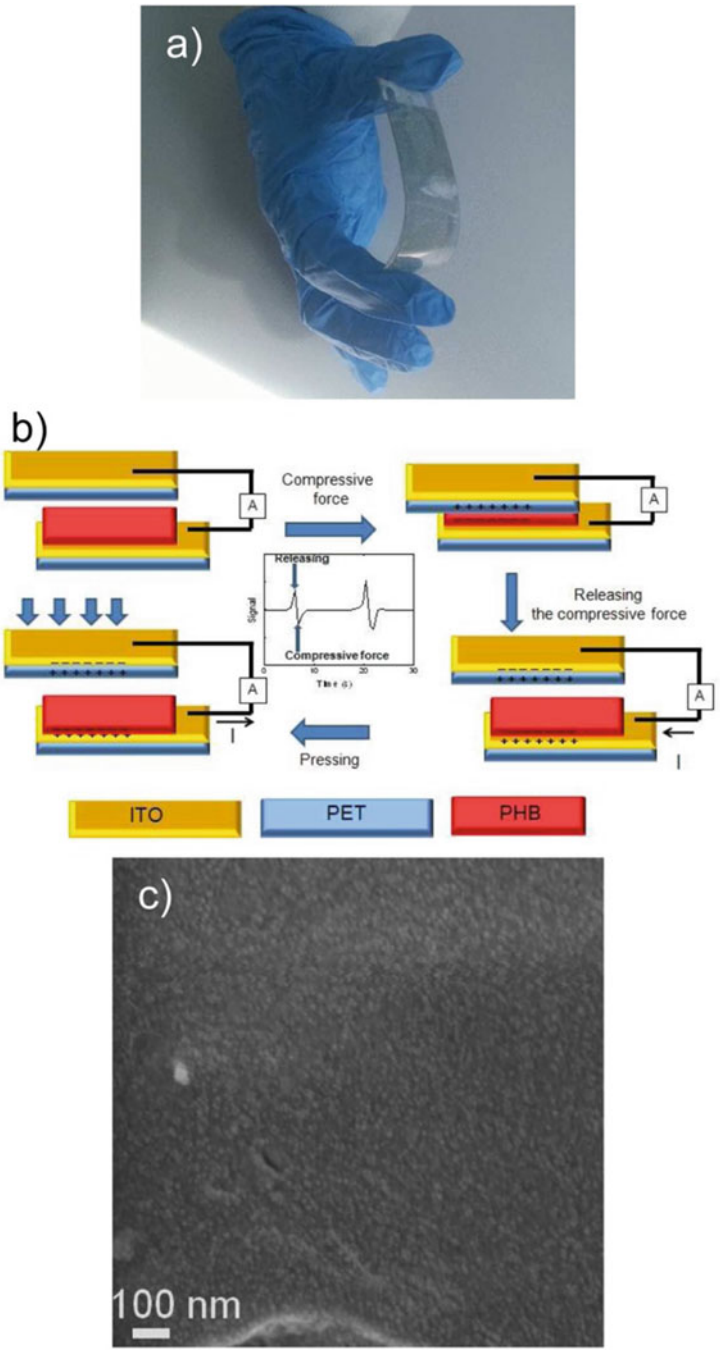

Fig. 2 (**a**) Photograph of the fabricated Triboelectric generator (TEG). (**b**) Proposed mechanism of the TEG device. (**c**) SEM image of the PHB surface

the indicator, since the biodegradation of PHB film using enzyme was considered to be a zero-order reaction. Because of such pseudo-zero-order kinetics, as the PHB film thickness increases a proportionate rise in time will be observed for complete dye release without influencing other kinetic parameters [27].

However, being an enzyme-based indicator, factors such as pH and storage temperature may affect the response. Usually, enzyme activity increases with temperature till it reaches its optimum activity beyond which the enzymatic activity declines sharply. Thus, for enzyme-based TTI temperature is an important aspect, particularly the storage temperature to which the product will be exposed such that the enzyme remains active in the required temperature range and time scales. Considering this, depolymerase enzyme used in this study was thermostable having optimal activity at 70 °C. Therefore, this enzyme is appropriate for applications such as food products that would not be stored at higher temperatures. There are several advantages of enzyme-based TTI that can generate visual signals (color change). These are biodegradable, low-cost alternatives, simple in design, possess good reliability, and are not dependent on secondary mechanisms (e.g., pH change or secondary chemical reaction). One major concern is that the enzyme must remain active before its deployment in the packaging material. This can be achieved by using appropriate storage conditions or by using additives.

Composite materials are suitable for various applications due to their unique properties that can be customized by changing the type of polymeric substance and its composition. In the past decade, the usage of graphene and its derivatives were explored in detail. In recent years, polymeric composites supplemented with nanomaterials made up of graphene for applications such as sensors, actuators, and degradable electronic devices are of much interest. These composite materials are sensitive to experimental conditions and it is imperative to learn various processing conditions and effects of aging on different properties before using them for sensing applications. When developing conductive polymer composites and active devices using a biodegradable polymer such as PHA, the effect of aging becomes important as the polymer crystallinity (and therefore the resistivity of the composite material) may vary significantly with time [41].

2.4 Strain Sensor

It is challenging to attain high electrical conductivity with the useful mechanical properties of nanocomposite materials. In an attempt to achieve this, polyhydroxybutyrate film supplemented with reduced graphene oxide (rGO) was prepared from the solution separately. First, the graphene oxide was reduced by three different reducing agents—L-ascorbic acid, hydrazine, and sodium borohydride [28]. It was observed that reduction by L-ascorbic acid (at 8% loading) led to the highest electrical conductivity of 30 S/m, and low electrical percolation, which is at par with the known values for polymer/rGO composite. In addition, the composite material had good mechanical properties. Based on their mechanical and electrical properties, the composites were used to develop biocompatible sensors and actuators

(i.e., a strain/bending sensor) [28]. The unique properties of this composite were attributed to the good dispersion of the nanofiller within the matrix, large sheet size, and higher intrinsic conductivity of rGO. In situ reduction of GO is therefore suggested for the preparation of polymer/GO composite from solution. The PHB-rGO composite prepared using L-ascorbic acid had low toxicity and thus had the potential to be used as flexible piezo-resistive sensors for bioapplications, e.g., tracking the movement of any organism/body. Since the preparation involves solution casting, printing methods may be adopted to make composite patterns for other similar applications such as wearable sensors, degradable bio-circuits, and actuators.

Based on this knowledge, sensors were prepared on ink-jet printed silver electrodes coated with PHB-rGO composite. They showed superior selectivity to temperature (with respect to pressure and moisture) with 6 × 7 arrays of sensing elements as observed by thermal mapping [30]. Flexible devices having twisty patterns were also prepared using direct ink writing. Therefore, by the assessment of stretchable and flexible thermal sensors, it was demonstrated that PHB-based composites can be used to develop health monitoring devices. Piezoelectricity is the main functionality required in any biomaterials for bone tissue regeneration. Yet, combining biodegradability, biocompatibility, and 3D structure with a prominent piezoresponse is quite challenging. Recently, a hybrid 3D scaffold was developed using PHB and rGO flakes. Homogenous distribution of PHB fibers with better surface potential was obtained by increasing the content of rGO to the tune of 1.0 wt % (a zigzag chain formation between paired lamellae of PHB fibers). The hybrid 3D scaffold showing enhanced piezoresponse could also be used for tissue engineering applications [23].

3 Detection of Gases and Chemicals Using PHA-Based Sensors

Generally, in a living system, biological macromolecules containing redox proteins or enzymes are used to shuttle electrons. Thus, understanding these electron transfer pathways and the function of the proteins through in-depth electrochemical methods will help develop useful biosensors and similar devices. Interestingly, the transfer of electrons between the macromolecules and electrodes is generally prohibited. Thus, attempts were made to understand the underlying mechanisms, including those involved in electron transfer mediators and modified electrode surfaces for the development of an interface compatible with electron transfer. Since, some enzymes are naturally bound to lipid membranes, casting of proteins on biomimetic macromolecules such as lipids, surfactants, or other biopolymers was explored. These macromolecules were modified on electrodes for direct exchange of electrons thereby avoiding chemical mediators. Thus, enzyme-coated electrodes could be constructed for biosensor and biomedical applications. One of the earliest bio-based sensors made up of PHA were those used to detect glucose and organophosphorus pesticide [36]. Before using PHA polymer for sensing application, its durability, degradation, modulus of elasticity, yield strength, and maximum stress to

fracture were also checked. Nanofabrication of patterned PHA polymer was carried out on interdigitated array (IDA) gold electrode by thermal evaporation, photo-lithography, and etching process. An enzyme, Acetyl-choline esterase was linked to IDA by sol–gel process (tetraethylorthosilicate and polydimethylsiloxane were used as precursors). The sensitivity was assessed by an impedance analyzer. The resulting biosensor was sensitive to the analyte (conc. Range of 0.1–50 mM). The utilization of sol–gel immobilization method coupled with photo-lithography was found advantageous for large-scale production of a durable organophosphorus pesticide biosensor [36] (Table 1).

On a similar theme, two proteins namely hemoglobin and myoglobin were incorporated/immobilized on PHB film for enhanced electron transfer [32, 33]. It was found that myoglobin-PHB film modified on a pyrolytic graphite electrode enhanced quasi-reversible electron transfer and catalytic activity towards hydrogen peroxide (H_2O_2). Such devices can act as a model to construct third-generation H_2O_2 sensors. In an acetate buffer solution (pH 5.0), the apparent formal potential of myoglobin was about −260 mV, while the anodic and cathodic peaks were at −224 and −284 mV, respectively. The detection limit of myoglobin-PHB-based sensor was lower (~3.3×10^{-8} M) compared to that of the hemoglobin-PHB sensor. No interference was observed by compounds such as catechol, uric acid, ascorbic acid, epinephrine, and dopamine with the determination of H_2O_2. In addition, the developed biosensor showed reproducibility and could remain stable for upto 7 days when stored in a refrigerator. Since PHB is prone to biodegradation, such biosensors must be stored in a sterile environment until use.

The excretion of waste metabolites (including urea) is the major function of kidney. Therefore, urea level in body fluids is used to indirectly assess kidney health. An abnormal level of urea in the blood could be a result of renal dysfunction. Thus, the determination of urea is important in the area of medical diagnostics. Besides, urea determination is equally important for the analysis of seawater, drinking water, and agriculture water where urea is used as fertilizer. In this context, the urease enzyme was immobilized on PHB substrate for its use as a urea sensor. A combination of etching, photo-lithography, and thermal evaporation was used to prepare PHB patterned with gold interdigitated electrode arrays (IDA) as a transducer. Here, the enzyme was covalently linked to functionalized gold-IDA. The fictionalization was done by 10-carboxyl-1-decanethiol (CDT) and CDT-polypropylenimine dotriacontaamine (DAB), separately. In vitro assay for urea was done by both the immobilization methods and was compared for pH stability, linear dynamic range, and repeatability. CDT-Urease biosensor showed high affinity to urea (K_m of 0.7 mM) compared to CDT-DAB-Urease sensor (K_m of 2.2 mM). In contrast, CDT-DAB-Urease biosensor displayed a dynamic linear range in the range of 0.10–300.0 mM (30-fold higher compared to CDT-Urease). The effects of dendrimers linked urease to the sensing electrode were positively related to its analytical (sensing) performance such as temperature, pH, high sensitivity, broad linear dynamic range, and fast response rate of the biosensor [34]. Thus, the CDT-DAB immobilization method along with microfabrication technology would be helpful for large-scale production of PHB-based urea sensors. Such biosensors

could also be potentially used for the assessment of other clinically important analytes. However, further improvements are required to address the issues of storage stability, sensitivity, and interferents. Nevertheless, such a sensing device allows convenient, inexpensive, and biodegradable alternatives to produce sensors using an easy beaker chemistry technique.

Detection of gases and other volatile compounds present in the air is highly important particularly in industries as well as for ecological studies. Detection of fluctuating levels of gases on a real-time basis is also required for environmental monitoring. Previously, a lot of emphasis was given to the development of portable and handy gas-measuring inexpensive instruments that can quickly analyse a mixture of gases based on sensors with broad sensitivity. These were among the early versions of mechanical olfactory systems or electronic nose systems.

A PHB-based eco-friendly conductive sensor was developed to measure volatile organics and gases in a moist environment. A blend of PHB and polyaniline was used (different proportions of PANi/PHB) to prepare a biodegradable scaffold of electrospun nanofibers [35]. Several features of the PHB nanofibrous layers generated via electrospinning were studied. A highly porous membrane was formed when this PHB polymer was spun on chemoresistors. The effect of humidity on the sensing behavior of the resultant blended scaffold was analyzed using ammonia and other chemicals that interact differently with PANi. The resulting sensor was also checked for its stability and reproducibility. It was observed that due to the electrospun nanofibrous structure, in the presence of water vapors chemical interactions between the selected compounds were significantly improved. This finding suggested that such scaffold materials have implications for developing chemosensors that can work efficiently in humid environments.

Use of biodegradable polymers in making such sensors also started recently. Mesoporous graphitized carbon (MGC) was used as conductive nanomaterial to develop a chemical sensor that can be used to detect a broad range of gases and volatile organic compounds (VOCs). Indeed, graphitized carbon nanomaterials have been well-studied for sensing devices. Previously, graphene and its derivatives were used (both oxidized and reduced form) with or without doping with metal-oxides, other conductive polymers, nanomaterials, etc., depending on their potential applicability [42]. Gas adsorption on these materials is mainly because of weak van der Waals forces that can be manipulated by changing the functional group present in the nanomaterial. Gases such as NH_3, CO, and NO_2 had been detected (with partial selectivity) by sensors made up of graphene or related materials. Partial selectivity of gas molecules may be attributed to the gas adsorption of graphene surfaces on which the adsorbed gases act either as donors or acceptors of charge. Consequently, the transfer of charge altered carrier concentration, and, therefore, the conductivity of sensors [43, 44]. Based on this, the graphene-based sensors could display either p-type or n-type semiconductor behavior. In a unique study, nanocomposite polymer and nanofibrous layer were explored to develop such sensor whose sensitivity and selectivity were tuned by the working temperature. Two thermoplastic polymers were used (PHB and polystyrene) due to their versatile usages, eco-compatibility, recyclability, and resistance to thermal processes. In addition, both the polymers

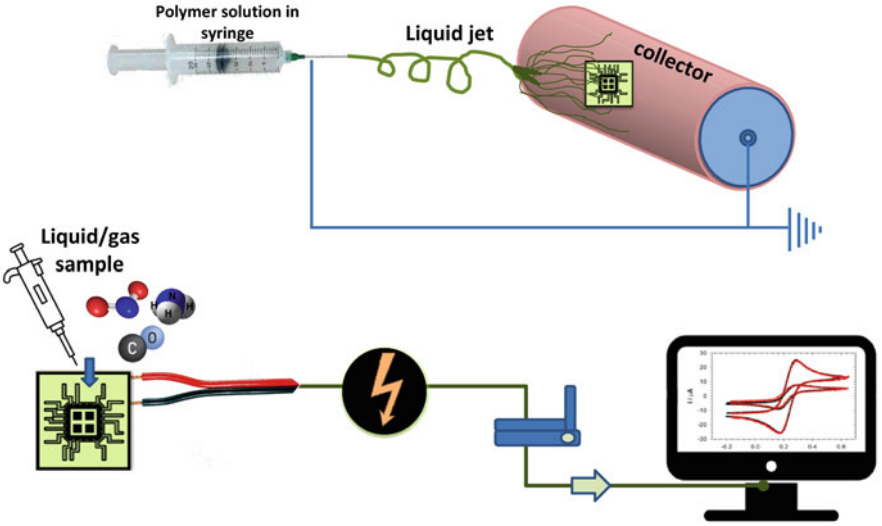

Fig. 3 Scheme of developing PHA-based sensor. The mixture of PHA and other composite material loaded syringe (top left) was allowed for electrospinning and the cylindrical collector having a pre-designed interdigitated electrode (IDE) gets coated with the biofibers. The fabricated IDE can be used as transducer (bottom) and connected to a PC for sensing current changes upon interaction with analyte (Adapted from [25])

were soluble in chloroform but insoluble in water, meaning that an easy yet unique electrospun material can be generated using polymer solutions prepared in chloroform. On the other hand, due to water insolubility, the resulting electrospun fibers can be used under a broad range of humid conditions without major structural transformations [43]. A simple one-step method was developed to deposit a peculiar layer of electrospun nanofibers (a mixture of PS and PHB) onto interdigitated electrode micro-transducers (Fig. 3). The nanofibers were thus designed to be porous and rough to achieve a higher surface exposed to the permeation of VOCs and gases while the MGC worked as conductive nanofillers. The MGC layer was a crystalline structure largely made of sp^2 carbon linked hexagonal honeycomb leading to unique properties such as high thermal stability and mechanical strength, superior carrier mobility, and large surface area. The microsensor developed by this method was able to work on a broad temperature of 40–80 °C with no evident degradation. The sensor was found to be highly selective and sensitive to CH_3COOH at 40 °C, which declines sharply when the operating temperature was kept at 80 °C. A similar observation was made with other VOCs (amine, acetone, and toluene). In contrast, sensitivity towards gas such as NO_2 increases with the rise in operating temperature (limit of detection—LOD reaches up to ~2 ppb). This shows the influence of operating temperature on the selectivity of sensors based on nanocomposite polymers [43]. Later, a similar device was prepared using a combination of porphyrin and MGC (Table 1). The porphyrin macromolecule resulted in a smooth and thin device that is more resistive at lower temperatures and more conducting at high

temperatures of 60–70 °C. The diffusion and adsorption of the analyte were governed by the combination of porphyrin present in the composite fiber. This was evident by the higher response rates for toluene and CH_3COOH [29]. Such reversal of the effect caused by temperature suggests the key role macromolecules will play in developing broad-spectrum biopolymer-based sensors.

4 Biomedical Applications of PHA-Based Composite Materials

4.1 PHA-Based Scaffolds

Solvent-based casting and assisted dispersion of composite material (a mixture of medium chain length-polyhydroxyalkanoate or mcl-PHA and multi-walled carbon nanotube or MWCNTs) led to wider mechanical and electrochemical properties. The nanocomposite film was seeded with rat primary mesencephalic cells for electro-stimulation study. The cell viability, morphology, and function were found to increase with higher content of MWCNT. This suggests the possibility of utilizing a green thermoplastic composite (PHA/MWCNT) in neural interfacing applications [45]. On the other hand, a similar mixture of PHB/MWCNTs was fabricated by *electrospinning and hot-stretching treatment*. Higher β-form crystals were present in the composite material compared to pure PHB nanofibers. Additionally, the presence of MWCNTs led to enhanced piezoelectric performances. The composite nanofibers of PHB/MWCNTs were advocated for the development of "smart" membranes to be used as sensors, actuators, and in other biomedical applications [46]. With the increasing fabrication by *3D printing* of biopolymers, designing devices using functionalized and tailor-made polymers have become frequent. Several reports are available on PHB composites prepared with electrically conductive MWCNTs. PHA is a piezoelectric and biocompatible polymer that can be mixed with MWCNT in different ratios to generate nanomaterial of desired morphology, dispersion, electrical, mechanical, and thermal properties [47]. The use of MWCNTs in the composite material increases Young's modulus and reduces strain at break. Such composite could be easily processed by 3D printers to prepare scaffolds having porous structures and stretchable meandering conductive traces. The biocompatibility and cytotoxicity of the resulting scaffold were tested using MRC-5 cells (fibroblasts), which suggested that these electrically conductive composites can be used as biomedical devices or electro-active materials for tissue engineering applications.

4.2 Detection of Antibiotic, Body Fluids, and Microbes Using PHA-Based Sensors

Being a hydrophobic molecule with unique polymer properties, it is easy to mold, electrospray, and imprint the biodegradable film to construct small sensing devices.

Some of the unique devices made using PHA polymer are already described in the previous section. Here, we will discuss some of the unique applications of PHA-based sensors for the detection of antibiotics, body fluids, and microbes. It must be noted here that the biomedical application of PHA is desirable for several beneficial reasons: being biodegradable hazardous medical wastes may be easily handled, generate more revenue from medical applications compared to other industrial applications (e.g., packaging industry as the competition from the synthetic polymer is very high), being a biocompatible thermoplastic polymer it can be blended and used with other polymers.

There are several analytical techniques used to detect the presence of drugs or any specific compound such as a spectrophotometer, antibody-based assay (e.g., ELISA), HPLC, GC-MS, and LC-MS. However, very limited methods have been developed to exploit electrochemical devices for real-time and quick detection of biomolecules. Phukon et al. demonstrated the use of PHA-based composite material to develop sensors that can detect antimalarial drug artemisinin [37]. A PHA/AuNP/HRP/ITO nanocomposite was used in this study. At first, PHA polymer was extracted from stationary phase culture of *Pseudomonas aeruginosa* BPC2 (isolated from the crude oil contaminated soil of Assam Asset, ONGC, Assam, India). The extracted biopolymer was mixed with $HAuCl_4$ to prepare PHA-AuNP nanocomposite and cast over indium tin oxide (ITO) glass plate. Subsequently, the nanocomposite material was used to immobilize horseradish peroxidase (HRP) enzyme using adsorption technique (Table 1). The electroactivity of the composite and HRP adsorption on the film was assessed by analytical techniques, viz., electrochemical impedance spectroscopy, cyclic voltammeter, and scanning electron microscopy. Pure samples of artemisinin and real body fluids were tested to check the sensitivity of the nanocomposite. The electrochemical activity of artemisinin in the tested condition was suggested to be an irreversible process controlled by diffusion. There was an increase in signal with a rising concentration of artemisinin (a range of 0.01–0.08 μg/mL was tested) in a linear fashion. In addition, the sensitivity of the biosensor towards the antimalarial drug was quite high with a LOD of 0.0035 μg/mL and 0.0036 μg/mL in bulk and spiked serum, respectively. The process was significantly reproducible and repeatable with accuracy showing the potential of this biosensor in the pharmaceutical industry and medical diagnosis. If developed in a refined manner, such biodevices could become a great eco-friendly alternative in comparison to other detection methods used to identify artemisinin and other compounds in body fluids [37].

A unique device was fabricated based on mcl-PHA to design an inductor-capacitor having an interdigitated capacitor with electrodes and an inductor as a planar layer, unlike other spiral inductors. The developed sensor was used to remotely detect liquids such as artificial saliva or gastric fluid [5]. Wireless coupling was based on an antenna coil that allows multiple advantages—it avoids the use of wires, eliminates chances of contamination, and is non-destructive for tested components. Capacitance was used for the measurement; it is lowest in air and increases with the applied samples such as saliva or gastric acid. The capacitance is inversely proportional to resonant frequency based on the following equation:

$$f_{\text{res}} = \frac{1}{2.\pi\sqrt{L.C}}$$

Due to the different compositions of the liquid samples, there were differences in the relative permittivity. Therefore, the variations in the dielectric constant were followed to change capacitance and thus the resonant frequency [5]. By using the theory of wireless coupling and quantifying the changes in the resonant frequency of the device chosen liquid samples were detected successfully. Such devices will be helpful in specimen detection and can later be fine-tuned for specific body fluids that may vary in normal and patients having some ailments.

Microbes are omnipresent and some cause infections leading to disease. Thus, it is also important to know their presence in our body fluids, on our body, food, and the outside environment. Several culture-based methods and molecular techniques are available to identify the presence of pathogens in any sample. However, a PHA-based sensor to identify the presence of food-borne pathogen *Escherichia coli* O157:H7 was recently developed [40]. Here, PHB polymer was used to immobilize engineered bacteriophage PP01 containing nanoLuc gene (encoding for luciferase enzyme) responsible for bioluminescence. The electrospun fiber or solvent cast PHB films were first prepared and then incubated with the recombinant phage for immobilization. The polymer with immobilized phage was then used to detect the presence of *E. coli* in a liquid sample and non-woven PHB fiber was compared with solvent casted films. Electrospun fiber showed higher infectivity (higher phage density) and thus had good sensitivity and response time as desired for any biosensor. This was further evident from the test conducted with contaminated milk and tryptic soy broth containing 10^5 CFU/mL and 10^1 CFU/mL with a successful detection within 1 h and 5 h, respectively. This demonstrated that such PHA-based biosensors can be an inexpensive tool for the detection of various microorganisms and can be used for food safety assessment [40].

Even viruses were detected efficiently by PHA-based biosensors. Electrospun fibers of PHB were coated with polymethyl methacrylate-*co*-methacrylic acid (poly—MMA-*co*-MAA). This platform was used to immobilize antibodies specific to the dengue virus and consequently detect enveloped dengue viruses by enzyme-linked immunosorbent assay (ELISA). Here, the fiber structure of PHB allows a large surface area for the interaction of biomolecules. On the other hand, the use of co-polymer (poly—MMA-*co*-MAA) coating on the surface inherits the permanent presence of –COOH group (from MAA segment) that can be used for covalent linkage and physical immobilization of proteins. The concentration of reactive surface (having –COOH group) can be manipulated by changing MAA concentration within the co-polymer. Immobilization of dengue antibodies was done by both physical attachment and covalent linkages based on carbodiimide chemistry. The antibody immobilization was confirmed by high-end microscopy, UV-VIS titration, and water contact angle. The developed sensor was efficient in terms of sensitivity and higher signal intensity as compared to the conventional ELISA method [39].

As discussed above, the purified PHA polymer is used alone or in combination with other composite materials to design a unique sensing device. However, the

naturally occurring intracellular PHA granules have also been explored for their potential role in protein purification, studies related to protein-protein interaction, and diagnostics. Indeed, a platform technology was established using the PHA depolymerase substrate binding domain (SBD) as a fusion partner to attach the desired protein on PHA surface. Thus, protein fused to the depolymerase gets immobilized on PHA microbead, thin polymer film, and on a printed PHA surface. This device was demonstrated to monitor the interaction between PHA and SBD. Proteins such as red/green fluorescent proteins, SARS coronavirus surface antigen, and single chain antibody against hepatitis B virus (preS2 surface protein) were studied in detail. This platform technology can effectively be used to learn and understand protein-protein/biomolecule interactions [38]. Recently, an Advanced proteoLytic detector PHA (AL-PHA) beads were developed as a low-cost alternative to protease sensors. A library of about 20 PHA-based biodegradable biosensors was prepared utilizing PhaC-reporter fusion proteins that remain attached to the outer layer of natural PHA granules [22]. When any proteases are present in the vicinity, the superfolder fluorescent reporter protein gets cleaved from the bead leading to a loss of fluorescence. Such AL-PHA beads were demonstrated to detect the presence of Tobacco Etch Virus (TEV), cancer-associated metalloproteinases in extracellular vesicle and cell-conditioned media samples, and cercarial elastase from Schistosoma mansoni-derived cercarial transformation fluid (SmCTF).

5 Conclusion and Future Perspectives

This chapter presents the potential usage of PHA polymers as a supporting material to fabricate low-cost sensing devices. Being a thermoplastic, biocompatible, and biodegradable polymer, PHA is well-suited for applications in biomedical diagnostics and related applications. In addition, the piezoelectric property of the polymer provides additional utility in developing any miniaturized sensing device. Several approaches can be adopted to fabricate PHA and PHA-based composites that include—solvent casting, electrospinning, 3D-printing, etc., making it a versatile substrate to develop high-quality novel bio-tools for broad implications. Polyhydroxyalkanoates (both extracted polymer and natural granules) provide an effective platform to immobilize a plethora of bioactive molecules. Therefore, a vast opportunity is available with PHA-based sensors that are cost-effective, portable, lightweight, miniaturized, and eco-friendly (a requisite for any typical on-site detection devices). Here, we have collected a few unique optical and electrochemical sensors developed using various types of PHA and other materials. Such devices were used for the detection of temperature, pressure, strain, chemicals, body fluids, proteins, bacteria, and viruses. Due to the unique feature of PHA polymer, it is believed to be more exploited and used for real-life applications and become considerably successful in the near future. However, these polymers are yet to be applied in other areas of monitoring such as food safety, biodefense, and forensic applications. The hidden opportunity of modifying and exploiting different monomers (present with PHA polymer) such as ter-*co*-polymer and molecular

weight of the polymer are yet to be explored. As of now, only lab-scale devices have been developed, and thus more efforts are required toward research innovation for commercial-scale fabrication of PHA-based sensors for automated and real-time biosensing devices.

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An Overview of Immunosensors and Their Application

Anil Kumar Gupta, Sambhavi Animesh, and Amit Singh

Abstract

A key challenge in clinical healthcare is meeting the need to detect a disease at an early stage. Early and accurate diagnosis not only cuts the treatment cost but can also reduce disease burden, mortality rate, and social inequalities. Therefore, researchers are always searching for a method that allows rapid, simple, sensitive, selective, and cost-effective detection of the target biomarker (peptides, proteins, or nucleic acid). Immunosensors are one such point-of-care diagnostic device that can play an important role in almost all clinical healthcare fields. They are a promising alternative to the traditional immunoassays and state-of-the-art affinity sensors to diagnose clinically important analytes/antigens due to their high affinity, versatility, compact size, fast response time, minimum sample processing, and the measurements' reproducibility. For many decades now, significant advancement has been made in the immunosensor field in which the use of nanomaterials for increased sensitivity, multiplexing, or microfluidic-based devices may have the potential for promising use in clinical analysis. This chapter will provide an overview of the currently available immunosensor technology, its

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types that are currently being developed, and the limitations and future directions of immunosensor technology for the clinical laboratory.

Keywords

Immunosensor · Immobilization · Antigen · Antibody · Communicable and noncommunicable disease

1 Introduction

Biological and biochemical processes are paramount in clinical diagnostics, medical applications, bioreactors, food quality control, agriculture, industrial wastewater, mining, and the military defense industry. However, conversion of the biological data directly to an electrical signal is challenging. The application of biosensors has increased significantly due to improved procedures and gadgets, especially in the conversion of biological signals to electrical signals. Nowadays, numerous biosensors have been produced industrially and are being utilized to develop large-scale multi-valued sensing systems. The first biosensor was developed by Clark and Lyons [1] to quantify glucose level in clinical samples using electrochemical detection of oxygen molecules via glucose oxidase electrode. From that point forward, remarkable advancement has been made both in innovation and the uses of biosensors with inventive methodologies including electrochemistry, nanotechnology to bioelectronics [2, 3]. The bioelement (antibody, aptamer, etc.) immobilized on the transducer's surface binds to the target molecule and passes signals to the transducer. The superiority of biosensing elements in the generation of real-time signals is incredible. They can detect the target molecules even in the picogram quantities and are therefore considered a powerful tool to detect pathogens at their initial infection phase [4]. This unique feature of biosensors has motivated researchers to develop new biosensing technologies continuously and the industry is now worth billions of dollars [4, 5].

Immunosensors are one of the most important biosensors classes, widely accepted as an analytical instrument, especially in the healthcare section due to their excellent detection efficiency and accuracy. The latest advances in immunosensor make it possible to combine detection with the current digital technology and miniaturize them without compromising the performance [6]. This book chapter will provide an overview of the immunosensor technology currently available, its types that are currently being developed, and also address the limitations, challenges, and future directions of immunosensor technology for the clinical laboratory.

1.1 Biosensors

The biosensor is a self-contained analytical device, combined with a biological element (biosensing components) and a physicochemical component (transducer component) [4] (Fig. 1). It detects changes during the biological process and converts them to an electric signal. Typically, a biosensor consists of three basic components as follows:

1. Detector (detect the biomolecule and generate impetus).
2. Transducer (convert the impetus to output signal).
3. Signal processing system (Process the output and present it in a user-friendly form).

1.2 Classification of Biosensor

The biosensors can be categorized on the basis of physicochemical transduction action or the type of biorecognition element. It can be classified as electrochemical, mechanical, and optical biosensors according to transducers used. Further, electrochemical biosensors can be reclassified as amperometric biosensors— measure current produced during oxidation/reduction of reactant, potentiometric biosensors— measure the potential of the biosensor electrode with respect to a reference electrode, and conductometric biosensors— measure the change in conductance arising due to the biochemical reaction [7]. An overview of biosensor classification is shown in Fig. 2.

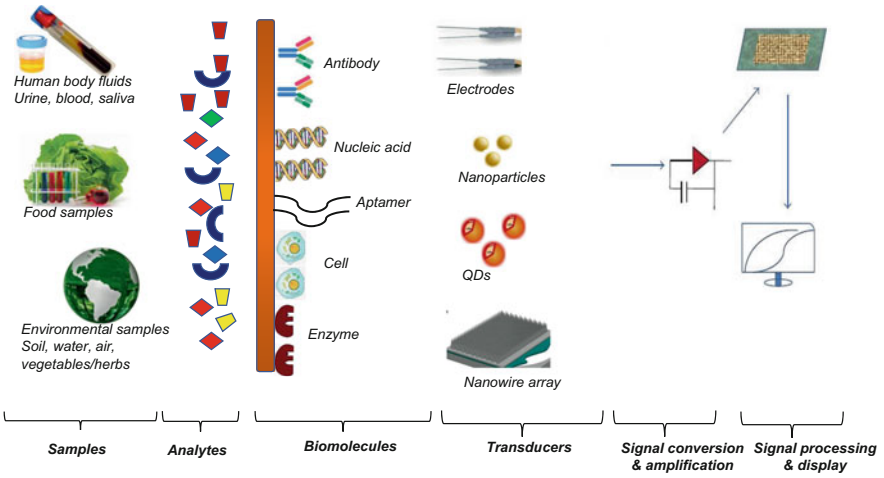

Fig. 1 Schematic diagram showing the components of a biosensor

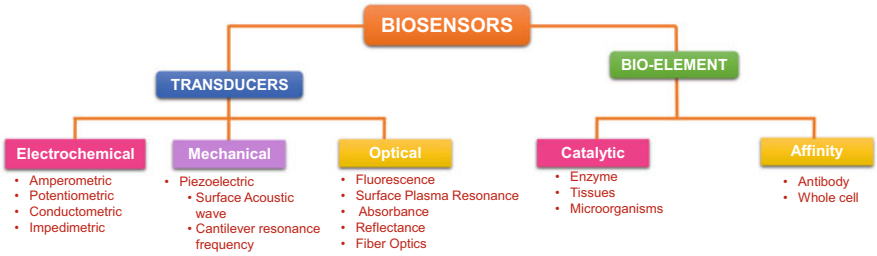

Fig. 2 Classification of biosensors

1.3 Immunosensor

The term "Immunosensor" is specifically employed to designate the entire instruments, i.e., immunoreaction-based biosensors. Immunosensors are solid-state devices in which the signals of immunochemical reaction are captured by a transducer. In the immunosensor design, the sensing component is formed by the immobilization of antigens/antibodies, and the binding events are being transformed into a measurable signal by the transducer [8].

1.4 Principles of Immunosensor

The immune system is a complex network of cells and proteins, which guards the body against infection. It keeps a record of every infectious agent (microbes) defeated once to recognize and destroy the microbe quickly; if it enters the body again. In the presence of foreign substances (*i.e.,* antigens), cells of the immune system produce specialized immunoglobulins (*i.e.,* antibodies) that bind specifically to these antigens. This phenomenon has many applications, including the development of sensors. "*A sensor that is based on the concept of immunology uses an antibody (as a bioreceptor) for specific molecular recognition of antigens and subsequently forms a stable immunocomplex are known as an immunosensor.*" An immunocomplex formation is determined and measured by coupling this reaction (signals) to a transducer's surface. The electrical device detects the signals and converts it to an electrical signal where it is processed, recorded, and viewed (Fig. 3). The produced analytical signals are directly proportional to analytes' concentrations [6, 9].

At the time of immunochemical reaction, the highly specific recognition of an antibody's variable regions with the epitopes of an antigen occurs via different types of bonding such as hydrophobic and electrostatic interactions, van der Waals force, and hydrogen bonding. The produced antigen–antibody complex is generally reversible due to a weak force holding the antibody and antigen together. The antigen–antibody complex formed would dissociate with a slight change in the reaction environment (e.g., pH or ion strength). The strength of an antibody binding to an antigen is generally characterized by its affinity constant (K). The high affinity and

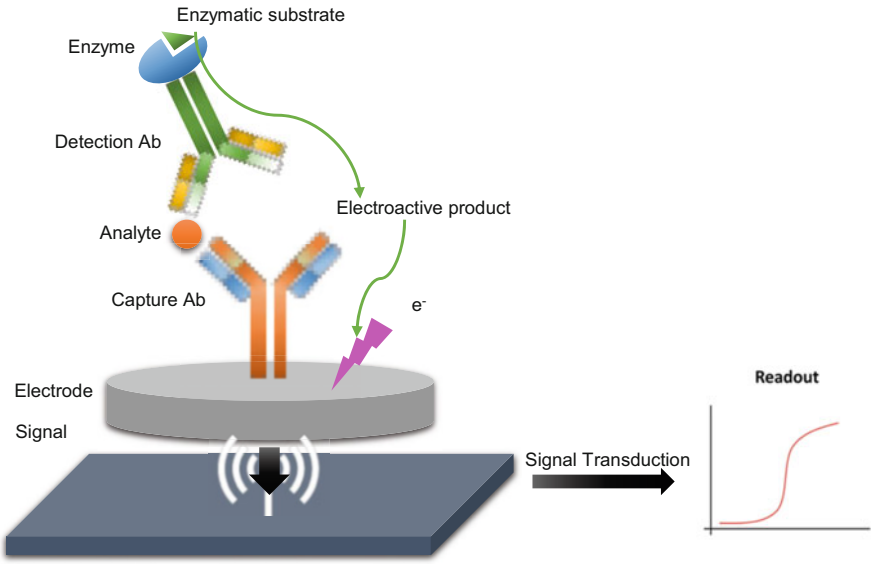

Fig. 3 A systemic diagram of immunosensor

specificity of this antigen–antibody complex determine the key feature of the immunosensor [6, 10]. An ideal immunosensor should be designed with the following specifications:

1. Should identify target antigens very quickly.
2. Should be able to generate antigen–antibody complexes without the requirement of supplementary reagents.
3. Should be able to produce results with high accuracy and reproducibility.
4. Able to detect the target in real samples easily.

1.5 Structure of Immunosensor

The immunosensor is mainly composed of three elements: bioreceptors, transducers, and electronics. The structure of the immunosensor is depicted in Fig. 3.

- Bioreceptors are biological recognition elements (antibody, enzyme, enzyme-substrate, aptamers, haptens, or nucleic acid) capable of detecting a particular target analyte such as enzyme-substrate, complementary DNA, antigen, or ligands. These elements are either integrated insight or intimately related to a physicochemical transducer.
- The second and most critical component of the immunosensor is the transducer, used to convert biochemical signals produced by the analyte's interaction with the receptor into an electrical signal. The intensity of the signals produced via biochemical reaction is directly or inversely proportional to the concentration of

the analyte. Electrochemical transducers are most commonly used to develop immunosensors. These systems provide some advantages, i.e., simple and unique design, low cost, and compact size [6].

- The electronic part is considered as the third component of the immunosensor, which is used to amplify and digitalize the physicochemical output signals from the transducer devices such as "electrochemical (potentiometric, conductometric, capacitive, impedance, amperometric), optical (fluorescence, luminescence, refractive index), and microgravimetric devices" [11, 12].

1.6 Why Immunosensor Is a Better Choice Than Other Sensors?

Since the discovery of biosensor in the early 1950s, it has become very important tools in the fields of agriculture, industrial processing, food processing, pollution control, and environmental monitoring. The immunosensors have certain advantages that make them superior to other states of the art sensors due to their compact size, low cost, quick response time, higher sensitivity, and selectivity [13]. Further, they offer easy-to-use and easy-to-automate, digitize, and miniaturize. They may bypass some inherent problems of traditional analytical methods [14]. Therefore, immunosensor is increasing focus of researchers in immunochemical studies due to their immense clinical diagnosis potential [15, 16], environmental analysis [17], and monitoring of the biological process. A great deal has been achieved in diagnosing certain diseases by measuring markers or pathogenic microorganisms responsible for the illnesses such as proteins, enzymes (glucose oxidase), microorganisms (HIV, Toxoplasmosis, Syphilis, Leishmania, Malaria), and hormones (TSH, LH) using fast and responsive immunosensor. For example, an amperometric immunosensor was recently developed, which detects *Trypanosoma cruzi* (*T. cruzi*)-specific antibodies in patient blood samples and tracks the anti-*T. cruzi* antibody decay during the treatment of chagasic patients [18, 19]. The applications of the immunosensors have been discussed in the latter part of this chapter.

2 Immunosensing Elements

An immunosensing element (biorecognition element) comprises a molecular probe to detect the target/analyte present in the samples. It is the most critical part of the immunosensor as each biorecognition element has its advantages and disadvantages that determine the overall performance of the immunosensor. The analyte specificity is mainly dependent on the selectivity and robust affinity between the biorecognition element and the target analyte [12, 20]. "A sensor based on the concept of immunology where the antibody is used as a bioreceptor for the specific molecular recognition of antigens and subsequently forms a stable immunocomplex are known as Immunosensor." The most prominent biorecognition elements used in the development of immunosensors are antibodies (Ab), antigens, and aptamers.

2.1 Antibodies

Antibodies are naturally occurring proteins of approximately ~150 kDa in size. Typically, an antibody consists of a light chain and a heavy chain which are linked by disulfide bonds to form the characteristic Y-shape. As shown in Fig. 2, the ends of each arm carry two identical variable regions (Fv) of the antibody for the recognition of the antigens, whereas the rod-like part containing constant sequences (Fc) is essential for the physiological functions of antibodies. (Fig. 4). The variable region encompasses three hypervariable areas, known as complementarity-determining regions (CDRs), responsible for the specific antibody–antigen interaction. This region encodes a unique recognition pattern which binds with the analyte with very high accuracy and affinity; therefore, they are used as a biosensing element. The diversity in CDRs allows the endless supply of Abs with different specificity and binding strength (affinity). Of the many immunoglobulins classes (i.e., IgE, IgM, IgG, etc.), the immunoglobulin G is the most prominently used class in the biosensing field [21].

The antibodies bind with its target with variable stringency which mainly depends on whether the antibodies are monoclonal or polyclonal. Monoclonal antibodies are highly specific recognizing only one epitope of a target molecule. On the contrary polyclonal antibodies can recognize totally diverse epitopes of the identical target. Because monoclonal antibodies are specific to a single epitope, they are less prone to cross-reactivity than polyclonals; however, polyclonals' cost is more economical, so polyclonals are still used. Along with the whole antibody, single-chain Fv fragments (scFv) and antibody fragment-antigen binding (Fab') units are widely used in the development of Immunosensors. An scFv is a fusion protein consisting of a variable region of heavy (V_H) and light (L_H) chains joined together by a flexible peptide linker of 10–25 amino acids [22, 23]. The Fab' is a region on an antibody consisting of one constant and one variable domain of each heavy and the light chain. It is mainly responsible for binding to the antigens [24]. Although antibodies have won enormous popularity, they still suffer from certain limitations. The antibodies are

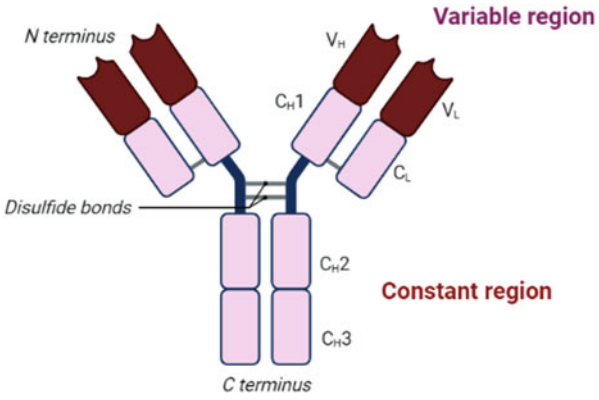

Fig. 4 Structure of immunoglobulin (IgG)

produced in animals, which is costly and time-consuming, limiting new antibodies' discovery.

Further, once an antibody is discovered, the isolation and purification procedures can be expensive. As antibodies are proteinous, they are highly sensitive to pH, ionic strength, and temperature, affecting their activity. The antibodies are produced in a living organism therefore display batch to batch variation. Additionally, the sensor regeneration is challenging in immunosensors because the dissociation of the Ag–Ab complex from the sensor surface often requires a drastic change in conditions such as low pH and high ionic strengths which denatures the antibodies. Moreover, antibodies cannot be produced against a non-immunogenic target [21, 22].

2.2 Antigens

Antigens are defined as molecules that can elicit an immune response in the body against any foreign substances. They contain distinct sites known as epitopes recognized and interacted with various immune system components such as antibodies. Sometimes antigens are immobilized on the sensor as a biosensing element to detect antibodies in the samples [25]. These immunosensors are most commonly used in serological assays to detect infections, pathogens, viruses, etc. Laila and coworkers have developed novel competitive electrochemical immunosensors for the simultaneous detection of different types of coronavirus (CoV), such as Middle East respiratory syndrome corona virus (MERS-CoV) [26]. The AuNPs electrodeposited carbon disposable array was used as an electrode. The human corona virus (HCoV) or MERS-CoV antigens were immobilized on the electrodes. The biosensor's main principle is the indirect competition between the free virus in the sample and the immobilized MERS-CoV protein. The sensor shows a linear response in the concentration range from 0.001 to 100 ng.mL^{-1} and 0.01 to 10,000 ng.mL^{-1} for MERS-CoV and HCoV, respectively. It can detect in 20 min with a detection limit as low as 0.4 pg.mL^{-1} for HCoV and 1.0 pg.mL^{-1} for MERS-CoV. The method is single-step, sensitive, and accurate [26].

The Antigens' utility as a sensing element has a significant advantage of detecting antibodies against any infection. But, it also has disadvantages similar to antibody-based sensors such as stability, specificity, and immobilization concerns. The antigens are often proteinous in nature, either purified or synthetically prepared for immobilization on the sensor platform [27]. These proteins might not undergo correct folding while expression and do not have the correct structure similar to native proteins, for which the antibody was generated in the body. These factors might affect the sensitivity and specificity of the sensor. However, careful consideration involving assays to validate the protein structure at the start of development will significantly help in creating a viable sensor.

2.3 Aptamers

Aptamers are tiny-sized, single-stranded oligonucleotides, either RNA or DNA or peptides. It folds into a well-defined 3D structure, which provides high specificity while binding to their corresponding ligands by complementary shape interactions. Aptamers are selected from a randomly synthesized initial library containing up to 10^{15} different oligonucleotides molecules through a combinatorial chemistry procedure termed Systematic Evolution of Ligands by Exponential Enrichment (SELEX) consisting of repetitive cycles of selection and amplification (Fig. 5) [28–30]. Aptamers recently emerged as a new class of biorecognition probes that can be used in the biosensor. Such biosensors are known as "Aptasensors." Aptamers offer a broad point of interest over other existing biological recognition components in terms of stability, design flexibility, robustness, and cost-effectiveness [31, 32]. As aptamers are either DNA or RNA, they can be easily tailored and more readily engineered with various reporter molecules like fluorophores, quantum dots, methylene blue, etc., without affecting their affinity. Aptamers generally undergo a change in conformation on binding with their target. This property provides the advantage of designing unique switchable aptasensors. Aptasensors are more stable than immunosensors and can be easily regenerated for reuse [31, 33–36].

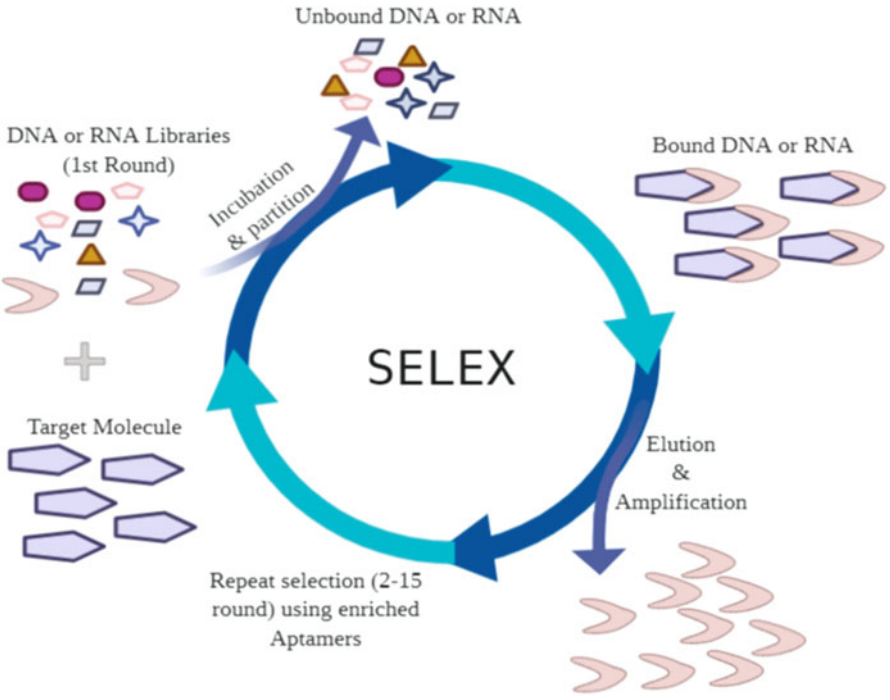

Fig. 5 Systematic evolution of ligands by exponential enrichment (SELEX)

Researchers are combining antibodies and aptamers to develop novel "Apta-immunosensors" in a sandwich format [37–39]. For example, a capacitive aptamer-antibody sensor was developed by Qureshi and coworkers for the detection of "vascular endothelial growth factor-165" (VEGF) in human serum. The anti-VEGF aptamer was immobilized on the sensor surface, followed by sandwiching with VEGF antibody-coupled magnetic beads, enhancing the response signal by 3–8-folds [38]. In another example, Zhu et al. [39] demonstrated the successful detection of "human epidermal growth factor receptor 2 (HER2)" and HER2-overexpressing breast cancer cells by an electrochemical sandwich sensor, where antibodies were used as a capture probe and aptamers as an indicator probe. In another example, Guo et al. developed aptamer-antigen-antibody sandwich biosensor based on LSPR [40]. They used Au nanorods containing coagulase binding aptamer as a capture receptor and tagged anti-thrombin protein as an LSPR signal amplification probe. The developed sensor was reusable and its LOD increased from 18.3 to 1.6 pM.

Overall, on comparing the scFv, Fab, and aptamers as biosensing element, scFv fragments-based immunosensors display the highest customizability, i.e., functional groups, immobilizing peptides, etc., due to their recombinant synthesis techniques. On the other hand, if time and cost are an issue in developing the biosensor, Fab' fragments should be chosen as they are relatively cheap and can be produced quickly from whole antibodies which takes several days. Nevertheless, if sufficient funds and time do not seem to be a factor, aptamers should be utilized as they show the best affinity toward their target analytes and are incredibly stable (excellent biosensor renewability) [41]. Additionally, aptamers generally undergo a change in conformation on binding with their target. This property provides the advantage of designing unique switchable aptasensors.

3 Immunosensor Format

Based on the detection format, Immunosensors can be direct (non-labeled) or indirect (labeled) Immunosensors.

3.1 Direct or Non-labeled Immunosensor

Direct immunosensors involve the linking of biological elements "intimately" (e.g., affixed, adsorbed, chemically bonded, adhered, coupled to, or otherwise in direct physical contact) with the transducer for the "direct detection" of the binding event to occur. In this type of immunosensor, labeling is not needed; thus, the sensors can be used for quick and real-time analysis. However, the label-free immunosensors have certain limitations of nonspecific adsorption of antibodies on the surface, leading to an increased background signal. Hence, it is vital to use a proper blocking agent such as bovine serum albumin (BSA), surfactants (Tween 20, polyethylene glycol), casein, and thionic compounds for gold surfaces [10, 42, 43].

3.2 Indirect or Labeled Immunosensors

The indirect immunosensors use a labeled secondary antibody for the detection of the signal. In an indirect detection format, signals are generated from the biorecognition elements' labels. The most unremarkably used labels are enzymes, i.e., peroxidase, alkaline phosphatase, glucose oxidase, catalase, or luciferase. Some other labels such as electroactive compounds (ferrocene, Prussian blue or In^{2+} salts), fluorophores (rhodamine, fluorescein, Cy5, ruthenium diimine complexes, phosphorescent porphyrin dyes, etc.), metallic nanoparticle (gold or silver), and quantum dots are also used [14]. Compared to label-free, labeled immunosensors have advantages like higher sensitivity and lower nonspecific adsorption, leading to lower background noise. However, it has some drawbacks, like the labeling process might affect the antigen–antibody binding efficiency [10, 42, 44, 45].

Indirect immunosensors can be further divided into two other types of formats: competitive format and non-competitive format. In competitive immunosensors, the sensor is first incubated with a mixed solution containing a known amount of labeled antigen and an unknown sample, where both compete to bind to the limited number of available antibody binding sites. The signal obtained from the labeled analyte is inversely proportional to the concentration of the sample analyte. The sample analyte is quantified by determining the amount of labeled analyte–antibody binding reaction. Competitive assays are commonly used to analyze small molecules because their small size limits the number of antibodies that can bind to the analyte due to steric hindrance [45, 46].

In a non-competitive format, the secondary antibody is labeled and the detection is in a sandwich format. Thus, the antigens' prerequisite criteria in this format are that it must possess at least two epitopes binding to two specific antibodies. In this assay, excess amounts of primary and secondary antibodies are used, and the analyte is sandwiched between two antibodies. An antibody immobilized on the solid substrate surface is called a capture antibody (primary) that captures the sample's antigen. Another one is the secondary antibody, a labeled antibody that binds to the other epitope of antigen and generates a signal for the detection [10, 45].

4 Classification of Immunosensors

The immunosensors are composed of an antigen-antibody reaction that generates an analytical signal converted into a transducer's physicochemical response. In the immunosensors, the target could be either an antigen (Ag) or an antibody (Ab). Nevertheless, the most popular approach involves detection of Ag using antibodies, but some works also report Ab detection, such as determining specific antibodies against pathogenic infection and autoimmune diseases. Immunosensors can be classified into three main classes, including electrochemical, mass-sensitive, and optical, according to the sensing platform used in the sensors [10]. However, there are immunosensors based on other transduction mechanisms like thermal changes (thermometric).

4.1 Electrochemical Immunosensors

The electrochemical immunosensors combine the antigen–antibody reactions with electrochemical measurements. The biorecognition elements, i.e., an antigen or antibody, are fabricated on an electrode's surface. The binding of the recognition element with its target biomarker/proteins results in electrochemical current/or voltage changes, which are measured [6, 47]. Thus in this immunosensor, the electrochemical signals are generated only by antigen–antibody complex and are not influenced by the concentration of unbound detecting secondary antibodies. Therefore, it is unnecessary to remove an unbound detecting agent, significantly reducing operation time [44]. Typically, an electrochemical biosensor is either a three-electrode or a two-electrode. The three-electrode format consists of a working electrode, a reference electrode, and a counter electrode.

On the contrary, the two-electrode system consists only of a working electrode and a reference electrode. However, the three-electrode system can protect the change in the reference electrode's half-cell potential because the charge from electrolysis passes through the counter electrode [47]. On the other hand, the two-electrode system is more straightforward and cheaper.

Electrochemical biosensors comprise the largest group of chemical and biological sensors [48]. Electrochemistry for analyte detection in immunosensors has several advantages; for instance, it is cost-effective, easy to operate, portable, and simple to construct. Besides, as electrochemistry is a surface-based method, minute samples are only required for detection purposes; hence, the reaction volume does not matter [49]. Further, the electrochemical immunosensors are classified into amperometric, potentiometric, impedance, and conductometric based on their transduction mode [50, 51].

4.1.1 Amperometric

Amperometric immunosensors measure the current output generated due to oxidation and reduction reactions of an electroactive species at a constant voltage. If the current is measured throughout controlled variations of the potential, it is stated as *voltammetric*. In amperometric, the measured current is proportional to the concentration of the analyte of interest. Amperometric immunosensors might be direct, where the sensing elements are non-labeled and the natural changes due to immune complex formations are detected. However, only a few applications implicate direct sensing since most of the analytes (protein) are not intrinsically able to act as redox molecules. Therefore, indirect amperometric immunosensors are most commonly used, where an electrochemically active label is needed for the analyte's electrochemical reaction at the sensing electrode. Enzymes, such as horseradish peroxidase (HRP) and alkaline phosphatase (AP), are the active labels most commonly used to catalyze the reaction of substrates to form electroactive products [52, 53]. Indirect amperometric immunosensors are more sensitive and versatile [10]. It is widely used as a cancer biomarker and cancer cell detection.

4.1.2 Potentiometric

These immunosensors mainly measure any potential changes that occur due to immunocomplex formation between antibody and antigen. Potentiometric devices measure any change in the accumulation of a charge potential at the working electrode against a reference electrode at zero current [54, 55]. In alternative words, potentiometry provides data regarding ion activity in an electrochemical reaction. The fundamental principle of all potentiometric transducers is governed by the Nernst equation [56]. According to this equation, potential changes are logarithmically proportional to the precise ion activity.

$$\text{EMF or } E\text{cell} = E^\circ \text{cell} - \frac{RT}{nF} 1nQ$$

E_{cell} represents the observed cell potential at zero current; this is sometimes referred to as the electromotive force or EMF. E°_{cell} is a constant potential contribution to the cell, R *is* the universal gas constant, T *is* the absolute temperature in degrees Kelvin, n is the charge number of the electrode reaction, F is the Faraday constant, and Q is the ratio of ion concentration at the anode to ion concentration at the cathode.

The potentiometric immunosensors have the advantages of simplicity of operation by automation and miniaturization of sensors. It also offers low LOD (between 10^{-8} and 10^{-11} M), which is important for cancer detection as biomarkers' concentration is very low in the early cancer stages. However, these sensors' major limitation is their sensitivity, which is lower than other immunosensors because, in most of the immunoaffinity reactions, the change in potential is relatively small [10]. Another challenge is the nonspecific effect of binding or signaling due to the other ions present in the sample. This often leads to a high signal-to-noise ratio, which is difficult to circumvent [57].

4.1.3 Impedimetric

In the impedimetric immunosensor, the impedance consists of a resistive and a capacitive part due to a complex interaction with a small amplitude voltage signal as a function of frequency, and the resulting current is recorded. Impedimetric immunosensors function by applying electrochemical impedance spectroscopy (EIS) to a biosensor platform using antibodies as receptors, which provide excellent sensitivity and selectivity. Unlike amperometric and potentiometric systems, impedance biosensors are label-free and do not depend on any specific enzyme for the analyte detection [15, 58–60].

4.1.4 Conductometric

Conductometric immunosensors are based on altering electrical conductivity at a constant voltage caused by immunoreaction that specifically generates or consumes ions. When a biorecognition element binds to its analyte, it changes ion species' concentration. This further leads to a change in the conductivity of the solution or current flowing through them. The signal generated due to such changes is generally

measured by an ohmmeter or multimeter. Conductometric immunosensors have several advantages, including low driving voltage, large-scale production, and miniaturization suitability without a reference electrode [61].

4.2 Optical Immunosensors

In optical biosensors, the analyte-antibody reactions are integrated with an optical transducer system, enabling the visible response whenever an analyte of interest is present in the sample. These are based on the quantification of chemiluminescence, absorbance, phosphorescence, reflectance, or fluorescence emission in the UV, visible, or near-infrared (NIR) or any color change [62–64]. Typically, in optical immunosensors, the light either comes from a diode, laser, or white-hot light bulb. Any alterations in the light's attributes reflected from or passed through the sensor are measured. Optical immunosensors have the advantage of allowing a safe non-electrical remote sensing of materials and usually do not require reference sensors since the comparative signal can be generated using the same source of light as the sampling sensor [65]. Moreover, when optical sensors respond in the visible light range, it removes the need for any equipment to read results, making them less expensive, portable, and easy to use [66, 67].

The detection schemes employed in the optical immunosensors are either label-free methods, such as surface plasmon resonance (SPR) and quartz crystal microbalance (QCM) measurements, Raman spectroscopy, and electrochemical impedance spectroscopy, or labeled such as fluorescence, chemiluminescence, electrochemiluminescence (ECL), field-effect transistors, and nanoparticles. Label-based detection often requires a combination of specific sensing elements fabricated with the detecting antibodies or the target. The color change can be visualized with the naked eye in colorimetric designs and does not require any sophisticated equipment. However, labeling makes the assays more complicated, time-consuming, and laborious. Moreover, this process is costly and often results in the denaturation of the modified biomolecules [68]. Among the various methods of optical detection, fluorescence is by far the most exploited. This is because of the existence of diverse fluorophore collections, which are highly sensitive to environmental changes. Additionally, they are easy to build and can facilitate detecting multiple compounds in a single device. But the instrument used for signal readout in fluorescence-based immunosensors is usually expensive and more suitable for laboratory settings.

On the contrary, the "label-free" sensors do not require covalent labeling to either the analyte or the biorecognition element. "Label-free" strategies are one of the foremost effective and promising methods for quicker, simpler, and additional convenient detection since they avoid the high-priced and tedious labeling method while retaining the activity and affinity of the antibodies [69]. SPR is an excellent example of a label-free optical transducer system. It is based on a phenomenon that occurs when light is reflected off thin films of metal and can be verified by the arrangement based on the Kretsch Mann configuration [70].

4.3 Piezoelectric Immunosensor

Any minor changes in the mass due to an analyte's binding to the antibody immobilized on a piezoelectric crystal can be detected by piezoelectric (PZ) immunosensors. The operation principle is based on the propagation of acoustic shear waves in the substrate of the sensor. The specific adsorption of antibody molecules influences the acoustic wave phase and velocity on the sensor surface [10]. Acoustics immunosensor is based on the piezoelectric effect and is highly effective in determining protein affinity on functionalized surfaces.

The piezoelectric crystals have a characteristic oscillation frequency in the presence of an electrical field. This frequency mainly depends on the crystal properties, such as its weight. A precisely cut quartz crystal slab is generally used for measuring very small mass quantities. The measuring device is known as quartz crystal microbalance (QCM). When QCM are coated with biorecognition elements such as antibodies, such sensors are known as QCM-based immunosensor [71]. The analyte binding causes a tiny change in mass on the electrode surface, resulting in the change of crystal frequency, which can be measured. QCM-based immunosensor is also an acoustic wave sensor that utilizes a thickness-shear mode vibration with the complete substrate's vibration.

The major drawback of piezoimmunosensors is nonspecificity due to the sensing layer's anomalous adsorption and nonspecific binding of proteins to the antibody test surface.

4.4 Others

Most biological reactions involve the release or absorption of heat. This concept is exploited in the development of an immunosensor known as thermometric immunosensors. It detects any temperature change, i.e., either released or absorbed heat due to the specific analyte-antibody/antigen reaction, and the temperature variations are converted to an electrical signal [72]. The transducer usually preferred for the thermometric sensor could be a semiconductor with an extremely high negative temperature coefficient of resistance. The thermometric immunosensors often are more stable in the long run as the analyte and transducer are distinctly placed and are not in contact with each other. It is also cost-effective and is generally unaffected by the unsteady optical or ionic impact of sample attributes. But this type of sensor has the inherent disadvantage of not being specific in its detection [72].

The temperature-based detection mode is often coupled with an enzyme thermistor because almost all enzyme reactions involve enthalpy changes [73]. In common practice, the thermometric immunosensors are combined with enzymatic reactions via the flow-injection assay (FIA) method. One such example is the thermometric immunosensor developed by Bari and coworkers [74] to detect tumor necrosis alpha (TNF-α), a protein associated with Alzheimer's disease, cancer, and inflammatory-related diseases. The calorimetric scheme consists of a microfluidic device integrated with an antimony/bismuth thermopile sensor to quantify TNF-α. The sensor is in a

sandwich format where the anti-TNF-α monoclonal antibody captures the analyte, while the glucose oxidase-conjugated secondary antibody was used for detection. The heat is generated due to the enzymatic reaction between glucose oxidase and its substrate. This heat was consequently transformed into an electrical signal by the thermoelectric sensor [74].

5 Immobilization of Immunoactive Elements

For the efficient performance of an immunosensor, biological components should be appropriately connected to the transducer. Biosensors are typically designed with a high loading of biomolecules to obtain sufficient biocatalyst activities and provide an appropriate molecular environment to enable biological activity [25]. The local chemical and thermal atmosphere can have a significant impact on the stability of the biomolecule. Various factors such as the analyte's physical chemistry characteristics, the existence of the biological elements, type of transducers, and sensing environment need to be assessed during the immobilization process (Table 1). Also, it must be essential that the biological elements should display maximum activity in their immobilized microenvironment. Generally, antigens can be immobilized by two methods, namely chemical and physical methods. The physical method is characterized by weaker, mono-covalent interactions like hydrogen bonds, hydrophobic interactions, van der Waals forces, affinity binding, and ionic binding of the catalyst with the support material. In the chemical method, the formation of covalent bonds is accomplished via the ether, thioether, and amide/carbamate bonds formed between the enzyme and support material [76]. Four methods such as adsorption, covalent bonding, cross-linking, and entrapment are commonly used for the immobilization of biomolecules [25, 77].

Table 1 Factors influencing immobilized enzymes' performance (Adopted and modified from [75])

S. No.	Factors	Implication of immobilization
1	Hydrophobic partition	Enhancement of the reaction rate of the hydrophobic substrate
2	Microenvironment of carrier	Hydrophobic nature stabilizes the enzyme
3	Multipoint attachment of carrier	Enhancement of enzyme thermal stability
4	Spacer or arm of various types of immobilized enzymes	Prevents enzyme deactivation
5	Diffusion constraints	Enzyme activity decreases and stability increases
6	Presence of substrates or inhibitors	Higher activity retention
7	Physical posttreatments	Improvement of enzyme performance
8	Physical nature of the carrier	Carriers with large pore size mitigate diffusion limitation, leading to higher activity retention

5.1 Adsorption

Adsorption is the best and economical method for the immobilization of biomolecules. However, interaction is weak, and the life of the electrode is very short. The adsorption process can be divided further into two classes: physical and chemical. Physical adsorption is weak and mediated primarily via van der Waals forces, whereas chemical adsorption is much stronger and requires covalent bond formation. The biomolecules (antibody, enzyme, protein, etc.) are resuspended in an aqueous solution and then placed on the solid support for a fixed period under appropriate environmental conditions [75]. The unabsorbed/unbound molecules are then removed from the surface by washing with buffer. It is generally considered a non-destructive method in terms of biomolecule activity due to the non-involvement of any functional group as supporting agents. However, this technique has some serious drawbacks: enzymes are loosely bound to the support by weak physical bonding, i.e., van der Waals forces. Any minor changes, i.e., temperature, pH, or ionic strength, may affect the result due to the biomolecules' desorption/leaching. In contrast, absorbed biomolecules-based biosensors have displayed poor functioning and storage stability due to nonspecific adoptions on the surface of the transducer can cause contamination and interference with the signal and liquidation of biomolecules, especially enzymes [77].

5.2 Covalent Bonding

Immobilization by covalent bonding is one of the best and widely accepted methods in the scientific community. It forms stable complexes between a functional group (–NH_2, –COOH, –OH, C_6H_4OH, and –SH.) of the biomaterial such as antibody, protein molecules (antigen), and an auxiliary matrix through a covalent bond [78]. The functional group of antibodies, which could be used as a covalent coupling agent includes amino group, carboxylic group, phenolic group, sulfhydryl group, thiol group, imidazole group, indole group, and hydroxyl group. Various chemistries have been developed for the covalent immobilization of Ab to the substrate. The establishment of covalent immobilization requires a mild environment, including low temperature, low ionic strength, and pH in the physiological range. Many traditional coating materials, such as polyethyleneimine [79], (γ-aminopropyl) trimethoxy silane [75, 80], and the copolymer of hydroxyethyl- and methyl-methacrylate, are often used as the mediate layers for immunoreactive molecule immobilization [77]. The major advantage of covalent immobilization is providing strong bindings between antibody and support matrix and reducing chances of enzyme leakage from the activated support. However, due to chemical modification, there is a higher risk of enzyme denaturalization, resulting in reduced enzyme activity in affinity reaction and poor reproducibility may be observed.

5.3 Cross-linking

Immobilization by cross-linking is an irreversible methodology performed by the formation of intermolecular cross-linkages between the antibody by covalent bonds. In this method, the biomaterial is chemically attached to solid supports/material to significantly increase the attachment [27, 81]. The most commonly used interactions are based on the use of cross-linkers that cross-links the functional groups present on the antibody to the functional groups present or induced on the substrate. An overview of the commonly used reactive group of antibodies is listed in Table 2.

The binding of a thiolated antibody to the gold-coated matrices is one of the most frequently used methods today. Because it offers a leach-proof, covalent binding of the antibody to matrices and requires minimal immobilization steps; however, it involves minor antibody modification before immobilization.

In another method, glutaraldehyde (GLD), a homo-difunctional cross-linking agent, cross-links the amino groups ($–NH_3$) available on the antibody. During immobilization, imine bonds are formed by aldehyde groups, induced on a GLD-functionalized substrate attached via lysine (amine groups) in antibody, resulting in the reversible Schiff bases. The antibody immobilization requires an initial ionic exchange in a low ionic medium with the amino groups on the substrate, followed by covalent binding, while in a high ion medium, the antibody is directly bound to slower immobilization kinetics [82]. Due to strong GLD binding with antibody molecules, leakage is minimal. However, GLD can cause significant conformational changes in antibody and could lose antibody affinity and specificity as well. This may be minimized by using inert proteins such as gelatin and bovine serum albumin during immobilization [27, 83].

Table 2 Commonly used cross-linker reactive groups for antibody (Ab) immobilization

S. No.	Reactivity	Functional group	Reactive chemical group
1	Amine-reactive	$–NH_2$	NHS ester, imidoester, epoxide isothiocyanate, aldehyde, pentafluoro-phenyl ester, hydroxymethyl phosphine
2.	Carboxyl-to-amine-reactive	–COOH	Carbodiimide
3.	Sulfhydryl-reactive	–SH	Maleimide, haloacetyl, pyridyldisulfide, thiosulfonate, vinylsulfone
4.	Aldehyde-reactive, (oxidized sugars)	–CHO	Hydrazide, alkoxyamine
5.	Hydroxyl	–OH	Isocyanate
6.	Azide-reactive	$–N_3$	Phosphine

5.4 Entrapment

In the entrapment immobilization, the biomolecules are not directly connected to polymeric materials' surface but entrapped insight into the polymeric network. It only allows crossing the substrate and products via micro/nano matrices pore, leading to a delay in the reaction. However, it retains the antibody affinity and specificity. The solution is coated on the electrode by various methods. The commonly used gels include starch gels [84], nylon, and conductive polymers such as polyaniline (PANI) [85]. The whole entrapment process is directed via two steps: (1) mixing of the antibody in a monomer solution, followed by (2) polymerization of monomer solution by the chemical reaction or changing experimental conditions. There are various methods available for the entrapment of biomolecules, depending on the type of entrapment, such as electro-polymerization, photo-polymerization, a sol–gel process for lattice fiber type, and microencapsulation for microcapsule. [75]. Although, this method offers to immobilize antibodies in their native conformation, leading to enhance their stability and protect from denaturation. However, poor substrate diffusion has been observed due to an increase of gel matrix thickness, resulting longer time consumed by the substrate to reach the enzyme active site [77]. Furthermore, the entrapped enzymes are most likely to suffer from leakage if the size of the support matrix's pores is too large.

6 Immunosensors as Diagnostic Tools

Immunosensors provided a new direction toward developing novel diagnostics in diseases, drug detection, and food quality control. Immunosensors can be designed for the detection of biomarkers, autoimmune diseases, cardiac diseases, etc.

6.1 Immunosensors for Detection of Biomarkers

The utilization of immunosensors for the detection and observation of biomarkers is presently a noteworthy area of research. Recently, the development of these novel techniques has assisted in the discovery of many new markers and provided a deeper insight into their disease role.

6.1.1 Cancer Biomarkers

Cancer is a devastating disease with the second most common cause of mortality and morbidity in developed countries. It is crucial to detect cancer at an early stage so that specific treatment may be applied as soon as possible and lead to better outcomes and prolonged cancer patients' survival. The development of novel molecular diagnostic tools has changed cancer's overall landscape in the last few decades. Enormous technological improvements in the genomics and proteomics field have identified several biomarker proteins whose over-expression can direct normal cells' oncogenic transformation into cancerous cells. A biomarker is defined

as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention" [86]. Cancer biomarkers can be of various molecular origins, including free DNA, RNA, or protein/glycoproteins (i.e., antigens, cytokines) or circulating tumor cells. Recently, cancer-derived exosomes have drawn much attention as a biomarker for the early diagnosis and drug sensitivity analysis of cancer as they carry the cargo reflective of genetic or signaling alterations in cancer cells of origin [87, 88].

Diagnostic biomarkers are those markers that help in detecting the disease, while prognostic biomarkers are indicative of disease reoccurrence. On the other hand, Predictive biomarkers measure the response to undergoing treatment. The different phases of cancer progression are marked by the changes in the different cell's specific biomarkers and their expression level. These tumor markers are considered one of the most valuable early cancer detection, classification, staging, and progression monitoring tools. Generally, some of the tumor markers are present in blood at very trace levels in the absence of a tumor. However, the markers' levels rise upon forming a small tumor, so very low limits of detection (LODs) of the developed immunosensors are essential for the early screening of a small tumor. Also, most cancers are heterogeneous and multifactorial, involving more than one marker; therefore, the use of panels of tumor markers can be more productive in their detection and diagnosis [89]. Several types of immunosensors have been developed for the detection of cancer biomarkers. In the table, immunosensors for different cancer targets are listed along with their transduction mode (Table 3). The achieved detection limits and linear detection ranges are also delineated.

Electrochemical immunosensors are most commonly used for the detection of cancer biomarkers. For example, Kim et al. [115] developed amperometric electrochemical immunosensors for diagnosing lung cancer by detecting Annexin II and MUC5AC biomarkers. The probe of the sensor was fabricated by electropolymerizing conducting polymer (poly-terthiophene carboxylic acid; poly-TTCA) onto a gold nanoparticle/glassy carbon electrode (AuNP/GCE) and a dendrimer (Den). The assay format is based on the principle of competitive reaction between label-free proteins and glucose oxidase-labeled proteins. The final sensor design was obtained by covalently attaching an antibody (anti-Annexin II) and hydrazine (Hyd), which is a catalyst for reducing H_2O_2 generated by glucose oxidase onto the Den/AuNP-modified surface. The use of dendrimer increased the sensor probe's sensitivity two or three times. The interaction of Annexin II and MUC5AC with the antibody was examined using quartz crystal microbalance, impedance spectroscopy, and amperometric ways. The detection limit of the proposed technique was 0.051 ng/mL.

Several potentiometric immunosensors are also developed for cancer detection and biomarker monitoring [49, 106, 116]. Jia and coworkers developed new techniques for detecting human phosphatase of regenerating liver-3 (hPRL-3), a prognostic biomarker of liver cancer. In this work, hPRL-3 can be detected in the concentration range of 0.04–400 nM, and the mammary adenocarcinoma cell (MDAMB231) in the concentration range of 0–105 cells/mL [49].

Table 3 Some examples of immunosensors for detection of cancer biomarkers

S. No	Biomarker	Cancer	Immunosensor type	Detection range	LOD	Reference
1.	CYFRA21-1	Lung cancer	Optical	0.05 pg/mL–100 ng/mL	0.05 pg/mL	Chiu and Yang [90]
2.	PSA	Prostate cancer	Electrochemical	0.2–1.0 ng/mL 1–40 ng/mL	20 pg/mL	Salimi et al. [91]
3.	PSA	Prostate cancer	Electrochemical	0–0.1 U/mL	0.0016 U/mL	Johari-Ahar et al. [92]
4.	PSA	Prostate cancer	Electrochemical	0.05–30 ng/mL	0.04 ng/mL	Shen et al. [16]
5.	PSA	Prostate cancer	Electrochemical	2.0 pg/mL−10.0 ng/mL	0.5 pg/mL	Yang et al. [93]
6.	PSA	Prostate cancer	Optical	5–500 ng/mL	9.9 ng/mL	Xiao et al. [97]
7.	ErbB2	Breast cancer	Electrochemical	1.0–200.0 ng/mL	0.22 ng/mL	Zhong et al. [98]
8.	ErbB2	Breast cancer	Electrochemical	0.01–100 ng/mL	0.01 ng/mL	Sharma et al. [99]
9.	p16INK4a	Cervical cancer	Electrochemical	15.62 ng/mL−0.25 μg/mL	0.49 ng/mL 28 (HeLa cells)	Duangkaew et al. [94]
10.	p16INK4a	Cervical cancer	Piezoelectric	50–1200 ng/mL	10 ng/mL	Yang et al. [95]
11.	CA15-3	Breast cancer	Electrochemical	1.0–150 U/mL	0.3 U/mL	Amani et al. [96]
12.	CA15-3	Breast cancer	Electrochemical	0.1–20 U/mL	0.012 U/mL	Li et al. [100]
13.	EGFR	Breast cancer	Electrochemical	1 pg/mL–1 μg/mL	0.88 pg/mL	Elshafey et al. [101]
14.	EGFR	Breast cancer	Electrochemical	2–14 fg/mL	2 fg/mL	Asav and Sezgintürk [102]
15.	EGFR	Breast cancer	Electrochemical	1 pg/mL–100 ng/mL	1 pg/m	Vasudev et al. [103]
16.	tPA	Breast cancer	Electrochemical	0.1–1.0 ng/mL	0.026 ng/mL	Vasudev et al. [103]
17.	UBE2C	Breast cancer	Electrochemical	500 pg/mL–5 μg/mL	7.907 pg/mL	Jayanthi et al. [104]
18.	MDM2	Brain cancer	Electrochemical	1 pg/mL–1 μg/mL	0.29 pg/mL	Elshafey et al. [101]
19.	CEA	Cancer	Thermometric	7.81–500 pg/mL	0.6 pg/mL	Ma et al. [105]
20.	CEA	Cancer	Electrochemical	1 pg/mL–1 μg/mL	0.3 pg/mL	Wang et al. [106]
21.	SOX2	Cancer	Electrochemical	25 fg/mL–2 pg/mL	7 fg/mL	

(continued)

Table 3 (continued)

S. No	Biomarker	Cancer	Immunosensor type	Detection range	LOD	Reference
						Aydın and Sezgintürk [107]
22.	RACK-1	Cancer	Electrochemical	0.01–2 pg/mL	3.1 fg/mL	Aydın et al. [108]
23.	CD146	Cancer	Electrochemical	0.0050–20 ng/mL	1.6 pg/mL	Wang et al. [109]
24.	IL6	Cancer	Electrochemical	$4.0–8.0 \times 10^2$ pg/mL	1.0 pg/mL	Wang et al. [110]
25.	DHEAS	Pediatric adrenocortical carcinoma	Electrochemical	10.0–110.0 μg/dL	7.4 μg/dL	Lima et al. [111]
26.	HE4	Ovarian cancer	Electrochemical	3–300 pM	0.06 pM	Lu et al. [112]
27.	CA 125	Ovarian cancer	Optical	0.1–40 U/mL	0.1 U/mL	Suwansa-ard et al. [113]
28.	CA 125	Ovarian cancer	Capacitive	0.05–40 U/mL	0.05 U/mL	Suwansa-ard et al. [113]
29.	CA 242	Pancreatic and colorectal cancers	Electrochemical	0.001–10,000 U/mL	1.54×10^{-3} U/mL	Du et al. [114]

Abbreviations: *UBE2C* Ubiquitin-conjugating enzymes 2C, *SOX2* SRY (sex determining region Y)-box 2, *RACK 1* Receptor For Activated C Kinase 1, *CYFRA21-1* cytokeratin fragment 19, *PSA* prostate-specific antigen, *ErbB2* epidermal growth factor receptor 2, *CA15-3* carbohydrate antigen 15-3, *CEA* carcinoembryonic antigen, *EGFR* epidermal growth factor receptor, *CD146* cluster of differentiation 146 antigen, *DHEAS* dehydroepiandrosterone sulfate, *tPA* tissue plasminogen activator, *MDM2* Murine double minute 2, *HE4* human epididymis-specific protein 4, *IL6* interleukin-6, *CA 125* cancer antigen 125, *CA 242* carbohydrate antigen 24-2

In a fascinating study, a convenient immunosensor for detecting carcinoembryonic antigen (CEA) was developed using just an ordinary thermometer as a readout [105]. The concept of generating enormous heat due to the exothermic reaction between the water and calcium oxide is exploited in this immunosensor. The immunosensor is in a sandwich format where an anti-carcinoembryonic antigen antibody acts as a capture antibody and a biotinylated antibody for detection. The biotinylated detection antibody is labeled with streptavidin-functionalized platinum nanoparticles. It catalyzes the decomposition of H_2O_2 into O_2, increasing pressure inside the reaction bottle. This further pushed the water flow into the exothermic reaction bottle containing calcium oxide. Then the water reacts with the calcium oxide to generate a large amount of heat in the exothermic reaction bottle, which was recorded by a standard thermometer.

The detection and diagnosis of cancer are very challenging due to several reasons. Firstly, cancer biomarkers are generally present in patients' biological fluids, such as blood or urine. Thus, the developed immunosensors should be sensitive enough to detect and quantify biomarkers' presence in a contaminant-ridden environment. Secondly, cancer involves multifactorial changes. At present, the most commonly used indicators for cancer diagnosis are morphological changes and histological characteristics of tumors or biomarker detection. A plethora of molecular biomarkers has recently been used for the development of single-analyte biosensors such as carcinoembryonic antigen (CEA) [117–120], α-fetoprotein (AFP) [121], epidermal growth factor receptor-2 (HER-2) [122], interleukin-6 (IL-6) [123, 124], and interleukin-8 (IL-8). But, most cancers have more than one marker associated with their incidence. Therefore, the development of multianalyte sensors will be more helpful in diagnosing and monitoring cancer patients. Then again, it has another limitation as performing such large numbers of multianalyte assays possesses various other technical and practical challenges, making it difficult to quantify a specific analyte accurately. In these cases, each antibody must be first characterized for performance and specificity by itself and again in combination with the additional reagents and antibodies. Moreover, the differences in shelf lives, stability, and binding affinities of the reagents and analyte concentrations may vary widely and may create additional difficulties [118].

Electrochemical immunosensors can be a strong candidate for performing multianalyte protein analysis because of their high sensitivity, selectivity, cost-effectiveness, quick response time, and simplicity. They can achieve excellent detection limits with tiny analyte volumes [125]. Furthermore, electrochemical sensors can be miniaturized and mass fabricated, which makes them better point-of-care diagnostics. In this regard, Wilson et al. developed an electrochemical immunosensor for simultaneous measurement of concentrations of seven important tumor markers: AFP (α-fetoprotein), ferritin, CEA (carcinoembryonic antigen), hCG-β (human choriogonadotropin β), CA 15-3 (carbohydrate antigen), CA 19-9, and CA 125 [126]. It consists of an array of immunosensing electrodes fabricated on a glass substrate, with each electrode containing a different immobilized antigen. Each electrode was capable of measuring a specific tumor marker using electrochemical enzyme-based competitive immunoassay. The secondary anti-IgG

antibody was labeled with alkaline phosphatase (AP). The hydroquinone diphosphate was added at the end of the assay, which is the enzyme-substrate. The oxidation current generated was measured simultaneously for all the electrodes after applying a potential of 320 mV. The developed multianalyte sensor had outstanding precision and accuracy and was comparable in performance to single-analyte ELISAs. In another example, Wu et al. [89] developed a simple, automated, and convenient multianalyte detection system based on screen-printed and flow-injection techniques. It is an electrochemical immunosensor that allows simultaneous detection of carcinoembryonic antigen, α fetoprotein, β-human choriogonadotropin, and carcinoma antigen 125 in clinical serum samples with concentrations up to 188 μg/L, 250 μg/L, 266 IU/L, and 334 kIU/L, respectively. The detection limits were 1.1 μg/L, 1.7 μg/L, 1.2 IU/L, and 1.7 kIU/L. The immunosensor arrays were stable for up to 1 month.

6.1.2 Cardiovascular Disease Markers

Cardiovascular diseases are the world's biggest killer for both men and women [127]. According to the World Health Organization (WHO), 17.9 million deaths are attributed to this disease in 2015, with 7.3 million being due to acute myocardial infarction (AMI) [128]. Early detection of patients with a high risk of acute myocardial infarction (AMI) is very important. Some of the indicators of elevated risk of AMI are creatine kinase MB (CK-MB), Myoglobin (Mb), myeloperoxidase (MPO), and cardiac troponins (cTn). To predict cardiovascular events; C-reactive protein (CRP) is the best-known biomarker, followed by cardiac troponin I or T (cTnI/T), myoglobin, lipoprotein-associated phospholipase A, interleukin-6 (IL-6) [129], interleukin-1 (IL-1), low-density lipoprotein (LDL), myeloperoxidase (MPO), and tumor necrosis factor-alpha (TNF-α) [111]. A cardiovascular patient's effective treatment strongly depends on a quick and rapid turnaround time, i.e., the time taken between acquiring the patient's blood sample and its results. The standard time for the diagnosis of chest pain should be between 30 min and 1 h. Therefore, handheld biosensor devices are the ideal tools for this setting, where testing can be conducted on-site, assisting in diagnosing the condition [127]. Suprun et al. developed a label-free immunosensor that can detect cardiac myoglobin in just 20 min and can be used to establish the diagnosis of acute myocardial infarction [130]. In a very exciting study, a group of researchers has developed an electric aptasensor that can detect cardiac troponin I (cTnI), a protein excreted by the heart muscle into the blood following a heart attack. It can provide the result in 1 min using just a single droplet of blood [131]. Over the last decade, many immunosensors have been developed to detect a wide variety of cardiac markers [130, 133–138]. In one such example, Khan et al. developed an impedimetric immunosensor for quick, sensitive, and selective detection of myoglobin (Mb). It incorporates a screen-printed multiwalled carbon nanotube electrode for signal amplification on which the anti (anti-Mb-IgG) antibody was immobilized.

The developed immunosensor is highly specific and sensitive, with a detection limit of 0.08 ng/mL [139]. Similarly, Ko et al. [140] developed an immunosensor for troponin I detection with a LOD of 148 pg/mL. Mattos et al. [141] developed an

amperometric immunosensor for cardiac troponin T (CTnT) detection in human serum. The antibodies are covalently bonded on a stable carboxylic film. It showed good operational stability performance, measuring it 100 times every 2 min. Recently, the detection of multi biomarkers has witnessed a significant boost. One such example is a sandwich-type antibody immunosensor for multiplexed detection of seven cardiovascular diseases (CVD) risk markers—SAA (serum amyloid A), TNF-α R1 (tumor necrosis factor-α receptor 1), IL-6R (interleukin-6 receptor), ICAM (intracellular adhesion molecule), VCAM (vascular cell adhesion molecule), MYO (myoglobin), and E-Sel (E-selectin). The multianalyte immunosensor can reliably quantify SAA, VCAM, and MYO, similar to ELISA [142].

6.1.3 Autoimmune Disease Marker

In autoimmune diseases(Ads), the immune system mistakenly attacks and damages the body's tissues, organs, and cells. It is estimated that ADs affect 5–10% of the general population [143, 144]. Due to the limited knowledge of ADs' pathogenesis, the medical treatment modalities are mainly based on managing the symptoms rather than curing the disease. Therefore, it is essential to detect and treat the disease early to obviate the symptoms' severity and the irreversible damage to organs or joints. But the biggest challenge is the sensitive detection of specific antibodies directed against various substances produced by the body. ADs are usually diagnosed based on the symptoms and the laboratory tests confirming the presence of serological and genetic biomarkers, such as autoantibodies or complement proteins [145, 146]. Some biomarkers are more sensitive and specific for a particular type of ADs. For example, systemic lupus erythematosus (SLE) diagnostic criteria include a list of autoantibodies, i.e., anti-dsDNA antibodies, lupus anticoagulants, anti-Sm antibodies, and antiphospholipid [147]. On the contrary, other autoimmune diseases involve different autoantibodies, such as anti-citrullinated protein antibody (ACPA) for rheumatoid arthritis (RA), anti-neutrophil cytoplasmic antibodies (ANCA) for inflammatory bowel disease (IBD), anti-transglutaminase in celiac disease, and ANCA-associated vasculitides [148, 149], anti-annexin II and V antibodies for antiphospholipid syndrome (APS) and systemic sclerosis (SS) [150]. Others have reviewed a more detailed spectrum of autoantibodies for ADs diagnosis [151–154].

The advent of highly sensitive immunosensors has helped in detecting these biomarkers at the early stages. As the diagnosis of autoimmune diseases mainly involves the detection of autoantibodies in the serum of affected patients, generally, the biorecognition element used in the immunosensors are the antigens [130, 132–138]. Neves et al. developed a disposable electrochemical immunosensor to detect celiac disease using the CV method [155]. In this work, the biorecognition element is immobilized on screen-printed carbon electrodes (SPCE) nanostructured with carbon nanotubes and gold nanoparticles. The carbon-metal nanoparticle hybrid conjugation aids in amplifying immunological interactions. The immobilized tTG on the transducer's surface was exposed to the sample and subsequently, alkaline phosphatase-labeled anti-human IgA or IgG antibody were added. The electrochemical signal was then generated by the anodic redissolution of enzymatically

generated silver. Because diagnosis often relies more upon qualitative results, this SPCE-based disposable biosensor may be an excellent point-of-care diagnostic device. In a similar example, Yerga et al. developed a blocking-free one-step immunosensor using 8-channel screen-printed arrays to detect celiac disease biomarkers, i.e., anti-transglutaminase IgA antibodies [156]. The dynamic linear range was 3–40 U mL^{-1}, with a limit of detection of 2.7 U mL^{-1}. The response time of the immunosensor was 80 min and was stable at least for 1 month when stored at 4 °C [156]. In another example, a sensitive and label-free impedimetric immunosensor was developed to detect anti-myelin basic protein autoantibodies in human cerebrospinal fluid and serum samples from MS patients [157].

Due to autoimmune diseases' heterogeneous nature, it is crucial for the detection of different antibodies simultaneously. For this purpose, Bleher et al. developed a label-free optical immunosensor involving multiple antigen–antibody interactions relevant to diagnosing antiphospholipid syndrome (APS) [158]. The proteinogenic antigens (β2-glycoprotein I) and amino-functionalized cardiolipin were immobilized on a glass surface using 11-aminoundecyltrimethoxysilane. The developed immunosensor could detect a broader antibody pattern of each patient within one single measurement. Each measurement is less time-consuming than standard ELISA procedures and can provide results in less than 20 min, depending on the measurement protocol.

6.2 Immunosensor for Detection of Metabolites

6.2.1 Glucose Detection

Diabetes is a metabolic disease that causes an abnormal blood sugar level, which activates several metabolic pathways related to inflammation and apoptosis events. This disease has no cure thus far. Hence, patients with diabetes systematically need to monitor their blood glucose levels to avoid complications. The glucose concentration can be monitored using a glucose meter for peripheral blood samples drawn by a finger prick. Most of the glucose meter is enzyme-based, relying on the substrate decomposition by an enzyme such as glucose oxidase to detect the product. However, since the enzyme rapidly degenerates, the sensor needs to be calibrated several times a day, and its lifetime is limited [159, 160].

Moreover, these meters are invasive and painful as the blood needs to be repetitively withdrawn for daily profiling. The antibody-based sensors provided a new direction toward the development of novel immunosensors for glucose monitoring. Electrochemical biosensors are most commonly used for the measurement of glucose. This is partially historical, but the primary reason for the success of devices of this type is that they offer suitable sensitivity and reproducibility and, importantly, can be manufactured in great volume at low cost [3].

Paek et al. [161] developed an alternative glucose analysis method based on antigen-antibody binding, which may be active over an extended period. In this study, mice were immunized with dextran chemically conjugated with keyhole limpet hemocyanin to produce monoclonal antibodies. Then, the sugar-specific

antibodies were screened. The antibodies showing typical binding characteristics toward epitopic sites and rapid reaction kinetics were selected for eventual use in blood glucose monitoring. The produced antibodies are very selective and can discriminate biological glucose compounds with a 1,4-linkage. The antibodies are immobilized on a solid surface to develop a label-free sensor system. When glucose was added to the medium, the sensor signal was inversely proportional to the glucose concentration in a range between 10 and 1000 mg dL^{-1}, which covered the clinical range. The response time was approximately 3 min for the association and 8 min for dissociation based on a 95% recovery of the final equilibrium under optimum conditions [161].

6.2.2 Cholesterol Detection

Cholesterol is an important sterol synthesized by liver cells. It is an essential component of cell membranes that acts as a precursor for synthesizing hormones, vitamin D, and bile acids. The high cholesterol level in serum is connected directly to various diseases such as cardiovascular diseases, hypothyroidism, nephrotic syndrome, diabetes, and liver diseases [162]. Several methods such as chromatographic colorimetric, enzymic colorimetric, spectrophotometric, and microphotometric are commercially available to detect cholesterol in serum. However, these methods have several disadvantages: tedious, costly, labor-intensive, less specific, and less sensitive [163]. Various type of electrochemical-based immunosensor has been designed using chemical agents such as microfluidic, nanoparticles (gold, silver, graphene, boronic acid, silica, and polymer) to detect cholesterol directly from clinical samples, i.e., urine, blood, etc., which are listed in Table 4).

Rahman et al. [171] developed a simple and cheap cholesterol biosensor by immobilizing cholesterol oxidase (ChOx) and horseradish peroxidase (HRP) onto a poly(thionine)-modified glassy carbon electrode. The immobilized HRP has shown admirable electro-catalytic activity in reducing H_2O_2 produced by the cholesterol during the enzymatic reaction of ChOx. Under standard conditions, the minimum detection limit (LOD) and sensitivity were 3.0 μM and 6.3 μM, respectively.

Gold nanoparticles have also been explored to develop biosensor for cholesterol detection. Umar and his coworker [172] developed a highly sensitive and selective amperometric cholesterol biosensor using properties of gold and bismuth subcarbonate ($Bi_2O_2CO_3$) nanoplates, which were synthesized by the hydrothermal process at a lower temperature. The invented biosensors exhibit a high and reproducible sensitivity of 139.5 μAmM^{-1} cm^{-2}, a large linear variation from 0.05 to 7.4 mM. Also, the sensor displays a fast response time of ~4 s, and a low detection limit of 10 μM ($S/N = 3$) for cholesterol sensing [172]. Some other components such as polyaniline, Au/hollowed-TiO_2, and Cu/Ni graphene have also been explored to develop an effective immunosensor.

6.2.3 Creatinine Detection

Creatinine (2-amino-1-methyl-5H-imidazol-4-one) is the end product of disruptive creatine metabolism. The quantification of creatinine level in human blood and urine is clinically important since it partially represents the nephritic, muscular, and

Table 4 Some examples of creatinine immunosensors

S. No.	Electrode type	Linear range (nM)	Limit of detection (nM)	Sensitivity	Test accuracy (%)	Reference
1	ChOx/PDMS/NiO/Pt	0.12–10.23	0.1	45 $\mu AmM^{-1}\ cm^{-2}$	1.25	Kaur et al. [164]
2	ChOx/CHER/AuNPs/SPCE	0.012–10.23	0.0078	–	5.2	Huang et al. [165]
3	ChOx/PBNPs/SPCE	0.15	0.2	2.1 $\mu AmM^{-1}\ cm^{-2}$	Not determined	Cinti et al. [166]
4	ChOx/GO/AuNPs/SPC	0.000025–12.93	0.000002	0.084 $\mu AmM^{-1}\ cm^{-2}$	4.95	Huang et al. [165]
5	Apo-ChOx/PTBA/FAD/PGE	0.0008–0.0048	0.0003	0.21 $\mu A\mu M^{-1}$		Huang et al. [167]
6	Apo-ChOx/PABA/FAD/PGE	0.0008–0.0056	0.0004	0.022 $\mu A\mu M^{-1}$	4.17	Huang et al. [167]
7	ChOx/Poly (CBNP)/PGE	0.0025–0.0275	0.0002	1.49 $\mu A\mu M^{-1}$	4.4	Vidal et al. [168]
8	ChOx/PB/GCE	8–4.5	4	0.54 $\mu AmM^{-1}\ cm^{-2}$	6.7	Singh et al. [169]
9	ChOx/Epoxyresin/P	1.0–8.0 mM	0.1	0.63 μA/mM	Not determined	Pundir et al. [170]
10	ChOx/PB sol–gel	0.001–0.08	0.00012	0.329 $\mu A\mu M^{-1}$	Not determined	Vidal et al. [168]

thyroid functions. Unlike urea, creatinine density in the body fluids is not affected by protein intake; thus, its level is a more accurate and reliable indicator of kidney function [173]. The commonly used methods are Jaffé's reaction and enzyme colorimetry to detect creatinine in clinical samples [174]. However, colorimetric methods are adversely affected by the presence of numerous metabolites/drugs in body fluids, whereas enzymic assays are cumbersome, complex, and costly.

In the past decades, various biosensors such as electrochemical, potentiometric, amperometric, and nano-sensor have been developed around the globe. However, they show variable sensitivity and accuracy [175].

Yang and his coworkers developed an electrochemical using gold nanoparticles and iron oxide-mediated sensor for the detection of creatine in clinical samples. The sensor was not useful clinically due to poor sensitivity and complex steps required for signal conversion [98]. Tang et al. explored Surface Plasmon Resonance Imaging and Near-Infrared Quantum Dots technology for the detection of creatinine. The sensitivity and specificity were quite better but observed poor reproducibility and test accuracy [176].

In this sense, electrochemical amperometric immunosensors can overcome these limitations due to fast miniaturization and rapid, precise, sensible detection. In label-free electrochemical immunosensors, the sensing platform involves the transport of the redox species to produce the amperometric signal, which is directly proportional to the diffusion of species on the electrode surface hindered by immobilized antigen/antibody. The redox species are essential for label-free electrochemical detection and are the most common approach to detect redox species in electrolyte solutions [177]. The key weakness of this approach is that it involves several steps, including multiple washes, which hindered their utility in point-of-care detection [178, 179]. Many strategies based on nanomaterials have been applied in the development of immunosensors to generate a new version of immunosensor to enhance their performances and accuracy [99]. Since then, many nanomaterials metal oxide nanoparticles, noble metal nanoparticles, and carbon-based nanomaterials have been explored to develop novel sensing platforms [180]. Recently, Erika Trindade et al. [181] developed an electrochemical immunosensor for creatine detection using redox probe-free probe technology. The sensor displays a positive response from 0.1 to 1000 ng/mL and the limit of detection (LOD) was 0.03 ng/mL, which shows high accuracy and specificity.

6.3 Immunosensors for Detection of Infectious Disease

Infectious diseases have been increased rapidly in recent years, causing high fatality rates due to incorrect diagnosis, delay in therapy, and many other complications [182, 183]. Globally, it accounts for approximately 40% of the overall 50 million annual deaths and is the main cause of death in many developing nations. Pathogenic microorganisms such as bacteria, viruses, and fungi are responsible for infections. Most infections can spread easily with the possibility of outbreaks or pandemics [184].

To identify a pathogen, rapid and accurate diagnostic devices/instruments are the prime concern. An accurate, rapid diagnosis can minimize hospitalization needs with a high impact on medical costs. Most of the existing POC tests consist of immunoassays: agglutination, immunochromatographic, and immunofiltration tests [185]. The diagnostic devices based on immunosensor could be more useful at home or in doctors' offices, as it permits rapid diagnostics, allowing the quick establishment of treatment and fast recovery by the patients. Most of the biosensors listed in the literature and available in the market are based on electrochemical techniques. The majority of these devices use nucleic acids as biorecognition components derived from the pathogenic agent, based on hybridization processes with a complementary DNA strand immobilized onto transducers, intercalating redox mediators in double-stranded DNA, or label-free detection mainly based on impedimetric measurements [186].

6.3.1 Influenza

Influenza is the most common acute respiratory infection caused by influenza type A, B, and C viruses. "Influenza viruses are classified into type A (infecting a large variety of species together including humans, pigs, horses, ocean mammals, and birds), type B (mostly infects humans), type C (almost exclusively infect humans), and type D (only infect cattle) types." Among these, influenza A viruses are the foremost serious and are liable for seasonal epidemics [187]. Extensive efforts have been made in the detection of the flu virus by health organizations, government agencies, academia, and independent laboratories worldwide. Over the past few decades, these efforts are starting to drive a move in strategy, i.e., from culture-based serological assays to genetic characterization methods and new optical and electrical biosensors. Among these methods, the polymerase chain reaction (PCR) and related techniques, including reverse transcription PCR and real-time PCR (rtPCR) have been broadly connected in biomedical research facilities for recognizing and measuring gene expression profile of the flu virus [188].

Various immunosensors have also been developed in the last two decades for rapid POC detection. In probe molecules-based detection, specific antibodies (monoclonal/polyclonal) are attached to the sensing plates, which bind to corresponding targets via antibody–antigen interactions [189]. Recently, the use of monoclonal antibodies in the development of immunosensor has increased dramatically. Many studies have also shown human monoclonal antibodies with neutralization ability and its assorted reactivity for subtyping influenza HA proteins or entirety infection particles [145, 186, 190].

Many impedimetric immunosensors were developed by exploring the principle of high-affinity antibody–antigen interactions for influenza viruses. Su et al. [191] demonstrated the utility of specific monoclonal antibodies for the detection of influenza A virus from swabs samples using the wash-free magnetic bioassay method. The LOD was 0.3 nM for nucleoprotein and 250 TCID50/mL for the spiked protein of influenza virus A.

Jarocka et al. [192] developed an impedimetric immunosensor with immobilized recombinant HA antigens, which can detect up to picograms of anti-HA antibodies

against influenza A H5N1 viruses in biological samples. It provides excellent sensitivity and very low limits of detection. The polyclonal antibodies were produced against the M1 protein (biomarker for influenza virus) and were used to detect all possible subtypes of influenza A viruses via electrochemical impedance analysis. The sensor shows quick and excellent activity with 1.0 fg/mL of LOD in saliva, corresponding to 5–10 viruses per sample [193].

6.3.2 Tuberculosis

Tuberculosis (TB) is a serious infectious disease caused by the aerobic, gram-positive bacterium *Mycobacterium tuberculosis* (*M. tuberculosis*). It is generally curable if diagnosed correctly and in a timely manner. However, the lack of effective and accessible point-of-care (POC) tests hindered the systematic screening of TB [182, 183, 194, 195]. Tuberculosis required more emphasis on the development of newer, more rapid, and sensitive diagnostic methods. Till now, various diagnostic methods have been developed for rapid detection of *M. tuberculosis*, like PCR, ELISA, Line probe assay, automated culture system, flowcytometry, etc. [182]. Since last two decades, various immunosensor technologies, i.e., screen-printed carbon electrodes [196], graphene-polyaniline mediated electrochemical [197], immunofluorescence microtip sensor [198], nanoparticles mediated sensors [199, 200] were developed for the detection of tuberculosis. Although, these sensors provide quick and fast detection of antibodies specific to *M.tuberculosis*, that could be used as POC test in primary health care center [183, 201–204].

6.3.3 Sexually Transmitted Diseases (STDs)

Sexually transmitted diseases (STDs) are viral or bacterial infections, transmitted from one person to another via unprotected sexual contact. HIV/AIDS, hepatitis B, herpes, and human papillomavirus seem to be the most common viral STDs, whereas gonorrhea, chlamydia, and syphilis are diagnosed as the largest bacterial infections [205]. A series of immunosensor has been developed against organisms responsible for STDs, i.e., HIV [206], *Neisseria gonorrhoeae* [207], syphilis [208], Chlamadia [209], etc.

6.3.3.1 Human Immunodeficiency Virus

The "human immunodeficiency virus (HIV) causes acquired immunodeficiency syndrome (AIDS)" and is transmitted primarily by unprotected sexual intercourse or the use of contaminated syringes [205]. A rapid, label-free capacitive immunosensor has been developed by Teeparuksapun et al. for the detection of p24-Ag (HIV-1 capsid protein) in serum soon after infection. The linear association was observed from 2.4×10^{-6} to 2.4×10^{-3} pg/mL with a LOD of 7.9×10^{-8} pg/mL [210]. Another electrochemical ELISA-type immunosensor was developed using HIV-1 gp41 and HIV-2 gp36 to detect HIV-1 and HIV-2 antibodies from patient serum/plasma. The sensor detects over a wide range of antibody concentration range (0.001–1 μg/mL), with a LOD of 1 ng/mL (6.7 pM) for both HIV-1 and HIV-2 [211].

6.3.3.2 Hepatitis

Hepatitis is an inflammatory condition of the liver caused by several types of hepatitis viruses designated from A to E. Hepatitis B and C are chronic diseases responsible for cirrhosis and liver cancer [212]. An optical immunosensor was designed at the beginning of 2005 to detect hepatitis C virus-specific antibodies using the photo immobilization method. Since then, significant progress has been made toward the advancement of sensing device development [213].

Recently, an electrochemical immunosensor was invented based on Fe_3O_4 nanoflowers (Fe_3O_4 NFs) and heterogeneous chain reaction (HCR) signal amplification technology for the fast and efficient detection of hepatitis B surface antigen (HBsAg) in serum samples. The developed sensor displays a wide linear detection range of 0.5 pg mL^{-1}–0.25 ng mL^{-1}. The low detection limit was 0.16 pg mL^{-1} with admirable constancy, accuracy, and reproducibility [214].

6.4 Immunosensors for Drug Safety

Therapeutic monitoring is essential for drug adjustment to reach optimal efficiency and minimal toxicity of the drug. Ideally, the concentration of drugs should be quantified at the location of the receptor but owing to its inaccessibility; drug concentrations are measured in body fluids such as serum, plasma, saliva, urine, or cerebrospinal fluids [215]. The most widely used drug detection techniques such as HPLC, GC-MS, LC-MS, radioimmunoassay, and chemiluminescence are time- and reagent-consuming, require trained staff, and complex pretreatment of biological samples. Thus, there is a need to develop a modern, minimally invasive, and handheld drug detection system that could be useful in homes, laboratories, and clinics.

Yang et al. [93] developed a simple immunosensor to detect clenbuterol using gold nanoparticles and QDs for enhanced signals. Using EIS and ECL emission, AuNP/ovalbumin-clenbuterol/anti-clenbuterol-QDs sensor was characterized that achieved LOD of 0.0084 ng/mL in the range of 0.02–50 ng/mL. An impedimetric immunosensor mediated 3-mercaptopropionic acid SAMs was developed to detect ketamine, a drug used for anesthesia. The developed sensor can detect 0.41 pmol/L of ketamine in clinical samples [216]. A similar approach has also been used to develop a sensor for detecting ciprofloxacin, a drug widely used for the treatment of pulmonary, urinary, or digestive infections, with a LOD of 10 pg/mL being obtained [217].

Developing innovative technologies for the rapid quantification of the drug is often essential in managing drug formulations in the pharmaceutical industry. A label-free immunosensor was developed using graphite oxide as an immobilization platform for antibodies specific to acetaminophen. The sample preparation steps were examined by several electrochemical techniques such as SWV, EIS, and EQCM, and the LOD found by SWV measurements was 0.17 μM [19].

Another major social and health issue is drug abuse and nobbling. The most commonly used doping agents are “beta-blockers, steroidal hormones, growth

hormones, theophylline and derivatives, peptides and methamphetamines." There is a growing interest in developing quick, sensitive, and specific screening tests, especially in sports, for the on-site detection of doping agents. Currently, numerous kits are commercially available to detect such drugs from body fluids such as saliva and urine. For example, Oratect, a gold particle-based ICTs immunoassay is used to detect marijuana (THC), cocaine, amphetamines, opiates, and methamphetamines [218]. A simple and sensitive electrochemical sensor was invented by scientists for the real-time detection of two lying drugs: morphine and methamphetamine. The sensor was designed using the immobilization of 3-mercaptopropionic acid on a gold electrode, which helped bind antibodies specific to the targeted drugs. "The sensors precisely detected morphine and methamphetamine in the linear range of 4–80 pg/L and 20–200 pg/L, respectively, with a LOD of 0.27 pg/L and 10.1 pg/L" [189].

6.5 Immunosensors for National Security

Immunosensors are used in military and defense to detect biological or chemical warfare agents, including a wide variety of synthetic chemicals, natural or animal toxins, and bacterial exotoxins capable of damaging or killing humans [219]. For example, "*Bacillus anthracis, Francisella tularensis, Brucella* sp., *Yersinia pestis, staphylococcal enterotoxin B,* botulinum toxin, and orthopoxviruses are typical biological warfare agents." Immunosensors are also used to detect various types of explosives/bombs and thus save millions of lives worldwide [220, 221]. "Military explosives mainly constitute nitroexplosives, such as 2,4,6-trinitrotoluene (TNT), dinitrotoluene (DNT), and hexogen (RDX)". Due to its low vapor pressure at room temperature, the identification of nitroexplosive vapors remains a problem. Immunosensors may also be used to identify volatile nitro vapors at a highly sensitive and precise location. Immunosensors can also track soldiers' health, response to the dietary shift, fatigue, environmental factors, etc., to improve soldier efficiency. Immunosensors will play an essential role in future military operations because of their sensitivity, selectivity, lower costs, scale, weight, and versatile on-site deployment [222].

7 Challenges and Prospects of Immunosensor

The number of immunosensors and their implementation have been expanded with modern approaches such as "magnetic nanoparticles, quantum dots, carbon nanotubes, noble metal nanoparticles, or hybrid nanomaterials," either as labels or immobilization platforms [175]. These materials provide important advantages, such as strong biocompatibility that preserves bioreceptor function, higher surface-to-volume ratio, i.e., rises in the number of immobilized bioreceptors, and exceptional conductivity and optical proprieties. The higher sensitivity and specificity have made it possible to use these immunosensors for in vitro as well as in vivo applications. Even though significant advancement has been made in the field of immunosensors,

novel techniques are still required to boost the sensitivity, specificity, and simplicity of these devices that would satisfy the exigent criteria of clinical diagnosis or industry. The vast majority of immunosensor mentioned in this chapter and used today are focused on using antibody, antigens, and aptamer as a recognition component. The high selectivity is a key advantage of the immunosensor, while inconsistency, cost, and the need to use them in combination with a mediator system limit their uses in clinical science. Another challenge is a need for miniaturization and integration on a computer platform that can detect biomarkers or pathogens in real time. In the last decade, remarkable progress has been made in the field of nanotechnology; novel sensor could be designed with new technologies of nanoparticles and nanostructured surfaces for a wide range of antigen detection using electrochemical strategy. Further, portable, cost-efficient, and accurate sensors may be produced via multiplexing of various antigens/protein detection devices, especially those coupled with microfluidics that could guarantee accurate detection, especially panels of cancer biomarkers detection in blood, urine, saliva, or other body fluids. Though incorporating electrochemical immunoassays in microfluidic platforms can produce a scalable platform for the construction of devices for clinical diagnostics, ultimately, the development and advancement of these systems would lead to faster clinical decision-making, reducing the patient's stress, and lower costs for healthcare.

8 Summary

We have discussed the various aspects of the immunosensor in this chapter. Although, the concept of straightforward detection of the binding event is simple and elegant, the advancement of such a gadget is overwhelming. The concept is very elegant, but requires a multidisciplinary approach that combines the expertise of immunologists, immuno-chemists, engineers, and materials scientists to develop the intimate interface between the biologic component and the transducer. Some critical parameters; (1) selection of high-affinity antibodies would be a critical parameter, failing with loss in sensitivity or specificity of immunosensor; (2) interface component also could become contaminated on contact with body fluids, leading to increased response times; (3) Behavior of biological material with selected transducer should be understood well under experimental conditions.

It is worth noting that scientists should also think about commercializing expect such as affordable and reliable P.O.C. devices to facilitate biomedical care in developing countries. Intrinsically, it will add an extra advantage to public health and reduce the healthcare sector's financial load.

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Live Cells as Biosensors

Sarita Devi

Abstract

The biosensors are seemed as a potential device to monitor and hit upon ecological pollutants, contaminants, and, more commonly, chemical or organic markers of potential intimidations to human well-being. They are essentially composed of a sensor element made up of either biologically active molecules or whole cells (live) coupled to a reporter/transducer technological element. The live cell biosensors may be based on animal tissues, unicellular microorganisms, or eukaryotic microorganisms, e.g., microalgae and yeasts. Various biosensors based on live cells have been disclosed in the prior arts for the past many years and these studies have revealed the prodigious prospective of their usage in the environmental pollution detection areas and in biomedical diagnostics. The particular characteristics of live cell biosensors lie in their potential to detect stress, toxicity, and bioavailability in situ, in addition to the benefits of easy use, quick response, sensitivity, portability, and low cost. All these factors make live cell biosensors an attractive device for health-associated applications. This chapter centers around bacterial live cell biosensors for diagnosis and treatment of cancer, innovation and identification of antibiotics, and assessment of fitness hazards. This chapter additionally considers the future insights and challenges of biosensors in clinical practices.

Keywords

Live cell · Biosensor · Bioavailability · Enzymes · Cofactors · Health benefit · Diagnostics

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1 Introduction

For detection and identification of a component present inside a cell, tissue, or organ of the body, a device called "biosensor" can be used. It is made up of various forms of chemical or physical transducers and biomolecule recognition elements [1, 2]. Based on their interdisciplinary construction nature, their developmental studies have been published in different fields of information science, chemistry, biology, and physics [3–6]. On the basis of variations in their cellular, tissue, and molecular sensing elements, the biosensors can be categorized into three different classes, i.e., molecular, cellular, and tissue [7]. The utilization of various biologically active molecules, e.g., antibodies, antigens, enzymes, DNA, and biofilms as reporter elements have been reported in molecular-based biosensors [8]. The foremost advantage of the molecular-based biosensor is its high selectivity [1], but the shortcomings like the short functional lifetime of the recognized molecules, expensive isolation costs of macromolecule, and restricted detection capability have limited applicability of this kind of biosensor [9]. The live cell biosensors that are produced from cells or intact tissue, in comparison to molecular-based biosensors, have seen rapid growth in novel immobilization and microfabrication methods, and these very recent vicissitudes have given specific and unexploited benefits to these types of biosensors [10]. The query that arises is then: why is it desirable to move deep into live cell (whole) based biosensors? A living cell is considered as a natural bio-catalyst factory and the process for obtaining these bio-catalysts (e.g., enzymes) involves a process of separation and purification of required bio-catalysts from the microbial strain or tissue, which is resource as well as time-consuming process [11]. The live cells produce a metabolic aggregate of enzymes, coenzymes, and cofactors, constituting a precise mechanism to guarantee chemical reactions which might be essential for their function, further, they self-control the recycling procedures for such materials; analog methods may be observed in tissues, but the necessities associated with preservation and price for microorganisms culturing are underneath from those of tissue cultures [12]. One benefit of utilizing live cells is that it is possible to achieve very complicated reactions by coupling several enzymes in one single step [13]. Therefore, the choice of an appropriate live cell for a bio-sensing application, basically, could obey the features rendered by means of the chosen microorganism on perceived surroundings, to set an instance, the organisms that live in harsh surroundings result in metabolic activities that involve the performance of specific compounds and are profusely available on the targeted environs. The prior example is not a limitation, inherent live cell is not confined to organisms that are harvested beneath extreme environments, and organisms present in friendlier surroundings can react to very precise stimuli, such circumstances allow the screening of various organisms as prospect candidates for a preferred bio-sensing application [14]. Even though the live cell biosensors are not susceptible to different ecological vicissitudes as molecular-based biosensors, they are possibly altered through simple recombinant techniques to facilitate their utilization to perceive a sequence of composite responses inside a live cell [1]. It is feasible to modify the genetic configuration of live cells consequently modifying a given organism's

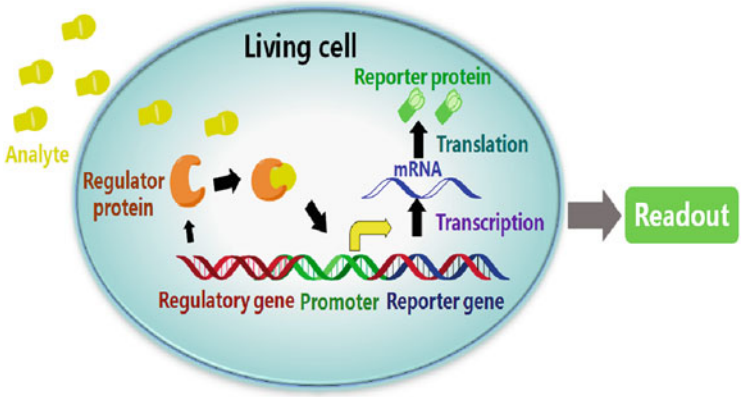

Fig. 1 Diagrammatic representation of a typical live cell-based biosensor [1]

enzymatic expression, transmitting the opportunity to respond to various substrates or to encompass responses that may be effortlessly examined, the induced reaction is arbitrated for what is called a bio-reporter gene [15, 16].

Further, the overall performance of a live cell biosensor involves the choice of reporter gene and the selectivity and sensitivity of the molecular recognition that occurs whereas target analytes are bind to their respective regulator proteins [17]. The detection of specific analyte species and amplification of this identification into an electrical and optical signal through a processor is the main mechanism of a typical live cell biosensor (Fig. 1). By the usage and immobilization of live cells or microbes as the component that offers the elements of molecular recognition, this readout process is detectable. The live cell-based biosensors, unlike a conventional biosensor, can perceive a broad variety of materials and therefore are more susceptible to modification in the tissue sample's electrochemical state, other cells or in the surroundings, for the reason that they can genetically alter and can function in a wider range of conditions that involve different pH and temperature values [18–20]. Due to the evident benefits of live cell biosensors like their high selectivity, good sensitivity, and their ability for in situ detection (high-throughput), they have been implemented efficiently in arenas including pharmacology, food analysis, environmental monitoring, and drug screening [17].

In this chapter, the summarization is done of what is befallen recently within the development and design of live cell biosensors by highlighting their utility for biomedical diagnostics and environmental pollution monitoring, two regions where these biosensors are most usually applied. The consecutive segment of this chapter relates to the association between chemical transduction and live cell (whole) transduction, benefiting from the suggested grouping of the latter segment, it aids a bridge among various disciplines, and it is here clarified the criteria to be mindful of both interactions. The discrepancies between electrochemical effects in live cells (amperometric, potentiometric conductometric, and impedance sensors) and physiological effects are overcome, either because of respirometry, external stimuli, or the

findings associated with bio-reporters. This chapter delivers a literature precis on the choice of host cells, regulatory proteins, reporter genes, and multi-functionalization for detecting various pollutants present in surroundings such as organic waste and heavy metals. It also provides an overview of current advances in the utilization of biosensors based on live cell in the area of micronutrient detection, precision medicine, and diseases diagnosis. In addition, this work offers an overview of the current problems and possible opportunities for the functional implementation of live cell-based biosensors. The last segment presents the latest innovations and the commercially available alternatives using live cell biosensors, further closing this chapter with the confab of the possible prospects and obstacles.

2 Live Cell-Based Biosensors: General Principles

The live cells have the capability to make critical vicissitudes on various substrates via a very well-described sequence of reactions; such modifications are benefitted as energy or as indispensable elements for the cell's dynamic processes. An individual species can interact at the same time with numerous substrates, each of which is driven in every instance by a very specific sequence of chemical reactions that compose a metabolic pathway. The initiation of certain chemical reactions is mediated by the enzymes and they are programmed to complete a chain of events that ensure that metabolic and physiological responses are accomplished by the live cells to ensure survival.

A naturally occurring event that presents a modified energetic pathway would be an enzyme-catalyzed reaction [21]. The production of an enzyme is simultaneously controlled by the living cell's genetic code, a distinctive imprint for every one-of-a-kind strain. The genetic integrity information is stored in what may be equated to the DNA (storage unit), i.e., the genome. Although, every subject has a different genome but with same basic functional unit, which will eventually suggest the uniqueness of the given strain. As a complete programming code, the resulting sequential amino acid arrangement constitutes the enzymatic structure [21, 22]. Through the coiling and folding of amino acids, the enzymes' very complex 3D structures form fragments that serve as pockets prepared for the substrate's coupling, known as active sites, which are fitted with a particular geometric shape and an atomic arrangement complementary to those of the substrate. Via the "lock-and-key theory" [23], a general method for enzyme-substrate binding mechanisms can be envisaged: Emil Fischer in 1894, proposed the rigid structures, enzymes would act as a lock with a particular shape; on the other hand, the substrate resembles a key: if an active site with correct shape is presented, it will act as an adequate key hole, so it will "unlock" the consequent enzymatic reaction ([24]; Fig. 2).

David Koshland presented an alternative to Fischer's postulate in 1958, by stating that the enzymes, rather than sturdy, are flexible; therefore the active site is actively altering its shape to adjust to the substrate and if there are some chemical bonds formed among the substrate and the active site in the enzyme, and are aligned to the catalytic groups, the reaction would only take place. The substrates can bind via

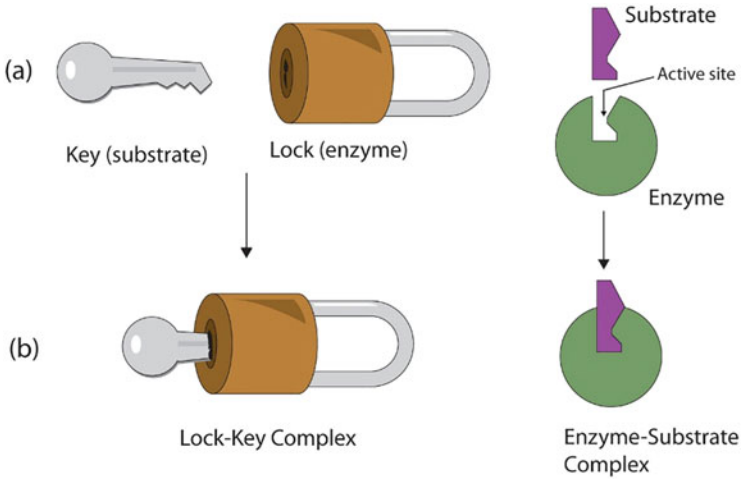

Fig. 2 Schematic representation of the lock-and-key hypothesis (https://socratic.org/questions/58f64d5c11ef6b44e4d659b6)

covalent and hydrogen-bonds; ionic- or van der Waals forces, these interactions are usually weak, but it forms a solid binding with many of these weak interactions taking place at the same time. The postulate given by Koshland is acknowledged as the "induced-fit theory" [21, 23]. In order to understand selectivity, the necessary conditions are enclosed by the distinctive binding mechanism and the central of the enzymatic technique depends on the acceleration of the chemical reaction rate. Both mechanisms rely on the development of molecular interactions with the substrate, as is inferred from the induced-fit theory. Although the enzyme-substrate interaction at initial stage is weak, the growing number of bindings produced among the enzyme's active site and the substrate, which is merely possible if there is a suitable substrate, will prompt structural vicissitudes on it until the firm attachment of the substrate to the enzyme is there; further, multiple substrates, coenzymes, and cofactors will be arranged by analogous mechanisms. Any of the four acceleration mechanisms could be caused by the correct alignment, to wit, covalent catalysis, approximation of the reactants, the introduction of distortion or strain in the substrate, or general acid-base catalysis [25, 26]. In terms of the thermodynamic characteristics of the system, a reaction can be understood, based on the first and second thermodynamics laws, which are energy conservation and increasing entropy. It was proposed by Willard Gibbs in 1873, a model, similar to the potential energy in classical mechanics, taking into account that to produce work under constant conditions of temperature and volume in a given closed system, would have a thermodynamic potential, namely Gibbs (G) free energy, originally referred as available energy [21, 27, 28].

In any chemical reaction, the free energy (maximum amount) derived, is determined by the difference between the free energy of the products and the reactants (ΔG), as soon as the energy is consumed by the reaction, i.e., the ΔG of reactants is less than that of the products, it is believed to be an endergonic reaction, i.e., it needs

an external energy source, e.g., heat. But it is said to be exergonic when the energy is released from the reaction; it is thus named a thermodynamically favorable reaction, implicating that it may occur spontaneously. Then, when a spontaneous (exergonic) reaction occurred, if the energy is not delivered to the system ($\Delta S > 0$), the entropy (S) will increase. Then again, if there is no change in entropy, the energy release can best be related to the system's internal energy, a feature termed as enthalpy (H), as it is generally calculated as heat, the difference among the resulting and the initial reaction state (ΔH) is acknowledged as the heat of a reaction. Consequently, it is termed as exothermic ($\Delta H < 0$), when the resulting process of the system releases energy, in contrast, it is named endothermic ($\Delta H > 0$); it is required to pressurized that not every exergonic reaction is inevitably exothermic, i.e., the released energy is not always as heat, so a reaction with $\Delta G < 0$ may have $\Delta H < 0$, $\Delta H = 0$ or $\Delta H > 0$. In Gibbs fundamental equation, these features were correctly explained as follows:

$$\Delta G = \Delta H - T\Delta S \tag{1}$$

The equation (Gibbs) describes a functional state, which shows that it relies exclusively on the equilibrium state of the system, irrespective of how it reached that state. Catalytic processes however pass within a tighter boundary of the chemical reaction procedure, not influencing the initial or final reaction state, but affecting the direction from one end to the other. The enzymatic process decreases the required free energy for the reaction to happen (Fig. 2). The frequency of unpremeditated chemical reactions is hindered by the influence of energetic barrier, designated as activation energy; in order for any reaction to happen, sufficient energy must be supplied to the system to out-perform it in a non-catalyzed reaction into a transition state; this can be done by heating up the system. This perilous moment signifies a state where the products and the reactants concurrently exist, due to the bonds concurrence, both from the product state and the reactant state. Such molecular form is exceptionally unsteady and hence associated with a huge quantity of free energy. The enzyme-catalyzed reaction would represent an alternative way for the reaction to happen [21, 25, 29].

It is easier to explain a predictable outcome through analytical applications devoid of much regard for the specifics of a particular metabolic pathway by considering the catalytic process as an energy-state transformation. Under constant temperature conditions, let us consider the Gibbs fundamental free energy equation again, as given in Eq. (1). Also consider, an exergonic reaction, such that there is neither energy delivered to the system, nor is it delivered by external means either because of a preceding enzymatic reaction to the metabolic pathway. In the simplest case, it should be concluded that from the system, there is no energy release, the merely probable result would be an upsurge in the system's entropy. Dissociation of the elements can be estimated as soon as the substrate is consisting of molecules created by numerous chemical elements, and in certain situations, one or more of the molecules can be easily identified by an acknowledged technique of chemical sensing. If this is the situation, it might be believed that the sensing strategy would not be aimed at the substrate, but at a related reaction or a by-product. This situation

Table 1 Comparison of the stimuli response-based and biotransformation-based strategies: advantages and disadvantages [21]

	Stimuli response-based strategy	Biotransformation-based strategy
Advantages	• Direct assessment of electrical measures • Quick response • Less composite measurement mechanism. No requirement of additional reagents/elements for measuring • Lower probability for occurrence of side-effect reactions • Swiffer usage for the assembled device	• Yield a ready amplified signal • No need for immobilization of cells • Wide variation of response signals. Some cases do not require to utilize measurement equipment
Disadvantages	• Low-level signals • High noise to signal ratio (NSR) • Need for an output signal external amplifier for portable applications • Need for precise biomass control and immobilization • Need for measuring devices to read the output signal	• Indirect measurement • The output signal is mediated by complementary reactions • Response dependent on the reaction and metabolic pathway time • Usual need for additional reagents/elements for measuring • Higher probability for the occurrence of side-effect reactions

will be referred to as a biotransformation-based strategy. A distinctive circumstance would be considered when the outcome of such biotransformation produces a readily detected bioluminescent signal, often considered as bio-reporter [16, 21, 30].

If no entropy change is considered, an additional case can be elucidated therefore the mere effect on the resulting free energy equation will be caused by an enthalpy modification. If the initial conditions agreed on those formerly suggested, the exothermic reaction would happen and the outcome would be an energy release that can be calculated by appropriate means. This method is classified as a stimuli response-based strategy. The actual situations of the reaction that are catalyzed by enzyme are a lot more composite than those considered above, the metabolic pathway creates a complicated reaction network that may be interconnected with different pathways, some of which are active during the course of the cell vital routines, others associated to the precise social behavior of various species, in both inter and intra species responses [31, 32]. Such situations combine diverse features on the enthalpy and entropic properties of the reaction, and the reaction's exergonic-endergonic nature. But such combinations would result in a comparable analysis, favoring either the expression of the by-products (biotransformation-based strategy) or by energetic measurable changes approached (stimuli response-based strategy). Table 1 lists some benefits and drawbacks of using both strategies.

3 Live Cell Sensing Technique: Transduction

In the preceding section, two bio-sensing strategies based on the specific contributory thermodynamic effects of each term were introduced, namely Biotransformation-based strategy (BtB strategy), which is based on entropy-related intake (molecular-related response); and Stimuli response-based strategy (SRB strategy), which is based on the concept of enthalpy (energy-related response). In this segment, transduction is defined as the process by which the presence of a specific substrate causes an appropriate reaction to an information-translated measurable unit. For both BtB and SRB techniques, the notion presented in this part is closer to that posed by the analytical procedure of the physical context; nonetheless, few insights into the physics and thermodynamic foundation of some concepts are provided, which are frequently overlooked in the literature.

3.1 Biotransformation-Based Strategy

In the current scope, the biotransformation-based strategy is considered as a collection of by-product-mediated sensing methods. The dissociation and consumption of various compounds can be stringently considered in the chemical realm of many reaction outcomes. Different methods developed for specific substances can easily detect the transformation occurring within the living cell and not the generated energy straight inside the live cell. Table 2 lists several examples of the electrodes used.

The chemical bond conformation, from the viewpoint of the molecular interaction, resulting from specific pathways of the metabolism, functions as an additional choice for substrate targeting. The approaches provided by the living cell might be only possible if they are complemented with a different substance, e.g., dependence of aerobic organisms on the consumption of oxygen for completion of the processes within their complete metabolic network, the volume of oxygen on a contained environment can be utilized as a measurement unit of the living cell activity. Besides the optical, colorimetric, and electrochemical microbial biosensors, baroxymeter microbial sensor, based on manometric bacterial respirometry, is used to detect the

Table 2 Examples of transducers utilized under the scope of the Biotransformation-Based Strategy (indirect electrodes, [33])

Transducer	Species detected
Amperometric electrodes	O_2, H_2O, NADH, I_2
Field-effect transistors	H^+, H_2, NH_3
Ion-selective electrodes	H^+, NH_4^+, NH_3, CO_2, I^-, CN^-
Piezoelectric crystal	Mass adsorbed
Photodiode (in conjunction with a light-emitting diode)	Light absorption
Photomultiplier (in conjunction with fiberoptics)	Light emission or chemiluminescence

pressure change, and the infrared analyzer-based microbial biosensor is used for the detection of the microbial respiration product CO_2 [34].

Further steps beyond strain-specific reactions will be needed for the transduction of a biotransformation-based strategy; the biological assignment is restricted to the intermediate fabrication of agents to release additional chemical reactions that could, for example, be converted into signals (electrical), chemically similar to the approaches proposed for the transduction of the stimuli response-based strategy. The quantification and recognition of the by-product created must conform with simple procedures. The use of bio-reporters, defined by means of the unique reporter gene responsible for controlling the substance production on a particular metabolic pathway, could address the targeting of the production and intake of substances for reporting the existence of a substrate given [9]. The expression "bio-reporter" is commonly referred to in terms of very particular products that can be recognized, e.g., by fluorescence and phosphorescence-based optical instruments [21].

Various approaches leveraged from genetic engineering can insert bio-reporter genes into the cell genome [16, 21], they are involved in precise metabolic pathways because of the sequential nature of the deployment of the reactions within various pathways of the metabolism. The activation of the bio-reporter is happening only when the corresponding pathway and specific reporter gene have reached the same stage thus reporting on the sought substrate's presence. The molecules released become the focus of different chemical sensing approaches, connecting the translation for substrate quantification, in an identical way, the electrical response to the stimuli response-based technique is proportional to the quantity of substrate detected, as there is less/more substrate concentration, this molecule will be consumed/released.

3.2 Stimuli Response-Based Strategy

The stimuli response-based strategy is supposed underneath the energy intake/release principle, the promising example of an exothermic reaction. In view of this primary glance, when acting together with a substrate, such an approach may be considered to be based on the living cell (whole) heat production as a responsive event. But, while the principle involves a direct situation to the thermal characteristics of the reaction, the energy intake/release is not inherently of thermal nature, it is also important to consider the effects on the electric domain. For the consideration of the stimuli response-based strategy, the reactions powered by the enzymatic behavior of the live cell are based on ionic effects, such as ionic transport occurrences [35], that cause alterations over an adequate substrate, namely electrode, freely measurable on the electric domain.

The most common reaction involved in biological methods is named as redox (reduction oxidation) reaction. When it is deployed, the reactants undergo an electron transfer process, that produces Gibbs' free energy (fractional quintets, [36, 37]), which is released in the form of heat. The breaking, forming, and reconfiguring of the atomic bonds often contribute to the heat production, both

effects imitate the metabolic activity of the specific strain when interacting with particular substrates. Thermal vicissitudes react straight to these chemical reactions [28]. The released energy in these reactions is effortlessly benefitted by using the temperature detection approaches relevant to biological interactions through microcalorimetric techniques, defined initially by Rubner [38], considering the heat flow measurement of biological process, that advances correspondingly as chemical interactions happen [39]. Akin interaction takes place on the interfaces of the electrode with live cell and this electrode acts as an effective redox reaction electron acceptor/donor set up on the substrate's existence. The ionic clusters released transport to a lower concentration region from a higher concentration region; production of a diffusion transport effect due to the concentration gradient [28]; the potential of the cell was first reported by Nernst, who interrelated such potential to the free energy state (Gibbs) of the reactants [40]. There is a free energy-dependent alteration for every chemical reaction [41]; the changes prompted on the electrode transform its electric structure and produce electrical units (readily measurable); usually, such reaction attains either a potential or charge accumulation (potentometric), a difference in current (amperometric), modifies the conductive characteristics among surfaces (conductometric), creates vicissitudes on the impedance (impedimetric) or potentiometric vicissitudes on a gate electrode (field-effect, [35, 42, 43]).

The microbial fuel cells (MFC) biosensors provide an additional interaction for the considered reaction [21, 44]. The ensuing ions that are required by the products of live cell enzymatic reactions or which are disconnected from them may be swapped across the cell membrane, and the ionic concentration gradient creates a quantifiable electromotive force as an electric energy difference. On the other hand, not all cell has the capability to transfer the ion cluster to the electrode directly because of the membrane's non-conductive nature; a membrane is usually utilized that will assist as a selectively transfer protons/electrons mediator to the electrode. Hitherto, certain electrochemically active species [45], such as *Rhodofoferax ferrireducens* [46], *Aeromonas hydrophilia* [47], *Enterococcus gallinarum* [21], *Geobacter sulfurreducens* [48], *Desulfoblus propionicus* [49], *Clostridium butyricum* [50], and *Shewanella putrefaciens* [21], would be capable to provide a microbial fuel cell (mediator less). Since the reaction is driven by the substrate transformation, the resulting electrical gradient is proportional to the concentration of the substrate; therefore, the output signal is a quantitative indicator that depends precisely on the quantity of the substrate that interacts with the cell surface [21]. Under the current concept, the detection proposed for the utilization of the stimuli response-based strategy is arbitrated only by an electrode usage, interfaced directly with the live cell or arbitrated through an electron/proton exchange membrane. The stimuli response-based strategy is considered for some of the exposed circumstances where the given substrate interaction creates a measurable change over an electrode, interfacing the live cell; these strategies generally include the cell immobilization on the electrode surface, cell trapping techniques for immobilization of polyvinyl alcohol (PVA, [51]), immobilization crosslinking method [52], hydrogel immobilization [53], and physical confinement [54], among others.

The two characteristics shared by the approaches set out in this strategy are the direct use of electrodes for the evaluation of proportional electrical measurement and the prevalence of the contribution of the system's enthalpy to the total reaction. The consideration of using a stimuli response-based strategy with an acceptable live species is beneficial if as a non-mediated direct response, a one-reaction one-response measure is essential, resulting in quicker quantity acquisition, less composite data transduction systems, and less possibility of side-effect reactions to enhance noise to the measure.

4 Live Cell Biosensors: Recent Advances

The selection of an appropriate approach for biosensor development reacts to different features hindered by the selection of the strain utilized. One of the key obstacles is to identify the particular substrate that can be directed by different species, or which species is ideally suited to a particular target. A thorough analysis of some cases is performed in Table 3, compiling different whole-cell strains, differentiating the recorded genetically modified strains, and documenting different targets alleged to be recognized for those strains.

The cell (whole) biosensors show a potential alternative to utilize in early warning screening because of their quick reaction to toxins according to the US Environmental Protection Agency [89]; some technologies are also disclosed in the same report, under the recent commercial application, but in accordance with the definition of biological test, including BioTox™ [90], ToxScreen-II (currently III) [90], POLYTOX™ [91], DeltaTox® [92], and microMAX-TOX, the latter is a potential device proclaimed by the Italian company Systea S.p.a. [93] that would shield the probable features of a biosensor based on the whole cell for continuous and online monitoring. Some of the commercially available biosensors based on whole cell are given in Table 4.

For commercial use, the recent preference for the use of the biotransformation-based strategy is clearly observed, while researchers have made efforts to build biosensors on the fringes of the stimuli response-based strategy. A novel generation of biosensors focused on the possibilities of different strains to change the given electrical structures of the electrode will lead to the trend posed by the current developments within the framework of the stimuli response-based strategy. In addition, the benefits of the excessive use of chemicals and the quick response will undoubtedly function significantly in favor of the inclusion of the stimuli response-based strategy biosensors development. The imminent innovations will involve the use of Archaea as a promising means for the highly efficient stimuli response-based strategy biosensors development, an important area to explore is the affinity of such domain-type live cells with different substrates and the potential it provides for strong electrode reactions.

Table 3 Various whole-cell strains for utilization in biosensing along with the targets, detection method, and attributed strategy [21]

S. no.	Microorganism	Target	Detection method	Strategy	References
1.	*A. aceti (IFO 3284)*	Ethanol	Amperometric	SRB	Ikeda et al. [55]
2.	*A. globiformis*	Choline	Amperometric	SRB	Stoytcheva et al. [56]
3.	*Arthrobacter.* sp. *JS 443*	*p*-Nitrophenol	Amperometric	SRB	Lei et al. [57]; Lei et al. [58]
4.	*A. peroxydans*	Hydrogen peroxide	Amperometric	SRB	Sumathi et al. [59]
5.	Activated sludge (mixed microbial consortium)	BOD	Oxygen electrode/flow injection system (entrapped in dialysis membrane)	BtB	Liu et al. [60]
6.	*B. ammoniagenes*	Urea	Conductometric	SRB	Jha et al. [61]
7.	*B. subtilis* (heat killed)	BOD	Amperometric	SRB	Tan and Qian [62]
8.	*Bacillus* sp.	Urea	NH_4^+ ion selective electrode	BtB	Verma and Singh [63]
9.	*C. parapsilosis*	BOD	Amperometric	SRB	König et al. [64]
10.	*Candida vini*	Alcohol	Oxygen electrode (porous acetyl cellulose filter)	BtB	Mascini et al. [65]
11.	*Comamonas testosterone* TI	Non-ionic surfactants	Amperometric	SRB	Taranova et al. [66]
12.	*E. coli* bearing *fab A':lux* fusions	Pollutants/toxicity	Luminescence	BtB	Bechor et al. [67]
13.	*E. coli HB101 pUCD607* containing *luxCDABE* cassette	Water pollutants/toxicity	Luminescence	BtB	Horsburgh et al. [68]
14.	*E. coli HMS174* harboring *mer-lux* plasmid *pRB27 or pRB28*	Hg2+	Luminescence	BtB	Rasmussen et al. [69]
15.	*E. coli K12*	Mono-and/disaccharides	Amperometric	SRB	Held et al. [70]
16.	*Flavobacterium* sp.	Organophosphates	pH electrode	SRB	Gäberlein et al. [53]
17.	*G. sulfurreducens*	Acetate	MFC	SRB	Tront et al. [71]
18.	*G. suboxydans*	Ethanol	Amperometric	SRB	Kitagawa et al. [72]

19.	*H. polymorpha*	L-lactate	Amperometric	SRB	Smutok et al. [73]
20.	*K. oxytoca AS1*	BOD	Amperometric	SRB	Ohki et al. [74]
21.	LAS degrading bacteria isolated from activated sludge	Anionic surfactants (linear alkyl benzene sulfonates—LAS)	Oxygen electrode, (reactor type sensor, caalginate)	BtB	Nomura et al. [75]
22.	Microbial consortium	BOD	Amperometric	SRB	Rastogi et al. [76]; Liu et al. [77]; Liu et al. [78]; Dhall et al. [79]
23.	*P. aeruginosa*	Cephalosporins	Potentometric	SRB	Kumar et al. [170]
24.	*P. alcaligenes*	Cafeine	Amperometric	SRB	Babu et al. [52]
25.	*P. putida SG10*	BOD	Amperometric	SRB	Chee et al. [80]
26.	*R. erthropolis*	2,4-Dinitrophenol	Amperometric	SRB	Emelyanova and Reshetilov [81]
27.	*Rhodococcus* sp. *DSM 6344*	Chlorinated and brominated hydrocarbons (1-chlorobutane and ethylene bromide)	Ion selective electrodes (alginate)	BtB	Peter et al. [82]
28.	*S. cerevisiae (I) (II)*	Cu^{2+}	Amperometric	SRB	Tag et al. [83]
29.	*S. typhimurium*	2-Amino-3methylimidazo [4, 5-f] quinoline	Amperometric	SRB	Ben-Yoav et al. [19]
30.	*S. marcescens LSY4*	BOD	Amperometric	SRB	Kim and Kwon [84]
31.	*T. bacteria*	BOD	Amperometric	SRB	Karube et al. [85]
32.	*T. cutaneum* and *B. subtilis*	BOD	Amperometric	SRB	Jia et al. [86]
33.	Yeast	BOD	Amperometric	SRB	Chen et al. [87]
34.	Yeast SPT1 and SPT2	BOD	Amperometric	SRB	Trosok et al. [88]

BtB Biotransformation-based strategy, *SRB* Stimuli response-based strategy

Table 4 Commercially available biosensors based on cells

S. no.	Biological system used	Technology developed	Strategy	References
1.	Algae	Aquasentnel	BtB	Aqua Sentnel [94]
2.	Bacteria	Amtox	BtB	Upton and Pickin [95]
3.	Bacteria	Baroxymeter	BtB	Baroxymeter [96]
4.	*Vibrio fscherei* (bacteria)	BioTox Flash Test	BtB	Aboatox environmental analysis [97]
5.	*Scendedesmus subspicatus* (algae)	Fluotox	BtB	Fluotox [98]
6.	*Pyrocystis lunula* (algae)	Lumitox	BtB	Stfey and Nicolaids [99]
7.	Algae and bacteria	Cellsense	SRB	Farré et al. [100]
8.	*V. fscherei* (bacteria)	LUMIStox	BtB	Hach-Lange UK-LUMIStox [101]
9.	*E. coli* (bacteria)	MetPlate	BtB	MetPLATETM [102]
10.	*Sinorhizobium mellioti* (bacteria)	Sinorhizobium melliot Toxicity Test	BtB	van der Schalie et al. [103]
11.	*Saccharomyces cerevisiae* (yeast)	GreenScreen EM	BtB	Keenan et al. [104]

BtB Biotransformation-based strategy, *SRB* Stimuli response-based strategy

4.1 Live Cell-Based Biosensors in Medical Diagnostics

The ion channels, enzymes, and receptors are a few of the molecular recognition components that are available and expressed by whole cells. These compounds are frequently vulnerable to their respective analytes due to their inherent biological mechanism [105]. Biosensors based on living cells can thus be utilized to monitor and analyze a range of physiological indicators in real time. As a result, these whole cell-based sensors can be utilized at the cellular level to understand biological metabolic states and other disorders, resulting in their widespread use in biomedicine, such as cellular physiological analysis, pharmacological evaluation, and medical diagnosis.

4.2 Precision Medicine

The latest investigation goal of precision medicine is to understand the genomic alteration of the biological target of a pharmacologically active medicinal compound, e.g., G-protein-coupled receptors and enhancements in their response towards drug. In order to characterize drug responses mediated by G-protein-coupled receptors in lymphoblastoid cell lines, a new biosensor based on the live cell was designed (label-free, [106]). This suggests that they could be used as a cellular model system to investigate the pharmacology of G-protein-coupled receptors in vitro in precision medicine. By means of a bullfrog fibroblast cell line, Feng

et al. [107] invented a cell-based biosensor expressing G-protein-coupled receptors as its basis, to assess these receptors' activity and determine the adrenaline quantity they secreted. At this point, a dominant downstream target gene p21 (tumor suppressor gene) of the activated p53 protein was utilized because it is vulnerable to carcinogens and can thus serve as a sensor. In another study, a human hepatoma cell-based biosensor was tested by Zager et al. [108] that under a p21 promoter regulation, utilized a plasmid encoding enhanced green fluorescent protein (EGFP) to detect genotoxic agents easily and quickly.

4.3 Detection of Micronutrients

The live cell-based biosensors have another area of application in the arena of micronutrients. The lack of a crucial vitamin for human well-being, i.e., riboflavin can lead to serious ailments, e.g., cataracts, metabolism disorders, and some cancers [1, 109]. It is also harmful to excessively intake riboflavin which further contributes to oxidative damage in light-exposed tissue [110]. To resolve this, Si et al. [111] described a biosensor based on a whole-cell (bio-electrochemical) system for the amperometric detection of riboflavin. A bio-electrochemical wire was developed, consisting of cytochrome C strung and riboflavin between *S. oneidensis* MR-1 as shown in Fig. 3. The addition of riboflavin to the bio-electrochemical wire system resulted in an electrochemical response. A 200-fold increase in electrochemical signal output was observed compared to traditional chemical biosensors. There was a wide linear range (5 nM–10 μM, 3 orders of magnitude), a high sensitivity (2.2 nM, $S/N = 3$), and a high resistance to signal interference in the cell-based biosensor.

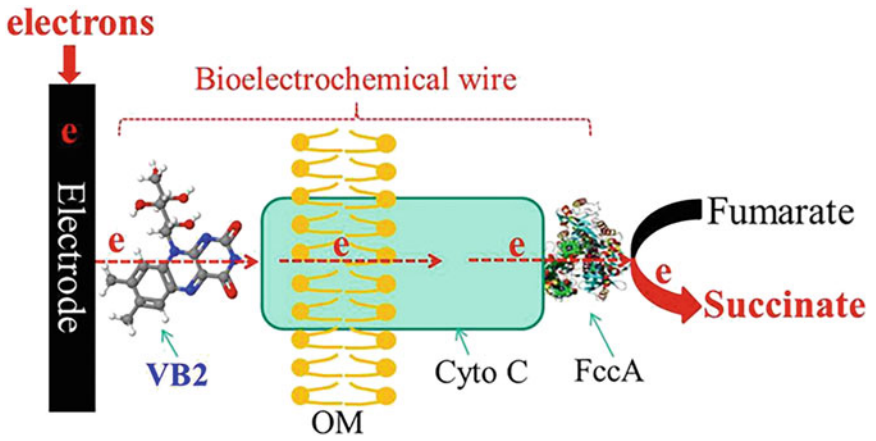

Fig. 3 Diagrammatic representation of an electron transfer pathway utilized in biosensors based bio-electrochemically on live cell. (*OM* outer cell membrane, *Cyto C* cytochrome C proteins, and *FccA* fumarate reductase, [111])

4.4 Diseases Diagnosis

A significant objective in the disease diagnosis is the fast and reliable identification of pathogens. As microorganisms in patient urine and blood samples must initially be pre-cultured to adequate quantities for their detection, traditional approaches of microbiology may take a number of days to weeks. Thus, for the precise detection of microorganisms that did not involve this lengthy culture stage, a new method based on the biosensors (whole cell) was developed [112]. As seen in Fig. 4, through impedimetric detection of *E. coli,* the cell-based biosensor, which comprised bacteriophages involved as recognition receptors, was immobilized to a functionalized carbon electrode (screen-printed) covalently.

In the diagnosis of diseases, biosensors based on cells may offer high-content screening and analysis. For instance, the microbial genome (4–10%) and its proteome (more than 20%) were affected by microbe quorum-sensing molecules, suggesting that quorum-sensing was correlated with the basic metabolic processes as well as the production of the modulate virulence factor [1]. The biosensors based on the cells have also been utilized as noninvasive procedures to assess quorum-sensing molecules in physiological samples collected from patients suffering from bacterial gastrointestinal disorders [113]. Detection limits of quorum-sensing molecules in biological matrices have been improved to the nanomolar level [114]. This was significant because the key reason for human morbidity and mortality observed in sub-Saharan African at-risk adults and young children is invasive non-typhoid *Salmonella* [115]. A cell-based electrochemical immune-sensor, hosted in yeast, was also documented by Venkatesh et al. [116] to detect invasive non-typhoid *Salmonella* antigens. The yeast cells were genetically engineered in that study to display on their surfaces, gold-binding peptide as well as single-chain

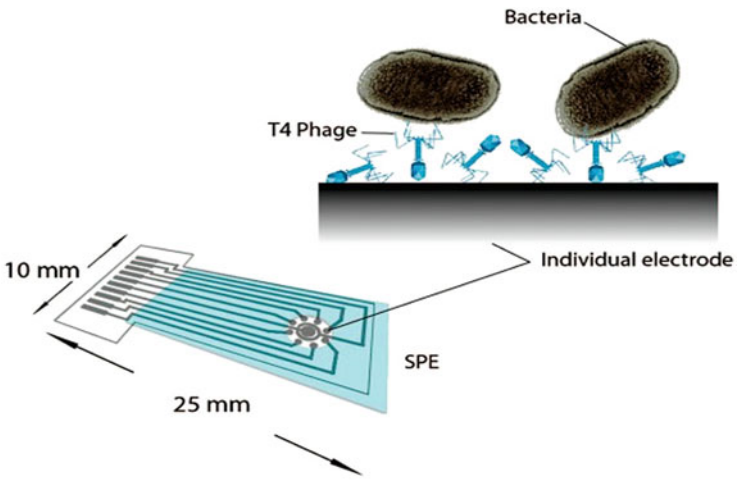

Fig. 4 Diagrammatic representation of the assay for immobilizing phages onto electrochemical electrodes [112]

variable fragment (scFv) antibodies. A wide dynamic range with high nanomolar sensitivity was shown by the resulting cell-based biosensor and was capable of detecting invasive non-typhoid *Salmonella* OmpD antigens [116]. The advantages of cell-based biosensors have evolved in part due to their ease of use and quick deployment for the diagnosis of a variety of illnesses. As a result, biomedical diagnosis techniques based on live cell-based biosensors showed great promise in the field of biomedical engineering.

5 Application of Cells as Biosensors Against Environmental Analytes

5.1 Bioavailability Detection

A substantial research subject is the rapid identification of environmental pollutants and the assessment of their health impacts. To determine the exact content and composition of pollutants in a given sample, traditional chemical and physical-based analytical approaches may be highly reliable and sensitive, but only limited varieties of pollutants may be examined for their bioavailability, genotoxicity, and toxicity [117]. In many circumstances, when using living cells, it is only possible to measure some important parameters [118]. Two distinctive benefits of the biosensors based on the cells include: (1) the effortlessness with which they can be field trialed and (2) the effortlessness with which the samples containing a bioavailable pollutant can be detected.

An example of this is given in Fig. 2, where a live cell-based biosensor *Pseudomonas putida* and a high-performance liquid chromatography were investigated and compared for their capability to detect phenanthrene, added to red soil samples [119]. At variable ranges from 10 to 60 mg/kg, the initial concentration of phenanthrene was measured. It was found that high-performance liquid chromatography detected approximately 80% phenanthrene. The application of cell-based biosensor *Pseudomonas putida*, on the other hand, has been able to bioavailable fraction detection at levels much lesser than those detected with the total phenanthrene material. During high-performance liquid chromatography measurement, this was largely due to the sample extraction process. Peltola et al. [120] have checked the copper and lead concentrations bioavailability in natural soil using an analogous bioluminescent live cell-based biosensor in addition to the identification of organics. Again, this finding was consistent with the findings shown by the phenanthrene study; the cell-based biosensor was used to achieve a much higher selectivity. The inorganic study was also reported to be costlier, tedious, and allowed the procedure to be performed in a specialized laboratory. Finally, it has been shown that the cell-based biosensors can continuously monitor, in real time and in the sample (in situ), the bioavailability and concentration of toxic compounds [121].

5.2 Reporter Genes

Usually, to detect environmental pollutants, the efficiency of live cell-based biosensors depends on the reporter genes selected for transcriptional contaminant regulation and the nature of regulatory protein allied with these promoters. A reporter gene present in the living cells utilized as a sensor may translate its biotic response into a physicochemically detectable signal. The live cell-based biosensors' selectivity and sensitivity are essential for this process. There are several commonly operated reporter genes that have been revealed to integrate effectively into biosensors, e.g., firefly Luciferase (luc), bacterial luciferase (lux), β-galactosidase (lacZ), and green fluorescent protein (gfp). It can be difficult to pick which reporter gene to use because there are a large number of the same to choose from [1]. A few of the benefits and drawbacks of widely described reporter genes employed to generate a biosensor based on cells are listed in Table 5.

The reporter gene (Gfp) codes for the green fluorescent protein (GFP) and it does not need an ATP or a substrate to emit as it autofluorescence [127]. But the intrinsic fluorescence of some cells (host) usually upsurges the background fluorescence besides this reporter, and this may result in signal interference. Therefore, biosensors based on gfp are typically unable to investigate with as much sensitivity as other lacZ and lux-based biosensors [128]. Moreover, in order to emit a stable fluorescence, GFP takes a longer period, decreasing its maximal detection activity [129].

Thus, live cell biosensors based on gfp are usually incompatible with rapid contaminants detection. Similarly, the bacterial luciferases (lux) largely suffer from dimeric protein interference and thermal lability and also restrict its application

Table 5 Some reporter genes used in sensors based on cells

S. no.	Genes	Method of detection	Advantages	Disadvantages	References
1.	lux	Bioluminescence	Quick response and easy measurement	O_2 requirement and thermal lability	Hakkila et al. [122]
2.	gfp	Fluorescence	No substrate requirement, high stability	Low sensitivity, lag-time for stable fluorescence and autofluorescence	Sagi et al. [123]
3.	lacZ	Bioluminescence, fluorescence, colorimetry, electrochemistry	High stability, wide variety of detection methods, detection by naked eyes	Substrate-dependent, low permeability	Mascher et al. [124]
4.	luc	Bioluminescence	Quick response, high sensitivity, thermal stability	O_2 and ATP requirements, low permeability	Gutiérrez et al. [125]
5.	crtA	Colorimetry	Detection by naked eyes	Substrate dependent	Chong and Ching [126]

Table 6 Comparison of different types of whole cell-based biosensors on the basis of the sensitivities

Reporter gene	Host chassis	Target analyte	Detection sensitivity	Reference
lacZ crtI	*D. radiodurans*	Cadmium	1–10 mM 50 nM–1 mM	Joe et al. [133]
luxAB	*B. sartisoli*	Naphthalene, phenanthrene	0.17 μM	Tecon et al. [134]
luxAB	*E. coli*	Benzene, toluene, and xylene	0.24 μM	Tecon et al. [134]
luxAB	*E. coli*	C_6–C_{10} alkanes	10 nM	Sticher et al. [135]
luxCDABE	*E. coli*	Tetracyclines	45 nM	Korpela et al. [136]
Luc	*E. coli*	Benzene, toluene, and xylene	40 μM	Willardson et al. [137]
luxCDABE	*E. coli*	Arsenic	0.74–69 μg/L	Sharma et al. [138]
lacZ	*E. coli*	Arsentate	<10 μg/L	De Mora et al. [139]
Gap	*E. coli*	Chromate	100 nM	Branco et al. [140]
Gfp	*E. coli*	Zinc, Copper	16 μM 26 μM	Ravikumar et al. [141]
luxAB	*P. putida*	Phenol	3 μM	Shingler and Moore [142]
lacZ	*S. typhimurium*	Single-stranded DNA	10 nM mitomycin C	Nakamura et al. [143]

as a mammalian cell's reporter gene [1]. The luc (firefly luciferase) reporter gene was often fused into mammalian cells to avoid these limitations in view of its wide linear range (up to 7–8 orders of magnitude) and high sensitivity [130]. A well-characterized bacterial enzyme, β-Galactosidase (lacZ), another reporter, similarly was widely utilized in molecular biology as it provides an outstanding transfection efficiency monitor. For identification using either fluorescent or colorimetric methods, the lacZ has some specific advantages as its usage with a sample is easy and quick [169]. The broad availability of lacZ electrochemical and chemiluminescent substrates also offers the benefits of ultra-high sensitivity, an extensive dynamic detection range, and low detection limit (as low as 2 fg, [1]).

CrtA, a unique type of reporter gene created by Fujimoto et al. [131], is responsible for carotenoid production in another reporter system. When applied to a sample, without the addition of a supporting substrate, the crtA-based live cell biosensors shift the culture media's color from yellow to red and are therefore appraised as a good choice for quick detection in emergency circumstances [132]. For the identification of environmental contaminants, e.g., organic and waste heavy metals, Table 6 compares the bio-sensitivity of developed biosensors based on cells in recent times. In these systems, a number of cell lines and reporter genes have been used. Sharma et al. [138], for example, utilized *E. Coli* and luxCDABE to construct biosensors based on live cells which were demonstrated to have a detection sensitivity of 0.74 g/

L when arsenic was introduced to water (10 g/L), which is well below the EU and US minimum safety criteria for arsenic. Furthermore, the sensitivity of live cell biosensors for detecting organic waste was shown to be exceptional, with enormous potential.

5.3 Regulatory Proteins

The complex interactions between regulatory protein and the target analytes of the contaminants of interest are important for the sensitivity and specificity of the biosensors based on live cell. There are several research studies elsewhere with the metallo-regulatory protein's discovery in recent years that have used these biosensors in soil and water samples to detect heavy metals. Compared to traditional biosensors, they have revealed improved sensitivity, increased detection ranges, and greater selectivity, e.g., a MerR family's regulatory protein (GolS protein), was described to have a great discernment for Au ions [144]. It was also observed that the detection ranges of GolS-based live cell biosensors were enhanced by including in the GolS protein, a single amino acid at the position 77 [145] and became ideal for the detection of cadmium, lead, mercury, and/or gold ions (Fig. 5).

A cysteine-rich peptide (Metallothionein, MT) was disclosed to possess a great affinity towards numerous heavy metals and contained five isoforms encoded by genes of *T. thermophila* [146, 147]. Further, a biosensor based on a live cell with MTT1 and MTT5 promoters (parted from MTs) was fabricated by Amaro et al. [118] and reacted sturdily and quickly to the heavy metal contaminants' existence. The *Sinorhizobium meliloti* chpA promoter was stably stimulated by the pesticide chlorpyrifos, according to Whangsuk et al. [148], who used the transcriptional activator ChpR. The biosensor based on a promoter (chpA) was introduced in *E. coli* in another study utilized for the chlorpyrifos detection of chlorpyrifos, over a linear response range of 25–500 nM [149]. Elsewhere, an extremely sensitive biosensor based on cells containing the ars operon's promoter region and a reporter gene, the crtA gene was developed by Fujimoto et al. [131] and was found to detect arsenite efficiently. And when the arsenite concentration was present at 5 μg/L, the color change was clearly recognized by the naked eye. Ars operon has also been reported to be associated with resistance towards arsenite, whereas the crtA gene, that regulates the *Rhodovulum sulfidophilum*' carotenoids synthesis, was responsible for shifting the color of the culture from yellow to red as the arsenite concentration changed [132].

DNA microarrays technology can be used as a high-throughput process for positive regulatory genes selection for biosensors as these arrays comprise several proteins, one of which usually reacts to the existence of the contaminant of interest in a sample, e.g., the use of DNA microarray information to choose suitable biomarker genes that were strongly induced following the paraquat toxin's exposure [150]. An alkane-inducible (AlkSp protein) biosensor, for short-chain alkanes detection was fabricated via two rounds of directed evolution of the transcriptional regulator, and

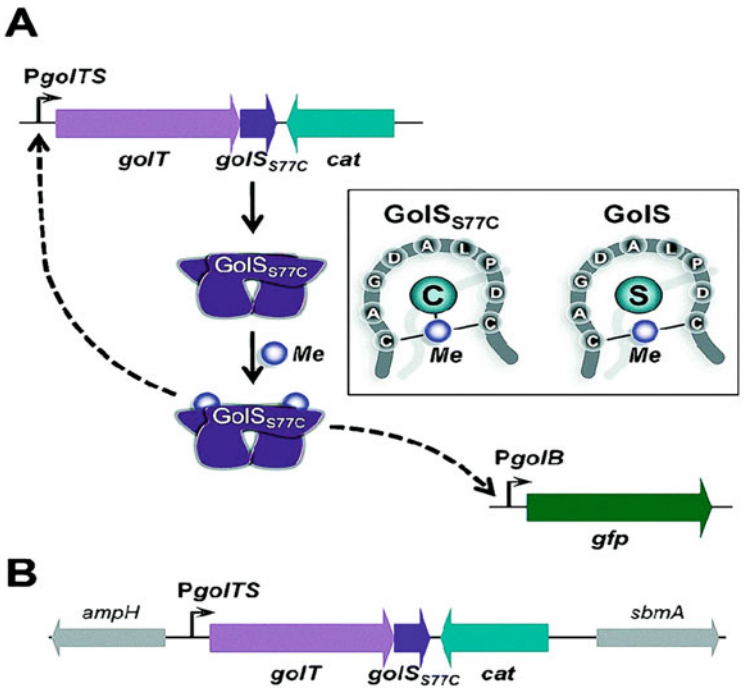

Fig. 5 (**a**) GolS77C-based biosensor platform, the sensor protein GolSS77C is expressed utilizing its chromosomally encoded gene with an operon with golT encoding the P1B-type Au (I) transporter (**b**) Genetic organization of the site selected for the golTSS77C-cat locus insertion in the *E. coli* chromosome [145]

the inventors were capable to demonstrate a fivefold increase in the emission in its reporter signal [151].

5.4 Host Cells

The host cell type choice is also vital as the sensitivity, specificity, and time-response of a biosensor can be greatly affected by the host cell type utilized as the sensing vehicle. In the meantime, there is a great resemblance between a host organism and eukaryotic-based biosensors in terms of genome, metabolism, and cellular organization, about 85% of live cell (eukaryotes) based biosensors are presently utilized for metal detection [152]. For the perception of monocyclic aromatic compounds in various ecological samples, Hernández-Sánchez et al. [153] constructed many cell-based biosensors utilizing different host cells, on the other hand, the same recombinant regulatory framework. The *Alcanivorax borkumensis* SK2 biosensor was reported to have a higher salinity tolerance but a lower pollutant tolerance and showed the best performance for the detection of contaminants at low concentrations in seawater samples. Because of its highest solvent resistance at high concentrations,

the biosensor based on *P. putida* DOT-T1E was established as the best alternative for highly polluted conditions before it became saturated [151]. Because of the emulsifying capacity and low accessibility of the *E. coli*-oil mixture to oil droplets, obtaining tiny oil droplets in the *E. coli*-oil mixture was difficult. However, A. baylyi ADP1 and its derivative ADPWH-alk (the circles in Fig. 4) were definitely an oil-water interface adhesive and could emulsify both crude and mineral oils into 10–80 m diameter oil droplets [154]. These characteristics make ADP1 an exceptional microbial substrate for the live cell-based biosensors' production to detect a wide variety of carbon chain length alkanes and alkenes (C7–C36) contained in water, seawater, and soils samples [154–156].

The *Alcanivorax borkumensis*-based biosensor, specializing in assimilation of linear alkanes, revealed a fourfold lesser fuel octane detection sensitivity (0.5 μM), in comparison with the biosensors that used *E. coli* as a vehicle. When evaluating the very low concentrations of petrol or pure alkanes in the samples, this performance improvement was most apparent [157]. Brutesco et al. [158] prepared *Deinococcus deseri* (a radiation and desiccation-tolerant environmental bacterium)-based functional biosensor and stated that after 7 days of storage, these sensors were able to detect arsenite. In potable water, for nickel detection, numerous cell-based biosensors have also been prepared from different *E. coli* strains. The *E. coli* (TD2158 wild-type) exhibited a ten times greater sensitivity and activity than *E. coli* K12 (W3110) equivalent, even supposing the identical mechanism was utilized with a natural target promoter (rcnA) fused to the luc reporter genes and RcnR Ni/Co metallo-regulator was used [159]. The limit of detection for nickel was stated to be very low (80 nM) in this analysis and was therefore accounted to meet the necessary quality requirements for most potable water. These analyses have shown that the host bacteria selection has a major effect on the efficiency of a fully fabricated live cell-based biosensor.

5.5 Multi-functionalization

Despite the fact that live cell-based biosensors generally exhibit enhanced performance of sensing than traditionally available biosensors based on chemicals, the subject prior art has focussed on enhancing the sensors' accuracy, sensitivity, and applicability. This concern probably reveals an understandable response to the extensive and enhanced pollution levels [160]. Diverse types of functional cell-based biosensors have recently been mixed together and it was found that it showed improved identification and measurement of contaminants than a single type of biosensor, e.g., for the monitoring of water samples, a combination of a yeast-based estrogenic activity assay and bioluminescent bacteria-based toxicity screening was applied [161]. Via a regulatory proteins mixture, such as ZntR, CadC, and ArsR, numerous biosensors were prepared, that then reacted concurrently when numerous different metals were added [1, 162]. In another example, a tailored biosensor set based on live cells was produced by examining an *E. coli* sensor set utilizing binary linear and regression equations for the refining purpose of the accurate and specific

bioavailablity recognition of Pb, As, and Cd in co-polluted surroundings, to reduce the signal interference generated when diverse metals were presented (e.g., Pb, Cd, and As, [163]). In that study, the sets of sensors were categorized into two different groups in accordance with their particular response to Pb, Cd, and As. Group 1 had pzntRluc and pcadCluc sensors to detect the bioavailability of Pb and Cd, while Group 2 had a parsRluc sensor to detect the particular bioavailability of As. The higher concentration ranges of mixes resulted in a linear increase in the relative light unit. To evaluate the bioavailability concentrations of Pb, Cd, and As in samples of soils from a polluted mine site, two sensor groups with three binary liner equations were used. With Group 1, using a linearly improved relative light unit, the coinciding ranges of concentration (0.1–1.0 μM) of mixed Cd-Pb were calculated. The overlapping concentration ranges for Group 2 were found to be 5.0–10.0 μM for Cd and 0.1–1.0 μM for As. These findings showed in this study that the bioavailability of the detected heavy metals appeared to be overestimated by a traditional single target cell-based sensor device. When a multiple cell-based biosensor was applied, more detailed bioavailability data was obtained.

This chapter, in some way, stems from growing trepidations about the alleged "antibiotics crisis" involving too much unnecessary pesticides, antimicrobials utilization, and their unregulated release into the environment [164]. There is a crucial need for biosensors based on live cell fabrication for the steady and quick detection of various antibiotics in samples obtained from tremendously contaminated environment, e.g., the *P. putida* DOT-T1E-based microbial biosensors have presented a detection capability for a broad range of structurally different antibiotics because of its environmental adaptability and its tolerance to numerous toxic organic compounds [165]. Camanzi et al. [166], on the other hand, found that following rehydration many months after freeze-drying, bacteria transmitted a steady light signal. Prévéral et al. [167] also report the fabrication of a cell biosensor based on arsenite that kept its performance for the sensitive detection of arsenite 7 months after lyophilization.

While compared to their traditional chemical equivalents, the live cell-based biosensors have usually shown superior efficiency, their commercialization realization is still a major challenge. Stability and reproducibility are still not sufficiently effective during long-term storage and transport to meet the requirements for large-scale production. However, Camanzi et al. [166] discovered that several months later, after their rehydration, freeze-dried microorganisms released a stable luminous signal. Another study proposed a flow-through biosensor based on disposable modular biochips including agar-immobilized bioluminescent recombinant reporter bacteria for online and continuous monitoring of water toxicity [168]. This cell-based biosensor was revealed to function properly in continuous flowing water over several days.

6 Future Perspectives and Conclusion

Due to the speedy advances seen in cell immobilization and in microfabrication, the biosensors fabricated from active cells have become an attractive arena for investigators. By translating signals that produce their homeostasis part into outputs (optical and electrical) that may then be detected, the biosensors based on live cell can quantitatively recognize data related to the position of live microbial or animal cells. These live cells can offer rapid, unique, and real-time information streams of the cell's homeostatic position and, by implication, and its microenvironment, with very high selectivity and sensitivity compared to traditional chemically based biosensors. This study explored recent progress in the production, use, and implementation of live cell-based biosensors across numerous arenas, with a specific emphasis on the arenas of medical diagnostics and ecological pollution monitoring. The biosensors based on live cells have numerous benefits, but their use is also limited. A huge number of contaminants and other naturally occurring molecules are present in most environmental samples examined and together they cover the signal from the analyst of interest. The toxic nature of the samples is another issue, as they may comprise organic contaminants or heavy metals and their presence may restrict the option of cells used to resist the action of those microbes. Finally, as these cells experience diffusion or leakage, emission from sensors based on live cell employed for prolonged time periods may become unstable over time. The potential prospects of biosensors based on live cells can prove exciting. Another possible goal for live cell-based biosensors is the development of precise and multi-functional biosensors for speedy and real-time detection in extremely unfriendly environments with extreme acidity, alkalinity, severe temperature, high salinity, and poisonous substrates. For intense environmental research, it could be envisaged that several strains (cells), e.g., halophiles, thermophiles, and alkaliphiles, etc., could be utilized as host cells for their application as live cell-based biosensors.

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Part IV

Biomaterials-Based Sensors for Agricultural and Biomedical Applications

Magnetic Nanoparticles-Based Novel Sensors for Select Biomedical/Biological Science Applications

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Abstract

This chapter deals with the design and development of select biosensors employing a novel approach and also using magnetic nanoparticles (MNPs) to unravel the significant challenges including rapid detection and precise measurements of protein biomarkers, cells, small molecules, pathogens, etc., in biomedical and life science applications. Therefore, the advancement of versatile and robust biosensing platforms needs to take into account the different requirements and consequences in medicine, pharmaceutical drug progress, clinical diagnostics, and genomic areas and proteomic research. Nevertheless, MNPs are of great attention in recent years for biological and life science applications because of their unique properties like biocompatibility, stability from the physical and chemical point of view, inexpensive to synthesize, large magnetic susceptibility, and environment friendly and safety, and, hence, MNPs-based sensor is used in biological samples for different applications such as biosensor, drug

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delivery, and thermal ablation therapy. This chapter is endowed with: (1) diagnostic magnetic resonance (DMR), (2) surface-enhanced Raman scattering (SERS), and (3) surface plasmon resonance (SPR) techniques having different mechanisms by using MNPs that are considered as the detection platforms for numerous applications in biomedical science. Although DMR technique comprises several sensing principles with assay configurations, the key basis for DMR is to use MNPs as proximity sensor which is exploited in clinical magneto resonance imaging (MRI) scanners or benchtop nuclear magnetic resonance (NMR) relaxometry. Currently, a chip-based NMR detector system has been explored in DMR technology for advancement and capable of functioning in extremely sensitive calculations on microliter sample capacities and in a multiplexed arrangement. DMR biosensor technology and its use to detect numerous biomolecules in biomedical science applications are discussed in this chapter. Albeit absorbance or fluorescence-based optical approaches have been employed since decades, comprehensive information about the usage of MNPs in surface-based optical techniques namely SPR and SERS is presented in this chapter. These surface-based optical techniques have been advancing very rapidly for potential applications in the biosensing platform.

Keywords

Magnetic nanoparticles · Biosensor · Diagnostic magnetic resonance · Surface plasmon resonance · Surface-enhanced Raman scattering, etc.

1 Magnetic Relaxation Properties

Through the controlled and optimized synthesis approaches, MNPs with a size range from 10 to 300 nm have promising biomedical science applications, since the size of biological molecules limits falls in such nanometer scale range. For instance, biological molecules, viruses, ferritin, and lipoprotein (LDL) are in the same nanoscale range and their properties can be finely tuned with the properly selected and controlled chemical synthesis process. MNPs are a class of nanoparticles (NPs) which can be influenced by using magnetic fields, as seen in Fig. 1, the external magnetic field with strength H is when applied to the MNPs then the materials reciprocate with magnetization M, defined by $M = \chi H$ where χ is the magnetic susceptibility. Nonetheless, in the case of superparamagnetic NPs, each particle act as a single magnetic domain rather than multidomain systems that commonly appear in ferromagnetic systems (Fig. 1). Consequently, the individual magnetic dipole moments of superparamagnetic NPs, due to the coupling and coalignment of electron spins in the single domain, lead to larger magnetization values than that of paramagnetic materials paving way for the high magnetic susceptibility. The magnetic moments of superparamagnetic NPs, in the absence of a magnetic field easily hop between the anisotropic axis (see Fig. 1d), and the assembly of superparamagnetic particles displays feeble magnetic moment as well as negligible

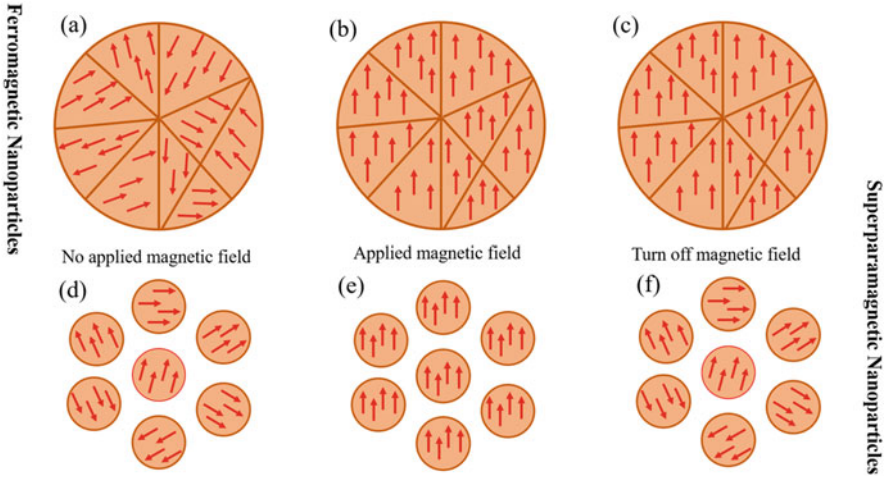

Fig. 1 Schematic representation of the nature of magnetic moments in ferromagnetic nanoparticles (**a**–**c**) and superparamagnetic nanoparticles in (**d**–**f**) in the absence/application/turn off of the external field. In the absence of an external field, the moment of both ferromagnetic and superparamagnetic particles in the time mean is zero (**a** and **d**). In the application of external field, the domains in ferromagnetic nanoparticles are aligned in the direction of the applied field (**b**), and the moments of superparamagnetic nanoparticles are also aligned in the applied field direction (**e**). Once the magnetic field is switched off, the remnant magnetization presents in case of ferromagnetic nanoparticles (**c**); however, the moments of superparamagnetic particles became chaotic that results in more or less no remnant magnetization (**f**)

remanent magnetization. Briefly, superparamagnetic NPs used to be magnetized in a relatively weak magnetic field (H) as it possesses high magnetic susceptibility (see Fig. 1e). Moreover, the magnetic moments of superparamagnetic NPs again randomized when the external magnetic field is turned off (Fig. 1f), leading to the unimportant of remnant magnetization because the moments cannot withstand the thermal agitation termed as Nèel relaxation process. On the other hand, the ferromagnetic particles have numerous magnetic domains in each particle compared to the superparamagnetic NPs (where each superparamagnetic particle acts as a single domain). When an external magnetic field is applied, the moments are aligned along the applied field direction (see Fig. 1b), similar to superparamagnetic particles. But the remnant magnetization remained when the magnetic field is turned off due to their moments that withstand thermal effect (see Fig. 1c) in contrast to the superparamagnetic NPs. In essence, NPs with superparamagnetic properties such as high magnetic susceptibility and lack of magnetic moment when an external applied magnetic field is turned off, favorably prevent the magnetic interaction/attractions among particles in a solution. More importantly, this feature creates superparamagnetic NPs to be suitable for many applications in biomedical sciences. It is distinctly evident from the above discussion that each superparamagnetic NPs engenders a big dipole moment on application of an external magnetic field. Thus, the developing gradient of the local field produces no uniformity in the external

magnetic field that triggers to kill the coherent precession of nuclear spins of adjacent water protons [1, 2].

Besides, the changes in the magnetic resonance signal can be identified by means of magnetic resonance methods such as MRI and NMR. Usually, superparamagnetic particles lessen the relaxation times in longitudinal (spin–lattice: (T_1)) as well as in transverse (spin–spin: (T_2)) of water protons in the MRI/NMR systems, nevertheless, T_2 takes advantage of DMR biosensing applications for the reason that the transverse relaxivity (r_2) for most MNPs is considerably larger than longitudinal relaxivity (r_1). Here, the relaxivity of particle "r" is defined as the change $1/T$ for a given change in particle concentration. It has to be noted here that the higher the relaxivity denotes that the smaller number of NPs is necessitated to engender a noticeable signal. More importantly, the relaxation properties are not only reliant on the "r" of the population of MNPs but also on the organizational state of the population of particles. It has been observed that the aggregation of NPs into clusters divulges the enhancement of net transverse relaxation rate ($R_2 = 1/T_2$) [3, 4]. This phenomenon is called as magnetic relaxation switching (MRSw) effect which means it is a cooperative process wherein the straight interaction of NPs instigates them to more efficiently de-phase the spins of neighboring protons in contrast to the dispersed MNPs (see Fig. 2). Of late, portable MR technology, termed as magnetic relaxation switch (MRSw), has been exploited to design a novel class of MR diagnostic sensor which has potential applications to offer selective, sensitive, immune-, and molecular diagnostics in point-of-care (POC) settings [5–7]. A recent hypothesis used the outer-sphere theory to expound on the rich mechanism of relaxation switching and predicted that a particle's relaxivity is clearly proportional to the area of cross section [8, 9]. But, when NPs are assembled to form clusters, then the effective cross-sectional area increases, which surpasses the additive contribution from each particle. Thus, the hefty effective cross-sectional area of particles in the form of clusters produces a larger and reasonably detectable dipole moment. Besides, the longitudinal relaxivity (r_1) is not influenced by the effect of aggregating, and consequently, it can be applied as a measure of total nanoparticle concentration [4]. It may be noted here that in order to favorably exploit the DMR sensing, firstly, one should prepare MNPs with a large magnetic moment and superparamagnetic characteristics to circumvent the aggregation to stimulate the prominent T_2 changes. Secondly, to prevent NPs' aggregation in an aqueous solution, the surface of NPs must be coated with a hydrophilic and biocompatible layer that will make them great impact/affinity to attach the molecules, for instance, antibodies, DNA, or peptides. Lastly, synthesizing the NPs with smaller size consequences means greater solution stability and no sedimentation. More importantly, they can be packed more compactly to augment the surface coverage for cell labeling applications. DMR assays configurations, as shown in Fig. 2, are designed based on two switching processes, viz., the first one corresponds to the designed MRSw assays root to the self-assembly of MNPs with respect to adding the molecular targets so-called forward switching/decreasing T_2, and the second one is the disassembly of organized clusters by enzymatic cleavage/competitive binding named as reverse switching/increasing T_2. Basically, cross-linked MNPs are used in forward MRSw assays to establish the

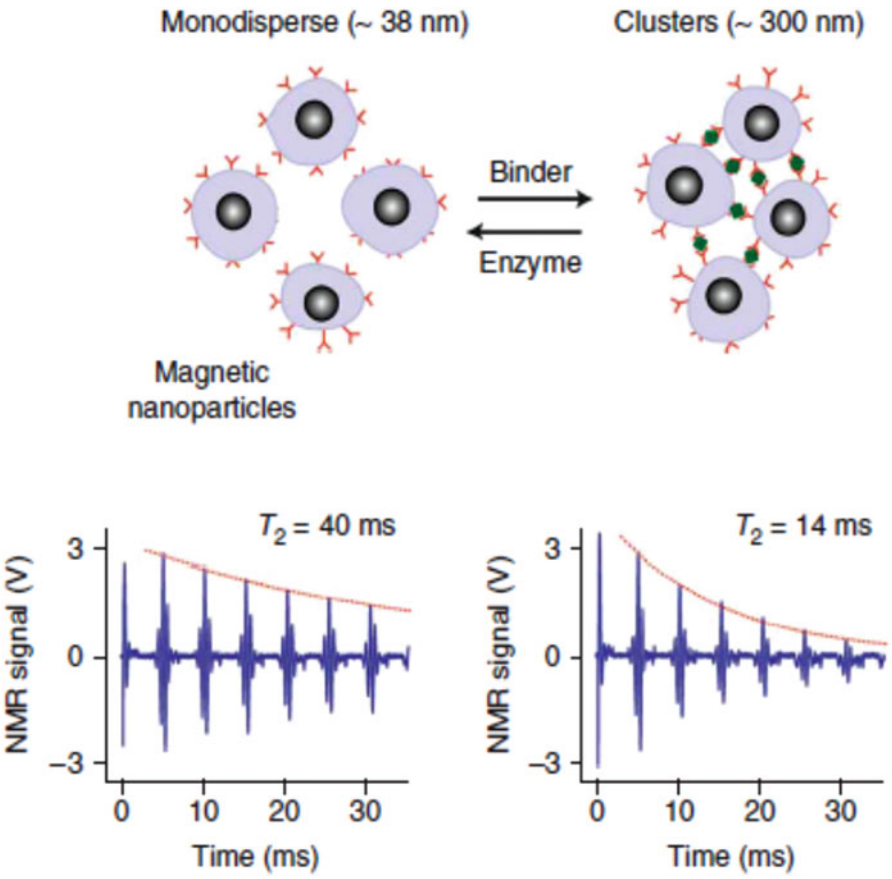

Fig. 2 Principle of proximity/magnetic relaxation switching assays: top panel shows the clusters of assembly of monodispersed magnetic nanoparticle by using a target biomarker as a cross-linking bridge, or disassembly of achieved clusters by using an enzyme. Below panel shows the changes of NMR signal w.r.t. time: clustered magnetic nanoparticles roots more effectively to de-phase the spins of water molecules shortening the spin-spin relaxation time (T_2), whereas the disassembly of clusters increases T_2 relaxation time [1]

clusters via molecular target bridges. For instance, small cross-linked iron oxide (CLIO) NPs are considered to be a good material for DMR biosensing applications supporting their outstanding biological properties [10]. CLIO NPs involve a 3–6 nm sized superparamagnetic monocrystalline iron oxide particles, precisely, it is comprised of maghemite (γ-Fe_2O_3) and/or ferrimagnetic magnetite (Fe_3O_4). Following that, the core of the iron oxide NPs is coated with biocompatible dextran which is consecutively handled with epichlorohydrin to shape the stabilizing cross-links and then activated with ammonia to set the amine functionality. These aminated CLIO (amino-CLIO) NPs in general will have a hydrodynamic diameter range of 25–40 nm on an average, and roughly 40–80 amines accessible for each particle

for biomolecule conjugation [11]. Moreover, these amine sets can make further reactions with a large number of reagents to add biomolecules through hydroxyl, thiol, carboxyl, anhydride, or epoxide groups numerous reagents [12]. Nonetheless, the surface functionalization lets the surfaces react with a variety of targets, the details can be found in the review paper reported by David and Lee [13]. Therefore, they are rather appropriate for detecting the small molecule analytes, for example, proteins, drugs, oligonucleotides, and metabolites as the short cross-links warranty the MNPs are positioned close enough to encourage the relaxation switching. More importantly, MRSw assays can be acted in turbid solutions like blood, and do not necessitate to the elimination of liberated MNPs emphasizing that there is no inevitability for the time-consuming separation or the capture strategies. Detection sensitivity has been realized to be enhanced by a factor larger than the rise in valency, fascinatingly, through augmenting the valency of a target via connecting to a protein or microparticle carrier [2]. This effect is attributable to the superior valency targets and can effectively foster nanoparticle clustering, consequently, liberating the MRSw technique from the equivalence principle (maximum sensitivity at equal-molar ratios of magnetic nanoparticle and target) that normally dominates. Prior to executing the switching assay to exploit this valency effect, it might be therefore advantageous to first capture targets with a particle. Nevertheless, for reversed MRSw assays, initially the clusters of NPs need to be created in a similar way as forward assay, and then adding the enzyme to the cluster system breaks molecular bridges at the precise sites or else can weaken the cross-links via useful binding molecule.

2 Design of Magnetic Relaxation Detection Devices for DMR Assay

Originally, NMR spectroscopy has been exploited to investigate small molecules and proteins, and MRI scanners to explore the detailed images of the body through magnetic resonance signals. These techniques were utilized to assess the spin-spin relaxation time (T_2) for DMR biosensing assays. Though MRI scanners provide super data acquirement, the instrument operating cost is extremely high, large in size, and requires a large amount of sample (hundreds of microliters) [2]. Further, benchtop-relaxometry has been considered as an alternative for DMR sensing due to the lower cost and operation at lower NMR frequencies (100 kHz–50 MHz), but the system necessitated a large amount of sample and also lack the capability to perform parallel measurements [2, 3, 14]. Thus, to carry out the measurements with less sample volumes, a chip-based μ-NMR component is further developed to operate in a multi-channeled based DMR system [1]. DMR system specifically consists of four major components such as (1) μ-NMR system comprises of micro-coils for radio-frequency (RF) excitation as well as for NMR signal detection, (2) a microfluidic network for sample handling, (3) an on-board NMR spectrometer, and (4) a small/portable permanent magnet [1]. In the first multi-channeled detection system which is displayed in Fig. 3a, the micro-coils were organized in an array-like structure for

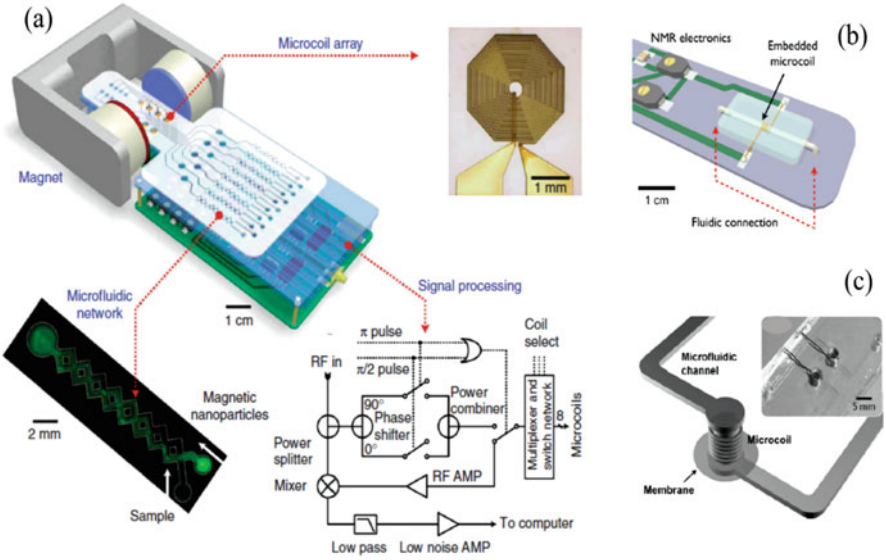

Fig. 3 Schematic representation of DMR system. (**a**) The system consists of an array of planar micro-coils for NMR measurements, microfluidic networks for sample handling and mixing, miniaturized NMR electronics, and a portable magnet for polarizing magnetic field generation [1]. (**b**) The second-generation μ-NMR with a solenoidal coil embedded in a microfluidic device, which could make maximizing the filling factor, diminished the signal-to-noise ratio, and lessen the sample amount to ∼1 μL [15]. (**c**) Illustration of a membrane filter which can be mounted at the coil outlet, enabling concentration of large samples and removal of smaller impurities [16]

parallel measurements to avoid the implementation of field gradient, and the microfluidic system facilitates to control sample volumes, possibly, each micro-coil held 5–10 μL of sample. Afterward, a solenoidal micro-coil embedded in a microfluidic structure based on a novel μ-NMR was established to increase the filling factor, reduce the sample volume nearly to 1 μL, and more importantly provide a consistent radio frequency magnetic field and less electrical resistance (Fig. 3b) [15].

Hence, the μ-NMR is a very much suited technique to uncover the many targets in bio-related samples and also to conserve the costly reagents. In such a novel μ-NMR system, the NMR electronics were monolithically integrated on a single CMOS-IC chip to prevail over the adverse conditions for NMR measurements originating from the miniaturization of the system. The field inhomogeneity sourced to small/portable magnet causes the fast signal decay (large T_2*) and small sample volumes lead to low NMR signal levels are the limitations of the miniaturization device which can be further succeeded by the development of an on-chip digital pulse generator for spin-echo sequences and implementing the low noise RF amplifiers with high voltage gain. These spin-echo pulse arrangements (T_2) compensate the unevenness of the polarizing magnetic field, and the relaxation times of the proton can be carried out by inversion recovery pulse sequences (T_1). One more component in DMR system is the microfluidic networks which facilitate to control and manipulate less amount of

sample, blend diverse flow streams, and restrict the sample to a highly sensitive area of a provided micro-coil. As seen in Fig. 3c, a membrane filter has been incorporated at the outlet of the solenoidal micro-coils to further look into the size selectivity. It means that the membrane filter can hold the large species like cells and eliminate the smaller contaminants like MNPs thus reflecting it to collect the cells from hefty amount of samples and accomplish cleaning steps on-chip [16]. In essence, the whole NMR platform can be considered as a hand-held unit for portable and point-of-care operations.

3 Biosensing Application of Magnetic Nanoparticles-Based DMR System

The most important advantage of MRSw sensors is that it has the ability to detect the analytes in opaque samples wherein the transmission of light through the sample is not required and thus MRSw assays are indifferent to the light-based interferences. Polyvalent interactions among NPs and targets' surfaces inside the solution cause increases in affinity and assay sensitivity, which is another advantage of MRSw sensors. More importantly, MRSw sensor can also be devoted to detecting the chemically diverse types of target analytes that are the surface of NPs and can be modified to detect proteins, enzymes, small molecules, nucleic acids, cells, etc. Though tremendous biosensing applications using MNPs have been reported previously [2, 13], we have discussed a few applications here below, and various detection of analytes such as proteins, DNA, RNA, small molecules, and pathogens using MNPs-based DMR assay technology are also presented in Table 1.

3.1 Detection of Proteins

MRSw biosensors were primarily exploited to see the T_2 changes with the protein molecule targets, wherein the MNPs were first treated with the biotin and then link with the avidin. So, the instigating clustered MNPs exposed T_2 changes with variation of an avidin concentration [3]. It is found that four orders of dynamic ranges regulating the concentration of MNPs demonstrate a strong working scale of the sensor. The detection of green fluorescent protein (GFP) was again experimented to corroborate the principle of MRSw biosensors. Here, GFP-sensitive NPs were first prepared by conjugating CLIO MNPs with avidin, followed by a biotinylated anti-GFP polyclonal antibody. Introducing the GFP into a solution of anti-GFP decorated NPs give rise to a rapid detection of GFP (<30 min), and was found to be a dose-dependent response in the observed T_2 values, down to the low femto-mole range (see Fig. 4) [3]. Michael Cima et al. group in another study explored the detection of the protein hormone through a slightly different strategy in which the beta subunit of human chorionic gonadotrophin (HCG-β) is a biomarker associated with prostate and ovarian [17]. Here, two different monoclonal antibodies on the HCG-β protein were appended to disperse the populations of CLIO NPs to engender a sandwich

Table 1 Summarizes the different analyte detection with magnetic nanoparticles-based DMR assay technology

Analyte	Target	Reference (s)
Proteins	GFP	[2, 13]
	Avidin	[3]
	HCG-β	[17]
	Telomerase	[2, 13]
	CA-125	[1, 2]
	VEGF	[1, 2]
	α-fetoprotein	[1, 2]
	HSA	[13]
DNA	Telomeres	[2, 13]
RNA	GFP	[3, 4]
Small molecules	Glucose	[19]
	Drugs, enantiomers	[20]
	Folate	[19]
	HA peptide	[19]
	Calcium	[22]
	Influenza Tag peptide	[2, 13]
Pathogens	Herpes simplex virus	[14]
	Adenovirus-5	[14]
	S. aureus	[2, 13]
	MTB/BCG	[16]

MRSw assay. Using this system, the HCG-β was effectively detected in a dose-dependent manner. More importantly, it has been noticed that the sensitivity can be enhanced when an HCG dimer is utilized as a target with increased antibodies amount per CLIO nanoparticle. Furthermore, MRSw biosensors are used to discover the different tumor biomarkers like CA-125, VEGF, and α-fetoprotein in the blood samples using MNPs coupled with μ-NMR device [1, 2]. Of late, different kinds of proteins and lectins particularly bind to the carbohydrates recognized by MRSw technique. In this case, specific carbohydrates functionalized the MNPs followed by the reaction with equivalent lectins. Essentially, the number of binding sites, topological arrangement of the binding sites, and disparities in the structure of proteins can cause a different initial accumulation rate with the carbohydrate-coated beads [18].

3.2 Detection of Nucleic Acids

Using oligonucleotides, the concept of MRSw was also demonstrated and firstly a biosensor was found to be very responsive to the manifestation of 24 base-pair target oligonucleotide arrangement [4]. Here, different complimentary 12 base-pair oligonucleotides were attached to the surfaces of the two separate CLIO nanoparticle biosensors. Further, the addition of a target oligonucleotide sequence causes a rapid aggregation of NPs and a consequent decrease in T_2 relaxation time. It has been seen

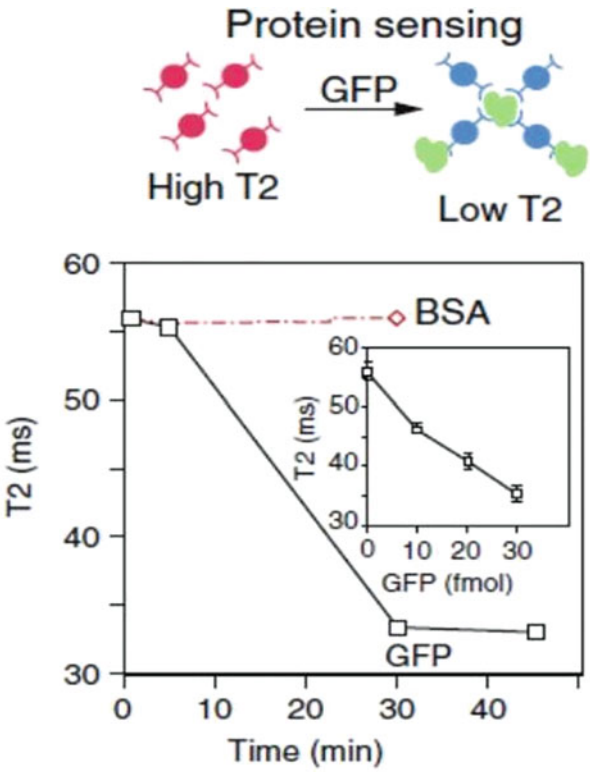

Fig. 4 Magnetic nanoparticles conjugated with a polyclonal antibody specific for GFP were incubated with GFP protein or BSA as a control. Spin-spin relaxation time decreased linearly with the amount of protein, getting a steady state in 30 min [3]

that the change in T_2 relaxation time varies linearly with regard to the concentration of target incorporated into the system, and the sensitivity threshold value was found to be seen in sub femto-mole scale. But by heating, the aggregation of NPs can be dissociated and the changes in T_2 will result in similar behavior while experimentation is made in a turbid solution. Interestingly, in the target sequence, the inclusion of even a single mismatch led to a noticeable change in signal compared with the perfectly matched sequence, but the change in T_2 was found to be ceased when scrambled oligonucleotide sequences were used. Hence, Perez et al. expounded a suitable technique for the sensing of nucleic acid and presented the capability of sensing sole-base pair mismatches [3], and it may prove valuable for mutational analysis. They also established the detection of a target mRNA from a transfected GFP gene in numerous eukaryotic cell lines [3].

3.3 Detection of Small Molecules

MRSw sensors are often used to detect the small molecules that involve modifying the surface of NPs with low-molecular-weight groups and then allowing them to accumulate with dimeric antibody, tetrameric Concavalin A, or tetrameric avidin.

Here, a competitive binding strategy has been exerted to demonstrate the glucose, folic acid, and influenza hemagglutinin peptide [19]. This means that initially the small molecules were attached to the surface of CLIO NPs and then the formation of clusters by using Concavalin A (glucose) or an antibody (folic acid and hyaluronic acid (HA)). After that, the addition of free analyte to the clusters causes a dispersion and a simultaneous rise in T_2. Most significantly, with the removal of the analyte from the solution, the change in T_2 was reversible, and resulted in reassembly of the NPs. This elicits a device with a semi-permeable membrane that is proficient for sensing the analyte amount in real time in regard to several addition and removal [19]. Moreover, MRSw sensors' versatility is capable of specifying between low-molecular-weight enantiomeric drugs [20]. Lastly, the pro-aggregation in MRSw designs has been established to detect the analytes like small calcium ions by using specific chelators [21], and the calcium-dependent interaction between the calmodulin and the M13 peptide resulting from rabbit myosin light chain kinase [22].

3.4 Detection of Pathogens

MRSw sensors by using antibody-conjugated MNPs have been magnificently developed to detect intact organisms, such as bacteria and viruses, and found more interesting as the targets are more or less the same size order or noticeably larger in regard to MNPs. Polyclonal antibody-conjugated CLIO detected antibody-conjugated MNPs targeting the adenovirus-5 and the herpes simplex virus-1 in serum and showing a lower limit of five viral particles in 10 μL [14]. Recently, μ-NMR device has been utilized for the detection of the bacterium Staphylococcus aureus and found that less sample volume (~10 μL) enabled the detection of as few as ten colony-forming units (CFUs) [1]. In another study, the cell tagging method was used for detecting tuberculosis (TB) bacterium [16]. Basically, sputum samples function for the investigation of TB diagnostic, and for such TB detection the standard procedure involves either culture or acid-fast bacilli (AFB) smear microscopy method. Nonetheless, Bacillus-Calmette-Guerin (BCG), a substitute for Mycobacterium tuberculosis, was spiked into sputum samples and then liquefied in a standard protocol, and followed by incubation with MNPs conjugated with an anti-BCG monoclonal antibody. Next, the second-generation μ-NMR device fitted with a porous membrane filter (~100 nm size cut-off) removed the unbound MNPs. Porous membrane further support allows the movement of NPs while not to the BCG thus abetting the BCG concentration from larger amounts of sample and deletion of NPs which are not bound (Fig. 5a). Demonstration of bacteria was presented in Fig. 5b.

Using CLIO NPs, nearly 100 CFUs can be discovered in 1-μL amount sample and further by highly magnetic Fe-core/ferrite shell NPs (cannonballs) it improved to around 6 CFUs [2, 16]. Eventually, by using the membrane filter it was shown that as small as 20 CFUs could be detected in a 1-mL sputum sample. At heart, the detection limit is considerably superior compared to that of AFB smear microscopy. Compared to the culture technique however it needed to function in minutes rather than weeks.

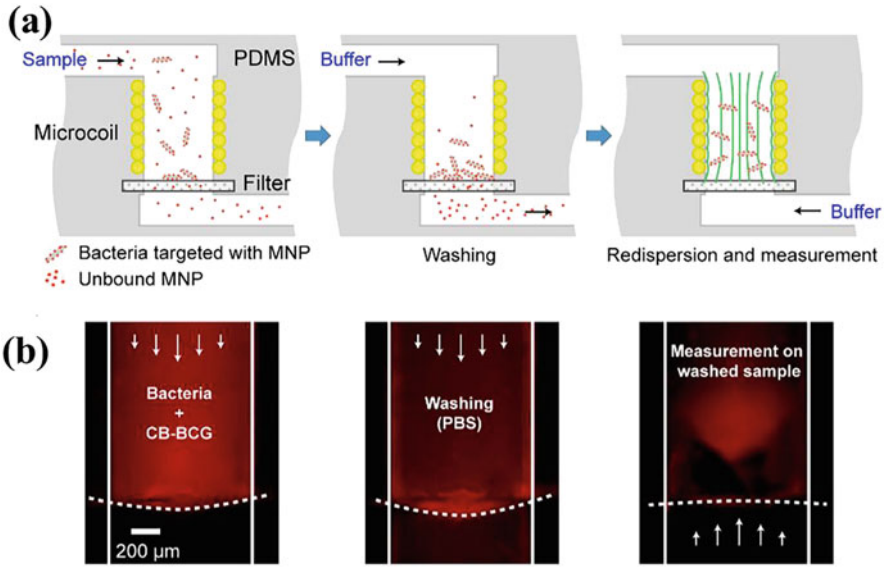

Fig. 5 Schematic representation of separation and concentration of bacteria (**a**) Membrane filter mounted at the outlet of the micro-coil probe can be used for concentrated bacterial samples, washed of excess magnetic nanoparticles, and resuspended prior to measurement of spin-spin relaxation time and (**b**) Illustration of bacteria capture [16]

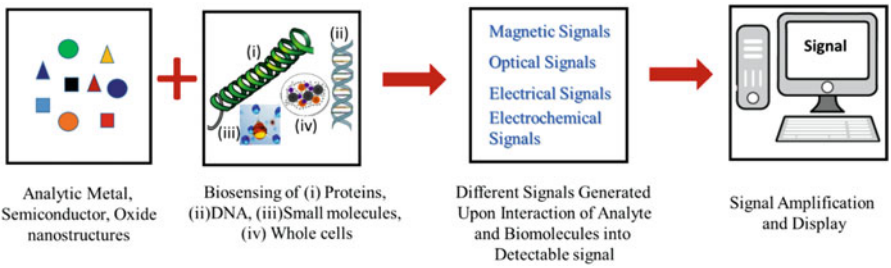

Fig. 6 Schematic representation for describing the detection of proteins, small molecules, and whole cells

In general, a biosensor is functionally composed of three components: (1) the biological element such as DNA and protein which is responsible for detecting the analyte (metal, semiconductor, oxide nanostructures) and generating a response signal, forms the first part of the biosensor, (2) the signal produced, which may be magnetic, optical, electrical, etc., generated upon the interaction of analyte and biomolecules is then converted into a detectable signal, and finally (3) the third part of the biosensor is the detector which amplifies and processes the signals before displaying it using an electronic display system. The various steps in signal processing of a biosensor, starting from the sensing to the generation of biosensing

signals till display, are schematically represented in Fig. 6. In the succeeding section, we will focus briefly on diverse sensors/biosensors that are established on the basis of different transduction principles, for instance, electrochemical, piezoelectric, and optical-based sensors (such as surface plasmon resonance and surface Raman scattering).

4 Electrochemical Sensor

In general, electrochemical signals such as current, voltage, and impedance are measured in electrochemical sensor devices. Moreover, the signals induced/altered when there is an interaction of analytes and electrodes linked with the biological molecules or biochemical materials or chemicals due to the progress of the activity of the surface [23, 24]. To create electrochemical biosensors, for instance, the immobilizing biological elements like DNA or enzyme or tissue are to be deliberated on the surface of the electrode, and then the signals are measured in the devices. More importantly, MNPs are functionalized on the surface of the electrode with biological-recognition elements causing the enhancement of the sensitivity of the electrochemical biosensors. Using MNPs, numerous electrochemical biosensor devices based on different detection modes such as voltammetry, electrochemiluminescence, potentiometry, amperometry, and electrochemical impedance are revealed in this chapterand therein [23]. Among various MNPs, Fe_3O_4 NPs are usually exploited in developing biosensors owing to their biocompatibility, superparamagnetic property, and ease of preparation. However, there will be a large chance of cluster formation of Fe_3O_4 MNPs because of the magnetic dipolar attraction and large surface area to volume ratio when exposed to biological solutions, but this problem can be resolved by some functionalization. It has been seen that a hefty variety of functionalized MNPs is used in biosensors. For instance, the functionalized Au-Fe_3O_4@SiO_2 core-shell MNPs are to be used for the detection of glucose (human serum) [25], Au-Fe_3O_4 composite NPs for organochloride pesticides (cabbage) [26], core-shell Fe_3O_4@SiO_2/multiwall carbon nanotube for the uric acid (blood serum, urine) [27], core-shell Fe_3O_4-Au NPs for the α-fetoprotein (human serum), etc. [28]. A comprehensive analysis and procedure for detection of various analytes using many detection modes are to be found in this review and therein [29].

5 Piezoelectric Sensor

While the piezoelectric device can be designed by quartz-crystal microbalance (QCM) and surface acoustic wave principles, the MNPs-based piezoelectric biosensor devices are essentially realized in the QCM-based transduction principle. In the QCM, the quartz-crystal disk, where two metal electrodes are used at every side of the disk oscillates under the effect of an electric field, and this engendered oscillation frequency dependent on the cut and the thickness of the quartz-crystal disk. Furthermore, the resonant frequency can be altered/manipulated when materials are

adsorbed or desorbed from the surface of the quartz crystal. Even though the QCMs are very much inexpensive, robust, and tiny in size, and more importantly, efficient for providing rapid response to a very less mass change of 1 ng, the problem of the device causes noise at the nanoscale size owing to the instability as increases of surface area to volume ratio. Note that, the disadvantages of QCMs are not only generation of noise with the decrease in size but also when it interferes with the atmospheric humidity then the difficulty arises for the determination of analytes in the solution [30]. Captivatingly, the MNPs with piezoelectric properties resolve these issues as it proffers striking transduction mechanism and bring frequency enhancement with advantages in cost-effectiveness and solid-state construction. The enhancement of frequency can be due to (1) some inherent piezoelectricity present in the MNPs, (2) the MNPs assist and bind the molecules at the surface of QCM, and (3) the MNPs can behave as matrix carriers to load the labels. A large number of piezoelectric-based QCM sensors using MNPs are to be found in this chapter [29], but here a QCM immunosensor for the detection of C-reactive protein (CRP) in serum is discussed in this section. Initially, a sandwich-kind immune reaction occurred among the silicon dioxide-coated Fe_3O_4 MNPs labeled with primary CRP antibody and the CRP and signal tag on AuNPs [31]. Then, the immunocomplex was subjected to hydrogen peroxide and amino-ethyl-carbazole (AEC). The schematic representation of the preparation and detection mechanism is presented in this chapter [31]. Interestingly, the capture probe containing the MNPs augmented the analytical signal because of magnetic separation and immobilization at the electrode surface. Because of the use of the MNPs, more importantly, the QCM-sensor surface can be used repeatedly and also be redeveloped easily.

6 Surface Plasmon Resonance (SPR)

Although optical-sensing methods such as surface-enhanced Raman spectroscopy (SERS), fluorescence spectroscopy and near-infrared spectroscopy and imaging (NIRS), and surface plasmon resonance (SPR) are used for biosensing applications, here in this chapter, we will solely focus on the utilization of MNPs in surface-based optical methods such as SPR and SERS for various applications in biomedical science. Surface plasmon resonance (SPR) is a complex physical phenomenon where conduction electrons at the surface of a metal layer are excited with the interaction of light at a certain angle of incidence. The collective oscillation of conduction electrons wave propagates parallelly on the surface metal termed as conditions of total internal reflection [32, 33]. However, this certain angle under constant wavelength and thin metal surface stimulate the SPR, and it is found to be reliant on the geometry of metal structures and also refractive index of the material. Thus, a change in the geometry of the structures of metal and refractive index will hamper the appearance of the SPR phenomenon. In 1983, Wijaya et al. firstly revealed that SPR is a powerful surface-based optical method designed for significant interest in biosensor applications albeit the SPR effect has been successfully explained previously in the year 1968 [34]. It is realized that SPR is a robust analysis

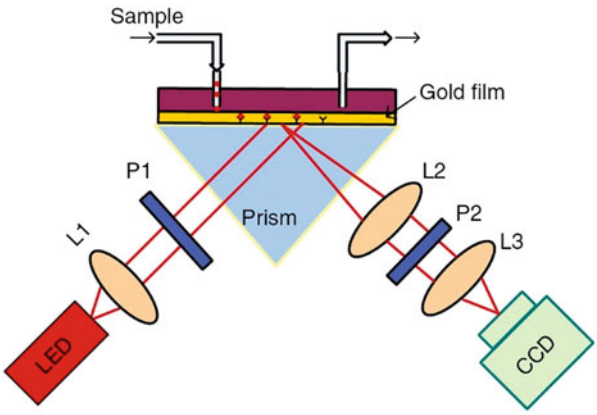

Fig. 7 Schematic representation of surface plasmon resonance assay [32, 36]

technique for the study of the interaction of biomolecules with the surface of metal layer because of its exceptional properties as an analytical tool like real-time, label-free, highly precise, a short time, and simplicity. Moreover, it is problematic to detect the low concentration, low-molecular-weight biomolecules regarding the conventional SPR technique due to the insignificant changes in refractive index in the binding process. Even though SPR techniques have been developed for various applications, nanomaterials are exceptionally useful for exploring and augmenting the strengthening of signal in nanoparticle-based SPR biosensors [35, 36]. Recently, it had been reported multiwalled carbon nanotubes (MWCNTs) are exploited as detection amplification labels for the creation of a SPR biosensor because numerous secondary antibodies can be modified onto the large surface area in MWCNTs, and hence augment the capture ability. Remarkably, Lisi et al. showed a 100-fold higher signal in tau protein case using MWCNTs technique with direct assay without amplification tags [36] (Fig. 7).

Nevertheless, decorating MNPs on the SPR surfaces was widely attested because of the considerable benefits like surface modification, low cost, and capacity to tailor with the external field. For instance, large refractive index and molecular mass of MNPs and ability to capture the target molecules from complex sample result in the SPR signal [37, 38]. A substantial improvement in SPR response using MNPs were made without using immobilized receptors on the sensor surface, selective detection of target molecules is possible as shown in Fig. 8 [39]. It is realized that superparamagnetic iron oxide NPs (SPIONs) undergo aggregation in the presence of target analytes, and then the aggregated SPIONs are attracted towards metal film by using externally applied magnetic fields (see Fig. 8). Thus, it creates a layer on the surface of the sensor with a different refractive index (Fig. 8). Besides, they found a substantial improvement in SPR response and allow for selective detection of target molecules without requiring immobilized receptors on the sensor surface, and, more importantly, the results of this study may contribute to make the SPR sensor chip reusable. Furthermore, Sun et al. testified the hollow gold nanospheres-based SPR biosensor, and then tweaked with MNPs for sandwich assay to detect rabbit IgG

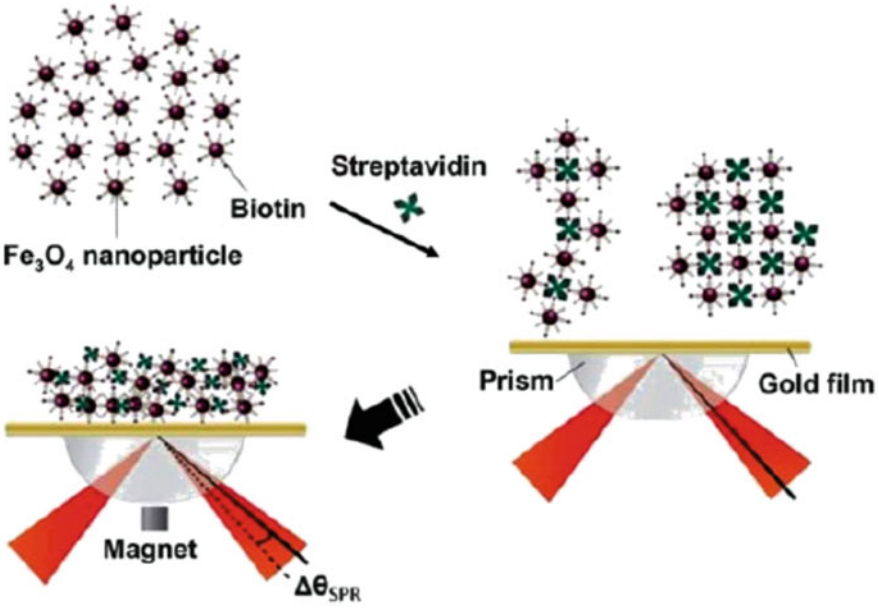

Fig. 8 Detection of surface plasmon resonance (SPR) response signal based on magnetic nanoparticles and magnetic field [39]

[40]. They exposed the detection of rabbit IgG by gold NPs, which brought it about 132 times lesser volumes pertaining to a conventional SPR biosensor. More importantly, the drastic variation in SPR signal due to magneto-hollow gold nanospheres sandwich immunoassay was shown to be 8 times higher in enhancement than with solely gold nanostructures [40]. Also, in another investigation aptamer immobilized Au NPs—MNPs conjugate-based SPR biosensors unveiled a significant response to the thrombin having LOD of 0.6 nM. But, a large shift of SPR signal is detected that is around 5 times at the 100 nM concentration as compared to a group particularly roots on the basis of SPR signal devoid of Au NPs—MNPs conjugate [41]. Many more applications of MNPs by unfolding the synergetic consequences of the SPR can be found in this chapter[42]. Essentially, the surface of SPR nanostructures modified with MNPs will be a suitable way for the biosensing application as it can be delved into to look at the variations in SPR signal.

7 Surface-Enhanced Raman Spectroscopy (SERS)

A surface-sensitive technique, surface-enhanced Raman spectroscopy (SERS), is particularly on the augmentation of Raman signal by means of chemical or electromagnetic enhancement mechanisms when molecules are adsorbed on the surfaces of metal or nanostructures. In general, chemical enhancement mechanism refers to the

charge transfer among the synthesized nanomaterials and the adsorbed molecules or targets, whereas the electromagnetic field effect of the adsorbed target causes the electromagnetic enhancement by exciting the surface plasmons in nanostructured materials [43–46]. Note that the surface-sensitive technique has been considered as an unceasing interest to look into the sensitivity of molecules and adsorbed targets, as the enhancement factor is so high (around 10^{11}-fold) that it can be used to trace the ultra-concentrations means to detect the single molecules, and more importantly, provide "fingerprint" information on chemical structure and conformation of the target molecule. It has been accomplished that nanostructured materials were exploited to advance the performance of SERS assay from diverse features [47]. The enhancement factor can essentially be modulated by changing the plasmonic properties by designing novel nanostructures with manipulation of size, morphology, composition, etc., that can promptly influence the sensitivity and selectivity of SERS [43]. Moreover, the SERS substrates, to be exact the plasmonic properties often found in noble metals such as Au and Ag thought out to be substrates, which can be effectively exploited for the detection of biomolecules, biochemical materials, cells, microorganisms, etc.

Even though SERS is a powerful tool for the detection of several orders of magnitude higher sensitivity than inherently weak spontaneous Raman scattering by exciting localized surface plasmon resonance on SERS substrates, there are still problems of SERS for biomedical use as the aforementioned detriments the reproducibility, uniformity, biocompatibility, and durability. However, the unique properties of MNPs along with simple synthesis process and large adsorption capability can be applied not only for the reproducibility augmentation but also for the substrate performance and examination of biological related samples. The report on the use of MNPs in SERS investigation had earlier established the detection of proteins [48, 49], microorganisms [50, 51], cells [52, 53], antigens [54], etc. Cheng et al. reported the detection of duple prostate-specific antigens by using SERS immunoassay hinged on magnetic beads and duo-kinds of Au nanoparticle labels [55]. They show technologically advanced magnetic beads dependent on SERS assay for prostate cancer analysis, as seen in Fig. 9, wherein the magnetic beads mutually be represented as the magnetic separation elements and the recognition elements explicitly once featured with antibodies. In this case, dual forms of antibody-conjugated Au NPs were tailored with two dissimilar Raman reporter nano-labels that form a sandwich type, leading to a concurrent revelation from both two prostate antigens [55].

He et al. in another study expounded an aptasensor which is founded on the SERS with MNPs for uncovering the microcystin-LR (MC-LR) [56]. Here, the surface of the synthesized Au NPs was modified with SERS reporter and followed by functionalization with MC-LR aptamer by means of SERS detection. Initially, MNPs were coated with silica for effortless immobilization of biomolecule. Further, the complementary DNA to MC-LR aptamer was enfeebled on MNPs' surfaces by biotin/avidin affinity as a capture examination. It has been seen that the established aptasensor dependent on magnetic SERS was magnificently addressed to the choosy and precise probe of MC-LR in tap water with the limit of detection (LOD), 2.0 pg/

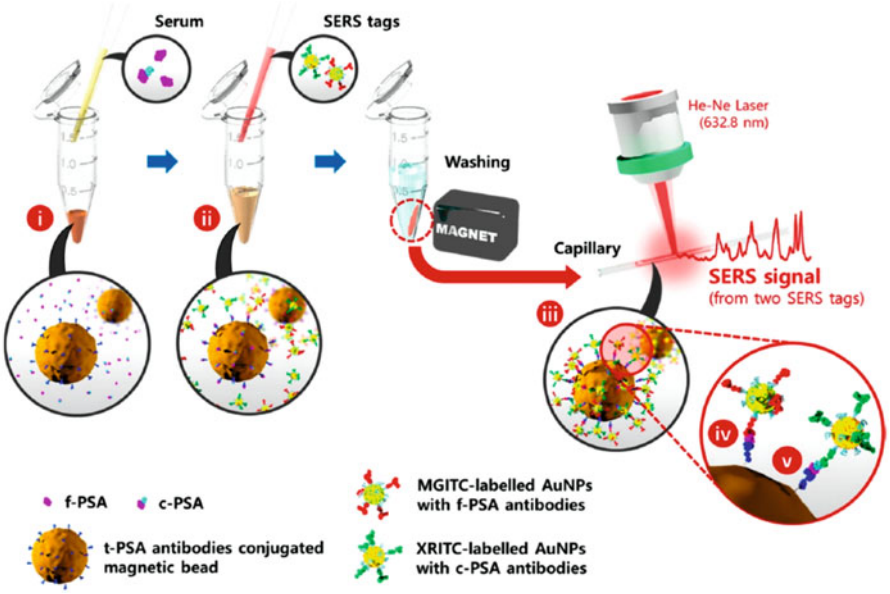

Fig. 9 Schematic illustration of a surface-enhanced Raman scattering (SERS) assay based on magnetic beads and two different types of Au nanoparticle tags [55]

mL. Yang et al. present a novel aptamer-based SERS sensor that used MNPs core-Au NPs satellite assemblies to detect the prostate-specific antigen (PSA) [54]. Firstly, they modified the MNPs with PSA aptamer for specific recognition of PSA. Though Au NPs were immobilized with Raman reporter molecules and DNA sequences, they were complementary to PSA aptamer. Thus, MNPs and Au NPs were cross-linked to obstruct SERS. Nonetheless, the robust interaction between the aptamer and PSA causes the dissolution of the core-satellite assemblies in the presence of PSA. Subsequently, it was found SERS signals from the supernatant corresponded to the concentrations of PSA. Pang et al. reported the detection of genes with silver (Ag) nanostructures by developing a functionalized Fe_3O_4@Ag magnetic nanoparticle-based SERS sensor [57]. They detect specifically micro-RNA (mi-RNA) in total RNA extracted from cancer cells. Here, they explored MNPs functionalized with the capture agent and functionalized with the SERS signal reporter and that causes great advantages of MNPs to be used to concentrate, capture and purify the target gene with the detection limit of 0.3 fM by using a single hybrid nanostructure. Basically, the unique properties of MNPs as abovementioned are of great interest for the analysis of biological fluids by reproducibility and stability of SERS substrates.

8 Interference-Enhanced Raman Spectroscopy (IERS)

Recently, Interference-Enhanced Raman Spectroscopy (IERS) has emerged as a promising tool for the detection of biomolecules on Raman-compatible surfaces using nano-metric metal-dielectric layers as signal boosting [58]. In this evolving methodology, Raman spectroscopy in combination with appropriate bi- or tri-layer sample preparation strategies to enhance the desired weak Raman signal, such as growth of bacteria on metals, is found to be useful for identifying infectious diseases. However, the development of the requisite chip/substrates is a very difficult task due to the lack of possible mechanisms, to be applied for quality control for the surface modification technique. The inherently feeble Raman signal produced by the biomolecules, transferred for the development of the microorganisms on the chip surface, usually does not allow for effective immobilization by means of a Raman spectroscopic method. In recent times, a simple aluminum surface allows, increasing the Raman signal of the biomolecules deposited on that surface. The change in the Raman signal strength is realized by manipulating the interference effects that are processed if aluminum of high reflective surface is reformed with thin layers of dielectrics such as aluminum oxide (Al_2O_3). However, depending on the biomolecule other thin dielectric layers (viz., TiO_2, ZrO_2, HfO_2, etc.) transparent to the Raman wavelength can also be employed in the geometry. The thicknesses of these layers for enhancement of the Raman signal are determined by optical interference-based theoretical considerations and calculations. In the recent work, it is shown that the interference effects can be used for the detection of biomolecules as well by investigating the siderophore ferrioxamine B. The observed degree of enhancement was approximately one order of magnitude.

9 Magnetic Nanoparticle-Based Optodes and Other Fiber Optic Sensors

An optode is a sensor based on optic principles like reflection, absorption, luminescence, evanescent wave, and surface plasmon resonance. An optode instrument consists of a light source, a detector, and an optical fiber connecting the source and detector. An optical fiber is a thin strand of glass within which light propagates due to total internal reflection. Optical fiber mainly consists of two different parts, core and cladding. Maximum light propagates in the core, while a small amount of light also passes to cladding called the evanescent field (EF). EF decays exponentially in the cladding. EF plays a significant role in optical sensing. The interaction of EF with the surrounding medium causes a change in the detector output. The detection sensitivity depends on how fast the detector output changes due to EF interaction with the surrounding medium where measurand is applied. A linear variation of the detector output with the measurand is required for better sensing.

Several optical fiber configurations are investigated to increase the EF interaction, such as clad removed optical fiber, U-shaped fiber, and Tapered fiber [59]. However, fiber optic biosensor based on surface plasmon resonance [60] has gained much

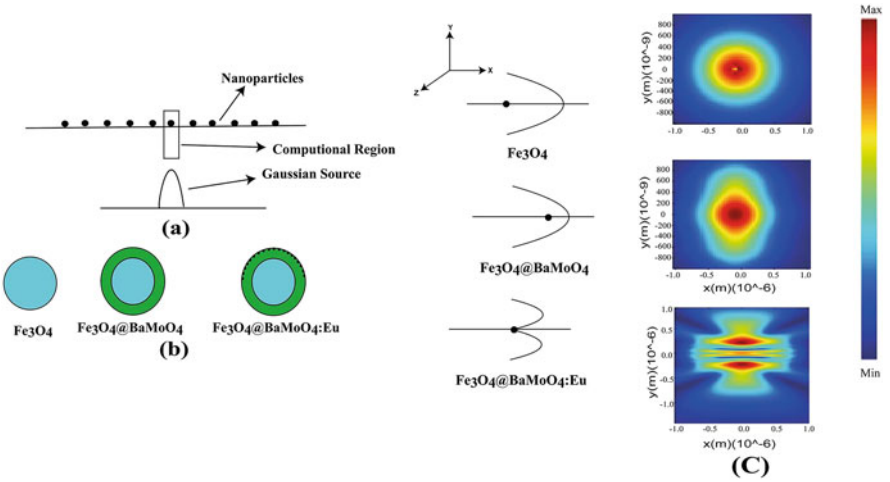

Fig. 10 (**a**) Schematic geometry to implement FDTD simulation. Nanocomposites are coated on a plastic (PMMA) substrate. Nanocomposite particles are assumed to be spherical, as shown in (**b**). (**c**) Results of FDTD simulation for Fe_3O_4, Fe_3O_4@BaMoO4, and Fe_3O_4@$BaMoO_4$: Eu composite [66]

interest. Here the conventional prism is replaced by an optical fiber core coated with a thin layer of metal such as gold and silver. Light entering the fiber at its one end such that the guided modes can create EF can excite the metal-dielectric interface's plasmons. For a given fiber geometry and metallic layer and dielectric constant of the surrounding medium, plasmon can oscillate in resonance for a given wavelength of the light signal. When this resonance condition is satisfied, the reflectance from the metal-dielectric interface suddenly decreases. The light propagating in the core changes accordingly, which can be detected at the other end of the fiber. As the dielectric constant of the surrounding medium changes, for example, when a given measurand is applied, there is a resonance wavelength shift. The shift of resonance wavelength is very sensitive to the dielectric constant of the surrounding. For a biosensor, an antibody must be immobilized on the metallic surface to ensure that the change in the dielectric constant of the surrounding is only due to the biological parameter to be measured [61]. Several modifications in SPR configuration were investigated [62–64] for biosensing applications. However, gold nanoparticles are used for sensing applications using a new principle such as optical manipulation method reported recently [65].

Recently another new principle, such as the emission properties of MNPs is used to realize a fiber optic refractive index sensor. In this work, the authors presented a new principle to enhance the initial evanescent field to increase detection sensitivity [66]. This principle is based on splitting mode field propagating in an optical fiber into almost symmetrically into two parts with the nanocomposite coating (see Fig. 10). Aided with simulation's FDTD, the authors showed that field distribution could be suitably changed with different MNPs. For example, when $BaMoO_4$ is

coated on Fe_3O_4, the spherical distribution of the mode field changes to elliptical distribution. Further, when Eu is dispersed on $BaMoO_4@Fe_3O_4$ core-shell, the field distribution splits into almost two parts about the optical fiber's core clad interface.

In the same study, the $Fe_3O_4@BaMoO_4$: Eu magnetic nanocomposite is coated on the optical fiber's clad to realize a refractive index sensor. This refractive index sensor can also detect biological parameters, coating the sensing parameter's antibody on the optical fiber similar to the procedure mentioned in reference [65].

10 Summary and Conclusions

Diagnostic magnetic resonance (DMR), electrochemical, piezoelectric, and surface-based optical surface plasmon resonance (SPR), and surface-enhanced Raman scattering (SERS) biosensors are all emerging and stimulating diagnostic sensing platforms because these techniques are suitable to detect the signal in a short period even with a small amount of sample volumes. A new principle based on certain nanocomposites' emission properties has been investigated for sensing optical fiber applications. New magnetic nanocomposite materials and new configurations on which the composite can be coated for sensing can be an exciting area of research in the near future. Nonetheless, MNPs' exceptional properties such as biocompatibility, low toxicity, chemical stability, high surface area, easy synthesis, high magnetic susceptibility, and modification have provided a strong motivation for developing advanced biosensing platforms. However, the most salient disadvantages like low sensitivity and selectivity, etc., of these sensors can be effectively overcome by using the MNPs with a magnetic field. Hence, these technologies have broad applications in biomedical science. The low-cost and hand-held device could help diagnose numerous public severe health issues, such as tuberculosis and HIV. Finally, the diversity of sensing technologies with MNPs provided unlimited possibilities for different applications in biomedical and life science.

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Electrochemical Biosensors in Agricultural and Veterinary Applications

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Abstract

Electrochemical biosensors are sensors which utilize some biological recognition element (for example, DNA/RNA, enzymes, cells, tissues, microorganisms, and antigen/antibody) to interact with the analyte, producing a detectable electrical signal. These biosensors have been successfully employed in a variety of applications. In the agriculture and veterinary areas, for example, they are explored in the detection of mycotoxins, herbicides, and veterinary drug residues. Due to its fast fabrication, sensitive, simple, compatibility with microfabrication techniques, and affordability, electrochemical biosensors can have their analytical performance significantly enhanced by modification of their surfaces with different materials such as metal nanoparticles, graphene, carbon nanotubes, magnetic particles, and quantum dots, among others. The utilization of nanomaterials favors electronic transfer and increases the analytical signal, improving the sensitivity. Electrochemical biosensors can be voltammetric, potentiometric, conductimetric, impedimetric, or coulometric, depending on the analytical signal to be measured. Among them, the voltammetric biosensors can be highlighted, mainly those based on amperometric detection, which is possible to verify a voltage-current-time relationship in an electrochemical arrangement consisting of three electrodes (biosensor as working electrode, auxiliary, and reference

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electrodes). Additionally, voltammetric biosensors can be associated with injection systems (sequential injection analysis, batch injection analysis, or flow injection analysis) which provide fast analysis, elevated throughput of samples, and high reproducibility. In this chapter, a short review of electrochemical biosensors and how they are applied to agricultural and veterinary analysis is addressed.

Keywords

Agricultural waste detection · Agricultural sensors · Biosensors · Electrochemical sensors · Veterinary sensors · Veterinary product

1 Introduction

Agriculture is one of the sectors of the economy that has significant participation in the world market when it comes to basic food to humanity and feedstock for industrialization, being considered a source of revenue and job for a large part of the populace, mainly for underdeveloped countries [1]. The advances in agricultural techniques have contributed to the increase in crop yields, by combating weeds and pests by the use of chemical herbicides, fertilizers, and pesticides. As a rule, these agrochemical products (with emphasis on organophosphates, carbamates, and organochlorines, among others), in most cases, are applied excessively in crops, leading to environmental imbalances with acute and chronic effects on humans (such as nausea, cancer, and Parkinson's disease, among others) [2–4]. In addition, the number of people who are poisoned by agricultural chemicals grows annually. The harmful effects of these excesses are not restricted to food but extend to air, soil, and water. In all these matrices, the detection of these species needs to be monitored which is a serious challenge. These agrochemical substances are determined mainly by widely complex, laborious, time-consuming, and expensive analytical techniques, such as chromatography (GC), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), ultraviolet-visible spectroscopy (UV-Vis), nuclear magnetic resonance (NMR), and Fourier transform infrared spectroscopy (FTIR) [1, 5, 6]. More complex analysis can require hyphenated techniques such as HPLC-mass spectrometry (HPLC-MS).

In many veterinary practices such as the use of antibiotics (chloramphenicol, penicillin, and others) and chemotherapy (sulfonamides, enrofloxacin, and others) in animal husbandry has caused the appearance of veterinary drugs residues in meals produced from animals. In addition, some components (organic or inorganic) are excreted via urine or feces from infected animals as well as the inappropriate disposal of medications not administered can directly contaminate the soil, water, and even humans [7–10].

All aspects mentioned above cause economic impacts, since conventional diagnostic methods, such as NMR and HPLC or GC coupled to mass spectrometry (GC-MS), require expensive instrumentation, time-consuming sample preparation,

and skilled labor [10, 11]. Recent advances in electrochemical biosensor technologies compared to conventional methods have the potential to provide diagnostics with greater sensitivity and selectivity, rapid analyses, simplicity of automation, and thus portability and relatively inexpensive since many of the compounds (for example, pesticides, antimicrobial agents, and hormones, among others) can be easily oxidized or reduced [12].

Electrochemical biosensors are devices that use a biochemical receptor (for example, enzyme, antigen/antibody, nucleic acid, and cell, among others) in direct contact with an electrochemical transducer (conductometric, potentiometric, impedimetric, coulometric, and voltammetric) to generate an electrical signal that is converted into analytical information on a given analyte [13–15]. The association of these sensors with Batch Injection Analysis (BIA) [16], Flow Injection Analysis (FIA) [17], or Sequential Injection Analysis (SIA) [18] will provide greater performance during chemical analysis, with increased sensitivity and reproducibility. In this context, this chapter is focused on the electrochemical biosensor applications in the field of agriculture and veterinary. Relevant examples from the literature are mentioned and fundamental information about electrochemical biosensors and their associations with injection systems (FIA, SIA, and BIA) are also addressed.

2 Electrochemical Biosensors

Biosensors are selective analytical devices capable of generating signals from biochemical interactions [19, 20]. These devices are made up of bio-receptors in touch with a signal transducer [19–22]. This transducer is the component responsible for recognizing the physical-chemical changes arising from the interaction that takes place in the bio-receptors (interaction between biological recognition element and analyte), generating a measurable analytical signal that can be related to the amount of the chemical species of interest [19, 23–26].

One of the first biosensors was designed by Clark and Lyons for the determination of glucose. This sensor was composed of the enzyme glucose oxidase in close contact with an oxygen electrode that aided in glucose monitoring (via oxygen consumption by the enzymatic reaction) [27, 28]. Biosensors can be classified according to biological component or transducer type (for example, electrochemical, acoustic, optical, or calorimetric) [22, 24], being the most reported those based on the process of recognizing electrical signals from biochemical interactions, called electrochemical biosensors [29, 30].

Electrochemical biosensors are devices that (generally) operate in conditions that do not require complicated sample pretreatment, present fast responses, ease of manufacture, and implementation in different fields such as environmental, health, and laboratory investigations [31]. They consist of (at least) three compact components, as any other biosensor, including (a) a biorecognition element, (b) an electrochemical transducer, and (c) an electronic data management system [32–34]. The representation of an electrochemical biosensor is shown in Fig. 1.

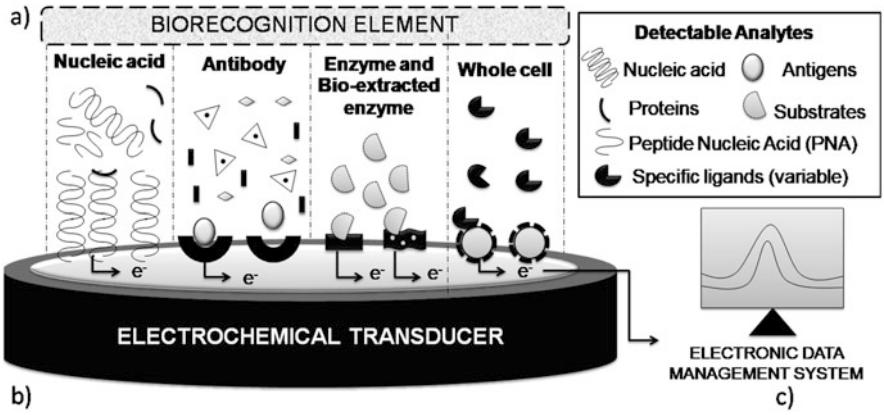

Fig. 1 Scheme of electrochemical biosensors showing (**a**) biorecognition element (for example, nucleic acid, antibody, enzymes, and whole cell), (**b**) an electrochemical signal transducer, together with (**c**) an electronic data management system (Figure based on [35])

The classic biorecognition elements are nucleic acids, antibodies, tissues, microorganisms, enzymes, and others [36, 37]. Such elements are important due to their roles in the recognition of the target analyte, directly affecting the sensitivity and selectivity of the electrochemical biosensor [32, 37]. Biorecognition molecules selectively bind to the target analyte to produce electrochemical responses [32]. The immobilization of biorecognition elements on the detector allows the construction of more compact and selective sensors. The responses of the voltammetric biosensors give information about the reaction rates, catalysis, or inhibition of enzymes in presence of the analyte [38].

Regarding the different types of biorecognition elements, there are many papers describing the use of one or more enzymes for the construction of biosensors, generally immobilized on different transducers and using various binding components [39]. However, many enzymes are expensive and some essential enzymatic reactions require cofactors that, if not regenerated, turn the use of enzymatic biosensors even more expensive [40].

Some researchers have explored tissues (animal, vegetable, or fungal) or crude vegetable extracts in place of purified enzymes for the manufacture of electrochemical biosensors. This choice occurs mainly due to the availability of crude tissues or extracts or even because the biosensors have greater stability due to the enzymes being naturally immobilized in the cells of the biological material. However, the use of tissues can lead to a decrease in the selectivity of the analytical method due to a large number of interferences and contaminants [41]. In the work developed by Rahimi-Mohseni et al. (2019), a low cost, disposable amperometric biosensor built from potato tissue extract was developed to determine phenol. The potato tissue containing polyphenol oxidase was immobilized on paper using physical and chemical adsorption and it was transferred to the top of the graphite SPE. The linearity range for phenol detection was found to be 0.1–300 μmol L^{-1} and the detection limit

found for the developed method was 0.042 μmol L^{-1}. This method was successfully used to determine phenol in water and effluent samples [42].

Sezgintürk and collaborators (2010) developed an amperometric biosensor using zucchini tissue (*Cucurbita pepo*) for the quantification of ascorbic acid in drugs and plants. According to the authors, the developed biosensor presented a linear range from 5.0×10^{-6} mol L^{-1} to 1.2×10^{-3} mol L^{-1} and the correlation coefficient found for the developed method was 0.9975 [43]. In the study carried out by Sekar et al. (2015), an amperometric biosensor was developed for the determination of hydrogen peroxide using turnip plant tissue as biological material. This sensor was constructed by immobilization via physical adsorption of the enzyme peroxidase from raw turnip on a cellulose paper, ideal and biocompatible with the enzyme. Potassium hexacyanoferrate mediator was also incorporated into the paper matrix along with the crude enzyme. The developed biosensor presented a linear range from 20 to 500 μmol L^{-1} ($R^2 = 0.999$) and the detection limit found for the developed method was 4.1 μmol L^{-1}. According to the authors, the biosensor retained 70% of its activity after a storage time of 25 days [44].

Lata and Pudir (2013) built an amperometric biosensor for determining L-amino acid from a goat kidney immobilized on the surface of the working electrode (glassy carbon). Studies carried out with the developed biosensor indicated that the analytical response was obtained quickly (5 s) with a wide linear working range (from 0.5 μmol L^{-1} to 100 mmol L^{-1}) and sensitivity of the biosensor was 79.31 nA cm^{-2} μmol L^{-1}. This sensor was applied in the quantification of L-amino acids in fruit juices and alcoholic beverages, presenting a fast response time, good durability over time, and insignificant interference during the analyzes performed [45].

In addition to animal and vegetal tissues, small molecules of oligonucleotides formed by simple strands, such as ribonucleic acids (RNA) and deoxyribonucleic (DNA), perform specific chemical bonds (high affinity) with various molecules. Such synthetic oligonucleotides are called aptamers [21, 46–48].

The high affinity and selectivity of aptamers are related to the proper selection of the oligonucleotide sequence, extracted from a set of random sequences. The process performed for the selection of the appropriate oligonucleotide sequence is carried out by the exponential enrichment (SELEX) [21, 49]. These molecules are similar to antibodies, acquiring a certain conformation, being able to join the analyte [49]. Nev Nevertheless, when the aptamers are used as elements of biorecognition in the construction of electrochemical biosensors they have not been able to completely replace traditional elements of biorecognition. This occurs due to several factors related to the characteristics of the aptamers, such as degradation, cross-reactivity, and low stability, among others [21, 46]. Villalonga et al. (2020) presented the use of an amperometric aptasensor for the quantification of carcinoembryonic antigen (CEA). This aptasensor presented a linear range concentration from 112 fmol L^{-1} to 11 μmol L^{-1} and the detection limit found for the developed method was 90 fmol L^{-1}. The analytical parameters of this sensor were evaluated in human serum samples and showed good stability, specificity, and reproducibility [50].

Electrochemical transducers are traditionally made of carbon (glassy carbon, carbon paste, and other carbon forms), metals (e.g., gold or platinum), or conductive

glass. All of them have the possibility to be chemically modified by a chemical agent composed of the selected biorecognition element [22, 36]. The analytical signals originating from the biological element and analyte interaction are received by an electronic system in which the generated data can be managed. Electrochemical sensors can be subdivided based on the way in which the physicochemical transduction is used during experiments. They can be voltammetric (special emphasis will be given to amperometric sensors in this chapter), potentiometric, impedimetric, coulometric, and conductometric. All of them present possibilities for the construction of Point of Care Testing (POCT) [23, 31, 36, 51, 52].

2.1 Voltammetric Biosensors

Among all types of existing electrochemical biosensors, voltammetric biosensors are widely used due to their analytical performance and popularity in the analysis of different matrices such as environmental, pharmaceutical, and biological samples, among others [36]. Voltammetric sensors are able to detect possible changes in analytical signals (currents) related to oxidation or reduction reaction of analytes that have the potential to oxidize or reduce (electrochemically active) [33, 53]. In many cases, the electroactive species will be identified in the voltammogram by the peak potential and the concentration of this chemical species can be determined by the peak current using an analytical curve. Different operating methods can be used in voltammetric sensors [54], which include:

- Amperometry;
- Cyclic voltammetry (CV);
- Normal pulse voltammetry (NPV);
- Differential pulse voltammetry (DPV);
- Square wave voltammetry (SWV)

In this chapter, special attention will be given to the amperometric biosensors since they are characterized by simplicity, sensitivity, portability, and reliability, as well as low consumption of samples, possibility of using microelectrodes, and performing experiments with high analytical frequency. Although amperometry has been one of the most used electroanalytical techniques during the use of biosensors, the cyclic voltammetry is a fundamental technique during initial studies to understand the redox behavior of the analyte [55–57]. Kokkinos et al. (2020) report in their review the potential and advances in the use of amperometric biosensors [58].

The amperometric biosensors are the devices most used commercially and the classic example of an amperometric biosensor produced commercially is the glucometer (Fig. 2). The amperometric glucometer generates an analytical signal based on the measured current when glucose oxidase facilitates the oxidation reaction (catalyzes) of glucose to gluconic acid thus generating the electroactive hydrogen peroxide too, or when glucose dehydrogenase catalyzes the oxidation of

Fig. 2 Commercial glucometer based on an amperometric transducer

glucose to gluconolactone and produces a reduced cofactor. In both cases, the products of the reaction can be related to the amount of glucose present in the blood. The resulting current is proportional to the glucose concentration and the device that will perform the analysis is calibrated to indicate the amount of glucose in the blood [59].

2.2 Amperometric Biosensors

Amperometric biosensors operate under a fixed potential applied on the surface of the sensor with monitored current in the function of time [36, 54, 60–63]. The potential applied across the experiment helps in the efficiency of electron transference from the biological system (oxidase or dehydrogenase enzymes, for example). In a certain concentration range, the observed current shows a linear function with the amount of the electroactive species [19, 62, 64–66].

The electrochemical system in an amperometric cell configuration can be composed of two or more commonly three different types of electrodes (working electrode-W.E, reference electrode-R.E, and auxiliary electrode-A.E) according to the system shown in Fig. 3, which represents experiments in hydrodynamic voltammetry mode [36, 67, 68]. The second electrode to compose the amperometric cell is the reference electrode, which in the vast majority of cases is composed of Ag/AgCl. This electrode provides a fixed potential against which the working electrode's potential is controlled and measured [36, 68]. The use of amperometric biosensors with two electrodes (working and reference electrodes) is limited since at high currents it is difficult to maintain the potential, resulting in a less linear range [36, 67]. In this sense, a third electrode is inserted into the electrochemical cell and acts as a counter electrode, another name for the auxiliary electrode. When three electrodes are utilized, the current passes through the working and the auxiliary electrodes, avoiding potential shifts of the reference electrode, preserving its stability [67, 69]. Amperometric biosensors are highly sensitive, have a quick response system, and are easy to operate [61, 66].

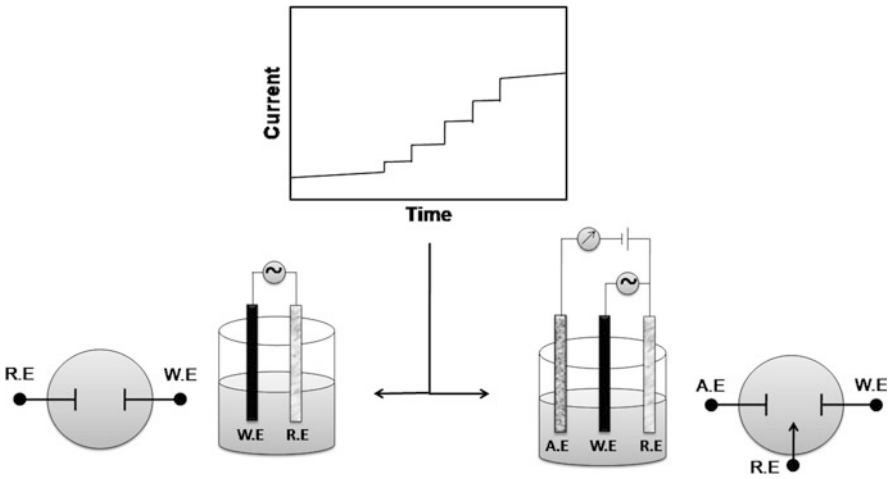

Fig. 3 Amperometric experiments with a configuration of two and three electrodes and their respective electrochemical signal (current vs time). W.E represents the working electrode, R.E represents the reference electrode, and A.E represents the auxiliary electrode (Figure based on [36])

The working electrode, detector, or transducer is composed of materials such as gold, carbon, and platinum, among others [36, 68]. These electrode materials ideally are inert and favor the transfer of electrons towards the biological system immobilized on the detector surface [19, 36]. The surface of the working electrodes, in many cases, contains conducting mediators, polymers, or nanomaterials (metallic nanoparticles, graphene, carbon nanotubes, quantum dots, among others) with the main purpose of improving electronic transference. Thus, the biological system can be covalently linked to the transducer surface covered with the functional groups or trapped in the polymeric layers [19, 70–73].

In the work developed by Zhang et al. (2019), organophosphate compounds were determined using an amperometric biosensor. For the manufacture of this biosensor, the compound 4,7-di(furan-2-yl) benzothiadiazole (FBThF) was electrochemically polymerized on the detector surface for the development of the electrochemical biosensor. Moreover, nanocomposite Ag-rGO-NH_2 and acetylcholinesterase (AChE) were modified in the membrane formed on the electrode surface [74]. This electrode explores the potential for inhibition provided by the enzymatic reaction between acetylcholinesterase and organophosphate compounds.

Recently, Ren and collaborators built an amperometric biosensor using enzymes for determining H_2O_2. The sensitivity of this sensor was 3.0×10^{-4}A/mol L^{-1} and the detection limit found for the developed method was 443 nmol L^{-1}. Moreover, the biosensor presented good reproducibility and stability too [75].

For improving the sensitivity of electrochemical sensors, nanomaterials have been incorporated into biorecognition materials [76]. These materials have the potential to build biosensors due to their thermal, optical, and electronic properties. Ideally, they have a high surface area, good electronic conductivity, and excellent

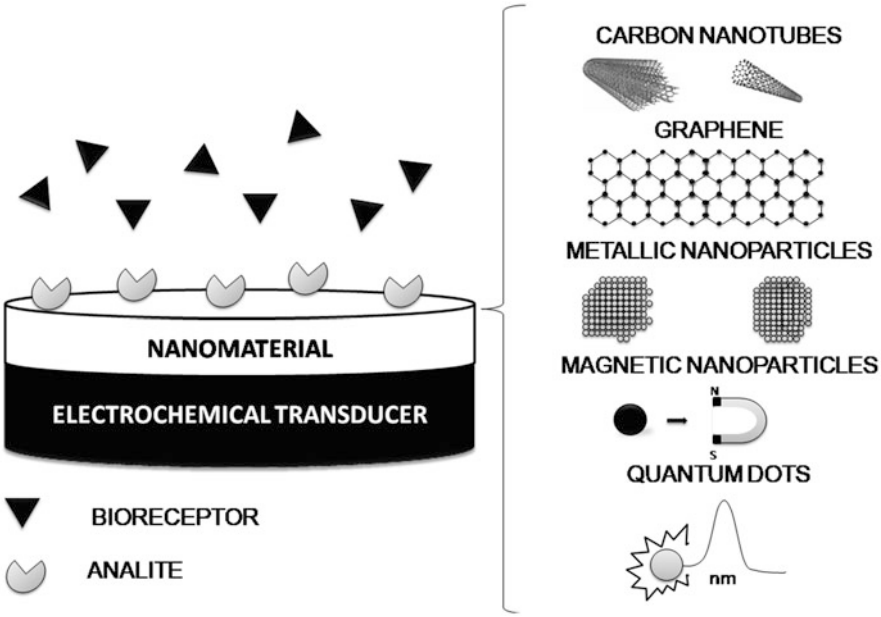

Fig. 4 Examples of nanomaterials applied in the construction of amperometric detectors (Figure based on [79])

chemical, magnetic, and physical properties [77, 78]. The nanomaterials most used for the development of amperometric biosensors are metal nanomaterials, carbon nanomaterials (graphene and carbon nanotube), magnetic nanoparticles, and quantum dots (Fig. 4) [77].

2.2.1 Carbon Nanotubes

Carbon nanotubes (CNTs) are found in two different ways: simple tubes-SWCNT and multiple concentric tubes-MWCNT. They are sp^2 hybridized carbon nanomaterials and are favorable for application in amperometric biosensors since they have a great surface area, in addition these materials present good chemical stability and remarkable properties—electronic and thermal [80, 81]. Also, CNTs can be functionalized with various chemical groups, favoring the connection of biomolecules or organic molecules with different biorecognition elements used in the development of biosensors [77, 82, 83].

Palomar et al. (2020) built an amperometric biosensor for the determination of two chemical substances, catechol and dopamine, with functionalized carbon nanotubes. In this work, functionalized tungsten disulfide nanotubes functionalized with carboxylic acid functions (WS_2-COOH) were used and so the tyrosinase enzyme presented better adhesion on the electrode surface and improved analyte detection. The developed method presented a good linearity in concentrations from 0.6 to 70 μmol L^{-1} and excellent selectivity with values of 10.7 ± 0.2 mA L mol^{-1}

for catechol analysis. For dopamine, the same method presented a good linearity in concentrations from 0.5 to 10 μmol L^{-1} and a good sensitivity with a value of 6.2 ± 0.7 mA L mol^{-1}. The performance of this sensor was considered within the average when compared to other studies using carbon nanotubes [84].

In the work developed by Ramonas et al. (2019), an amperometric biosensor was used for the quantification of glycerol. This sensor was built through immobilization of alcohol dehydrogenase from *Pseudomonas putida* on a graphite electrode. The graphite electrode was modified with carbon nanotubes and during the experiments a redox mediator tetrathiafulvalene was used. The evaluation of the proposed biosensor showed high sensitivity to glycerol (29.2 ± 0.9 μA mmol L^{-1} cm^{-2}), low limit of detection (18 μmol L^{-1}), and linearity for concentration values varying from 0.05 to 1.0 mmol L^{-1}, with good selectivity and stability [85].

2.2.2 Graphene

Graphene is an allotrope of carbon constituted by a thin sheet of atoms arranged in a 2D lattice. The carbon atoms (hybridized in sp^2) of this nanomaterial are arranged in a rigid network similar to a honeycomb [77, 86]. Graphene has good electron transfer capability, excellent thermal and electrical conductivities, mechanical resistance, large surface area, good flexibility, impermeability, and biocompatibility [77, 86, 87]. The thin thickness of the graphene sheet (theoretically) allows each carbon atom to interact directly with the analyte giving the biosensors a high sensitivity [88, 89].

A recent study reports the use of graphene in its own way of graphene oxide or incorporated into nanocomposites. In this work developed by Song and collaborators (2020), an amperometric biosensor for the quantification of ascorbic acid (AA) and dopamine (DA) was developed. It was built using nanobiocomposite derived from poly (aniline-*co*-thionine) (P(ANI-co-THI)) and modified with graphene oxide (GO). The analytical parameters presented a good linearity with concentration values that varied from 0.002 to 0.5 mmol L^{-1} with a limit of detection of 2 μmol L^{-1} for DA, as well as 0.5–5 mmol L^{-1} and a limit of detection of 242 μmol L^{-1} for AA. The designed electrochemical biosensor presented high selectivity, good stability, and reproducibility [90].

2.2.3 Metallic Nanomaterials

Metal nanoparticles are nanomaterials with specific physicochemical properties, which relate approximately to their size, composition, format, and structure. These materials are widely applied in the development of voltammetric biosensors because of the large surface area, excellent electron transfer kinetics, and many absorption sites for the attachment of biorecognition materials [77, 91]. The most common metallic nanomaterials used in the building of biosensors are gold (Au), silver (Ag), and platinum (Pt).

Gold nanoparticles (AuNPs) are very popular in the construction of electrochemical biosensors due to their biocompatibility and easy protein functionality [92]. Gold presents good compatibility and has the main characteristic of making strong bonds with enzymes [93, 94]. In this sense, enzymes can be easily

immobilized on the surface of AuNPs and in many cases, their activity is increased [94, 95].

Chiang et al. (2019) developed an amperometric biosensor of high sensitivity modified with AuNP for glucose determination. The proposed amperometric biosensors demonstrated a low limit of detection of 50 μmol L^{-1} and a wide linear range of concentration when modified with AuNP electrodeposition [96].

Platinum nanoparticles (PtNPs) have the specific characteristic of being able to catalyze the decomposition of H_2O_2. In this sense, this nanomaterial is widely used in amperometric biosensors since PtNPs act as a catalyst, increasing the biosensor response when compared to traditional platinum electrodes [94, 97, 98].

Wang et al. (2019) built an amperometric microbiosensor using carbon fibers for the determination of hydrogen peroxide. During the construction step of this amperometric sensor, the electrometallization of carbon-fiber microelectrodes and electrodeposition of PtNPs were used. The hybrid structure obtained with PtNPs and carbon fiber provided a sensitivity of 7711 ± 587 μA mmol L^{-1} cm^{-2} and a limit of detection of 0.53 ± 0.16 μmol L^{-1}. Moreover, it was possible to obtain a good linearity with concentration values that varied from 0.8 μmol L^{-1} to 8.6 mmol L^{-1}, and a response time of <2 s [99].

Silver nanoparticles (AgNPs) have the same advantages presented as in other metallic nanoparticles, such as amplification of the electrochemical signal and increased sensitivity [94]. Thought about these characteristics, Hou and collaborators (2020) built an amperometric biosensor for the quantification of wild-type p53 proteins. In this work, AgNPs were deposited in situ on the gold electrode surface for amplifying the generated signal. According to the authors, the biosensor exhibited a wide linear range of concentration and a detectable concentration as low as 0.1 pmol L^{-1} [100].

In the work published by Medyantseva et al. (2017), AgNPs and nanotubes immobilized in polyester polyols were used in the quantification of antidepressants. AgNPs and nanotubes were trapped on a carbon electrode using polyester-polyol. According to the authors, AgNPs extended the concentration range to 1.0×10^{-4} mol L^{-1} until 1.0×10^{-8} mol L^{-1} and decreased the limit of determination to 3.0×10^{-9} mol L^{-1} [101].

Metallic nanoparticles have important characteristics but are electrically unstable due to their susceptibility to salt concentrations that can cause their precipitation. In this sense, chemical and biological adjustments are necessary for the use of these nanomaterials in biological matrices with a high concentration of salt [92, 102].

2.2.4 Other Materials

Another class of nanomaterials explored for biosensing are magnetic nanoparticles (MNPs), that present specific characteristics such as large surface area, high mass transfer, specific physicochemical properties, biocompatibility with biomolecules, and easy production [77, 103, 104]. The singular and attractive aspect is that MNPs can be immobilized on the detector surface, or be dispersed in the sample for a fixed time and in sequence be attracted (through an externally generated magnetic field) on the sensor [77, 105].

The classic example of a magnetic nanomaterial is iron oxide (Fe_3O_4) nanoparticles. This material is characterized by the ability to enhance the sensitivity of biosensors constituted by oxidase enzymes [94, 106].

Magnetic nanoparticles are commonly found in amperometric biosensors modified with composites. In the work developed by Pakapongpan and Poo-arporn (2017), an amperometric glucose biosensor was built. This biosensor was modified with reduced graphene oxide (rGO) covalently conjugated to magnetic nanoparticles (Fe_3O_4NPs). The authors observed a rapid amperometric response (3 s) and linearity with concentrations that varied from 0.05 mmol L^{-1} to 1 mmol L^{-1}. The limit of detection found for the developed method was 0.1 μmol L^{-1} and the sensitivity found was 5.9 μA mmol L^{-1}. Moreover, this biosensor presented good reproducibility, high stability, and selectivity [107].

Another interesting class of nanomaterials is quantum dots (QDs). These nanomaterials are semiconductor crystals that have characteristic optical properties, such as wide absorption band, long-term photostability, with symmetric emission, among others. They can consist of carbon, graphene, or inorganic materials (CdS, ZnS, and CdT, among others) [77, 94]. In the study described by Fatina et al. (2020), quantum antimony nanodots were immobilized with catalase on the surface of the working electrode (glassy carbon). The amperometric biosensor was developed to determine hydrogen peroxide in human serum samples and showed good linearity (0.989) and the limit of detection found was 4.4 μmol L^{-1}. Amperometric measurements showed 95–103.4% recovery for H_2O_2 and electrochemical stability [108].

2.3 Potentiometric Biosensors

Potentiometric biosensors are devices that incorporate a biological element into a potentiometric transducer. The potentiometric measurement corresponds to the changes that occur between an indicator or working electrode and a reference electrode, as is schematically represented in Fig. 5. The first potentiometric biosensors were based on fixing enzymes on the surface of a glass sensor. In this case, the variations of the pH produced by the enzymatic reactions can be directly related to the amount of analyte present in the reaction medium. Many biosensors were constructed using ion-selective electrodes (ISE), mainly those based on polymeric membranes, or ion-sensitive field-effect transistors (field-effect transistors, FET). ISE are extensively used because they have the following advantages: they are more easily reproduced than FETS, are sensitive, provide quick responses, and simplicity in measurement, among others [22, 100]. They are able to record charge accumulation, in equilibrium condition created by specific chemical bonds on the electrode surface [65, 109–111]. The reference electrode provides a constant half-cell potential and the working electrode responds to the variation in the concentration of the electroactive species, indicated by a variable potential [68]. Unlike amperometric biosensors, in potentiometric biosensors, the change in potential

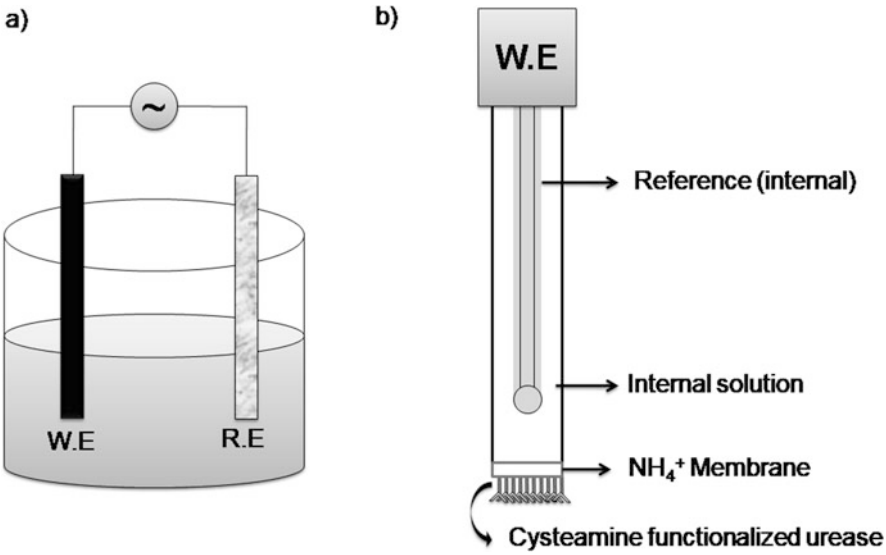

Fig. 5 Classical electrochemical cell with potentiometric sensor (**a**) and the representation of a potentiometric urea biosensor based on cysteamine functionalized urease immobilized on the membrane (**b**). W.E represents the working electrode and R.E represents the reference electrode (Figure based on [112])

comes from the concentration of electroactive analytes, occurring logarithmically according to the Nernst equation [68, 111].

Potentiometric biosensors are attractive for practical applications due to their ability to detect ions (Na^+, I^-, F^-,CN^-, K^+, NH_4^+, and Ca^{2+}) gases (NH_3 and CO_2) and pH [19, 22, 36, 113]. Potentiometry is the standard technique for determining biologically active species in biological fluids such as Na^+, K^+, Ca^{2+}, and Cl^- [114–117]. In the work developed by Toshiya et al. (2020), a potentiometric biosensor was produced with a focus on the determination of sodium ions and pH, using paper-based metal as transducers [118], with potential applications as wearable electrodes.

Potentiometric biosensors present some significant advantages including simplicity of use, low cost, great sensitivity, reduced size, as well as quick response acquisition [36, 113, 114]. However, low selectivity limits its use in some applications [36, 119]. This disadvantage is overcome by bio-modifications that promote specific molecular interactions and broaden the spectrum of analytes detected by potentiometric biosensors [63, 113]. In the study described by Mello et al. (2020) two potentiometric biosensors, for the quantification of glucose and urea, were proposed and their surfaces were biomodified with polyaniline films by entrapped enzymes. The produced films presented a linear dynamic range from 10^{-5} mol L^{-1} until 10^{-1} mol L^{-1}, for both analytes [120].

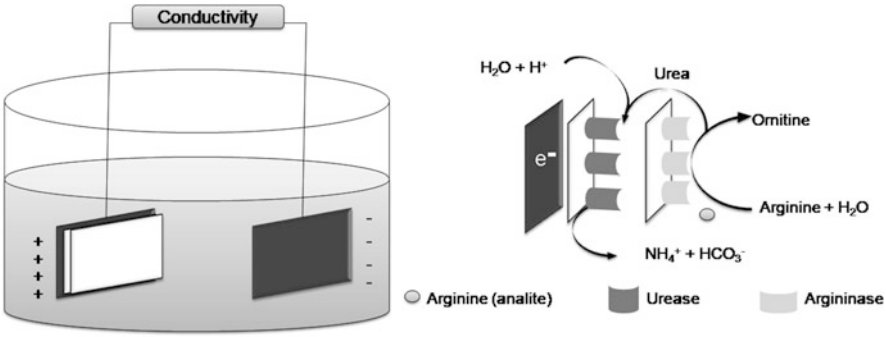

Fig. 6 Representation of a modified conductometric biosensor using two-layer membrane (Figure based on [123])

2.4 Conductometric Biosensors

Conductometric biosensors are devices that detect changes in electrical conductivity in solution from biological recognition reactions [19, 121]. The biological recognition reactions are responsible for the production or consumption of ions, resulting in the change of conductivity in solution [111, 122].

The conductivity measurements performed by the conductometric biosensors are made relatively quickly when compared to other analytical methods, being appreciable for different applications [119]. In the work developed by Soldatkina and collaborators (2018), a conductometric biosensor was developed to determine arginine in pharmaceutical products (Fig. 6). The biosensor was built in urease and arginase enzymes (UE and AE) co-immobilized on the electrochemical transducer (Fig. 6). The function of the arginase enzyme (AE) is to cleave arginine into two other molecules: urea and ornithine, through an enzymatic reaction. The urease enzyme (UE) has the function of transforming urea into carbon dioxide and ammonia. In this sense, reactions involving enzymes produced variations in the conductivity of the supporting electrolyte and the variations were proportional to the concentration of arginine. The results indicated that the conductometric biosensor presented a limit of detection of 2.5 μmol L^{-1}. The linearity of the method developed varied from 2.5 μmol L^{-1} to 500 μmol L^{-1} and the sensitivity for arginine detection was 13.4 ± 2.4 μS/mmol L^{-1}, with a response time of 20 s [123].

Braiek et al. (2018) built a conductometric biosensor for the determination of creatinine. The detection of creatinine in a sucrose bottom solution showed a limit of detection of 2.0 μmol L^{-1} and good repeatability. According to the authors, the proposed method represented an interesting alternative for the determination of creatinine in biological samples due to the low associated cost, speed, and practicality [124].

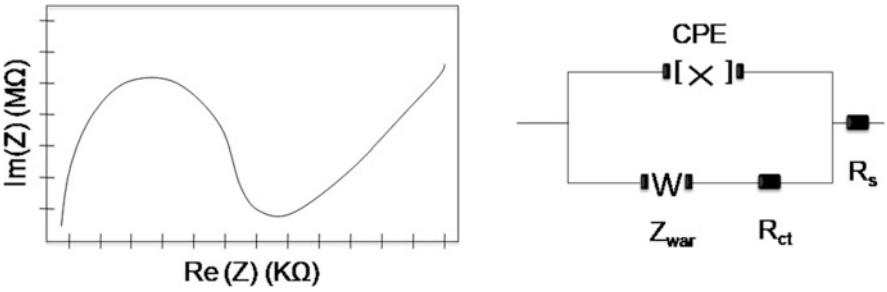

Fig. 7 Representation of impedance spectra at zero potential (**a**) and an equivalent circuit (**b**) (Figure based on [127])

2.5 Impedimetric Biosensors

Electrochemical impedance spectroscopy is an electrochemical technique of great utility to study the mechanisms of charge accumulation that happens at the interface of the conductive electrodes. The impedimetric biosensors are able to determine electroactive species by recording changes in the impedance value from biomolecular reactions on the electrode surface [121]. The basic principle of impedimetric biosensors is the provision of small sine wave disturbances over a wide frequency range. Thus, the current that is generated can be recorded and measured, informing the biorecognition phenomena found [36, 121]. More generally, the impedance describes the dependence of the current (at different excitation frequencies) which is directly influenced by the biorecognition system placed between two electrodes [64]. The representation of impedance spectra at zero potential of an impedimetric biosensor and its equivalent electrical circuit is shown in Fig. 7. The analysis of the results with these sensors is often performed through simulation with models of equivalent electrical circuits. In the work developed by Bhat et al. (2020), the equivalent circuit analysis was essential for the rapid monitoring of small pH fluctuations in the pathophysiological range of 7.35–7.45, with high sensitivity [125].

In the circuit model shown in Fig. 7b, CPE represents the capacitance of the electrical double layer in the surface area of the electrode in solution. R_{ct} corresponds to resistance to load transfer or faradaic impedance. The symbol W that equals Z_{wa} corresponds to the impedance that measures the resistance to mass transport of electroactive species. And lastly, R_s corresponds to the resistance of the solution between the working and reference electrodes. The simplest equivalent electrical circuit containing the components shown (CPE, R_{ct}, Z_{wa}, and R_s) is known as the Randles circuit [126].

Some authors consider that the impedimetric biosensors have low energy consumption, so they are inexpensive and can be easily miniaturized [128]. Probably the software used for data acquisition corresponds to the higher cost of common impedance equipment. Another aspect that some authors highlight as an advantage of the technique is the fact that they do not hinder most biorecognition interactions

from stimulation applied. This happens because the applied sinusoidal voltage is negligible being around 5–10 mV in amplitude [36, 129].

As shown by Rocha et al. (2020) in recent studies, an impedimetric biosensor was constructed for the quantification of staphylococcal enterotoxin A, from Staphylococcus aureus found in milk samples. The biosensor was developed by modification of the working electrode (glassy carbon) with reduced graphene oxide (rGO) and Anti-Staphylococcal Enterotoxin A (anti-SEA) to prepare sandwich-type electrochemical immunosensors. This sensor detected concentrations between 0.5 mg L^{-1} and 3.5 mg L^{-1} of the analyte by antigen-antibody binding. According to the authors, the proposed biosensor presented a limit of detection of 0.102 μg mL^{-1} for SEA analysis. The analyses of the milk samples showed good robustness, good specificity, and reproducibility to determine SEA [130].

2.6 Coulometric Biosensors

Coulometric biosensors are similar to voltammetric biosensors in terms of electrochemical principles, whereas in place of the maximum current generated coulometric measurements are based on the charge (= amount of electricity in Coulombs) required for the conversion of ~100% of a species by oxidation or reduction. The effectiveness in the conversion of the substance occurs since electrodes with a large active area are used during experiments. In the work presented by Tsujimura et al. (2009), a coulometric biosensor was built for the determination of D-fructose. This method was able to quantify D-fructose successfully at a concentration range from 1 mmol L^{-1} to 100 mmol L^{-1}, a much broader range than that attained by amperometry reported to date [131]. In coulometric cells, a reference electrode, a graphite counter electrode, and a mercury, gold, and carbon working electrode, among others, are used. To have good control of the potential applied in processes involving elevated currents, the reference electrode was positioned near the coulometric transducer (working electrode) avoiding variations of the applied potential due to the IR drop. Alternatively, a Luggin capillary can be used to measure the potential close to the electrode, without significantly blocking the current flow.

In the work proposed by Cao et al. (2020), an origami-based coulometric biosensor was designed to determine multi-metabolites with a single electrode (using 0.5 μL of samples). In order to build this biosensor, a screen-printed electrode and an origami device equipped with three folding flaps were used. The linearity intervals were of 0–10 mmol L^{-1} for L-lactate, 0–5 mmol L^{-1} for cholesterol, and 0–24 mmol L^{-1} for glucose. The electrolysis efficiency found for L-lactate, cholesterol, and glucose were 99% ± 3%, 99% ± 1%, and 100% ± 2%, respectively. The limit of detections were 0.25 mmol L^{-1} for L-lactate, 0.23 mmol L^{-1} for cholesterol, and 0.03 mmol L^{-1} for glucose [132].

In the work proposed by Liu et al. (2012), a coulometric biosensor was developed for DNA determination. The biosensor was built by means of enzymatic silver deposition on gold nanoparticle (AuNP)-modified screen-printed carbon electrode

(SPCE). The concentration range used was from 3.0×10^{-17} mol $L^{-1}$to 1.0×10^{-14} mol L^{-1}, and the limit of detection was calculated as 1.5×10^{-17} mol L^{-1} [133].

2.7 Point of Care Testing (POCT) as Biosensor

Due to its ease of use, Point of Care Testing-POCT has become widespread, since tests can be performed by the patients themselves without the need for specialization. The POCT, also known as self-test, has an advantage as minimum infrastructure requirements and excellent cost-benefit, among others [134].

The applications of POCT are beyond clinical analysis since it is currently used for environmental monitoring, food quality control, toxicological tests, and forensic sciences, aiming to minimize the time of diagnosis of diseases and contamination, among other functions [135].

He et al. (2020) designed a POCT by means of the capture of microdroplets on a tape previously sputtered with gold for rapid screening of Surface Enhanced Raman Scattering (SERS). The device was used to determine food contaminants. This type of POCT has the potential for military applications, consumer protection, and forensics, among others, as it is easy to operate [136].

Most of the POCTs have used screen-printed electrodes (SPE) during the construction of biosensors. SPE are disposable devices, that have a chemically inert surface that enhance the advantages of POCTs due to their versatility of design (from a single printed electrode to matrices of several electrodes), fast manufacturing process, good reproducibility, fast response and portability [18, 56, 137]. The surface of the SPEs can be modified with graphene, carbon nanotube, nanoparticles, and other materials, to significantly improve electron transfer and consequent increase of the sensitivity [56].

Due to their versatility, easy access, high-quality cameras, and wireless access, they open a new perspective for diagnostic applications with POCT. Thanks to these properties, researchers have developed electrochemical sensors for the quantification of some chemical species, reducing the size of the devices, dropping the cost, and simplifying the utilization of wireless biosensors. These biosensors can be linked to smartphones in order to determine biomarkers and toxic products produced by bacteria, [48, 138, 139]. In the study developed by Zhang et al. (2015), a smartphone-based POCT system was developed to detect 2,4,6 trinitrotoluene (TNT) by electrochemical impedance. In this study, a SPE was modified with peptides to produce impedance responses to TNT. This system was able to detect TNT at concentrations below 1.0×10^{-6} mol L^{-1}. The specificity of the POCT built was efficient to differentiate TNT from other chemicals [140].

Electrochemical biosensors can be associated with injection systems in order to provide high performance in the detection of different analytes, since these systems contribute to the improvement, mainly, the sensitivity and analytical frequency.

3 Injection Systems Associated with Electrochemical Biosensors

The search for analytical methods with greater sensitivity, selectivity, reproducibility, and low-value-economic equipment, in addition to shorter analysis time constitutes a wide and constantly expanding field of research, mainly, in the area of Analytical Chemistry. For greater mechanization and increased robustness of electrochemical analysis systems, injection devices can be implemented, such as Flow Injection Analysis Systems—FIA, Sequential Injection Analysis—SIA, and Batch Injection Analysis—BIA. These systems facilitate the transport of samples to the detector, providing several advantages during analyses, mainly low cost and high performance [141, 142]. The injection systems most commonly used in electrochemical analysis (FIA, SIA, and BIA) are briefly discussed below.

3.1 Flow Injection Analysis (FIA)

Flow Injection Analysis—FIA is a system widely explored in a large amount of analytical applications, providing simplicity of handling, low cost, use of versatile instrumentation, and greater precision, besides minimization of the waste of reagents and samples [142–144]. FIA was developed in 1975 by Ruzikca and Hansen [145] and this innovation was an important way for the automatic and accurate determination of several species in biological samples for clinical diagnosis [145, 146]. Initially, FIA was segmented, in which biological samples were transported to the spectrophotometric detector using bubbles to separate each injected sample. These bubbles were implemented in the system to decrease the dispersion of the samples and prevent contamination between successive experiments. However, these bubbles must be removed before detection and this can cause an increase in the analysis time, characterizing it as a major problem in a segmented FIA system. Over time, this segmented mode was gradually replaced by continuous flow in different applications [141, 144, 145].

Due to its efficiency and versatility, FIA has been extensively used in different detection systems in order to improve analytical performance in general [147]. This flow system can be defined as an automation process for analytical procedures. Its main function is to transport the sample within an analytical path to the detector, providing low detection limits and speed of analysis [148]. The most basic components of a FIA manifold are a propulsion system, an injection device, and a detection arrangement coupled to data analysis equipment. Its simplest mode of operation is that of a single line, as shown in Fig. 8.

The propulsion system has the function of boosting the fluid and promotes the continuous flow of solutions. In general, peristaltic pumps are preferred to propel the carrier fluids mainly due to the fact that they are multichannel devices. However, the peristaltic pumps produce pulsation of the flow (this process is called peristalsis) that occurs between rollers and tubes of its own pump, promoting noise and loss in sensitivity. Then, new alternatives have been developed and used to replace the

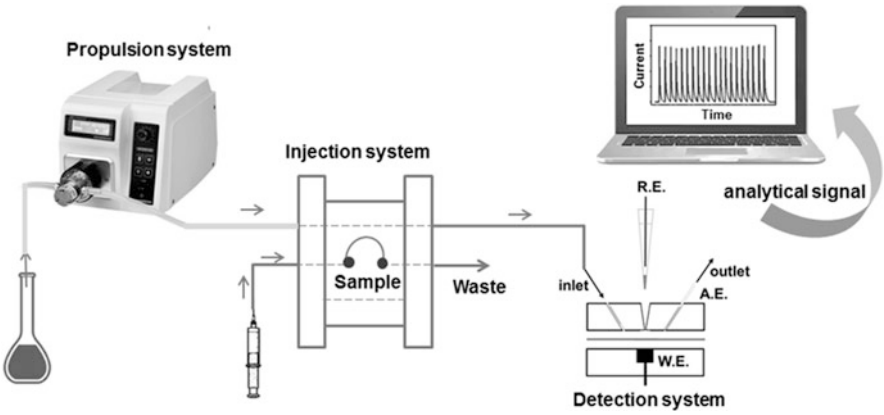

Fig. 8 Flow injection analysis manifold with amperometric detection: Propulsion system using peristaltic pump; Injection system using proportional injector; electrochemical cell constituted by W.E.—Working Electrode/A.E.—Auxiliary Electrode/R.E.—Reference Electrode

peristaltic pump [141, 142, 149]. These include the use of the force of gravity [150–153], piston or syringe pumps [154–156], pneumatic system driven by diaphragm pump [157, 158], or compressed gas [159], among others [160].

Moreira et al. (2014) developed a low-cost system with FIA and used gravity force to propel solutions and needles or metallic threads as sensors for amperometric detection. Therefore, the authors studied the behavior of $[Fe(CN)_6]^{3-}$ that has a well-established redox process, besides evaluating the stoichiometry of the compound formed by Cu^{2+} and EDTA and pH changes in the reaction between ascorbic acid and ferricyanide. According to the results, they developed a very satisfactory FIA system with particular emphasis on low cost and ease of handling [161].

Matos et al. (2001) used aquarium air pumps to propel solutions instead of peristaltic pumps. Aquarium pumps can withstand a pressure of 4 psi, equivalent to 0.28 bar, as well as it can be a simple and versatile way that allows continuous flow adjustment to achieve values of up to 12.5 mL min^{-1}. According to the results, the authors observed that in tubes with an internal diameter of 0.8 mm for a very long reactor (300 cm) the flow reaches 8.0 mL min^{-1}, while tubes with a smaller internal diameter of 0.5 mm in long circuits will lead to lower flow rates. In this way, the electrochemical experiments were always carried out with the advantage of the absence of pulsation of the propelled fluid observed with peristaltic pumps. In summary, the authors have proposed an efficient and low-cost propellant system to drive and aspire to flow solutions [160].

Regarding the injection system in FIA, it serves to guarantee the insertion of a defined volume of the sample (sample loop generally situated between 10 and 200 μL) into a liquid carrier flowing through a tube with an internal diameter generally less than 1.0 mm. Moreover, this defined volume is gradually dispersed into the carrier by radial and axial diffusion and by convection [141, 161]. Initially, a hypodermic syringe (Fig. 9a) was used to insert samples into the flow system that

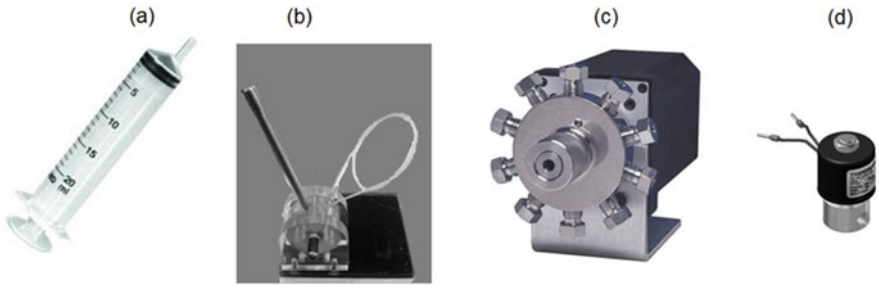

Fig. 9 Different ways for solution injection in FIA: Hypodermic syringe (**a**), Proportional injector (**b**), Rotary valve used in high-performance liquid chromatography (HPLC) (**c**), and Solenoid valve (**d**)

was loaded until carried to the detector. During the evolution of methods and the sophistication of materials, the components of the FIA manifold were gradually improved, showing greater repeatability and less dependence on the operator. Among these, injectors became popular, in which the main ones are: the proportional injector (Fig. 9b) that was created by a group of researchers from CENA—USP/Piracicaba, Brazil. Its operation is performed with the valve in the loading position, in which a defined volume is completed with the sample, and also in the discharge point, where the restricted volume in the loop is introduced in the carrier flow. Other types are the rotary valve (Fig. 9c), in which this valve is changed and the sample or carrier flow is directed to the detection system and the solenoid valves (Fig. 9d) that enable reproducible solution delivering in small volumes (for example, 10 μL) into the manifold [162, 163].

Aguiar et al. (2006) built a flow injection system with low operating cost and easy automation, aiming at the amperometric determination of iodide ions in commercial expectorant syrups. The method was carried out in an acid medium using the reaction of the iodide with nitrite ions. The propulsion system of this FIA system was performed by gravitational pressure at a flow rate of 4 mL min^{-1}. The aliquots of the samples and standard solutions were injected using a hypodermic syringe. As a result, the proposed method was fast (100 injections h^{-1}) and precise (RSD = 1.9%), with the detection of limit (LOD) calculated as 8.0×10^{-7} mol L^{-1} [164].

Dilgin et al. (2018) built a biosensor using electroanalytical techniques for glucose analysis, and the detection was performed by amperometry. For the construction of this sensor, a graphite electrode was modified by electropolymerization of polymethylene blue on its surface. To perform the FIA experiments, a single-channel peristaltic pump and a sample injection rotary valve were used for the injection system. Polyethylene tubes (0.75 mm) were used to connect all parts of the arrangement. Thus, they obtained a glucose biosensor with a detection of limit (LOD) of 4.0 μmol L^{-1} [165].

FIA is a very versatile system and allows association with different detectors [166–169]. As already mentioned earlier in this chapter, electrochemical detection, when compared to other detection systems, can offer many advantages such as the simplicity of application and reduction of sample preparation steps, in addition to low instrumentation cost with high precision and sensitivity [142, 170, 171].

Tvorynska et al. (2019) performed amperometric experiments in association with flow injection system—FIA for the quantification of choline (Ch). In this study, enzymes were immobilized covalently with glutaraldehyde in mesoporous silica powder previously covered by NH_2 groups. The detection of Ch occurred through amperometric monitoring of the oxygen consumed during the enzymatic reaction, which had a direct proportion to the concentration of Ch. The biosensor showed a linear range of concentration of 80–700 μmol L^{-1}, repeatability of 3.9%, in addition to high reproducibility for the detection of Ch [172].

Nikolaos et al. (2012) carried out studies involving the association between flow injection and potentiometric detection. They proposed a biosensor for the determination of uric acid manufactured by immobilizing uricase in stabilized lipid films, using as a transducer zinc oxide (ZnO) nanowires. Thus, they obtained a detection limit of 0.4×10^{-3} mol L^{-1} and good reproducibility (RSD lower than 5%) [173].

Chiriaco et al. (2019) proposed an impedimetric biosensor associated with FIA for the quantification of cholera toxin (CT). This system used two reaction chambers with eight detection areas for biorecognition among antibodies immobilized on the surface of gold electrodes. Standard tests for CT detection include the GM1 technique and the immobilization of antibodies on standard ELISA screening plates. A detection limit of less than 10 pmol L^{-1} was reached, thousands of times less than the lethal dose. The proposed biosensors presented a low price and fast response, besides allowing screening in crops, with clinical and medicinal applications [174].

Despite the FIA success described in the literature, there is a restriction regarding the use of these systems in industrial processes, due to the need for frequent maintenance of fluid transmission lines. Therefore, the need for a system designed to monitor different processes favored the emergence of sequential injection analysis (SIA) [175].

3.2 Sequencial Injection Analysis (SIA)

Sequential Injection Analysis—SIA was developed by Ruzicka and Marshall in 1990 [175], and presents the same important analytical characteristics as the FIA system, such as improvements in reproducibility, low consumption of samples and reagents, and minimal human interference. SIA presents advantages compared with FIA, between them the low need for maintenance of the SIA system, the lower consumption of reagents and samples, as well as greater robustness and stability, makes this system quite attractive [175, 176]. The main disadvantage is the price of its components. Figure 10 illustrates a typical SIA system, composed of a propulsion device (usually a syringe pump) and a precise injection valve (a rotating selection

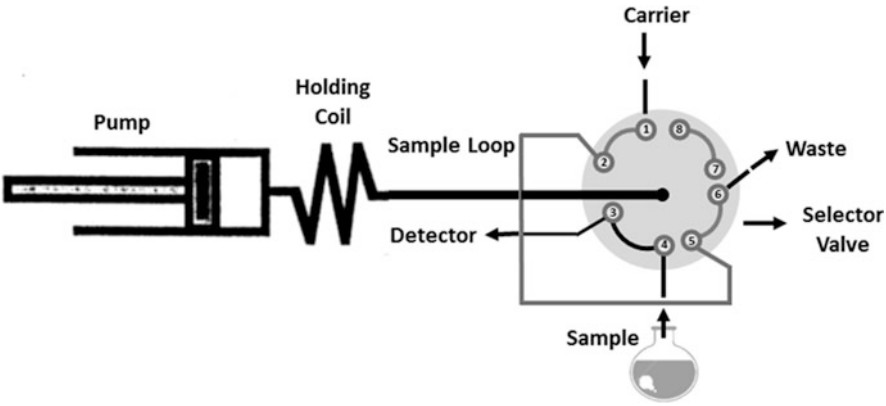

Fig. 10 Basic scheme of a SIA manifold with rotating selector valve consisting of eight ports

valve), responsible for the controlled dispersion and control of the reaction times with high repeatability, so as for the transport to the detection system [177].

In a typical SIA manifold (Fig. 10), samples and reagents are aspirated sequentially using a piston pump or a syringe pump, or even a peristaltic pump. Piston and syringe pumps provide greater precision in the aspirated or injected volumes. A rotary selector valve, which has many input channels, is used to select different solutions. The entire operation is controlled by a computer that monitors the synchronization between the pump and the valve. For carrying out an analysis, defined volumes of carrier and sample solutions are aspirated into the collector. After the position of the multichannel valve is changed and the flow direction is reversed, passing the solution to be pumped to the detector [176–178]. SIA has a low analytical frequency when compared to the FIA due to its operational characteristics. Additionally, some computer programming knowledge is required, making it a little more difficult to use for some electroanalysis users [176]. Figure 11 shows the popularity of FIA rather than SIA in association with electrochemical biosensors over the years.

Since its development, SIA has been used in the analysis of several types of samples, from food and pesticides to beverages, such as milk, juices, and wines. Applications involving the use of biosensors were also explored, although in smaller numbers than those involving FIA [141, 178]. Over the past 14 years, the number of papers involving the association between SIA and electrochemical biosensors was approximately 10. This small number of papers probably is proportional to the small number of SIA users.

In 2006, Staden et al. explored a SIA system to develop a carbon paste-based biosensor with amperometric detection for the simultaneous quantification of creatinine and creatine. According to the results, the proposed system can be used reliably for online detection of these chemical species in pharmaceutical products, with an analytical frequency of 34 samples per hour and RSD values better than 0.16% [178].

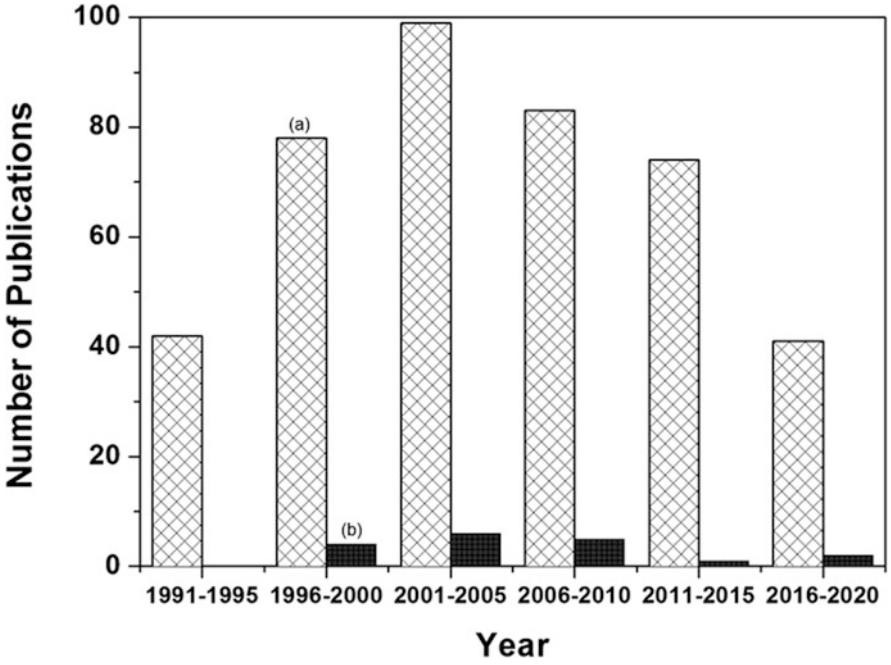

Fig. 11 Publications from the association between (**a**) Biosensors and FIA and (**b**) biosensors and SIA (Web of Sciense® database)

3.3 Batch Injection Analysis (BIA)

Batch Injection Analysis—BIA was developed by Wang and Taha in 1990, it is a tool which has been explored by some research groups as an option to perform, in a very simple way, quick analyzes. In BIA, an aliquot of the analyte solution is injected, using an automatic micropipette, in the central part of the working electrode, localized in the center of the bottom of the electrochemical cell, which was specially designed for this purpose (wall-jet cell) (Fig. 12a). In this experimental arrangement, the injection of the analyte produces a transient signal. The height or area of the peak is directly related to the concentration of the analyte (Fig. 12b) [141, 177].

Before the injection of the analyte, the cell is filled with electrolyte and the detection potential is applied. When the chosen potential is applied, initially the current is elevated (due to the charging of the electrical double-layer process). This current decreases exponentially and after a certain time, the signal tends to be very low (low microamperes or even nanoamperes) corresponding to the baseline. When the analyte is injected, an increase in current occurs due to the redox process. During the injection, the current generated increases rapidly and remains constant and maximum for a short time, and when the injection ends, the current drops very

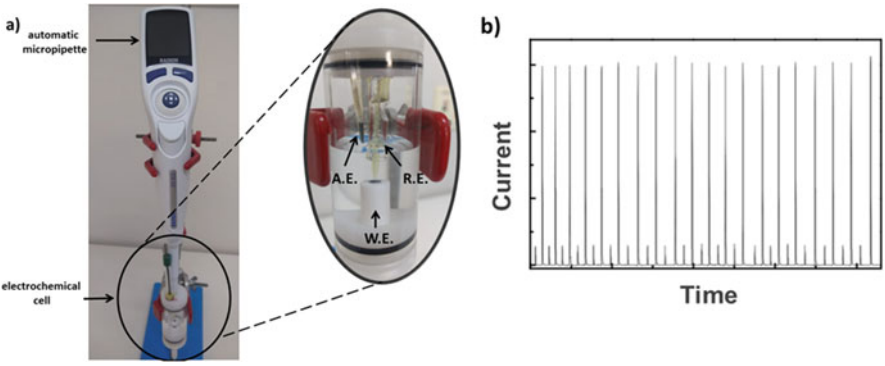

Fig. 12 (**a**) Representation of a BIA system with amperometric detection and in the detail the electrochemical cell constituted by: W.E.—Working Electrode/A.E.—Auxiliary Electrode/R.E.—Reference Electrode. (**b**) Analytical signals from alternate injections of 1.0×10^{-6} mol L^{-1} (smaller peaks) and 1.0×10^{-5} mol L^{-1} (larger peaks) ferricyanide standard solution during experiments of BIA with amperometric detection

rapidly. The signal decreases to almost the same minimal signal that the one that was recorded before the injection. Even having now, the analyte dispersed in the electrolyte, its contribution is very small compared with the signal generated by the injection of the analyte. This aspect was not clear at the beginning of the studies using BIA. In the first studies, cells with volumes of about 1 L were used. Over time, it was learned that the major difference in transport was represented by the injection of the sample (centimeters or even meters per second) and diffusion (somewhere around 10^{-6} cm^{-2}). As equilibrium is reached, the current stabilizes again until another sample or standard aliquot is injected on the electrode surface, leaving only a residual current in the system [141, 177, 179]. Thus, the transient signal is obtained during experiments (Fig. 12b).

The BIA system presents several advantages, such as the elimination of typical problems related to valves and pumps in the FIA or SIA systems, high sensitivity, greater analytical frequency, low consumption of electrolytic solution, and portability [180].

Despite the advantages of BIA, so far in the literature, there is no research on the development of biosensors in agricultural and veterinary applications that has yet adopted this system. However, there are some studies on electrochemical biosensors in association with BIA for the determination of glucose in saliva and urine [181] and hydroquinone in pharmaceutical samples [182], among others.

Baronas et al. in 2004 explored the association of an electrochemical biosensor within batch and flow injection modes. Chemometric models of the proposed biosensor were built to simulate amperometric responses for mixtures of compounds. For this, the authors used principal component analysis—PCA to optimize the calibration data, as well as used artificial neural networks to differentiate molecules or mixtures and predict the concentration of each molecule. The

amperometric biosensor in association with both flow injection and batch analysis showed a 99% prediction of each component [183].

The biosensor is an important device to diagnose, and, therefore, assist in the inspection of contaminants in soils, water, and food, in the monitoring of epidemics that are a serious cause of death of animals, among other applications. The next section will cover biosensor applications in association with electroanalytical techniques exploring agriculture and veterinary medicine samples.

4 Electrochemical Biosensors Applied in Agricultural and Veterinary Matrices

Toxic agrochemicals are used in crops to prevent or control the development of weeds, insect infestation pests, and diseases that can cause harm from the birth, growth, and production of the plant, or during storage, processing, or transport of food. However, pesticides can contaminate the environment with toxic effects on humans. Seeking to determine toxic agrochemicals, such as triazine and urea, Buonasera et al. (2010) proposed changes in the surface of carbon electrodes with photosynthetic organisms (*S. oleracea* thylakoid) for the manufacture of sensitive and low-cost biosensors. The developed device was applied in the quantification of different triazinic and ureic herbicides using amperometry and fluorescence spectroscopy as detection systems. During amperometric experiments in dynamic mode (FIA), a working potential of +0.20 V (vs. Ag/AgCl) was used and the current signal decreased with increasing concentration (from 1.0×10^{-8} mol L^{-1} to 1.0×10^{-10} mol L^{-1}) of herbicides, for example, atrazine, diuron, linuron, or terbuthylazine [184].

Gramberg et al. (2012) reported an approach using a membrane containing antibodies for the determination of cherry leaf roll/CLRV virus and tobacco mosaic virus/TMV. The detection principle of the bacteria biosensors was based on the measurement of changes in the membrane potentials as a result of bindings between virus and antibody. With this sensor, the limit of detection—LOD of 1 pg mL^{-1} was calculated for both TMV and CLRV [185].

Yang et al. (2014) proposed a sensitive biosensor of *Magnaporthe oryzae* in rice plants, using amperometric detection *M. oryzae* is an extremely effective plant pathogen because it can reproduce sexually or asexually, and it is very destructive to rice fields. The authors used a biochemical marker from M. oryzae's chitinases (Mgchi) and as a recognition probe a rice cDNA from Oryza sativa that encodes lectin related to mannose jacalinization (Os-mbl). Moreover, for the construction of the electrochemical biosensor, they modified a gold electrode with nanoparticles of palladium magnetic spheres in order to increase the sensitivity. The amperometric studies were performed using a working potential of +0.2 V (vs. Ag/AgCl). The proposed sensor allowed reaching the detection of 6.1×10^{-12} mol L^{-1} for Mgchi [186].

Tarasov et al. (2015) explored the capabilities of a biosensor with potentiometric detection for rapid analysis of the main viral pathogen in bovine respiratory diseases,

bovine virus-1 (BHV-1). During the construction of this sensor for application in bovine serum samples, the viral protein gE of the bovine herpes virus-1 (BHV-1) was immobilized on the gold surface to capture the BHV-1 antibody. In comparison to ELISA, it provided results much faster (~10 min vs 19 h) with high sensitivity and selectivity [187].

Cesarino et al. (2012) built an amperometric biosensor for the detection of carbamates in fruits and vegetables. The authors modified a glassy carbon electrode with a core-shell structure of carbon nanotubes and polyaniline for subsequent immobilization of acetylcholinesterase. During the experiments, values of LOD of 1.40 and 0.95 μmol L^{-1} were calculated for the carbaryl and methomyl toxins, respectively [188].

Crew et al. (2011) reported the use of electrochemical sensors for the determination of various organophosphates (such as chlorpyrifos-oxon, dichlorvos, naloxone, among others) in environmental and food samples. For this, six acetylcholinesterase enzymes were immobilized in gold arrays. These sensors were produced based on screen-printing technology for large-scale manufacturing and low cost, as well as they incorporated a neural network program. These organophosphates were determined by amperometry and a working potential of 0.0 V (vs. Ag/AgCl), with measurements within 10 s, was used. In this system, it was possible to obtain a wide linear range from 1.0×10^{-5} mol L^{-1} to 1.0×10^{-9} mol L^{-1} in phosphate buffer 0.05 mol L^{-1} (pH 8.0) [189].

Gong et al. (2013) developed a biosensor with amperometric detection associated with Flow Injection Analysis to detect organophosphate pesticides in environmental and food samples. During experiments, glassy carbon electrode was modified with inorganic layered double hydroxides and acetylcholinesterase. Next, it was dipped into saturated glutaraldehyde to enhance the stability of the sensor. The pesticide Methyl parathion-MP was used as a model and biosensor inhibition. The response of this sensor was linear in two distinct ranges from 0.002 μg mL^{-1} to 0.3 μg mL^{-1} and 0.3 μg mL^{-1} to 4.0 μg mL^{-1}, with correlation coefficients of 0.999, for both two ranges. Recovery analyzes were made from different concentrations of MP in cabbage, apple, and garlic samples and recoveries ranged from 97.2 to 104.6% [190].

Luo et al. (2010) functionalized the surface of nitrocellulose nanofibers with silver to construct a working electrode that was modified with appropriate antibody (with formation of sandwich complex) for bovine pathogenic detection. During conductometric experiments with this biosensor, it was possible to obtain detection limits of 61 CFU/mL and 103 CCID/mL for Escherichia coli bacterium and bovine viral diarrhea virus, respectively [191].

Ma et al. (2008) built an electrochemical biosensor from a gold disk electrode modified with self-assembled monolayers of *p*-aminothiophenol and nano-SiO_2 for immobilization and hybridization of DNA. This biosensor was constructed for detecting phosphinothricin acetyltransferase gene (PAT), which is an important indicator of transgenic plants. The impedance results indicated a high sensitivity of the DNA biosensor for the wide concentration range of the PAT, varying from 1.0×10^{-11} mol L^{-1} to 1.0×10^{-6} mol L^{-1} [192].

Song et al. (2011) developed a voltammetric sensor to determine carbaryl, an extremely toxic pesticide. For the construction of this sensor, a glassy carbon electrode (GCE) modified with chitosan and Prussian blue film was used, followed by the immobilization of acetylcholinesterase-AChE. The voltammetric results indicated that the changes in the concentration of the carbaryl were proportional to the inhibition of the pesticide on the action of AChE, with a correlation coefficient of 0.999 and a limit of detection—LOD calculated as 3.0 nmol L^{-1} [193].

Muhammad-Tahir et al. (2005) developed a conductometric biosensor for the detection of bovine viral diarrhea virus (BVDV) and other agents related to agricultural bioterrorism, using culture media and blood serum samples. The developed biosensor showed good sensitivity to a concentration of 10^3 CCID/mL of BVDV antigens. The authors indicated that it is necessary to make modifications in the proposed biosensor to obtain a rapid device that identifies outbreaks of infectious diseases in the livestock population in an agricultural terrorism event [194].

Wei et al. (2015) proposed an acetylcholinesterase-AChE biosensor for the determination of the dichlorvos pesticide in lettuce leaves. This biosensor was manufactured using a boron-doped diamond electrode. Next, this electrode was modified with gold nanoparticles grown in porous carbon material and ionic liquids to improve the dispersion and subsequently immobilization of AChE. During experiments using the differential pulse voltammetry technique, it was possible to observe linear responses in the interval of 10^{-6} g L^{-1}–10^{-10} g L^{-1} and a limit of detection—LOD of 6.61×10^{-11} g L^{-1} (2.99×10^{-13} mol L^{-1}) [195].

Durrieu et al. (2011) developed optical and conductometric biosensors based on the measurement of the metabolic activities of marine algae for quantification of pesticides (for example, diuron and glyphosate) capable of contaminating southern France. Algal suspension was immobilized on two identical pairs of interdigitated gold electrodes using the self-assembled monolayers during the construction of a conductometric sensor. The activity of the *D. tertiolecta* esterase algae varied in presence of diuron, leading to a change in the conductivity signal due to a decrease of ionic species in solution. One of the diuron pesticide inhibition results showed a residual activity of esterase algae of about 30% [196].

5 Concluding Remarks

In this chapter, a general view about electrochemical biosensors and the different forms of their utilization associated with different techniques, under quiescent and under flowing processes, was presented. The different electrochemical techniques covered in this chapter allow the analyst to choose the most suitable technique to be used. The association of voltammetric techniques with injection analysis techniques (FIA, SIA, and BIA) allows faster analyzes, with greater precision and more importantly, with enhanced sensitivity, thanks to the significant increase in the transport of the electroactive species to the surface of the working electrode. Besides the electrochemical methods and the flowing systems, many of the electrochemical biosensors are modified to improve sensitivity and selectivity according to the

analyte and type of sample, among others. Increasingly, the technology developed based on electrochemical biosensors is present in the life of the population directly or indirectly. Proof of this is the growing advances in the development of POCT that allows the rapid diagnosis of diseases and contaminants via smartphones. Significant advances are also noted in the monitoring, through electrochemical biosensors, of environmental contaminants from activities that affect the soil, water, and food, among others. Veterinary applications with electrochemical biosensors are also found in this chapter, presenting potential for the residual analysis of veterinary drugs, pathogens, and other compounds in complex matrices (blood, urine, feces, and so on). In this perspective, the potentiality of the electrochemical biosensors for applications in agricultural and veterinary areas is just starting. It can be expected that more and more studies will be carried out, seeking to quickly and effectively meet agricultural and veterinary needs with selective and sensitive electrochemical devices.

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The Applications of Biosensors and Biochips for Prognosis and Diagnosis of Diseases

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Abstract

The early diagnosis of chronic and infectious diseases is one of the top priorities globally. But the major obstacle is the detection and screening of minute amount

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of disease biomolecules or biomarkers circulating in the bloodstream and cerebrospinal fluid for the treatment of particular disease. The highly sensitive approaches are needed to detect targeted disease biomolecules or biomarkers at the concentration of pico- or femto-scale. Currently, sensing technologies available are confined to a thousand times larger concentrations. Using a pico- or nanoscale platform allows us to identify the lower concentrations of targeted biomolecules or biomarkers levels of diseases that will help in not only early diagnosis or beginning stages of diseases but timely treatment as well. For this purpose biosensors and biochips may play a pivotal role in the early diagnosis and screening of chronic and infectious diseases. Biosensors are composed of a wide variety of bioreceptors and tranducers. Commonly used bioreceptors are based on intermolecular interactions between antigen/antibody, nucleic acids, enzymes, cells (i.e., microorganisms), and synthetic bioreceptors (biomimetic materials). When biosensors are designed on integrated circuit microchips with transducers they are called biochips. To achieve higher throughput and speed, a collection of miniaturized test sites works together at the same time on a biochip. These miniature devices contain millions of biosensors. These biochips can detect chronic diseases, i.e., chronic kidney diseases (CKD), cancer, arthritis, neurological, and cardiovascular disorders, and infectious diseases as well. Pathogenic microorganisms are notoriously known for causing infectious diseases, i.e., protozoans, bacteria, fungi, or viruses. The current chapter provides an overview of the different types of biosensors and biochips for the diagnosis and screening of various chronic and infectious diseases along with significant advances.

Keywords

Biosensor · Biochips · Chronic and infectious diseases · Biomarker · Biorecptors

1 Introduction

Biosensors are used in a broad variety of applications to enhance people's quality of life. The vast applications of biosensor have actually attracted research attention globally. They have been effectively employed in many methods when it comes to improving the standard of living. The applications are now being dispersed in almost every area beginning from glucose testing to nuclear tests. The applications cover environmental monitoring, disease detection, food safety, defense, medication development, and more. The biomolecules that are detected, either marker of disease or therapeutic targets, are one of the most common uses of biosensors. Electrochemical biosensing methods, for example, may be utilized in the clinic to detect protein cancer biomarkers [1–3]. Food traceability, quality, safety, and nutritional value may all be monitored using biosensors [4, 5]. These applications come under the category of analysis tools known as "single shot," meaning they need cost-effective along with disposable sensor systems. While, an application like monitoring of pollution [5, 6] necessitates a biosensor that can operate for many hours to days. Biosensors

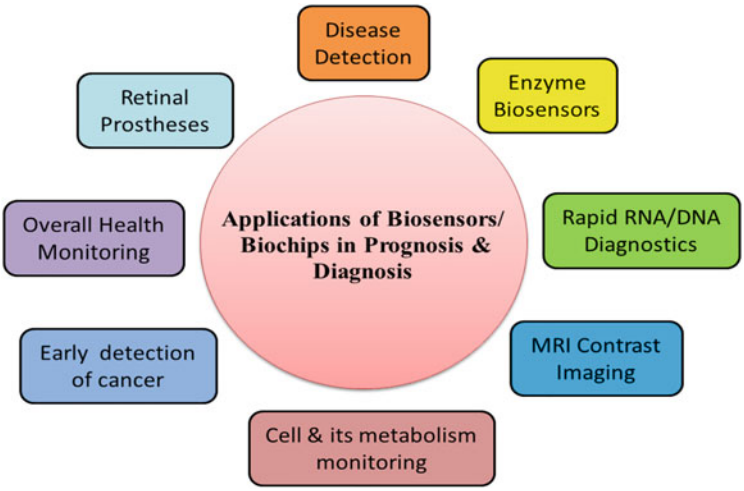

Fig. 1 Major areas of applications for biosensors and biochips in prognosis and diagnosis

like this are referred to as "long-term monitoring" analytical instruments. Biosensors are technologically advanced devices and are very useful in settings that are resource-limited and medically sophisticated set-ups, for example, in drug discovery applications [7–9]. They are useful to detect a number of toxic chemical and biological agents and materials of defense interest [10] and for utilization in artificial implementation. Electrochemical, optical, and acoustic sensing methods, as well as their integration into analytical instruments for different purposes, have all been used. Biosensors have been utilized in a variety of research fields, as shown in Fig. 1.

In this chapter, biosensors applications in diagnosis of disease have been assessed. Contemporary trends and the literature available at online platforms for the development of biosensors will lead to better diagnosis of diseases in near future. Mainly, a sensor is described as a device that is competent to determine a physical property or can measure it. It could probably also indicate or suggests and can respond to it. Thermometer, pyrometer for heat, microphone for sound, thermocouple and hydrometer for thickness, barometer for force, breathing analyser for substance, and anemometer for wind speed are some of the preferred examples of biosensors. The sensor that works on the element are biological entity that identifies the analyte for the recognition of several substances with additional susceptibility and specificity and is known as a biosensor [11]. They provide the introduction of new advancements in areas of biotechnology that is certainly including diagnosis of various diseases, health condition analysis, and health care to improve total well-being as well as commercial implementations [12], environment, and food industry. These are generally getting used as vital elements in forensic science [13]. These are typically generally employed primarily for treatment, tracking, and control, specifically beverage and food; secondly, medication for diagnostics, metabolites, and

hormones of the body; thirdly, military for battleground monitoring of poison gases; fourthly domestic for residence track of non-acute conditions [14].

Biosensor is a product that is promising integration of both the axioms of biology and electronic devices. Simply, it is the combination of the benefits of sensor technology [15] and also biology. The prosperity of setting up biosensors might count on efficient construction and architecture. It required skilled personnel having an understanding towards various fields, i.e., biology, chemistry, information science, electronics, and physics [13]. Biosensors consist of an element which is bio traces (analyte), a transducer along with an electric processor [16]. The reaction in the middle of bio constituent as well as the analyte or component guides to a change which is physicochemical properties and then converts to an electric signal by a transducer. The biological element could be any structure, i.e., organelles, cell receptors, microorganisms, chemical compounds, nucleic acids, antibodies, etc. Materials derived from biological sources are biomimetic connects (ties or recognizes) of the analyte under investigation [16]. Their specific ability differentiates the indication from the environment via specific biochemical reactions while the transducer converts the biochemical signal to an electric signal on an electric processor. Earlier techniques were capable for complementing the assessment nevertheless they have been lacking specificity and sensitivity [17]. Biosensors overcome these limitations [18] and will offer a great advantage that is certainly dependable and more specific along with low sensitivities.

2 Detection of Biomarkers

2.1 Protein Biomarkers

Protein biomarkers are used in several of the subsequent applications (Fig. 2). Biomarkers are "molecules that can be reliably and accurately quantified and are indications of normal or pathological biological processes and reactions to therapeutic interventions," according to the National Institutes of Health (NIH) in the United States [19]. Protein measurements in blood or cells give a "snapshot" of a patient [19]. Secreted proteins, membrane-bound proteins, antibodies, and small molecule metabolites are examples of molecular biomarkers for illnesses. Serum protein measures in cancer diagnostics offer early identification and monitoring of treatment and postsurgery remission [20, 21]. They are found in the tissue, blood, plasma, and serum of patients and offer great potential for early illness diagnosis [22, 23], prognostic evaluation [24, 25], and therapeutic efficacy monitoring [26]. Immunosensors, for example, are extensively used to detect proteins, antibodies, and a variety of other biomolecules.

Electrochemical sensors are a promising tool for detecting biomolecules (such as proteins, nucleic acids, and lipids), which are key targets for early illness and condition detection. The creation of a new kind of biosensor, allowing non-destructive and label-free detection of function, viability, and the genetic signature of entire cells, has been enabled by advances in electrochemical sensing

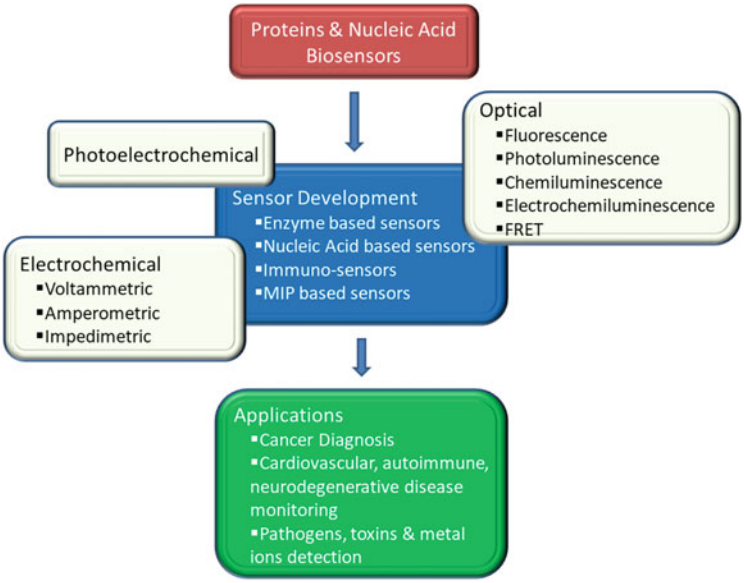

Fig. 2 Proteins and nucleic acids in biosensor development. *MIP* molecularly imprinted sensors

platforms. Sensitivity and selectivity in numerous researches have been conducted to improve the sensitivity of electrochemical sensors, that are the most important characteristics to consider when evaluating sensor performance. Metal and metal oxide nanoparticle graphene and its derivatives, carbon nanotubes, have all been utilized to increase sensor sensitivity by improving the electrical conductivity and electrocatalytic characteristics of working electrodes. Biomaterials have been used to further improve, i.e., aptamers, antibodies, extracellular matrix (ECM) proteins, and peptide composites sensor platforms for their selectivity and biocompatibility.

2.2 Nucleic Acid Biomarkers

Nucleic acid biomarkers are used in a wide variety of applications (Fig. 2). Quantifying particular disease nucleic acid biomarkers that are detected in aberrant amounts in bodily fluids or tissue when the disease is present can efficiently improve patient prognosis [27]. For example, microRNAs (miRNAs) are small nucleotide sequences (usually 20–25 bases) found in tissue, blood, plasma, or serum of patients and have shown promise in early illness diagnosis, prognostic evaluation, and treatment efficacy monitoring [26]. One of the most significant obstacles is that miRNA concentrations are generally ultralow, i.e., picomolar or below. Increases in the signal associated with the binding of these biomarkers to a sensor capture surface are still being worked on. However, if these techniques also increase the background response, the gains in analytical performance with real-world samples may be

insufficient. For instance, using a nanostructured material to increase the surface area of an electrochemical sensor increases the current for a redox probe in solution or the surface coverage of a labeled nucleic acid probe strand, but it also increases the background charging current against which the faradaic current must be measured.

3 Biosensors for the Detection of Whole Cells and Microbes

3.1 Tuberculosis

Tuberculosis is one of the globally infectious diseases caused by a pathogenic bacterium known as *Mycobacterium tuberculosis*. Early analysis associated with infection might be beneficial in medical viewpoint. Various types of biosensors were designed for tuberculosis to detect using the applications of optical, piezoelectric, and electrochemical maxims. Tuberculosis is the ninth reason for deaths accounting globally 10.4 million novel cases in 2016 with the TB-related fatalities 1.7 million. Much more, TB may be possibly one of the several causes that is regular in individuals infected with HIV. The efficacy of this is certainly limited to treatment and difficulty in getting an analysis which accurately led scientists to take into consideration much better recognition modalities. Numerous methods such RNA gene-probe evaluation, interferon-gamma-based assays, and urine-based test is recommended as recent TB examinations [28, 29].

Researchers at Massachusetts General Hospital (MGH) have developed a strategy for the early and fast detection of TB as well as other microorganisms [30]. The recognition strategy had been initially created to identify cancer cells [31] afterward it had been discovered that it is highly relevant to microbial detection [32]. The transportable microfluidic item was in fact predicated on nuclear magnetized resonance (NMR) recognition, which has been known as "diagnostic magnetic resonance (DMR)" [33]. DMR was considering metal that has superparamagnetic nanoparticles. Magnetic nanoparticles (MNPs) having diameter <20 nm having thermal variations could overcome the problem of anisotropy obstruction and suddenly flip the minute which is a magnetized particle [34]. This is certainly the possible lack of extra magnetic properties; the magnetized moment, nonetheless, develops with an increasing exterior magnetized field to provide superparamagnetism because of this, an assembly of MNPs has no magnetism. This means that MNPs do not unexpectedly aggregate under physiological conditions. A MNP is usually composed of an inorganic core that is magnetized by a biocompatible area layer and could be customized with useful ligands. The coherence can be efficiently destroyed by MNPs involving spin-spin relaxation of liquid protons. The effect derived is web of a modification of magnetic resonance sign, shortening of the T1 which is longitudinal, and T2 spin-spin leisure times [33].

DMR detection makes use of MNPs to target and modulate the spin-spin T2 leisure biological samples time. The DMR assays are contained in two kinds based on the measurements of the goal. A technique called magnetic relaxation flipping (MRS) is exploited when it comes to the recognition of tiny analytes. When MNPs

cluster in solution, the collections will assume r2 values this is actually various, causing t2 this is certainly corresponding. MRS assays are carried out without getting rid of the additional unbound MNPs, consequently allowing the recognition of little particles. The cells tend to be tagged with functionalized MNPs; then, unbound MNPs tend to be eliminated for larger biological goals for example microorganisms. Miniaturized NMR coils (so known as μNMR) [32] are incorporated into microfluidics chips [35]. Effective temperature control had been a challenge that is major moving DMR into a POC device due to the inherent heat susceptibility associated with the magnetized industry produced by the permanent magnet. The authors utilized an electronic digital strategy to get a feedback routine that instantly monitors the heat alterations and correspondingly reconfigures the dimension choices [36] in a device. The device ended up being configured for the detection of MTB [33]. Polymeric beads have been conjugated with MNPs linked to the sequences of oligonucleotide particular for MTB. These probes utilized artificial single-stranded DNA (ss-DNA) having 92-nucleotide (nt) sequence particularly as the part of the acyl-CoA de-hydrogenase MTB fadE15 gene [37, 38]. Sputum instances were mechanically liquefied and amplified polymerase chain reaction (PCR) that is utilizing on-chip, in that case, MNPs and buffer solutions had been loaded into valves gated individual chambers. The PCR items have been coupled with MNP-labeled capture beads certain when it comes to amplicons which are potential. Alterations into the transverse relaxation point (DR2) were assessed μNMR. The test quantity per measurement was in fact 1 μL. During experimental researches, ss-DNA and ds-DNA can be compared. ss-DNA ended up being discovered to become superior as ds-DNA required denaturation that is annealing that is extra.

3.2 Diarrhea

Diarrhea is a pathogenic disease caused by microbes, i.e., *Escherichia coli* O157:H7, *Salmonella typhimurium,* etc. Biosensors based on aptamers were designed to detect minute organisms by colorimetric test [39]. Significantly approx. 80 million instances of food-borne infections are reported every year in the United States of America and accounting for around 9000 fatalities [40] per year. *E. coli* 0157:H7 is among the pathogens mainly associated with these cases which can be dangerous and can be introduced into the system as a result of contamination by fecal bodies. The original observable symptoms consist of diarrhea, belly cramps, and temperature; whereas in latter it may lead to hemolytic uremic syndrome (HUS) and cause fatality. Mainstream antibiotics usually are inadequate. Detection of *E. coli* is reported use that is making of antibody-based and techniques which are DNA-based. Colorimetric-biosensing making use of antibodies specific for *E. coli* and are conjugated and fabricated with gold nanoparticles (Ab-AuNPs) have been found in a lateral flow assay (LFA) having a recognition time of 10 min. Capillary circulation permitted the microorganisms resulting in agglomeration for the Ab-AuNPs generated a blue color that is red from the report strip. LFA-based

systems reduce the susceptibility. The assay susceptibility could possibly be enhanced by magnetized bead pre-concentration and amplification from the cells that are bacterial [41].

In another example it was illustrated that the detection of *E. coli* cells was in fact immobilized on a multilayer substrate and permitted binding to antibodies labeled with Cy5; binding ended up being recognized because of the variety of photodiodes along with amplifiers, discriminators, and reasoning circuitry [42]. A group demonstrated a micro-electromechanical (MEMS) biosensor referred to as a monolayer that is "Self-Assembled Monolayers (SAM)" surface [43]. They reported the deposition of numerous electrodes for a Si wafer as well as a streptavidin monolayer that ended up being immobilized in the Au electrode area. Biotinylated single-stranded DNA (ss-DNA) probes were in fact then connected with Au-thiols which are making use of recapture rRNA from *E. coli*. A group is certainly second of ss-DNA probes was then included and followed by an anti-fluorescein antibody to peroxidase. The Enzymatic amplification permitted the device detection of 1000 *E. coli* cells without the requirement for PCR.

3.3 Cholera

Cholera is caused by *Vibrio cholera*, a pathogenic bacterium. It is an etiological agent for epidemic cholera, and contamination is serious and intestinal. A DNA biosensor is good for the amplification of PCR amplicons of *V. cholera*. A real-time multi-target NASBA assay was developed for the specific detection of diseases including cholera by *V. cholera.* Molecular beacons and primers have been aimed at five genomic sequences. In another study [44], the particles which work as biomarkers associated with disease caused by *V. cholerae* utilized an electrochemical microfluidic model to separate and trace D-amino acid (DAAs), i.e., D-Leucine (D-Leu) and D-Methionine (D-Met). One of the features of this study relied on when you look at the application of a processor that is quick of an electrochemical system this is actually microfluidic reasonable biological items ingesting. The microfluidic having DAAs can separate the mandatory response between each D-amino acid oxidase (DAAO) and the D-AA biomarker. On the list of advantages of the utilized processor chip which is microfluidic the yield is large split without consuming components; this stops covalent immobilization of enzymes at first of the channels chip. Utilizing this developed technique, effective separation of DAAs was attained and *V. cholera* in situ evaluation was carried out.

3.4 Salmonellosis

This is often a globally spread illness due to *Salmonella* sp. Determination of the gene that is *invA* of is vital and it is carried out by surface Plasmon resonance (SPR) recognition method. DNA biosensors have found its application once you glance at the analysis. *Salmonella* spp. is normally referred to as a food-borne pathogen. Fast

and sensitive detection is needed for the analysis [45]. Polymerase chain reaction (PCR)-based techniques represent earlier strategies implemented regarding the detection of *Salmonella*; these methods however need highly skilled personnel, consequently becoming costly [46, 47]. Some type of computer device is really smartphone-based already been used in combination with report-based microfluidic chip is a lightweight, simple to use, exceptionally delicate and precise way for the recognition of *S. typhimurium* [48]. Each microfluidic channel paper has been preloaded with submicrometer (920 nm) polystyrene latex particles which were anti-*Salmonella* antibodies conjugated.

4 Biosensors for the Detection of Viruses

Viruses are generally micrometer-scale pathogens that may trigger several diseases in human, animals, plants as well as microorganisms. These pathogens coated with proteins are nucleic acid-based just able to reproduce inside living cells. The Viruses are solitary or double-stranded and consist of either DNA or RNA which remains very infectious in certain circumstances. Viruses, bacteria, and other infectious microbes cause a significant amount of illness and mortality in humans. Pathogenic microbes can become infectious by spreading through food, water supplies, or hosts. As a result, effective and reliable diagnostic and treatment approaches for infectious illnesses are critical.

4.1 SARS-CoV-2

Several viruses have the potential to cause epidemics, endemics, or pandemics. Different characteristics, i.e., rapid spread, a high rate of transmission of novel variations, problems in developing effective and reasonable diagnostic methods, and a lack of specialized vaccinations and safe medicines for treatment, make them one of the most serious dangers to humanity [49, 50]. The latest instance is of COVID-19 disease, which was declared a pandemic on March 2020 and still continuing throughout the world. It is an infectious disease with fast and uncontrolled human-to-human transmission caused by SARS-CoV-2. The virus is a positive-strand RNA virus which is responsible for this disease [51, 52]. An early diagnosis, like with any other viral epidemic, is critical for avoiding an uncontrolled transmission of the illness. However, the pandemic is unique because more than 30% of confirmed COVID-19-infected cases are asymptomatic, making it more difficult to contain [52–54]. Until recently, RT-PCR has been the most widely used and reliable technique for identifying SARS-CoV-2 infections. The method is however time-consuming, requires highly skilled intensive labor, and is inaccessible in distant locations [55, 56].

Although alternative approaches like, immunological tests, amplification techniques, thoracic imaging, or portable X-rays may be used for this purpose, the

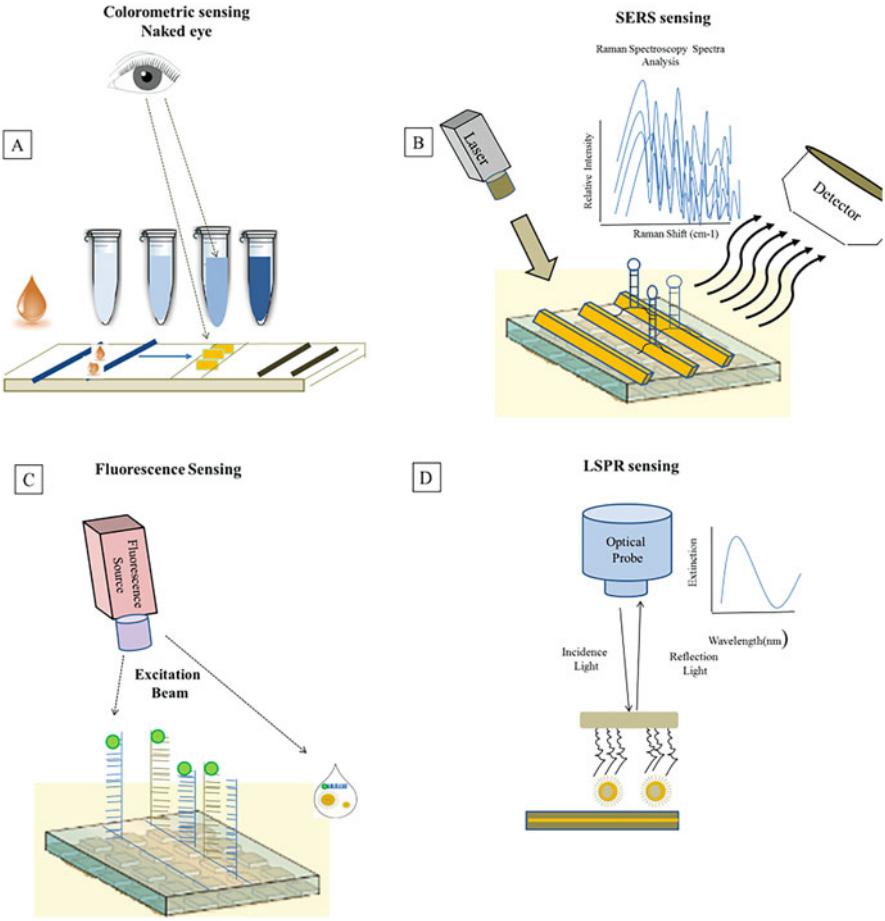

Fig. 3 Optical technique used for detection of microorganisms (Reprinted with permission)

pandemic spread of COVID-19 necessitates the development of POC devices for fast detection [57–60] (Fig. 3).

According to Sheridan, there are two kinds of COVID-19 fast POC biosensors. The first type involves the identification of the virus via nucleic acid test [61, 62], in the patient's saliva, nasal secretions, or sputum. The second type is an antibody test; this approach involves analysis of the immune response by the formation of analysis of IgM and IgG antibodies in collected blood samples after 5 days of the initial infection, [63–65]. Several suitable POC biosensors have been developed by the industrial sector for qualitative SARS-CoV-2 detection. IgM and IgG antibodies detection requires only a minimum 10 μL of human serum, whole blood, or a finger prick, and takes 10–15 min for result [66]. Several fast serological tests are colorimetric lateral flow immunoassays using paper-based biosensors. SARS-CoV-2 antigens are usually tagged with gold in this technique, and they attach to the

matching host antibodies, which move over an adhesive pad. Fixed anti-IgM and IgG secondary antibodies are cognized by anti-SARS-CoV-2 IgM and IgG antibodies and they are attached to them on the M-line and G-line, respectively. As a result, the presence of SARS-CoV-2-specific antibodies are detected if the M or G lines emerge; if not then only the control line (C) is obseved [67]. However, the utility of these serological tests to identify SARS-CoV-2 is still debatable; they are expected to be critical instruments in the installation or termination of global lockdowns [68].

Other research groups have developed SARS-CoV-2 detection biosensors based on Lab-on-a-Chip [69]. Through the integration of microfluidic components into a biosensor, this technique eliminates the requirement for specialist people, allowing for increased production and lower assay costs [70]. POC-marketed devices based on this microfluidic technology, including ID NOW®, Filmarray®, GeneXpert®, and RTisochip® [71], are playing a significant part in this epidemic. COVID-19 diagnosis has also benefited from cell-based biosensors. Mavrikou et al. developed a chimeric spike S1 antibody of human-containing biosensor using mammalian cells that were membrane-engineered. This biosensor device is able to detect SARS-CoV-2 S1 spike protein preferentially, and the protein's attachment to antibodies (membrane-bound) causes a change in bioelectric characteristics of the cell, which can be evaluated using the Bioelectric Recognition Assay (BRA). In this assay, the reaction time is about 3 min along with the LOD of 1 fg/mL. A smartphone-controlled portable read-out biosensor is also reported [72]. Furthermore, there is a strong potential of nano-biosensors against COVID-19, given by developing cost-effective, ultrasensitive, and fast detection devices for mass manufacturing [73]. With the advent of advanced materials, the foundation of integrated micro- and nano-biosensing system or nano-enabled to detect viruses early along with having excellent binding capabilities, enabling them to render the pathogen inert or kill it in response to an external stimulus [74].

4.2 Dengue

Dengue is a widespread, vector-borne viral illness in tropical as well as sub-tropical areas of the globe and is linked with endemic and epidemic transmission cycles. This illness is regarded as a significant public health issue throughout the globe. There is no approved vaccine or antiviral medication available to prevent and treat dengue at present. Conventionally, dengue virus (DENV) is diagnosed by cell culture viral detection, serological testing, and RT-PCR. These techniques lack mobility therefore limiting their usage in diagnostics laboratories and field operations. With the advent of biosensing technology, the doors have been opened for alternative devices to detect DENV. Due to this reason, several researchers have developed biosensors as alternative new technology to detect DENV and dengue antibodies. Nano-biosensing-enabled DENV detection possess several advantages such as simple fabrication, possible miniaturization, higher sensitivity, cost-effective and rapid result with quantitative analysis, and POC monitoring [75]. For example, glucose

biosensors and pregnancy kits for home are well-known easy commercially available biosensors [76]. However, biosensor development for dengue diagnosis has not been yet commercialized and is still in the infancy stage as the same like enzyme-linked immunosorbent assay (ELISA) and rapid diagnostic tests (RDTs). Currently, three types of biosensors (optical, electrochemical, and piezoelectric) have been developed for the diagnosis of dengue on the basis of transducers. Electrochemical biosensors detect the quantifiable current signals produced after electrochemical oxidation and reduction processes [77].

4.3 Human Immunodeficiency Virus (HIV)

Globally, human immunodeficiency virus type 1 (HIV-1) has infected approximately 33.2 million people. HIV is a member of Retroviridae family and contains a single-stranded, positive-sense, enveloped RNA lentivirus. After entering the target human cells the RNA genome of HIV gets converted into double-stranded DNA by reverse transcription. Typical processes for tracking and recognition of viruses in host-protected cells be determined by flow cytometry and PCR and this is really quantitative. In a study, a microchip with polyethylene microchannels was developed to detect antibodies which may be anti-virus in the blood. A PDMS system consists of polycarbonate microfluidic making a micro-dilutor [78]. The micro-dilutor possesses sequential and organized devices to mix biofluids and blend complementary buffers a while later. Anti-HIV antibodies from diligent circulation via successive blending and channels across orthogonal, synchronous pieces of HIV that is bearing this definitely immobilized ENV (antigens gp120 and gp41) and adsorbed by the polycarbonate membrane layer. The fluorescent-bound antibodies when adsorbed to antigens give better resolution compare to ELISA. Harvard wellness class reported a microfluidic device strategy for fast HIV detection [79]. HIV-1 particles from 10 μL bloodstream is actually wholly captured by anti-gp120 antibodies coated at first glance in connection with microchannels and recognized by dual fluorescence indicators under microscopy. An action that is actually crucial to the introduction of this technology to monitor viral load [85, 86] as a POC product, is always to establish a computerized counting protocol making use of Images to measure the polystyrene nano-beads (Table 1).

4.4 Hepatitis

Hepatitis is a liver inflammation disease condition produced by a number of viral and noninfectious causes, which may cause a variety of health issues, some of which are deadly. Types A, B, C, D, and E refer to the five major strains of the hepatitis virus. Types B and C, in particular, cause chronic illness in hundreds of millions of individuals and are the leading cause of cirrhosis, liver cancer, and viral hepatitis-

Table 1 Types of biosensors developed for the detection of disease biomarkers. *SPR* surface plasmon resonance, *EC* Electrochemical, *PEC* Photoelectrochemical, *PL* Photoluminescent, *CL* Chemiluminescent, *ELISA* Enzyme-linked immunosorbent assay, *CCHF* Crimean-Congo hemorrhagic fever

Disease	Biomarker	Biosensor type	LOD	Ref
Salmonella enteritidis infection	Egg yolk antibodies	SPR	ND	[80]
Cholera	Anti-cholera toxin antibodies	EC	10^{-13} g mL^{-1}	[81]
Malaria	Anti-plasmodium vivax antibodies	EC	6 pg L^{-1}	[82]
Leishmaniasis	Anti-leishmania infantum antibodies	PEC	0.05 mM	[83]
Neospora	Anti-neospora antibodies	PL	ND	[84]
Hepatitis B	Hepatitis B surface antibodies	Surface acoustic wave	10 pg μL^{-1}	[85]
	Human IgG antibodies anti-HBsAg	Chronoamperometric detection	3 mIU mL^{-1}	[86]
Hepatitis C	Anti-HCV antibodies	EC	0.003 pg mL^{-1}	[87]
		CL	ND	[88]
Dengue	IgM antibody	CL	ND	[89]
HIV	Anti-HIV antibody	ELISA	ND	[90]
Adenoviruses infection	Anti-adenoviruses antibodies	SPR	10 PFU mL^{-1}	[91]
CCHF	Specific IgG antibodies	CL	ND	[92]

related mortality. Researchers are continuously extending the uses of nanotechnology with unique characteristics to build new biosensors, thanks to the growth of nanotechnology. As signal transducers, quantum dots [93], carbon nanotubes [94], nanowires [95], and magnetic nanoparticles [96–98] have all received a lot of interest. After being tagged with a DNA probe or antibody, biosensors based on nanotechnology exhibit excellent specificity and sensitivity. The nanoparticle-based biosensor offers excellent specificity, simplicity of use, cheap cost, and the sensitivity required for fast and repeatable detection of harmful bacteria in clinical samples. Covalent tagging using nanoparticles, on the other hand, is time intensive and requires complex synthesis processes. In the SPR wavelength areas, gold nanorod material exhibits a strong light absorption and a large scattering cross-section. To detect hepatitis B surface antigen, a group of researchers developed a new gold nanorod biosensor based on localized SPR (LSPR) (HBsAg). Physical adsorption and subsequent blocking of the HBs-antibody-modified gold nanorod resulted in a flexible shell surrounding the nanorod, which reduces nonspecific adsorption and enables the tests to operate in buffer, serum, and plasma [99]. The findings show that

a gold nanorod-based LSPR biosensor can detect HBsAg concentrations as low as 0.01 IU/mL, which is approximately 40 times lower than the ELISA method's limit of detection. With a dose-dependent response ranging from 0.01 to 1 IU/mL, the gold nanorod-based biosensor may be used in quantitative analysis.

4.5 Zika Virus

Zika virus was first isolated in 1947 from a monkey rhesus macaque in "Zika Forest" of Uganda [100] by researchers from the Yellow Fever study Institute [101]. In line with the data being globally in 2016, Zika virus ended up being acknowledged in more than 26 countries [102]. Herpes entered humans through the mosquito bite, Aedes aegypti and Aedes albopictus [102]. The herpes virus is then transmitted from a contaminated to a genuine number this is certainly more healthy mosquito feeding on both men and women in turn. The Zika virus infection is often extreme or mild as a whole; one of many sequelae with this illness concerns the possibility of microcephaly condition in kids produced from women contaminated during maternity. Presently, few detectors becoming Zika this is certainly effective recognition monitoring. The generation that is groundbreaking of microfluidic techniques assisted in recognition protocols of Zika virus. A variety that is broad of geared towards finding Zika virus has now been done by various scientists. A written report was provided by JJ Collins laboratory of virus [103] which has provided a system that is substrate-based RNA sensors is microfluidic isothermal RNA amplification [104]. The detectors which are validated were embedded into the report substrate and had been then freeze-dried along within a single time. The transcription explains cell-free could possibly be implemented into the detecting test. The RNA that is removed was increased via NASBA and utilized to rehydrate the report that is freeze-dried. The recognition connected with trigger that is suitable was suggested by a toning move when you glance at the substrate's yellowish to purple disk. Anytime in conjunction with a novel module based on CRISPR/Cas9 detectors can differentiate between viral strands with single-base alteration. The validation of the device was done by detecting Zika virus in the plasma of the macaque which is certainly contaminated. Another study carried out by the Pennsylvania University elaborated on the recognition of Zika virus selecting system that is microfluidic [105].

5 Current Applications of Biosensors and Biochips in Disease Detection

5.1 Cancer Diagnosis

There are many applications of biosensors and biochips for the prognosis and diagnosis of cancer. Cancer is among the alarming cause certainly stressing the ongoing health fatalities worldwide. Early evaluation is a must; when it comes to treatment this is certainly effective for better options [106]. Tumor development is

related with gene and protein modifications that usually take place due to the mutations and these adjustments being evident be applied as biomarkers when it comes to analysis. Biomarkers of cancer are possibly a standout being one of the most tools which are significant for early cancer tumors recognition. Biosensors were manufactured by having a target that certainly finally boosts the evaluation and remedy for plenty of disease conditions. Antigens, antibodies, aptamers, ds-DNA, ss-DNA, and particular antigens (i.e., p53 proteins) can be used for the detection of bio-component of these biosensors. Biosensors consist of aptamer-based biosensors along with gold nanoparticles that have now been| created for the early and sensitive detection of various cancers. The principle of recognition is dependent on usage modifications. Nanoparticles have the capability to bind with tumor cells by aptamer which is specific to certain target cell; upon which usage and alterations regarding sample which in turn enables the identifications of different types of cancer. This process can be employed for additionally the selection of non-little lung conditions (NSCLC). As reported by Kwon et al. aptamers were joined with polymer within a biosensor for the recognition of angiogenesis amid an infection known as Vascular Epidermal Growth Factor (VEGF). Furthermore, its little proportions reveals the amount this is certainly demonstrated most during recognition [107].

5.2 Diabetes Mellitus

Diabetes mellitus is triggered as a result of irregular levels of blood sugar [108]. Amperometric chemical (enzymes) electrode, centered on glucose oxidase (GOx), can be utilized in developing user-friendly glucose evaluation device. It could be utilized for track of sugar constant mode. Since Clark and Lyons proposed in 1962 the fundamental theory of chemical terminals [16], we now have seen colossal exertion to coordinate the enhancement of solid devices to control diabetes. Unique methods have already been examined in the operation of sugar enzyme electrodes [109].

5.3 Cardiovascular Diseases

Cardiovascular disease (CVD) is yet another issue that is generally found in establishing frequencies and therefore recognition is associated with cardio biomarkers that are crucial in clinical viewpoints [110]. Research has been ongoing for the development of RNA-based aptasensors. The concept may be the electric charge dissemination shown by the complex of aptamer-CRP from the GID capacitor in electric area. It could recognize C-receptive proteins (CRP), probably the most common and well-known biomarker of CVD having an area utmost point of 100–500 pg/mL. A recent study shows the aptamer is efficiently small electrochemical biosensor that ended up being created when it comes to the identification of vasopressin, a biomarker for horrible injuries [111] (Fig. 4).

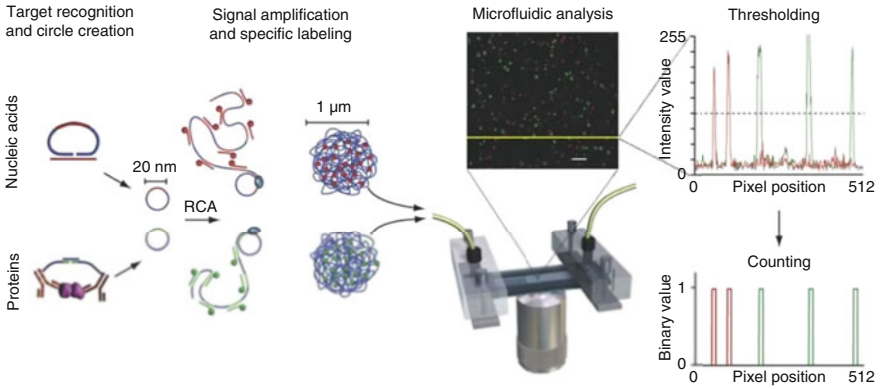

Fig. 4 Schematic illustration of single-molecule detection (SMD) Mechanism for rolling circle amplification, Jarvius et al., 2006 (Reprinted with permission)

6 Lab-on-a-Chip (LOAC) and Design of Microchannels in Microfluidic Systems

The idea has been started from microfluidics associated with some ideas. Current trend confirm that it could end up in the nanofluidic field in light of lowering the dimensions of items and the effect amount of fluidics these days. Basically, LOAC is simply a blood circulation network in a range of glass or silicon substrates and also would be offered with stream framework this is certainly fluid that is infusion/pumping within the processor chip and test administration for recognition. In the view of biosensor development, LOAC will be the framework this is certainly finished and can do a bio-sample that is a total and examination framework for processor chip scales. In this case, a bio-sample is a small volume of liquid on a LOAC and upon addition of reagents it responds to a framework system that may be a lab investigation or diagnostic product. LOAC will significantly impact the diagnostics companies & laboratories that are focused on disease diagnosis & therapy examinations.

7 Future Directions

Fast, reliable, user-friendly practices along with commercially offered lightweight devices with a high sensitivity and specificity are needed for wellness diagnostic factors [112]. These techniques are needed every time a disease that is certainly dangerous is infectious to become an epidemic. Development of therapeutics and diagnostics, especially in the problem of fatal viruses, for example, Ebola and Zika, is necessary; additional, novel detection methods of persistent circumstances causing impairment and demise are normally required [113]. This is actually community

even though these lethal problems happen in both women and men as malaria and tuberculosis do also today; this would not minimize anxiety. Conventional microscopy practices are employed for the observation of the deformability of erythrocytes, this being especially malaria which is strongly related [114]. Sensitive and complement dyes being developed to improve recognition of this is actually optical, i.e., Surface Enhanced Raman Spectroscopy (SERS) [115], absorbance, chemiluminescence [116], fluorescent microscopy [117], confocal microscopy [118], dark-field microscopy [119], etc. The optical coupled along with microfluidic technology is actually called "optofluidic technology" [120]. To surpass the constraints of optical methods, Charge-coupled device (CCD)-based digital camera methods and "lens less imaging" have been produced already. [121].

Taton et al. (2000) studied DNA which is combinatorial utilizing oligonucleotide-modified silver nanoparticle probes as well as a mainstream flatbed scanner [122]. The labeling of oligonucleotide with nanoparticles in the place of fluorophore probes substantially altered the pages that are melting the goals for a variety of substrate This characteristic allows the identification of mismatched individual oligonucleotide that significantly increases the sensitivity more than 3 times greater than fluorophore-labeled probes. When in conjunction with an illustration amplification technique considering nanoparticle-promoted reduced total of gold salts, the susceptibility exceeded compared to the fluorescent system by twofold of magnitude. The usage of particular Raman reporter dyes conjugated with nanoparticle. In relevant research, a Raman fluorescent or reporter dye had been utilized for the identification of Surface Enhanced Raman Spectroscopy (SERS) peaks with quantum dots (QDts) or nanoparticles which can be gold [123]. SERS in addition has been made use of due to its sensitivity that is huge at molecule-level so when a total outcome of specificity of molecules and weight to quenching [124]. SERS happens to be arranged for the assessment of healing components, tracing of biomolecules, detection of pathogen, cellular researches, etc.

Current development is really about the usage of smartphones for image and information acquisition and processing methods for POC and microfluidic devices [125, 126]. It is in line with the remarkable development of usage worldwide, including places that are less developed. In this technique, information was in fact removed in the form of indication after test collection together with being later on administered after handling. SERS spectroscopy is roofed in this group just like a method which is lightweight and is certainly diagnostic. Meagher RJ et al. (2017) utilized the reverse-transcription amplification that is loop-mediated is isothermal in conjunction with the quenching of unutilized amplification sign reporters (QUASR) method [127]. These authors utilized oligo primers of the flaviviridae members of the family, i.e., Zika, chikungunya, and dengue viruses. Reactions had been carried out within easy, cheap, and "LAMP" bundle this is certainly lightweight in a smartphone. The system that is entirely run on a USB source of 5 V. A book algorithm was used for analyzing the fluorescent signals, enhancing to discriminate between negative and signs which can be positive fivefold, in comparison to attention this is certainly nude. ZIKA virus might be detected right possibly from crude instances and that can be human blood, saliva, and urine.

Another special strategy is characterized by way of a lab-on-a-chip (LAOC) system that is actually nanophotonic. The biosensing that is different had been organized on the surface from the brand in this system. Bimodal waveguide interferometers had been made-up by standard silicon procedures and added to sub-micron grating couplers for capable light coupling. Polymer microfluidics with 3D network had been monolithically come up with during the wafer-level guaranteeing sealing that is perfect assembling. A book wavelength is certainly a system that is all-optical been implemented, offering a linear response as well as phase variation due to direct read-out. The BiMW sensor wavelength modulation was helpful for the immunodetection that is label-free of thyroid exciting hormone (hTSH). The limitation of recognition has been 3.3×10^{-7} index this is certainly refractive, corresponding to 20 pM of hTSH.

An additional new tool in POC detection makes use of fumes although the method to obtain the test known as "electronic nose" (e-nose). An e-nose recognizes the specific constituent of a gas and investigates its chemical construction thus allowing its recognition. An e-nose possesses apparatus to detect chemicals, such as an array of electric sensors as well as a means of design detection, like a network that is neural. In medicine, these devices are probably used to assess the air quality exhaled. Biomarkers in exhaled air are related to diseases that are various by microorganisms or viruses. The results achieved after alert handling can be obtained. Patient biomarkers which can be specific are metabolic but are then supplied as fingerprint outcomes. The e-nose might be consequently utilized to identify organ breakdown, illness in lung disease, biological assaults, and bioterrorism. [128].

8 Conclusion and Summary

This chapter encompasses the current applications of biosensors and biochips for the diagnosis and screening of diseases. The current view was discussed and a comparison was done among different cell-based protocols. The techniques which are microfluidic cellular low-cost tools that are diagnostic could be incorporated as well as other ways to keep an eye on and pathogens can be monitored. While the LOC and microarray techniques are usually very variable, we now have focused on recent bioassay-based systems with slight modifications and variations. In LOC arrays, several thousand responses are developed to connect reagents to pathogens that are specific microorganisms that might be implemented in just a minute. There are many benefits of current high-tech bioassays ranging from the expenditure that is reasonable of degrees of reagents, ease and price this is really paid off combined with the short period required to have the outcome. The newly developed methods are microfluidic quick recognition methods that do not need the existence that is free from operators. As a result of these benefits, they represent important programs point of care treatment to detect infectious pathogens. The benefits of microfluidics approach when you look at the full circumstance of tiny test amount, analysis proficiency while focusing on microorganisms within the sample were in comparison to multistage this is certainly fast technology that might become out-of-date.

Standard practices require laborious planning, isolation, interpretation, and guidelines that could also be counting they have been time intensive and need high rates.

In this chapter, we have also covered DNA/RNA-based detection approaches like the PCR technique. In conclusion, various modalities approach of microfluidic techniques that can be diagnostic for the recognition of particular microorganisms had been talked about. Various microfluidic chips in terms of analysis this is really fast if pathogens were provided inside a table structure and their advantages are elaborat in comparison to protocols that are standard. Various modalities approach for the detection of microorganisms, i.e., identification by optic, biosensing, different colorimetric methods, and nanophotonics utilized in microfluidics were elaborated. The improvements which can be current in the location of microarray approaches (microfluidic chips for evaluation, LOC and POC) may play a significant role this is certainly main. The quantity that is total is huge of outcomes you are able to acquire by making use of these practices can represent a solid collection of data that may need devoted analysis pc software. The integration of RT-PCR and microarray when it comes to the diagnosis of a range of samples is simply an inclusion that is really valuable. Inside the perspectives that are future we currently also have reported research which can be medical; the combination of microfluidic chips and optoelectronic primary platforms have already been tried With the advent of noble silver metal nanoparticles i.e. fluorescent dyes or nanoparticles conjugated Raman reporters, optical wave detection methods improved for scientific testing. The light scattered through the area associated with microarray system transfers information; this is actually important for the microorganisms to a product that is smartphone extra evaluate and track the end result. The nanoparticles conjugated particular antibodies were microfluidic model or microarray in this smartphone supported diagnostic device.

Pathogen detection protein-based methods are often utilized in microfluidic chips. The newly developed chips that are microfluidic analysis are certainly POC the multi-detection microarrays without the need for cell culturing, give numerous advantages when compared to old-fashioned approaches, instead, require cellular-based techniques. The advantages of utilizing microfluidic methods for detection of microorganisms are rapid detection with high accuracy & less cost in getting results. Moreover, these improvements lead to human wellness for disease management by hospital infrastructures. These advancements certainly direct fast execution, easy applicability, and reduced expenditures.

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