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Nanomaterials for Advancing the Health Immunosensor

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Abstract

Nanotechnology has exerted a significant impact in the development of biosensors allowing more sensible analytical methods. In health applications, the main challenge of the immunoassay is to reach the suitable limit of detection, recognizing different analytes in complex samples like whole blood, serum, urine, and other biological fluids. Different nanomaterials, including metallic, silica and magnetic nanoparticles, quantum dots, carbon nanotubes, and graphene, have been applied, mainly to improve charge electron transfer, catalytic activity, amount of immobilized biomolecules, low-background current, signal-to-noise ratio that consequently increase the sensitivity of immunosensors. Given the great impact of nanotechnology, this chapter intends to discuss new aspects of nanomaterials relating to immunosensor advancement.

Keywords: Immunosensors, immunoassay, nanosensor, nanomaterial

1. Introduction

A major challenge faced by health programs is the maintenance and availability of diagnostic tests that are required not only in inpatient or outpatient hospital but also for an improved epidemiological survey. In many cases, the absence of laboratory testing or delay of diagnosis generates negative economic impacts, resulting in unnecessary hospitalization, intercurrent,

and in some cases implications on the global life quality of patients and underreporting of surveillance. In this context, the development of practical, fast, and reliable analytical methods is imperative.

Biosensors have been considered one of the more attractive analytical methods. They are biodevices capable of transforming an interaction with specific analytes into an electrical signal by a transducer, including a biorecognition element. [1] Pharmaceutical industries and users of rapid tests from the United States and Europe are unanimous in stating that biosensors, mainly those based on point-of-care testing (POCT), or bedside testings are a practical technology, regarded as a short-, medium-, and long-term trend. Among several advantages, POCT can provide immediate responses (results in few minutes or in real time), samples do not need to be transported to the analytical phase (in situ monitoring) and require generally small volumes of samples, and the users can be skilled or unskilled and present better cost-effective analyses compared with conventional technologies used in clinical diagnostic (user-friendly technology). One of the most widely useful POCT is the glucometer, which measures glucose levels with accuracy by requiring a single drop of blood. The rapid glucose measurement is very important in trials to avoid serious adverse effects stemming from diabetes, including seizures, coma, or even death. Worldwide, some diabetic outpatients have been benefited by POCTs.

Although there is a great promising market dedicated to health for the detection of diseases and therapeutic monitoring, biosensors are not yet entirely broadcast, especially those devoted to nonenzymatic reactions, i.e., biosensors based on the affinity between antigen–antibodies, DNA–RNA, DNA–DNA, etc. So far, there are focused studies to develop affinity biosensors for a wide number of applications. Some of these include environmental, agriculture, veterinary, safety food analysis, and health diagnostic in attempting to detect pesticides in water, in monitoring of environmental pollutants in soil, and in determining contaminants and pathogens in food and many others.[2]

Regarding the health diagnostic, affinity biosensors devoted to immunoserological diagnosis have demonstrated to be more accurate, feasible, practical, and advantageous for POCTs than nucleic acid biosensors. First, the levels of antibodies or antigens circulating in whole blood, serum, or other biological fluids are in higher amounts compared to RNA or DNA sequences. Second, blood samples of immunosensors do not need cell lysis before measurements to release the analytes. Third, antigen or antibody samples do not need pretreatment before measurements as amplification by polymerase chain reaction (PCR) or transcriptase reverse polymerase chain reaction (TR-PCR). Fourth, antibodies are more chemically stable than RNA or DNA sequences that are easily contaminated by attacking the RNases or DNases enzymes present in digital samples.

Due especially to nanotechnology, biosensors dedicated to immunoserological diagnosis have emerged, in the last decade, with the possibility of very promising point-of-care diagnosis. The contribution of nanomaterials has made possible the development of new immobilization matrices with improved features, increased sensor surface area, greater amount of biomolecules per area/volume, and major electrical conductivity, making it possible to achieve a lower limit of detection compared to existing bulk biosensors. Currently, several studies have

highlighted the following nanomaterials: metallic, silica and magnetic nanoparticles, quantum dots (semiconductor nanoparticle), carbon nanotubes, graphene, and nanostructured surface.

Selective monoclonal antibodies, recombinant antigens, fragments, and aptamers associated with the nanomaterial advancements to mediate the antigen–antibody responses have also allowed several nanostructured devices with optical, piezoelectric, and electrochemical improved transductions, besides the integration of microfluidics and portable approaches.

Given the great impact of nanotechnology, this chapter intends to discuss new aspects of nanomaterials concerning to the development of immunosensors that resulted in more accurate, reliable, and practical analytical methods for health.

2. Important aspects for immunosensor development

Immunosensor technology has shown an exponential growth in the number of publications over the last decade (Figure 1(a)). Although, there were significant advances in all the areas mainly in the food analyses, immunosensors devoted to health still have huge challenges to overcome in order to yield commercial uses (Figure 1(b)). Some difficulties can be attributed to the biomolecules specificity, immobilization matrix stability, transduction mode employed and pretreatment of complex samples like whole blood, serum, or other fluidic biologics for a reliable detection.

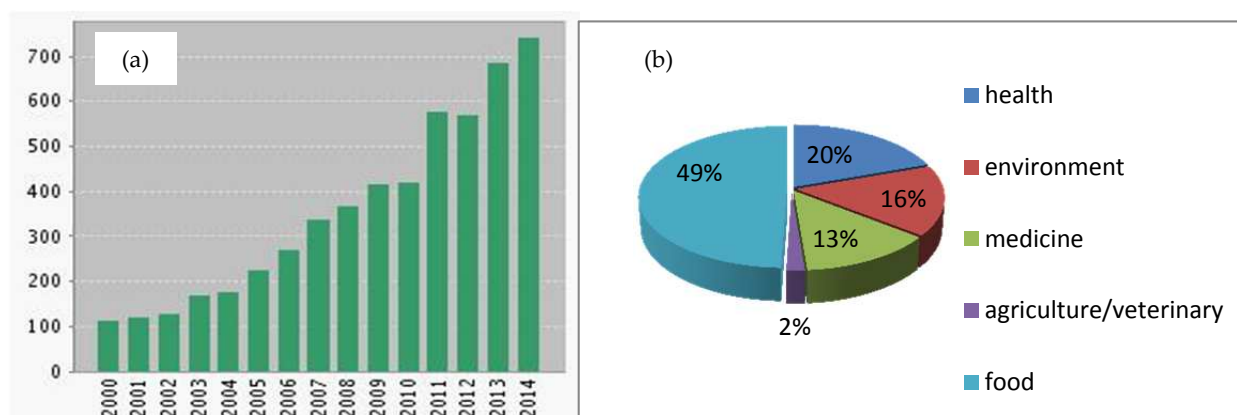


Figure 1. Immunosenors research published over the last decade (a) and main application areas (b). (Extracted from ISI of Knowledge base)

Three aspects are considered crucial in the development of an ideal immunosensor: (a) the bioreceptor, i.e., biomolecule used to recognize the antigen or antibodies in the sample; (b) the matrix assembled for immune compound immobilization; and (c) the transducer type employed.

The choosing of bioreceptors for analyte recognition is a fundamental aspect to ensure an optimal selectivity of immunosensor. Different immunomolecules have been used to detect antigens or antibodies in different samples, besides monoclonal or polyclonal antibodies, and

antigens, recently recombinant antibodies, [3] aptamers, [4] and antibody fragments [5] have been also assayed. Immunoglobulin classes IgG and IgM are the most commonly employed in immunosensors. IgG is a Y-shaped structure with two binding sites for antigens recognizing (two paratopes), with approximately 150 kDa. Meanwhile, IgM immunoglobulins are pentamers comprising of ten antigen sites, called natural antibodies. However, due to IgG being more prevalent and most abundant in the circulation (73%), this immunoglobulin is more frequently used in all immunoassays. IgM immunoglobulins are detected in specific assays when is important to identify diseases in their acute Phase, Kidwai et al. [6] developed a rapid immunochromatographic (ICT) assay detection for IgM and IgG detection in serum.

Immunosensor performance is directly dependent on the immobilization matrix used and orientation and density of affinity biomolecules (antibodies and antigens) on the electrode surfaces. There are different strategies used to immobilize the recognition element, either directly on the electrode surface or on other solid supports. [7] Conventionally, there are noncovalent and covalent techniques employed to immobilize antibodies, which are based on adsorption, encapsulation, and entrapment in polymers, covalent binding, and cross-linking of antibodies aggregates (Figure 2). Developments in these techniques have great interest and potential application in other areas of biotechnology, including purification of proteins, [8] medicine and drug delivery, [9] regenerative medicine, tissue engineering, and many other applications. [10]

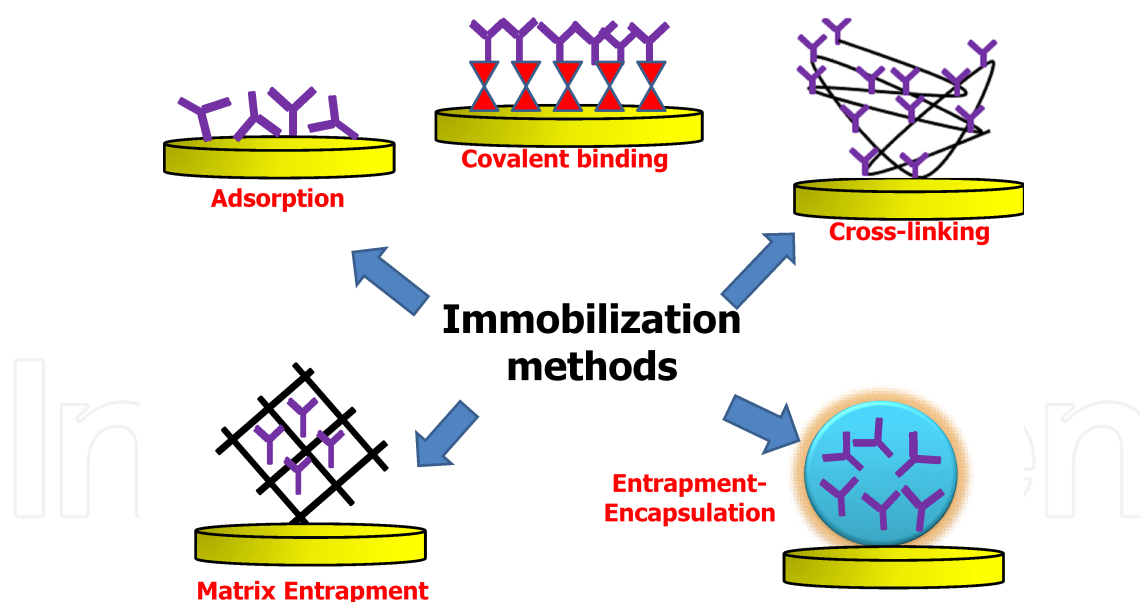


Figure 2. Illustration of different methods of antibodies immobilization.

Although sensor surfaces prepared with antibodies immobilized in a random manner yield satisfactory results, the site-directed immobilization of the sensing molecules significantly improves the immunosensor sensitivity. [11] In this sense, antibodies should be immobilized with optimal capability to recognize the antigens, while fully maintaining their preserved structures. The Fab region needs to be sufficiently free in order to be exposed to the medium,

i.e., epitopes of antigens. The best approach is to immobilize antibodies by their Fc regions. [12] This configuration has been achieved using different strategies, including by the use of protein A [13] or protein G, [14] via covalent immobilization through the oxidized sugar chains of the antibody, [15] and others. Besides orientation, it's important to consider antibody density on the electrode surface. A higher density increases the sensor response, however is likely to increase the steric hindrance on planar substrates causing a low immobilization efficiency and low assay sensitivity. To solve these problems, researchers are focused on modifying the substrates for forming the 3D network, which ensures high percentage availability of antibody binding sites. Nanomaterials contribute to increase the amount of protein immobilization because of their capability to form 3D nanostructured surfaces with innumerable cavities and valleys.

Choosing the transducer is another important and fundamental aspect to achieve the sensitivity and response time desired. Bioaffinity sensors (immunosensor) have been explored by using different transduction modes: optical, acoustic and electrochemical by using different approaches. Surface plasmon resonance, [16] localized surface plasmon resonance [17] and fluorescence resonance energy transfer (FRET) [18] are examples of optical transducers. Quartz crystal microbalance (QCM), also entitled mass-sensitive, is the most explored as acoustic transducer. Electrochemical transducers are comprised of different ways to generate an electrical signal, for instance, by amperometric, impedimetric, potentiometric and capacitive changes. [19] Regarding the response time, two classes of immunosensor operation mode are distinguished by, a) Label-free or nonlabeled immunosensors that readily convert the species interaction response with the complementary species into an electrical signal, denominated as direct transduction, and b) Labeled immunosensors that need a second antibody or antigen conjugated to chemical species to generate the analytical response, such as enzymes, fluorescent labels, etc. [20] Although labeled immunosensors are more time consuming than label-free immunosensors, they provide more specificity due to the second antibody which minimize the nonspecific binding negative effects.

The design of label-free affinity biosensors has been extensively studied in academy and industry. One source of stimulation is the demand of POCTs for health, which requires rapid response, lower cost-effective analyses and simplicity for potential analysis. The main technologies of label-free immunosensors currently in use or under development are: surface plasmon resonance (SPR) devices, mass-sensitive, field effect transistor (FET), and electrochemical sensors, including impedimetric and capacitive. Recently, due to advances of nanomaterial based-immunosensors, new categories of label-free amperometric sensors using screen printed electrodes have been successfully developed. [21, 22, 23, 24]

3. Immunosensor based on nanomaterials

Nanomaterial is composed of unique functional materials that display incomparable characteristics related to their shape, structure and size (in the order of 1 to 100 nm). Nanostructured materials are interesting because they can bridge the gap between the bulk and molecular

levels and lead to entirely new avenues for applications, especially in electronics, optoelectronics and biology. The contribution of nanomaterials has allowed powerful immunosensor assemblies, creating platforms with increasing detection limit. [25]

In recent years, various nanomaterials with different physical and chemical properties have been applied to achieve the immobilization of immunocompounds. They can modify the sensing surface, improving the immobilization of procedures and transduction properties of immunosensors. A great number of electrochemical advantages have been mentioned, such as possessing low-background current, high signal-to-noise ratio, and fast electron transfer, including an increased amount of immobilized biomolecules, with consequent increase on the sensitivity of sensors. Nanomaterials with zero-dimensional space (metallic, silica, and magnetic nanoparticles and quantum dots or semiconductor nanoparticles), one-dimensional space (carbon nanotubes), and two-dimensional space (graphene) have been shown as potential for different transducers in many immunosensor applications.

3.1. Metallic, silica, and magnetic nanoparticles

Nanoparticles (NPs) obtained from commercial sources or properly produced in laboratories have attracted much attention in biological studies due to their low toxicity, biocompatibility, and unique optical properties. Nanoparticles and nanospheres can be divided into magnetic, metallic, semiconducting, or insulating nanoparticles based on their conductivity.

NPs have high surface areas and unique physical–chemical properties that can be easily tuned, making them ideal candidates for developing immunosensors devices. The basic function of nanoparticles in an immunosensor can be summarized as follows: immobilizing the biomolecules on the electrode surface, catalyzing electrochemical reaction, enhancing electron transfer charge, and acting as a reactant or labeling biomolecules for further experiments, among others. [26]

Biological tests measuring the presence or activity of selected analytes become quicker, more sensitive, and flexible when nanoscale particles are combined, with numerous advantages over more traditional procedures. In recent years, various nanomaterials with distinct physical and chemical properties have been applied to improve the immobilization of immunocompounds. [27] These have many electrochemical advantages, such as possessing low-background current, high signal-to-noise ratio, and fast electron transfer, besides increased amount of immobilized biomolecules, with consequent increase on the sensitivity of sensors. [28]

Surface modification using nanoparticles composites have shown an increase of sensitivity and help adsorb a large amount of antibodies on electrode surface. Lu et al. [29] constructed an immunosensor based on a nanocomposite formed with CeO_2 and gold nanoparticles on the Au electrode via cysteine to detect a cardiac marker, the myeloperoxidase. [29] Thereat, the nanoparticles enhanced the active surface area available for antibody binding. The high stability of this sensor was attributed to the good biocompatibility of the composite. Another study shows an increase in immunosensor response. Fe_2O_3 nanoparticles were used in the construction of an electrochemical device to detect cancer biomarker prostate antigen (PSA) via horseradish peroxidase (HRP) signal. The high amount of nitrodopamine (film coated on nanomaterial to

immobilize the anti-PSA) anchored onto Fe_3O_4 increased the loading of biomolecules onto the surface, which increased the electrochemical immunosensor sensitivity. [30]

The carcinoembryonic antigen (CEA) is a protein used as a tumor marker and has been frequently investigated in immunoreactions. An elevated CEA level in serum may be an early indication of lung cancer, ovarian carcinoma, colon cancer, breast cancer, and cystadenocarcinoma. Recently, an interesting work was reported involving this protein investigation using an immunosensor constructed by Pt hollow nanospheres modified with anti-CEA as label for a 3D Au-TiO₂ hybrid platform. [31] The immunoassay exhibited a high sensitivity and a low detection limit compared with conventional label methods. Another way to detect CEA antigen was developed by Gao et al. [32] using a label-free voltammetric sensor with chitosan and gold nanoparticles (AuNPs) to immobilize anti-CEA on carbon surface. The detection is based on the variation of current responses before and after the immunoreaction. When the immobilized antibodies have bounded with antigens, the antigen-antibody complex formed on the surface inhibited the electron transfer. Then a decrease of the electrochemical signal was verified as the concentration of antigen on surface increased. Another method also using a composite of chitosan and AuNPs for CEA determination, but with multiwalled carbon nanotubes, was described by Huang et al. [33] The nanocomposite film exhibited high current response intensity, good biocompatibility, and high stability. Similar CEA detection was also performed using a gold nanoparticle-thionine-reduced graphene oxide composite that possesses as advantage fast electron transfer kinetics and large specific surface area. [34] Another work for CEA analysis described the use of silver nanoparticles on SiO₂ surfaces. [35] The high stability of the immunosensor was assigned to the stable nanocomposite produced.

The sensitivity of electrochemical immunosensors can also be improved by using the association of AuNPs and dendrimers that are three-dimensional macromolecules, with hundreds of functional groups at the periphery, for surface modification. This architecture was employed by An et al. [36] to detect α -synuclein, a very important neuron protein. The dendrimer (PAMAM)-encapsulated AuNPs were covalently bound on the poly-o-aminobenzoic acid (ABA) electropolymerized on a glassy carbon electrode surface to achieve abundant carboxyl groups, which allowed a highly dense antigen immobilization and facilitated the improvement of electrochemical responses as well. Subsequently, the enhanced gold nanoparticle labels were fabricated by immobilizing a horseradish peroxidase secondary antibody (HRP-Ab₂) on the AuNPs surface. After an immunoassay process, the labels were introduced onto the electrode surface to produce an electrocatalytic response with hydrogen peroxide. The presence of dendrimer Au not only increased the covalent coupling of more protein but also accelerated electron transfer when compared to immunosensor without dual signal amplification strategy.

The picogram detection limit of estradiol was achieved using an immunosensor constructed with AuNPs and protein G scaffold to modify a gold electrode. [37] Coupled with the amperometric determination of the hormone in a flow system, the device exhibited superior linear range, sensitivity, and stability in blood serum samples spiked with estradiol.

Other applications for metallic nanoparticle have included optical transduction. Krishnan constructed an optical immunosensor in a quartz glass surface for the detection of *Escherichia*

coli, using core-shell nanoparticles (silver-silica) anchoring labeled antibodies. The results show that changes in photoluminescent standards are consistent with the immobilization of various species. Thus, the optical immunosensor demonstrated improved sensitivity and specificity in comparison to the usual methods, detecting as low as 5 CFU/mL.

Using a great number of luminescence molecules as stabilizers coated on the surface of the AuNPs, Shen et al. [40] developed an electrochemiluminescence immunosensor to detect human cardiac troponin, an important acute myocardial infarction biomarker. First, the sensor was constructed by using streptavidin-coated gold nanoparticles as the immobilization matrix for biotinylated antibody. Meanwhile, the three-dimensional nanostructures increased the surface-to-volume ratio, allowing more biomolecules to be immobilized. The sandwich-type immunosensor was fabricated by reacting with antigen and AuNPs modified with luminescence molecules labeled with the secondary antibody, forming a nanoprobe. The enhanced sensitivity of the proposed apparatus mainly derives from the novel nanoprobe, which achieves a large amount of luminescence molecules loading toward each sandwich immunological reaction event.

Another strategy in immunocomplex detection involves the use of magnetic nanoparticles as solid support for biomolecule immobilization. The magnetic particles offer the convenience of magnetic separation. These particles respond to a magnetic field but demagnetize completely when the field is removed. Thus, the nanoparticles can easily be separated from the liquid phase with a small magnet but can be dispersed again immediately after the magnet is removed. The use of magnetic nanoparticles as solid phase for the immunosensor development improves the bioreaction performance due to surface area increase and has better immunoassay kinetics because the particles are in suspension and the target species does not need to diffuse very far. [41]

An interesting work was described by Shen et al., [42] who developed a device to detect *E. coli*, an intestinal pathogenic bacterium, using a quartz crystal microbalance (QCM) immunosensor based on beacon magnetic nanoparticles. A polyclonal antibody was immobilized on iron nanoparticles with subsequent addition of *E. coli*. AuNPs were inserted in the system to amplify the signal. Weakly bound biomolecules were removed with a magnetic plate. Finally, the crystal was modified with protein A and monoclonal antibody. The frequency shift of the QCM immunosensor is amplified using *E. coli* immobilized on to magnetic particles and enlarged gold particles for the bacterium detection. The signal was amplified three times, and the crystal was regenerated without difficulty and could be used at least 10 times. In a recent work, the use of magnetic nanoparticles as an amplification means for QCM signal for avian influenza H5N1 virus detection has been reported. [43] Polyclonal antibodies against the virus were immobilized on the gold surface of the crystal through self-assembled monolayer (SAM). Target H5N1 viruses were then captured by the immobilized antibodies, resulting in a change in the frequency. Magnetic nanoparticles coated with anti-H5 antibodies were used for further amplification of the binding reaction between antibody and antigen (virus).

AuNPs have a remarkably high extinction coefficient and strong distance-dependent optical properties. Different aggregation states of AuNPs correspond to distinctive color, which can be appreciably discerned with the naked eye and be used in immunoassay. Based on this, Yuan

et al. [44] developed a label-free colorimetric immunoanalysis for the simple detection of neurogenin3, a marker for pancreatic endocrine precursor cells, using glutathione functionalized gold nanoparticles. The antibody-conjugated AuNPs were formed through electrostatic interaction upon the addition of the antibody to the modified AuNPs solution. The antigen positively charged to the negatively charged AuNP antibody will minimize the electrostatic repulsion between nanoparticles by neutralizing the surface charge and then agglomeration is induced by an increasing NaCl salt concentration, noticeably revealed by the color change of the solution from red to purple or blue. The concentration of neurogenin3 can be conveniently accessed by the optical absorption spectra. Another important property of the AuNPs is that they could catalyze silver reduction and act as the nuclei for silver precipitation. [45] In this interesting work, the core mechanism of the method to quantify cardiac troponin is that the catalytic capability of the AuNPs was inhibited by immunocompounds covering their surface. This covering is influenced by the amount of reduced silver of the reaction, resulting in a color difference.

In state-of-the-art improved sensor devices for health applications, the possibility of assembling nanoparticles and biomolecules in different ways by using different sizes, formats, and compound types allow more sensitive, simple, robust, and especially faster analysis.

3.2. Quantum dots

Quantum dots (QDs) represent one class of nanostructured materials. They are spherical nanocrystals of semiconductor, 1–10 nm in diameters, made of elements of the IIB–VIA or IIIA–VA groups. The use of QD properties requires sufficient control during their synthesis because their intrinsic properties are determined by different factors, such as size, shape, defect, impurities, and crystallinity. [46, 47] Two of the most widely used commercial QDs come with a core of CdSe or CdTe and a shell of ZnS and emissions from 405 to 805 nm. [48, 49] The shell stabilizes the structure, helping to overcome quenching compared to a QD made only from a core and provides a large surface area available for further modification.

Analogous dimensions of QD and biological materials, such as enzymes, antigens/antibodies, protein receptors, or nucleic acids, show great promise as photonic labels for bioanalytical applications and suggest that electronic communication between the QD and the specific recognition site or biocatalytic processes of the biomaterials can exist. These electronic interactions may lead to the optical or photoelectrochemical transduction of the biological events. [47, 50]

Generally, monodispersed QDs are developed by introducing organic molecules that adsorb on the surface and act as capping agents. The efficacy of QD in a biological application is critically dependent on coating properties. The liabilities of these initial methods require the continued development of QD coatings. Important criteria for an ideal QD coating include high-affinity for the QD surface, long-term colloidal stability across a broad range of pH and ionic strengths capacity for bioconjugation, minimization of hydrodynamic size, and biocompatibility with nonspecific binding. [51] However, the selection of organic ligands that bond with surface atoms of the QD is a very delicate issue. In general, phosphenes or mercaptans (-SH) are the most widely used ligands. [52]

In order to make QD suitable for biological imaging and use in a biological environment, they also have to be rendered water soluble. This is done by capping the shell with a polymer layer that contains a hydrophobic segment facing inward, the shell, and a hydrophilic segment facing outward. The hydrophilic layer can be modified to include functional groups such as COOH and -NH_2 groups for further conjugation to proteins and antibodies or oligonucleotides. [49, 53] The single-step synthesis of thiolated cyclodextrin-modified CdSe/CdS core-shell QD resulted in a water-soluble QD, keeping the luminescence properties of the QD in aqueous systems. This is an important aspect since biorecognition events require aqueous environments for reaction. [54]

Thiol ligands and amphiphilic polymers are the most common types of QD coating available. They allow two essential design elements: a moiety that anchors to the QD surface and a hydrophilic functionality for aqueous dispersion. The selection of these groups determines the degree to which a QD coating can approach the ligand/amphiphilic polymer structures. [51] For example, small molecules with thiol groups can bind to the quantum dot surface, and distal carboxylated group provides aqueous colloidal stability. [55] Another strategy for QD coating that provides aqueous dispersion, improves the biocompatibility, and minimizes nonspecific binding was developed by Mattoussi et al. (2000). They combined dihydrolipoic acid, a dithiol ligand that binds the QD more closely, which is attached to a poly(ethylene glycol) oligomer.

Biomolecule conjugation on to the QD is achieved by different ways like electrostatic binding, noncovalent biotin–streptavidin bonding, or covalent bonding. The most widely used conjugation technique of all is the covalent bond formation between the QD surface and the biomolecules. Surface modifications on QD allow easier covalent bond formation. In one of the most widely used methods, amine-terminated QDs are used for conjugating antibodies. The amine-terminated QDs are activated with maleimide containing a cross-linker molecule, which can then be conjugated to a fragment or whole antibody molecule. Some of the most commonly employed QD conjugation methods are based on cross-linking reactions between amine and sulfhydryl groups, carboxylic acid, and amine and aldehyde and hydrazide groups. The carboxylic-amine bond has one advantage over all other methods, seeing as this method does not require any antibody modification before QD conjugation. In the case of amine and sulfhydryl bond formation, the antibody should be reduced to expose their interchain -SH bonds. In relation to aldehyde and hydrazide bonds, carbohydrate groups on the antibody Fc portion are oxidized. These modifications on antibodies may affect their performance to a certain extent. [56]

Functionalized semiconductor quantum dots have been used as fluorescence labels in numerous biorecognition events. For example, Liu et al. (2004) developed an immunosensor with simultaneous measurements of four proteins based on antibodies linked to the inorganic nanocrystal. Stripping voltammetric immunoassay was used to observe the response of a mixture containing microglobulin, IgG, bovine serum albumin, and C-reactive protein connected with ZnS-, CdS-, PbS-, and CuS-labeled antibodies, respectively. The system was obtained by using carbamate linkage for conjugating the hydroxyl-terminated nanocrystal with the secondary antibody. [57]

Li et al. (2011) [58] used a novel strategy to modify the surface of graphene quantum dots composites. A layer-by-layer assembling process was employed via electrostatic interactions

between negatively charged thioglycolic acid modified CdSe QD and positively charged graphene, which was noncovalently functionalized with poly (diallyldimethylammonium chloride) (PDDA) via an exfoliation in situ reduction of graphene oxide in the presence of PDDA. This process allowed excellent conductivity, extraordinary electron transport properties, and large specific surface area, which resulted in high electroluminescence (ECL) intensity and excellent film-forming ability and made it a promising candidate for the development of ECL immunosensors.

Luminescent quantum dots are viable optical markers and have been used in a direct assay for IgG. Protein A was labeled with CdSe/Zn QD (λ_{max} of 655 nm) and then was immobilized at the tip of an optical fiber. Once the immunoreaction with IgG occurs, a decrease in fluorescence intensity is observed as a result of the fluorescence resonance energy transfer from the QD to the bound protein. [59] Lingerfelt et al. [60] reported the preparation of QD-biotin conjugates and their use in immunochromatographic assays. The detection of immunoglobulin G was carried out on a glass chip through a sandwich assay approach using a secondary antibody conjugated to the QD. [61] A sandwich immunoassay for the detection of staphylococcal enterotoxin B was run using polyclonal sheep anti-staphylococcal enterotoxin B antibody conjugated with QD and microtiter plates coated with monoclonal staphylococcal enterotoxin B antibody. [62]

Kerman et al. (2007) applied conjugated QD streptavidin in a model immunoassay system for the detection of a total prostate-specific antigen cancer marker from the spiked and undiluted serum samples. Immunorecognition was carried out on a carbon substrate using a sandwich assay approach. After the recognition event, the substrate was exposed to the biotinylated secondary antibodies and, subsequently, fluorescence imaging of the substrate surface illuminated the QD. [63]

QDs based on narrow photoemission spectra, with high resistance to photobleaching and broad excitation spectra, are widely used as tags in immunoassay. A carcinoembryonic antigen immunosensor was fabricated using biofunctionalized QD probes. This immunosensor array was designed to detect a wide range of analytes using the inherent characteristics of QD and the flexibility of engineered elastin-like polypeptides. [64]

There are some studies based on thioalkyl-functionalized QD, which are pH sensitive, [65] suggesting many different biological applications. In this context, mercaptoacetic acid-CdSe/ZnSe/ZnS QDs have been used as an intracellular pH sensor by observing a quenching of fluorescent QDs in acidic pH. [66]

Another approach was based on the direct conjugation of CdSe/ZnS QD-IgG complexes using a genetically engineered tripartite fusion protein. This fusion protein was made up of a histidine tag for QD conjugation, an elastin-like peptide for stimuli-responsive purification and the protein L (cell-wall component of *Peptostreptococcus magnus*) that has high affinity to IgG. The functionality of this sensitive immunofluorescent probe was demonstrated in the detection of a representative tumor antigen. [64]

Despite recent progress, more work still needs to be done to achieve reproducible and robust surface functionalization and develop flexible bioconjugation techniques. The potential of QD in biology has just begun.

3.3. Carbon nanotubes

Carbon nanotubes (CNTs) can be highlighted as the most important nanomaterials for biosensors. Their excellent optical and mechanical conductivity, high surface-to-volume ratio, good chemical stability, biocompatibility, and easy functionality have revolutionized the biosensors area for the last decade.

Since their discovery by Iijima in 1991, CNTs are being used in large volumes for different purposes in many industrial areas, i.e., in nanocomposites for sporting materials, as a battery in supercapacitors, transparent films, and liquid crystal displays. Other limited-volume carbon applications include their use as components in wind turbine blades, scanning probe tips, membrane filters and sorbents, flat panel displays, memory devices, transistors, drug delivery systems, and other medical and analytical chemistry applications. [67]

Carbon nanotubes can be described as hollow cylindrical tubes of graphene sheets with high aspect ratios (length/diameter). [68] The structure of graphene is a planar atomic sheet consisting of covalently bonded carbon atoms. The atoms in graphene are sp^2 carbon units, forming a two-dimensional (2D) network with a hexagonal lattice. A graphene layer wrapped as a cylinder forms a single-walled carbon nanotube (SWNT). A multiwalled carbon nanotube (MWNT) is nothing more than multiple SWNTs packed in a tight concentric frame. All the carbon nanotubes have several nanometers in diameter and many microns in length. SWNTs have the smallest diameter (0.8–5 nm), whereas MWNTs have a larger diameter (~ 3 to >100 nm), both variable in length (from millimeters to tens of nanometers). The proper architecture is reflected in the highly anisotropic properties. Most of the extraordinary electrical, thermal, and mechanical characteristics are localized specifically along the axial direction. The strong sp^2 bonding between the carbon atoms in CNTs yields remarkable mechanical strength, making them one of the most resilient materials. Moreover, it is known that an SWNT presents metallic and semiconducting properties where such electronic features depend on its chirality. [69] They have three different structures: armchair, zigzag, and chiral. [70]

The applications of CNTs in biosensors have been hindered for a long time due to the drawback of insolubility. CNTs present a high molecular weight, an ability to entangle (tendency to individually interact with each other through van der Waals forces), aggregating into bundles and ropes. However, these bundles can be quite large that they become insoluble in any solvent; thereby, it can be difficult to disperse them in either aqueous or nonaqueous medium. [71]

Ultrasonication is an effective method to disperse CNTs in liquids that have low viscosity, such as water, acetone, and ethanol. However, most polymers are either in a solid or viscous liquid state, which require the polymer to be dissolved or diluted using a solvent to reduce the viscosity before dispersion of CNTs. [72] The simplest stable dispersions have been achieved by using a solvent able to efficiently interact with CNTs, such as phenylethyl alcohol, *N*-methylpyrrolidone (NMP), *N,N*-dimethylformamide (DMF), and *N,N*-diethylacetamide (DEA). An additional strategy to favor dispersion in organic solvents is to coat CNTs with a molecule characterized by a high affinity toward nanotube sidewalls and at the same time soluble in the selected solvent. Both small molecules and polymers formed by repetitive units

of alkyl chains and aromatic compounds have been used as dispersants. Thus, the adsorption of different polycyclic aromatic hydrocarbons such as pyrene, anthracene, tetracene, and phenanthrene on SWCNTs has been extensively investigated. In order to favor the dispersion of CNTs in water, the widely and most used approach is the adsorption of surfactants. These small molecules typically have a hydrophobic tail and a hydrophilic head group—the former is intended to favor adsorption onto the hydrophobic carbon nanotube and the latter to promote affinity with the aqueous solvent. Over the years, stable aqueous CNT dispersions were obtained with differently charged and nonionic surfactants such as sodium dodecylbenzene sulfonate (SDBS), cetyltrimethylammonium p-toluenesulfonate (CTAT), cetyltrimethylammonium bromide (CTAB), and sodium cholate (SC) enhanced by sonication. Additionally, polymers have been employed for CNT dispersion in water. The majority of polymers and block copolymers have been used to wrap CNT by exposing their polar domains toward the aqueous environments while favoring the contact of their hydrophobic domains with the nanotube surface. [73]

Strategies for the immobilization of biomolecules on CNTs have been widely explored aiming to improve sensitivity on an immunosensor. The high aspect-ratio of CNT allows a great amount of anchored biomolecules by noncovalent and covalent functionalization for different types of transduction (Figure 3).

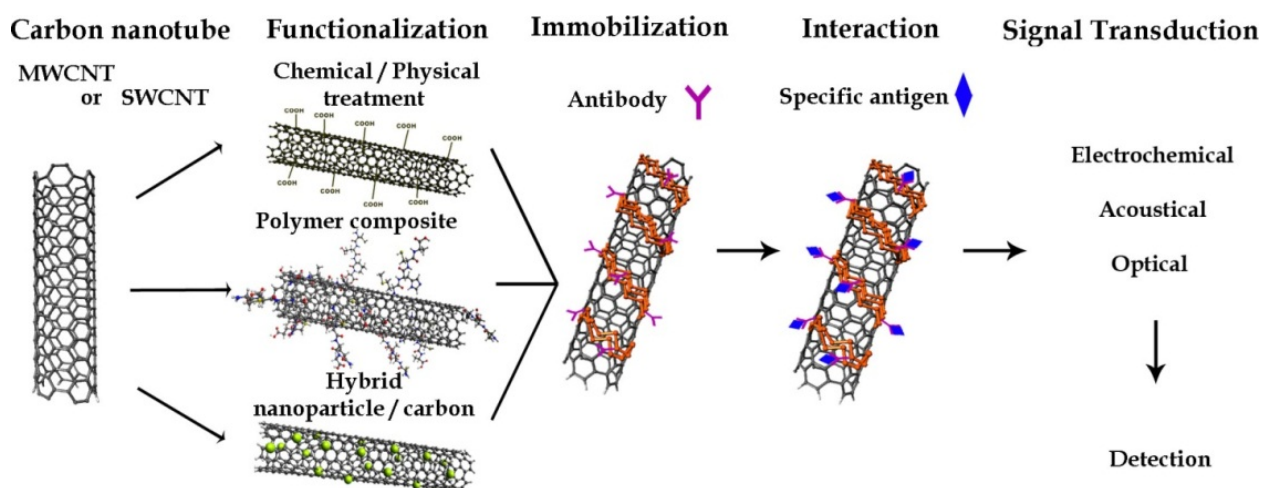


Figure 3. The schematic diagram steps and transducer types of immunosensors based on carbon nanotubes.

Noncovalent functionalization enables reversible adsorption of biomolecules on the CNT surface. For this purpose, CNTs are added to a dispersant solution, and the mixture is agitated in an ultrasound bath. The CNTs are mechanically debundled and then stabilized by dispersant molecules through noncovalent interactions. This does not cause changes in the chemical structures, electronic, and mechanical properties of the carbon nanotubes, and therefore it is a very attractive method. Surfactants, biomolecules, and polymers are widely used as dispersants and noncovalent modifiers. Among them, the polymers are quite efficient dispersants because of their long chain structure that can wrap themselves around CNTs by disrupting the van der Waals interactions between the walls of nanotubes. In biosensors, polymers are

particularly interesting and have been widely employed to prepare CNT composites for electrochemical detection, especially for conductive polymers due to their native electron trans-mediation, high conductivity, good environmental stability, and specific organic groups. Furthermore, they can be overoxidized to create an electrically insulating layer. Many reports have demonstrated that CNTs coated with polymers, including polypyrrole, poly(methylene blue), poly(neutral red), poly(acrylic acid), and poly(3-methylthiophene) have become a popular strategy [74]

The covalent chemical functionalization arises mainly from organic molecules reacting with carboxyl groups of CNTs treated by oxidation, which depends on the hydrophilicity/hydrophobicity of the species attached, which can make carbon nanotubes soluble in water or organic solvents. The modification of carbon nanotube surfaces by covalent attachments of soluble groups usually alters intrinsic properties such as conductivity, mechanical strength, and optical properties. [73, 75] Nevertheless, the functionalization involving the introduction of carboxyl, amine, thiol, and other reactive groups are attractive strategies because antibodies or antigens can be covalently immobilized, improving the stability and, in some cases, the sensitivity and selectivity of the immunosensors. Figure 4 exhibits different covalent and noncovalent methods of functionalization of the carbon nanotubes.

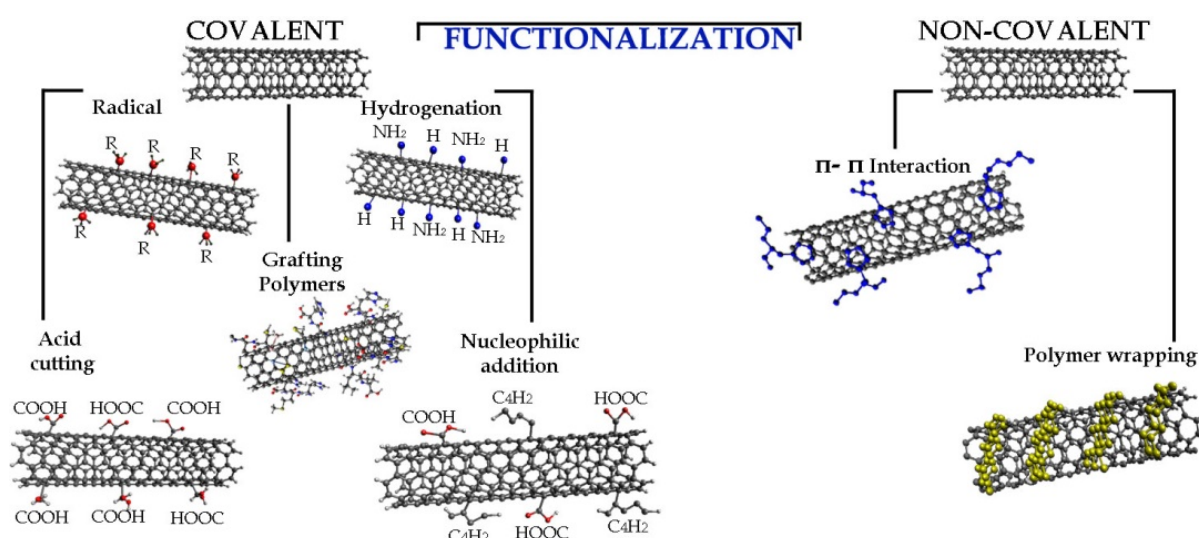


Figure 4. Illustration of different carbon nanotube functionalization methods.

In some practical applications, Sánchez and coworkers [76] have constructed immunosensors where the biomolecules are immobilized on an MWCNT–polysulfone composite film. The layer was applied onto screen printed working electrodes to provide a suitable immunosensor for the rapid determination of human chorionic gonadotropin hormone. The detection limit was 14.6 mIU/mL with a linear range up to 600 mIU/mL. Viswanathan et al. [77] developed another disposable electrochemical immunosensor based on CNTs for the detection of carcinoembryonic antigen with a detection limit of 1 pg/mL in saliva and serum.

For better detecting performance toward interleukin-6, in cases of oral cancer, Malhotra et al. [78] made an ultrasensitive immunosensor sandwich assay on an electrically conductive and

high surface area platform, featuring densely packed and upright SWCNTs with capture antibodies attached to their ends. This biosensor had the highest sensitivity at $19.3 \text{ nA/mL (pg IL-6)}^{-1} \text{ cm}^{-2}$ and the best detection limit (DL) of $0.5 \text{ pg/mL (25 fM)}$ for IL-6 in $10 \text{ }\mu\text{L}$ of calf serum. Similarly, Munge et al. [79] have presented a novel electrochemical sensor using a sandwich immunoassay for the detection of metalloproteinase-3, a cancer biomarker, based on vertically aligned SWCNT arrays. The multilabeled polymeric bead amplification method demonstrated a detection limit of 0.4 ng/mL in $10 \text{ }\mu\text{L}$ of calf serum. This showed great potential for these elements in future cancer diagnostics.

The self-assembly of oxidative SWCNTs on gold was attempted for the detection of bovine serum albumin, BSA, by cyclic voltammetry. This sensor has shown excellent sensitivity and dynamic linear response at the range of 0.1 to $1.2 \text{ }\mu\text{M}$. [80] A conductive multilayer composed of Nafion-coated MWCNTs, thionine (Thi), and AuNPs was prepared using an innovative self-assembly strategy to form an immunosensor for α -1-fetoprotein. This reagentless amperometric sensor presented broader linear response in two ranges between $0.5\text{--}20 \text{ ng/mL}$ and $20\text{--}200 \text{ ng/mL}$ with a detection limit of 0.26 ng/mL . [81]

There are many studies demonstrating that CNTs can provide high electrocatalytic activity to the electrochemical devices and minimize surface fouling effect. Their unique properties enable them to promote a fast electron transfer, play the role of a biomolecular immobilization platform, and be compatible with different materials for construction of different electrodes. The sensitivity of electrochemical sensors has been greatly enhanced due to these materials, which promotes high active surface area and conductivity. CNTs play an important role in recent trends for immunosensor fabrication. They can function as transducers, act as carriers and labels of immunoassay due to the transfer of large amounts of electroactive species for amplifying electrochemical signals, and also offer an easy way to protect and stabilize these bioactive species. [82] In this section, different strategies were described like the easy adsorption of CNT on the electrode surface, biomolecule immobilization by simple adsorptions and covalent binding, and preparation of screen printed electrode.

Based on a simple amino-functionalization method for MWCNT, Dutra's group developed an electrochemical immunosensor for the detection of human cardiac troponin T (cTnT), an important marker for acute myocardial infarction. It showed a broad linear range (0.02 to 0.32 ng/mL) and a low limit of detection, 0.016 ng/mL . [83] Another sandwich-type immunosensor for the detection of cTnT based on carbon nanotubes supported by a conductive polyethyleneimine film has achieved a low limit of the detection of 0.033 ng/mL and a linear range between 0.1 and 10 ng/mL . [24] Amperometric response is generated by peroxidase reaction with substrate in chronoamperometry detection. The high electronic transfer and catalytic response helped by the CNT was essentially important to dispense the mediator in order to generate the analytical responses. Due to the high conductivity achieved by incorporation of CNTs in screen printed electrodes, a label-free amperometric immunosensor was fabricated, presenting new strategies based on differential pulse amperometry. The immunosensing device for cTnT, with amine-functionalized carbon nanotubes incorporated in screen-printed

electrode ink, reached a lower detection limit of 0.0035 ng/ML, better than any previously described immunologic sensors. [21]

3.4. Graphene

Graphene is a two-dimensional material, formed by carbon atoms that are densely packed in a regular sp^2 -bonded atomic scale as a hexagonal pattern, [84] which was produced in laboratory for the first time in 2004. [85] This is the base construction block for other carbon allotropes such as fullerene, carbon nanotubes, graphite, nanoribbons, and others. [86] It is a transparent (optical transmittance of $\sim 97.7\%$), very thin sheet with large theoretical surface area ($2630 \text{ m}^2 \text{ g}^{-1}$), one atom thick, stronger than steel (mechanical stiffness of 1TPa). In addition, it is a good heat conductor (thermal conductivity of $500 \text{ W m}^{-1} \text{ K}^{-1}$), chemically inert, and a semimetal with high electron transfer (charge-carrier mobility of $250\,000 \text{ cm}^2 \text{ V}^{-1} \text{ s}^{-1}$ at room temperature). [87, 88] These properties make them attractive for many applications. [84] There are a variety of synthesis methods for obtaining graphene such as chemical vapor deposition, chemical vapor deposition by plasma, the graphite intercalation of metal sheets, mechanical or thermal exfoliation of graphite oxide, intercalation, and exfoliation of graphite, among other variants of these. Despite all these syntheses methods, the mass production is still difficult, making it hard to develop some applications. [88]

Graphene oxide (GO) synthesis has been an alternative to graphene mass production. It is produced from the oxidation of graphite and has polar oxygen functional groups. GO is rich in carboxylic acids at its edges, and epoxy and hydroxyl groups at basal planes, which grants many functionalization routes and good dispersion in water. [89] Furthermore, the functional groups are responsible for the exfoliation of graphite, seeing as they increase the interplanar distance due to the formation of hydrogen bonds between the graphite sheets. The hydrogen bonds are weak and can be easily broken by ultrasound bath, resulting in monolayer or a few sheets of carbon, known as GO. This is an excellent material for biological applications attributed to the functional groups that readily interact with nucleic acids, proteins, cells, and other organic molecules. However, GO is not a good electrical conductor because of the disruption of its sp^2 bonding as functional groups increase, which can narrow its nanobiotechnology applications. To overcome these difficulties, the reduced form of GO has been chosen as an alternative.

Reduced graphene oxide (RGO) has more commonly been used to form nanocomposites with nanoparticles or polymers to develop biomedical applications such as biosensors, controlled drug delivery, therapeutic modalities for cancer treatment, substrates for antibacterial effects, scaffolds for mammalian cell culture, and gene delivery among others. [90]

In the RGO synthesis, functional groups are removed, and the conductivity is increased again. This removal can be done in different ways such as electrochemical, optical, hydrothermal, microwave, or heating procedures. These methods for removing the functional groups form different shapes and therefore the conductivity recovery is variable. Also, they form different functional groups, becoming favorable in a wide number of applications. [91]

The most common method for obtaining RGO is chemical reduction, which is used in colloidal dispersing of GO. Hydrazine monohydrate is the most used reduction agent, seeing as it does not react with water, which makes it attractive for aqueous dispersions. The reduction process mediated by hydrazine normally occurs through the addition of H₂ groups and removal of N₂, and it is gentle enough not to affect the cyano and nitro groups. The second most used reducing agent is sodium borohydride (NaBH₄), which is more effective than hydrazine and easily hydrolyzed in water. The hydrolysis process should be slow enough so it does not affect the reduction process. The NaBH₄ reduces C=O species and has a low effect in epoxy and carboxylic acid groups. Other reduction agents such as hydroquinone, alkaline solutions, and gaseous hydrogen are also being described as mediators. [92]

Another low-cost mean of producing RGO is by thermally reducing GO, heating it in a furnace at 1050 °C, which creates thermodynamically stable oxide carbon species. Electrochemistry can also be used in the reduction process of GO, removing oxygen functionalities. Thermal and electrochemical reduction techniques have the advantage of avoiding dangerous reducers and the problem with their disposal, but they are still less used than chemical reduction. The reduction processes frequently provide RGO with functional groups, but in some cases, its functionalization is still necessary prior to use. Covalent and noncovalent methods for functionalization of RGO have been studied, whereas noncovalent bonds are the most common used, for instance, the physical adsorption of both polymers and small molecules via van der Waals interactions onto the basal planes of RGO sheets. [92]

An initial and successful approach using RGO to create biosensors was its combination with nanoparticles. An example is the work of Shan et al., [93] who used Au nanoparticles associated with RGO and chitosan as a nanocomposite film onto a gold electrode for developing an electrochemical glucose sensor obtaining a linear response range from 2 to 10 mM. Copper nanoparticles were also used to modify RGO sheets to create an electrochemical sensor for glucose obtaining a detection limit of 0.5 μM. [94]

Afterward, RGO was applied to the production of immunosensors, with and without nanoparticles. An example is the work of Mao et al., [95] who reported the use of RGO sheets coated with Au nanoparticles, which were initially functionalized with human immunoglobulin G (IgG) to create conjugates. These conjugates were immobilized onto a field effect transistors (FETs) biosensor platform for the detection of human proteins.

A developing area for immunosensors is the detection of cancer markers. It is a recent and very attractive field, with growing publication numbers, including the use of RGO for these. An example is the work of Zhong et al., [96] who used a gold nanoparticle enwrapped graphene nanocomposite on a glassy carbon electrode in a sandwich-type immunoassay format. The detection limit obtained for this assay was 10.0 pg mL⁻¹. Another CEA immunosensor was developed by Huang et al. [97] using Ag/Au nanoparticles coated with RGO in a clinical immunoassay for the detection of carcinoembryonic antigen (CEA). The nanoparticles were used as means for amplification of the signal and the method showed a detection limit of 8.0pg mL⁻¹ in human serum.

Different cancer markers were the focus of other works, such as the one developed by Tang et al., [98] which aimed to create an electrochemical immunosensor for the simultaneous detection of alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA), using biofunctionalized magnetic RGO nanosheets (MGO) coated with iron oxide nanoparticles as immunosensing probes, obtaining detection limits of 1 pg mL^{-1} for CEA and 1 pg mL^{-1} for AFP. Also, Teixeira et al. [99] created a chemically modified epitaxial graphene diagnostic sensor for the detection of human chorionic gonadotropin, which is a main marker for pregnancy and can also indicate some types of tumors. They obtained a detection limit of 0.62 ng/mL .

For optical transducers in cancer marker detection, RGO was used by Xu et al. [100] in a modified glassy carbon electrode using luminol to create a electrogenerated chemiluminescence (ECL) immunosensor for prostate specific antigen, using two antibodies in a sandwich immunoassay, which achieved a detection limit 8.0 pg mL^{-1} . RGO was also used in the development of an ECL immunosensor using CdTe quantum dots (semiconductor nanocrystals) along with Au nanoparticles for signal amplification in the detection of human IgG with detection limit of 0.005 pg/mL . [101]

4. Conclusions

The different concepts of nanomaterials applied to immunosensors have been discussed. Nanomaterials can be utilized for a wide variety of immobilization matrices intending to improve the immunosensor sensitivity, allowing lower limit of detection. The potential of nanomaterials on immunosensors has resulted in a positive impact on the clinical outcome of various diseases, including cancer, cardiac injuries, parasitic infections, and viruses, among others. It is well known that carbon nanotubes and graphene nanostructures are more favorable to amperometric transducers due to their electrochemical proprieties, which increase the electronic transfer charge and electrocatalytic activity. Metallic and magnetic nanoparticles have successfully been applied to different transducers, especially electrochemical, by enlarging the electroactive surface area. Quantum dots, a semiconductor nanoparticle, present a promising potential for many transducers mainly due to their photostability and luminescence characteristics. Nevertheless, more challenging studies involving nanomaterial sciences, biochemistry, electronic, and molecular engineering should be done in attempting to achieve faster, more practical, and more reliable biosensors. More specifically, biomolecules and a deeper knowledge in nanomaterial science associated to new electronic designs represent a promising field in the development of portable and integrated point of care devices for health applications and other areas of diagnostic.

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