Chapter

Dyes as Labels in Biosensing

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Abstract

Investigation and evaluation of dyes play a vital role in the process of introduction novel labels and their corresponding sensing methods, which signify opportunities for the development of biosensors. This chapter introduces applications of various dyes as labels in biosensing. Bio-recognition molecules with dyes transduce biological information into measurable optical, electrochemical, magnetic or other kinds of signals for quantification. The dyes used in this field were summarized and reviewed according to their signal types, namely colorimetric, fluorescent and electrochemical. Some dyes can transduce signals between multiple physical signals. For some most important dyes, detailed descriptions were given focused on their unique properties, labeling methods and sensing mechanisms.

Keywords: biosensors, labels, micro- and nano-particles, optical dyes, charge-transfer complexes

1. Introduction

Applications of, e.g., clinical diagnosis, drug development, environmental research, security and defense, require self-contained rapid analytical platforms to get rid of tedious operation processes and long turn-around times. In 1960s, applications of enzymes explored a new way in analytical chemistry to obtain specific, sensitive and ease-of-use assays. At the same time, ion selective electrodes have been developed for rapid non-reagental analysis of inorganic ions. In 1962, the concept of enzyme transducer was proposed and sooner the device was developed [1]. Following this idea of enzyme electrode, the first enzyme electrode-based glucose meter was commercialized in 1975. After that, self-contained analytical platforms based on different principles were developed including thermistor, [2] optical fiber, [3] piezoelectric crystal detector, [4] surface plasmon resonance, [5] etc. Today, such analytical platforms are regarded as biosensors. A biosensor is a self-contained integrated device that is capable of providing specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) which is in direct spatial contact with a transducer element [6]. Today, biosensors have been applied to a wide variety of analytical problems, e.g., medicine, environmental research, food control, and process industry [7].

Biosensors can be divided into two groups based on involving a labeling process during the detection or not. A label is a foreign molecule that is chemically or temporarily attached to the target (i.e., the molecule of interest) through a labeling process to detect molecular presence or activity. In the above-mentioned early

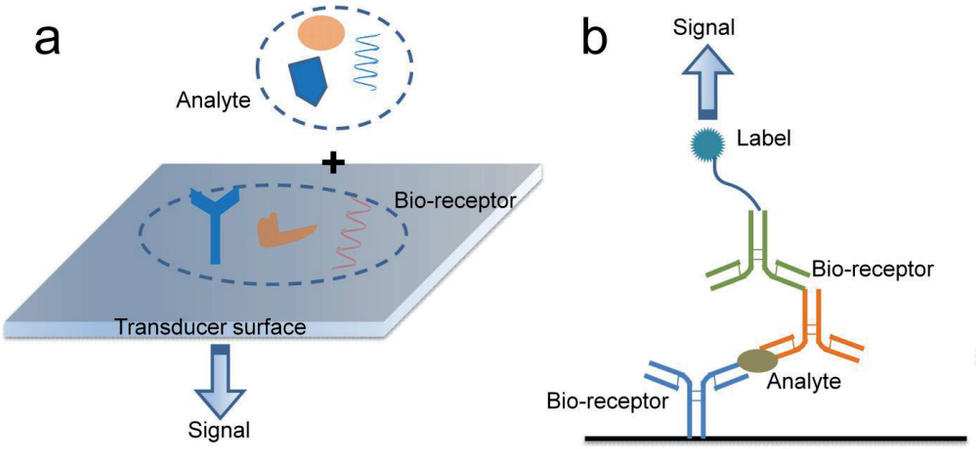

Figure 1.
Scheme of typical (a) label-free and (b) label-based biosensors.

period of the biosensor history, typical biosensing processes were usually realized by measuring the transduced mechanical, electrical, or optical signals without any labels, as illustrated in **Figure 1a**. Such label-free biosensors can provide direct information without complicated sample preparation steps. In contrast, label-based biosensors utilizing additional operation processes for higher signal-to-noise ratios and for a broader range of sensing/transducer systems. Conventional biosensing labels are optical molecules or radioactive elements borrowed from bio-analytical systems such as gel electrophoresis and enzyme linked immunosorbent assays. A typical strategy of sandwich assay for antibody-based detection is shown in **Figure 1b**.

Due to the fast development of nanotechnology and material science, nanomaterials are widely adopted as biosensing labels, many of which are dyes in senses of optics or electrochemistry. The special size of these nanomaterial dyes provides unique properties that greatly improve the performances of relevant biosensors. In this chapter, we focus on dyes used as biosensing labels and discuss their properties, applications and how they improve the biosensing properties.

2. Dyes in colorimetric biosensors

Colorimetry technique is a practical and direct analytical method to determine the concentration of colored analyte depending on the color change in solution. The colorimetric biosensing strategy based on this principle has become one of the most popular and important strategies due to its simplicity, visualization, low cost and non-destruction. Based on the strategies of signal generation, colorimetric assays can mainly be divided into two groups, i.e., assays based on enzymes for chromogenic reactions, and assays based on colored labels. Herein, we focus on the later group in which dyes play a crucial role.

2.1 Gold nanoparticles

As a successful example to employ nanomaterial and nanotechnology to solve the biological problems, colloidal gold has been introduced in the biosensing fields more than 20 years with lots of amazing works being reported. Colorimetric biosensors based on gold nanoparticles (GNPs) have greatly developed in both scientific study and commercial applications.

2.1.1 Sensing mechanism

Surface plasmon resonance. Various GNP-based colorimetric biosensors were built depending on the same principle, i.e., surface plasmon resonance (SPR). SPR is a prominent spectroscopic feature that results in an intense and sharp absorption band in the visible range of noble metal nanoparticles that have an adequate density of free electrons. Localized SPR is an effect that the electron cloud of the nanoparticle sense and start to oscillate at the same frequency as the electromagnetic field of the incident light (**Figure 2a**). During this process the incident light is scattered and converted into heat, both leading to the intensity attenuation of the incident light. Meanwhile, localized SPR produce an electric field on the nanomaterial surface, which can be utilized for labeling several kinds of biosensors. The SPR-induced color is determined by several factors including the size, shape, modified ligands and aggregation state. In colorimetric biosensors, the aggregation state of GNPs is interested. As shown in **Figure 2b**, in bioanalytical assays, the combination of bio-receptors (or targets) labeled with GNPs can induce the isolated GNP assembly as well as the interparticle coupling of the surface plasmon, resulting in the color of solution transferring from red to blue.

Surface-enhanced Raman spectroscopy. Based on the SPR effect, GNPs have also been used in other types of biosensors, e.g., surface-enhanced Raman spectroscopy (SERS) based sensing. SERS as a versatile finger-print vibrational technology has been widely utilized in analytical chemistry, [8] electrochemistry, [9] and media diagnostics. [10] However, the mechanism of SERS is still

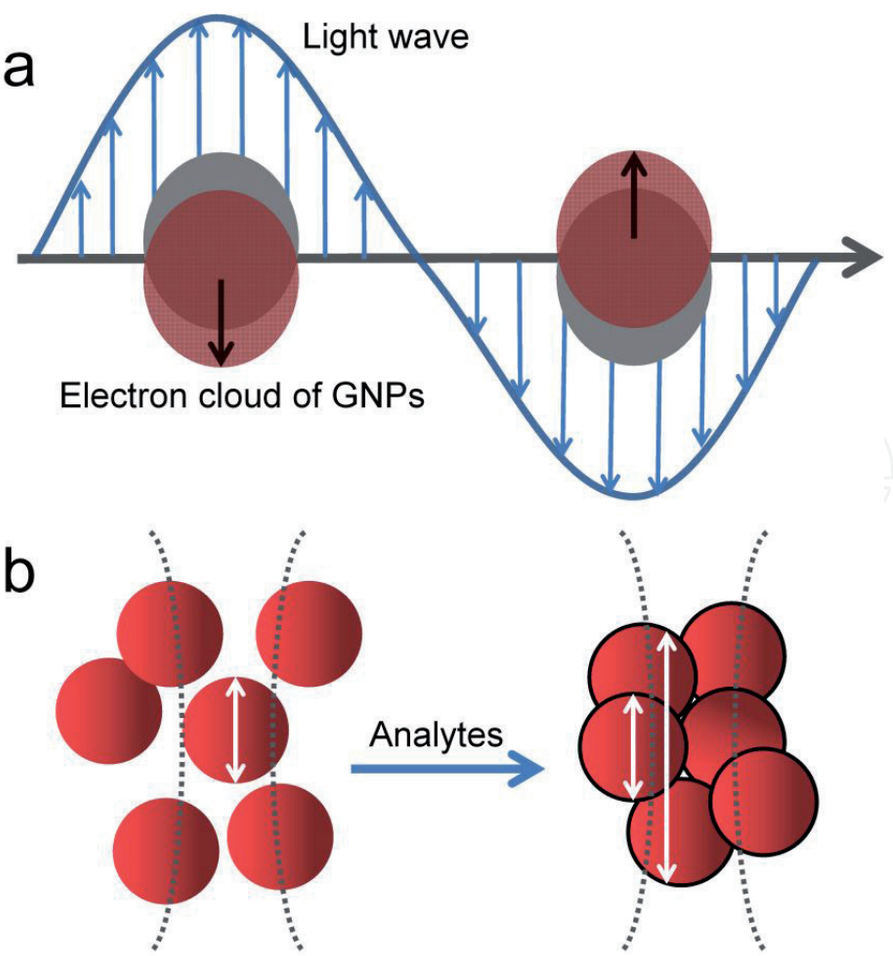

Figure 2.
(a) Localized SPR band formation. Red and gray circles represent negative and positive electron clouds, respectively. (b) GNP agglomeration leads to the shift of SPR absorption band.

under debating [11]. Regarding to the mechanism of SERS, there are two broadly accepted opinions: electromagnetic (EM) theory and chemical enhancement (CE) mechanism [12]. The EM enhancement considers the molecules as point dipoles, which react with the local enhanced electric field on or near the surface [13]. The CE mechanism attributes the SERS intensity to molecular resonance through the interaction with the metal surface, thereby changing the molecular polarizability, resulting in enhancement such as resonance Raman scattering [14]. SERS does not occur on any metal surfaces, among which GNPs are currently the most widely used SERS substrate. SERS-based biosensors involving GNPs have been widely used in the sensitive and selective detection of antigens, [15] aptamers, [16] tumor biomarkers, [17] as well as *Staphylococcus aureus* [18].

Lateral flow system. The most well known biosensing application of GNPs must be the pregnancy test sticks, which belong to the most commercialized type of biosensor, i.e., the lateral flow (LF) system. LF biosensors are paper-based devices permit low-cost and rapid diagnostics with moderate robustness, specificity and sensitivity. The ease-of-synthesize, stability, biocompatibility and tunable size make GNPs suitable label for LF systems. Moreover, the SPR effect endows GNPs an intense red color that can be either detected qualitatively by naked eye or measured quantitatively using spectrometers for lower detection limits. Because of these properties, GNPs are the most widely used optical label in LF systems [19]. A standard and conventional LF strip consists of four main sections made of membranes, papers or glass fibers, including a sample pad for sample loading, a conjugate pad impregnated with bioreceptor-modified labels (usually GNPs), a detection pad/membrane where test line (to show whether the target is exist in the sample) and control line (to show whether the LF assay works well) are printed, and an absorption pad at the end of the strip, as illustrated in **Figure 3**. Except for GNPs, other colored materials are also used in LF biosensors, including carbon dots and latex particles.

2.1.2 Labeling type

Three principle methods for modifying GNPs are briefly illustrated in **Figure 4**: (1) A simple and common way to immobilize the biomolecules on the GNPs is labeling through the sulfur-gold interactions; [20] (2) Ligands can be capped on the GNPs in the process of particle growth or though ligands replace after the synthesis; (3) Ligands can be absorbed on the GNPs surface directly via non-covalent interactions.

In 1997, the first GNP-based colorimetric biosensor was reported [21]. In this work, the hybridization of DNA probes capped on GNP surface and target DNA

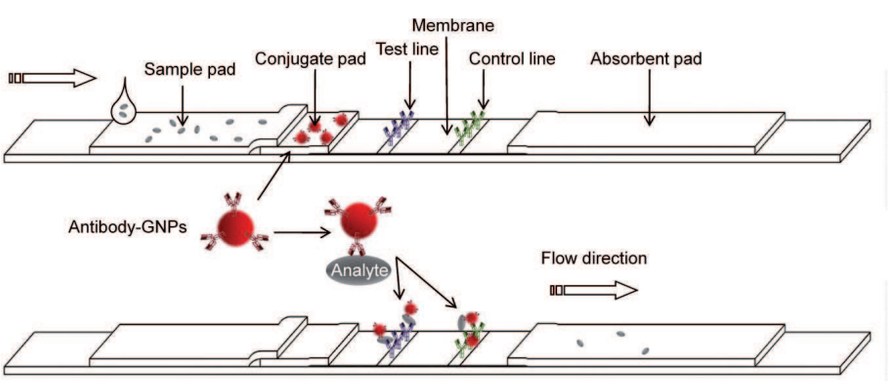

Figure 3.
Schematic of a typical lateral-flow immunological detection system.

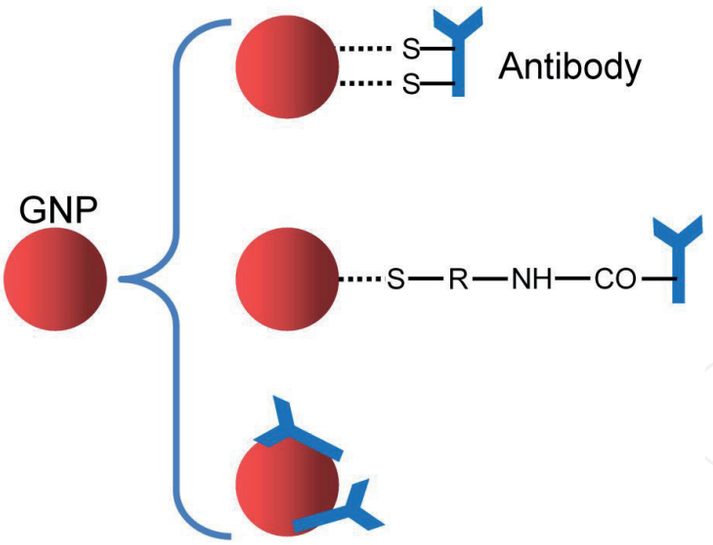

Figure 4.
Illustration of different GNP conjugation methods.

resulted in the formation of GNP agglomeration, accompanying a visually red-to-purple color change. This is the most typical biosensor that uses biochemical reactions to induce the cross-linked aggregation of GNPs. Since then, a new generation of medical diagnostic technology based on the nanomaterials has begun. Oligonucleotides, peptides, antibodies and aptamers have been labeled with GNPs for the colorimetric detection of different targets based on the similar principle. Such strategy designs usually achieve naomolar detection limits, which is limited by the signal-to-noise ratio related to the intrinsic properties of labels and sensors [22]. To amplify the signals and optimize the performance of biosensor, the biochemical and molecular amplification methods are introduced in the biosensing process. Via duplex-specific nuclease-assisted amplification method, a colorimetric method was developed for microRNA dection based on GNPs aggregation [23]. DNAzyme-assisted target recycling was utilized, combined with surface plasmons of GNPs coupling in the colorimetric biosensor, obtaining a fast and simple detection of genetic targets with 50 pM sensitivity [24]. Using imaging-based analysis instead of spectrographic analysis has higher signal-to-noise ratio and thus potentially lower detection limit. A dark-field microscope based methodology was applied for the sensing of GNP aggregation, obtaining a detection limit of 43 aM of DNA, which was 5–9 orders of magnitude lower than conventional colorimetric sensor based strategies [25].

2.2 Carbon nanoparticles

Carbon nanoparticles, also named colloidal carbon, can be visually detected in a qualitative or semi-quantitative manner and thus being used as colored labels. Compared to GNPs, carbon nanoparticles have several excellent properties, e.g., high stability, nontoxicity, ease-of-preparation, and ease-of-modification [26]. The dark black color of carbon dots endows a high signal-to-noise ratio, allowing sensitivities below the low picomolar range even by visual inspection [27].

2.3 Latex particles

Colored latex particles are also often used as labels in the colorimetric biosensor. Latex particles are natural or synthetic polymer nano- and micro-particles that suspend stably in water, and the polystyrene particles are used mostly.

There are three ways to prepare colored latex particles by dying latex particles with different types of dyes molecules: (1) co-polymerization of polymer monomer with dyes; (2) cross-linking the dyes on particles surface by covalent bonds; (3) physical embedding or absorption dyes in particles. After the dying, usually dyes on the surface are removed in order to functionalize the active groups (sulfhydryl groups, amino groups and carboxyl groups) on the latex particles for further labeling the biomolecules [28].

Benefit its wide variety of sources, low cost and easy to be functionalized, the latex particles are applied as probes in immunochromatographic analysis quite early [29]. The good properties enable them are still used now. A lateral flow immunoassay was developed by covalent functionalizing the antigens on colored latex particles for the visual diagnosis of canine visceral leishmaniasis [30]. A latex particles-GNPs composites labeled with antibodies were synthesized as probed for the immunochromatographic test. The nanocomposites amplified the binding capacity of GNPs with target antigens and improved the sensitivity 2 orders of magnitude compared with GNPs-antibodies probes [31].

3. Dyes in fluorescent biosensors

In the field of biotechnology, diagnosis and drug discovery, fluorescent assay is by far the most popular methodology because of not only its sensitivity and versatility but also the high commercialization of fluorescent labels [32]. In addition to the new fluorescent nanomaterials (e.g., upconversion fluorescent materials and aggregation-induced emission (AIE) materials that are described further below), new spectroscopic sensors have also been developed based on rising technologies such as fiber optics, LEDs and fast imaging devices, all of which contributed to the fast development as well as high interdisciplinarity of fluorescent biosensors.

3.1 Organic dyes

Organic fluorescent dyes are a class of organic molecules that contain a fluorescent core skeleton with a large conjugate system and some auxochrome or active group (such as carboxyl, amino, amide, etc). The fluorescent core skeleton enables them absorb a certain excitation light and emitting it as fluorescence. The auxochrome or active group is capable of altering wavelengths and enhance fluorescence or labeling them to bio-receptors for recognizing various biomolecules in biosensing [33]. Briefly, the fluorescent dyes labeled bio-receptors, also called fluorescent probes, can recognize various biomolecules and then convert the recognition events into fluorescent signal output to achieve biosensing or imaging.

Currently, there are many kinds of organic fluorescent dyes, most of which can be used to label bio-receptors for biosensing and imaging. Here, some major organic fluorescent dyes labels are introduced, including fluorescein derivatives, rhodamine derivatives, cyanine derivatives and other commonly used organic fluorescent dyes.

3.1.1 Sensing mechanism

The signal conversion mechanism in sensing process are various, such as electron transfer quenching or fluorescence recovery, fluorescence resonance energy transfer (FRET), or monomer-excimer emission conversion with pyrene fluorophores. The following will introduce the major signal conversion mechanism involved in the fluorescence biosensing process.

Nucleic acid intercalating dyes. Nucleic acid intercalating dyes [34–36] is a special kind of organic fluorescent dyes that have no fluoresce or the fluorescence is weak in solution, which may be caused by the quenching of solvent. However, when they are embedded in specific DNA structures, the fluorescence intensity will increase significantly, due to the protection of the hydrophobic groups of the oligonucleotide (**Figure 5**). Commercially available nucleic acid intercalating dyes for labels in biosensing mainly include ethidium bromide (EB), thioflavin T (ThT), N-methylporphyrin dipropionic acid IX (NMM) and triphenylmethane dyes. The most classical biosensing application is EB staining-mediated gel electrophoresis for nucleic acids detection [37]. Nucleic acid fragments can be separated in gel under the action of electric field, then EB contains a tricyclic planar group can insert between nucleic acid stacking bases, resulting in increased fluorescence intensity of EB for detection.

An emerging biosensing strategy is designed based on the G-quadruplex (G4) DNA structure and corresponding intercalating dyes such as ThT, NMM, etc. (**Figure 6**) [38]. G4 DNA structure is formed from DNA guanine-rich sequences, which has been confirmed to be stably present in human live cell [39]. Therefore, endogenous G4 DNA can be easily detected by using intercalating dyes targeting G4 DNA. Additionally, G4 DNA structures can be formed by the amplicons of any kinds of DNA amplification methods that produce single-stranded DNA, making G4 structure a convenient cascade amplification tool (a molecular amplification followed by a signal amplification) that can be applied in homogeneous and isothermal bioanalytical assays. Moreover, the formation or consume of the G4 structure after binding to the target molecules will change the interaction between G4 and intercalating dyes, resulting in increased or decreased fluorescence intensity for detection.

Fluorescence resonance energy transfer. Measuring the presence of labels always means employing tedious operation steps for separation and washing. Today, homogeneous reaction processes are highly preferred due to its potential for point-of-care applications. FRET assays are frequently used in biosensors due to achieve homogeneous reaction processes with high sensitivity.

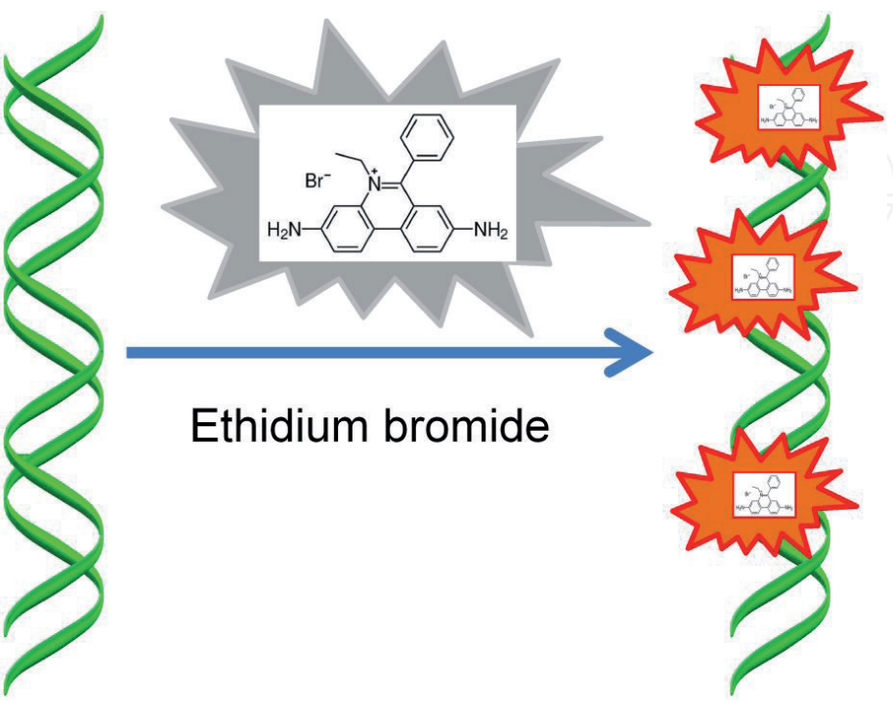

Figure 5.
EB staining for nucleic acids detection.

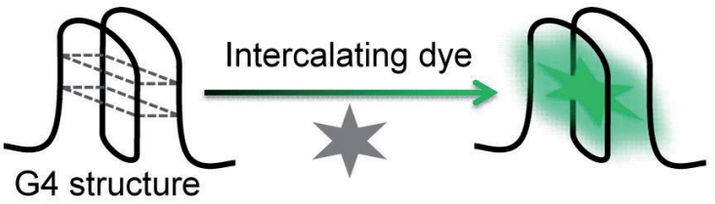

Figure 6.
Fluorescence enhancement based on the intercalating dyes and G4 structure.

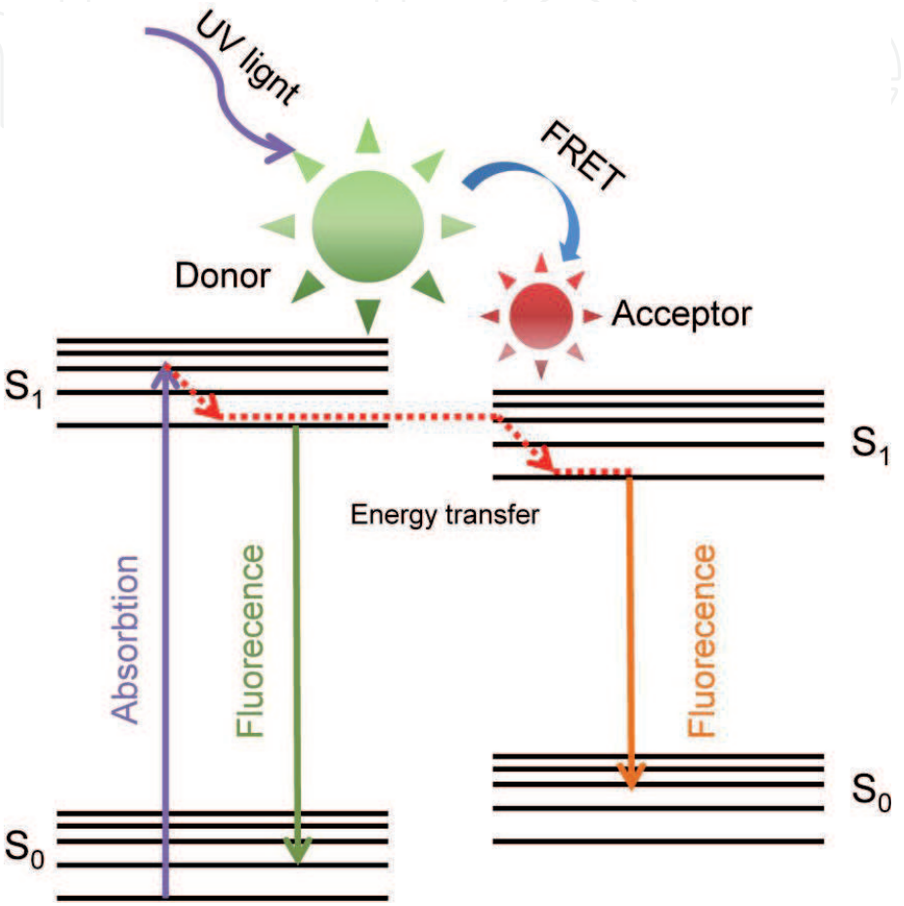

Figure 7.
Schematic illustration of fluorescence resonance energy transfer.

A FRET technique includes an energy transfer between two fluorophores, i.e., from high energy donor to a lower energy acceptor (**Figure 7**). The FRET occurs when the donor and acceptor are close to each other, approximately between 1–10 nm, and this distance meets the dimensions of biological molecules. Since the FRET is sensitive to the relative distance between donors and acceptors, when biological acceptor labeled with donors (or acceptors) comes close to the biological target labeled with acceptors (or donors), FRET signals can be detected.

Rather than labels the donor and the acceptor on different biomolecules, a molecular beacon (MB) utilized a donor linked with an acceptor through a biore-sponsive probe, further simply the design of FRET biosensors [40]. Typically, a MB is a single-stranded oligonucleotide probe labeled with a fluorophore at its one end and a quencher at the other. Due to the length and/or the secondary structure of the oligonucleotide, the probe holds the fluorophore and quencher close to each other, thus inducing a quenching. Upon the hybridization between the probe and the target, the distance between the fluorophore and the quencher changes, restoring the fluorescence [41] (**Figure 8**).

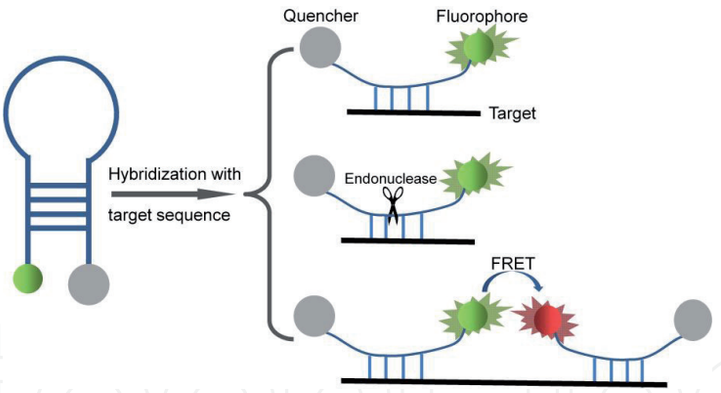

Figure 8.
Illustration of a molecular beacon and examples of its applications.

Monomer-Excimer. Generally, when the distance and position between fluorescent dyes with the same or different structures are appropriate, the excited state fluorescent dye and the other ground state fluorescent dye would form an excimer. Therefore, the fluorescence emission intensity of the original monomer weakens or disappears, and the fluorescence emission of the formed excimer appears [42, 43]. For monomer-excimer based biosensing process, the reaction between the fluorescent dyes-labeled probe and the target biomolecules will trigger or hinder the monomer-excimer process, causing the variation of fluorescence emission spectra.

3.1.2 Labeling type

The labeling type between organic fluorescent dyes and bio-receptors can generally be divided into two types: covalent and non-covalent (such as intercalation, groove binding or electrostatic interaction). Different labeling method has different fluorescent sensing mechanisms.

Covalently binding labeled fluorescent probes including single-labeled fluorescent probe and dual-labeled fluorescent probe. Single-labeled fluorescent probes are obtained by covalently binding single fluorescent dye molecules to bio-receptors. The single-labeled probe sensing mechanism may be summarized as follows: when the bio-receptor of the fluorescent probe recognizes the target molecules, the fluorescence properties of dyes would be changed, such as changes in fluorescence intensity and fluorescence anisotropy, thus converting the recognition process into a measurable fluorescence signal [44–46]. Dual-labeled fluorescent probes are obtained by covalently binding dual fluorescent dye molecules (or a dye and a quencher) to bio-receptors. The dual-labeled probes are all distance dependent, the rearrangement of the probe structure after binding to the target molecules will change the distance between the two labels, resulting in changes in the fluorescence properties of the system.

Non-covalently binding fluorescent probes mainly refer to nucleic acid fluorescent probes that obtained by the binding of intercalating dyes and DNA. As mentioned above, when the nucleic acid intercalating dye binds to a specific DNA structure, the fluorescent signal changes. Based on this principle, a series of biosensors have been developed. Compared with covalently binding fluorescent probes, the non-covalently binding fluorescent probes will not affect the binding affinity of the probe to the target, and also have the advantages of easy operation and low cost [47].

3.1.3 Fluorescein derivatives

Fluorescein and its derivatives are one class of xanthene dyes. Fluorescein was first produced by Von Bayer in 1871, which has a good rigid coplanar structure

and can produce strong green fluorescence under the action of laser. Due to its easy synthesis, low cost, low biological and cytotoxicity, high molar absorption coefficient, and high fluorescence quantum yield, fluorescein can be widely used in biological imaging and analysis [48]. However, fluorescein also has some defects, such as high pH sensitivity [49], small stokes [50] and poor light stability [51]. In order to improve the fluorescence performance of fluorescein, many important fluorescein derivatives have been developed by introducing functional group modification to fluorescein [52]. Additionally, fluorescein derivatives contain some active groups, which can bind with bio-receptors to obtain fluorescent dyes labeled probes with high selectivity, good stability and high sensitivity for biosensing [53]. Currently, commercially available fluorescein derivatives dye mainly includes 6-carboxy-fluorescein (FAM), 5-tetrachloro-fluorescein (TET), 5-hexachloro- fluorescein (HEX).

3.1.4 Rhodamine derivatives

It was discovered in the late 1980s that rhodamine and its derivatives are important fluorescent dyes and also belong to xanthene dyes. The molecular structure of rhodamine dyes is very stable, coplanar, and can produce strong red fluorescence under the excitation. They also can bind with bio-receptors to obtain fluorescent dyes labeled probes with high selectivity, good stability and high sensitivity for biosensing. Compared with fluorescein derivatives, rhodamine derivatives have stronger photostability, higher fluorescence quantum yield and lower pH sensitivity. Commercially available rhodamine derivatives dyes for labels in biosensing mainly include 6-carboxyl-x-rhodamine (ROX), tetramethyl-6-carboxyrhodamine (TAMRA) and Texas red.

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3.1.5 Cyanine derivatives

Cyanine dyes was first discovered by Williams in 1856, subsequently, Vogel discovered that these dyes have very good photosensitivity, which promote the development of cyanine dyes [57]. Cyanine dyes and derivatives have excellent spectral characteristics, such as high molar extinction coefficient, high fluorescence quantum yield, and long fluorescence emission wavelength. More importantly, the maximum emission and absorption of these dyes are located in the near-infrared region. In this region, the self-absorption and background fluorescence of biomolecules are relatively small [58]. Thus, cyanine dye derivatives have become the most commonly used fluorescent signal groups in protein, nucleic acid and other biological analysis [59]. Commercially available cyanine derivatives dyes for labels in biosensing mainly refers to N-carboxypentyl-5-sulfonate-3H-indocyanine dyes, including Cy3, Cy5, Cy5.5 and Cy7.

3.2 Quantum dots

Quantum dots (QDs) are spherical or quasiballistic semiconductor nanoparticles that bind excitons in the three dimensions, with a diameter no larger than twice the Bohr radius of the excitons of their corresponding semiconductor material, thus confining the motion of electrons, holes, or excitons in three dimensions. Due to the quantum confinement effects, the quantum dots display unique optical and electronic properties compared to the bulk materials.

QDs were firstly synthesized in glass matrix in 1970s and with their fluorescent properties reported [60]. Later some groups studied the novel properties of quantum dots and tried to investigate influences of quantum effects on the optical properties of QDs [61]. In 1980s, CdS nanospheres were synthesized in colloidal solution and the basic theory of QDs were studied [62]. In 1993, the high quality colloidal QDs were prepared first time with uniform size in the solution [63], which provided favorable materials for both theoretical study and practical applications. Since then, various types of QDs with different compositions and properties have been synthesized by the solution growth method.

According to the chemical compositions, QDs can be mainly divided to two groups. Single component QDs, such as metal chalcogenides, [64] attracted much attentions at the early stage of the QDs development. Due to the uniformity, optical and electronic properties of such QDs can be tuned by simply controlling their sizes. Multiple component QDs are either core-shell structural or alloyed. Core-shell structural QDs have a core with one component embedded in another material as a shell, such as CdSe/ZnS [65]. Usually, to reduce the nonradioactive recombination of electron–hole pairs, the material used as the shell has a larger band gap than that in the core, thus improving the fluorescence quantum yield. Coating the same core with different shells adjust the properties of the QDs. Alloyed QDs that have homogeneous or heterogeneous alloyed internal composition, for example, CdSxSe1-x/ZnS [66]. This type of QDs allows tuning the properties by changing the proportion of the component without changing the size. Interestingly, alloyed QDs not only exhibit the original properties of each component, but also display newly additional and adjustable properties because of the merge of the different composites. Now despite classical nanocrystals, there are various new species of QDs that have been prepared, such as perovskite QDs [67] and graphene QDs [68].

Generally, the size of QDs, i.e., in the range of 2–10 nm, endows these nanoparticles high surface-to-volume ratios. The large surface provides rich sites for further functionalization and immobilization of molecules, including nucleic acids and proteins. [69] Importantly, after functionalized with hydrophilic ligands, QDs are soluble and stable in aqueous solution, which is the common environment for biological reactions.

3.2.1 Sensing mechanism

The most obvious and widely utilized properties of QDs are the optical properties. Compared with organic dyes, QDs display higher fluorescence quantum yield and extinction coefficient, broader excitation spectra, longer lifetimes and tunable fluorescent emissions [70]. As the size of QDs decreases, the band gap between valence band (VB) and conduction band (CB) increases, which means more energy needed for electrons excitations (from ground state in VB to CB) as well as more energy released from the electrons de-excitation (from CB to ground state inVB), leading to the fluorescent emission shift to the high frequency range. In addition, the fluorescence wavelength can also be tuned flexibly by adjusting the structure

and chemical compositions of QDs as mentioned above. These stand out properties make QDs appealing for bio-medical applications including imaging and biosensing [71]. In biosensing systems, QDs, used with or without nano-sized quenchers, are transducers and functionalized with bio-recognition molecules (bio-receptors). Because of the tunable size and broad spectral width, QDs can play as either energy donors or acceptors in the FRET biosensor [72]. Furthermore, due to their high fluorescence intensity, photostability and long lifetimes than conventional organic fluorophores, QDs were also involved in the design of molecular beacons.

Bioanalytical systems using QDs as labels. QDs were first applied as bioanalytical labels in 1998, [73] an ultrasensitive bioanalytical system involving QDs was demonstrated for protein imaging. Since then, QDs have been widely applied in various bioanalytical methodologies, e.g., the enzyme-linked immunosorbent assay (ELISA), fluorescence resonance energy transfer (FRET) assay and cell tracking.

QDs can be simply used as fluorescent labels in immunosensors to quantify the biological targets through directly measuring the presence and/or the intensity of the fluorescence. CdTe/SiO2 core-shell structured nanoparticles were labeled with prostate-specific antigen (PSA) detection antibodies for PSA detection. And the fluorescent signals were measured after the specific recognition between PSA and the QD labeled antibodies followed by a magnetic separation to remove unbound QDs. This system represents a typical fluorescent biosensor detecting the presence of QD labels [74]. This strategy design shows great flexibility for employing a variety of fluorescence detectors, e.g., fluorescence spectrometry and handheld UV lamp tests. Furthermore, such design enables not only target quantification but also imaging multiple targets with different QD labels [75].

Imaging. QDs have also demonstrated their applicability in biomedical imaging, which is an important tool in diagnosis, visualization, treatment and prognosis of diseases [76]. QDs emit in the visible and near infrared ray wavelengths, with high brightness and excellent photostability, suitable for morphological studies. A representative examples of such applications encapsulated QDs in virus-lick particles as theranostic platforms to image viral behavior in living cells [77]. Furthermore, multifunctional SV40 virus-like particles was constructed encapsulating QDs bearing peptides recognizing early, developmental, and late stages of atherosclerosis, respectively, in live mice [78].

3.2.2 Labeling type

One of essential challenges to apply QDs in biosensors is immobilization of target recognition biomolecules onto the surface of QDs via stable bonding. In this part, methods for preparing QD-biomolecule conjugates will be presented and modified QDs applied as labels in biosensors are also summarized. Roughly, there are 4 strategies to prepare the QD-based bioconjugates:

1. Direct binding: proteins and nucleic acids can be immobilized on the QDs surface directly through interactions between the thiol-groups or imidazole-groups with the metal component of QDs, e.g., alkylthiol terminated DNAs are linked with QDs surface directly via dative thiol bond [79].

2. Conjugation via ligands: QDs can be functionalized firstly by ligands, such as carboxyl groups, hydroxyl groups and amino-groups, then covalently bond to biomolecules [80].

3. Conjugation via functional shell: QDs are capped with silane shell [81] or copolymers [82], then bond to biomolecules by the functional groups on the outer shell.

4. Conjugation via specific biological affinity: some types of biological affinity can be used to bond the QDs with biomolecules strongly and specifically, such as biotin-streptavidin interaction [83].

3.3 Upconversion fluorescent materials

Upconversion fluorescent materials are emerging fluorescent nanoparticles that can convert low frequent exciting light into high frequent emitting light by absorbing two-photons or multi-photons. The luminous mechanism of upconversion nanoparticles (UCNPs) is anti-stokes, which is opposite with the most fluorescent materials, including organic fluorescent dyes, quantum dots, fluorescent proteins, metal complexes, etc [84]. Because of the distinctive luminescence mechanism, UCNPs have some unique advantages, which make up the disadvantage of above other dyes. Firstly, UCNPs have improved biological tissue penetration. Secondly, UCNPs can reduce light damage on biological samples. Thirdly, UCNPs can effectively avoid the disturbance of autofluorescence from biological samples. Therefore, UCNPs have wide applications in biosensing and imaging [85]. The sensing principle of UCNPs-based probe is widely based on fluorescence resonance energy transfer (FRET) between UCNPs (donor) and other down-conversion fluorophores (acceptor). The reaction between UCNPs-based probe and the target biomolecules will trigger or hinder the FRET process, causing the quenching and enhancement of fluorescence for detection.

4. Dyes in electrochemical biosensors

Electrochemical biosensor is capable of providing specific quantitative or semi-quantitative analytical information using electrochemical transduction elements, e.g., charge-transfer complexes. Low-cost, energy efficient, portable, easy fabrication, and real-time sensing are major advantages of electrochemical biosensing platforms. Among the electrical signal molecules, there are several types of dyes with electrochemical activity, and they will be introduced in this part. In addition, the electrochemical signals generation mechanisms are explained and applications of these dyes as labels in the biosensors are also displayed.

4.1 Organic dye molecules

Methylene blue (MB) is a kind of derivative of phenothiazine and widely used a redox indicator and electron transfer medium in electrochemical analysis. For a typical DNA detection using MB labels, the distance between the MB and the electrode surface is adjusted by the change of conformation of the DNA probes labeled with MB, so that the peak current or the change in impedance can indicate the presence of the target DNA and quantitative concentration. This method is simple and versatile, however easy to be influenced by the solution environment. Despite MB, other organic dyes, such as gentian violet, ethyl green, Hoechst 33258 are also utilized in the electrochemical works. They are not as popular as MB, but show good performance in some biosensors.

4.2 Organometallic complexes

Organometallic complexes consist of centrally located metal atoms or ions and completely or partly coordinated organic ligands. The organometallic complexes with transition metals have the advantages of strong redox signal, good chemical

stability, low toxicity, and high structural flexibility. They interact with biomolecules via the Intermolecular interaction force and electrostatic interaction.

Ferrocene (Fc), is a yellow organometallic complexes with transition metal (Fe) and aromatic ligands (cyclopentadiene rings). Because Fc has two freely rotating cyclopentadiene rings, it can be labeled with the biomolecules, such as DNA via hydrophobic interactions. As an electrical signal molecule, in the combination of bio-receptors and target molecules, Fc generates electrical signals mainly by adjusting the distance between the Fc and the electrode surface to realize the change of electrical signal and achieve the purpose of detection.

K3[Fe(CN)6]/K4[Fe(CN)6], is a pair of dyes with bright red and yellow color, respectively. Mainly, they are used as electron transfer agents in amperometric biosensors, to replac the natural electron transfer agents of the enzymes. In the commercial blood glucose meters, the glucose in the blood reacts with glucose oxidase and K3[Fe(CN)6] fixed on the surface of the test strip to produce gluconic acid and K4[Fe(CN)6]. Applying a constant working voltage to the test strip, K4[Fe(CN)6] is oxidized to K3[Fe(CN)6], generating an oxidation current that is proportional to the glucose concentration.

4.3 Nanomaterials

4.3.1 Quantum dots

One of the most commonly used electrochemical biosensor is cadmium selenide (CdSe) QDs, which employ as electrical signal molecules for the labeling of nucleic acid strands [86]. The Pb^{2+} cleavage ribozyme sequence was modified on the surface of the magnetic beads, and designed an electrochemical biosensor for detecting Pb2+ by using rolling circle amplification reaction and a signal probe labeled with CdS QDs [87]. Based on Ni^{2+} cleavage ribozyme and CdSe QDs, The Ni^{2+} was detected and the detection limit was 6.67 nmol/L [88]. As electrical signal molecules, QDs have versatility and low background signal, which has great application prospects.

4.3.2 Graphene quantum dots

Graphene quantum dots (GQDs) are actually sheets of graphene with dimensions less than 100 nm with sp^2 hybridized honeycomb structures, and their shapes are mostly circular and elliptical, but square and hexagonal QDs are also available. Basically, GQDs are characterized as graphene-like, consisting of C, O, and H as well as carbonyl, carboxyl, hydroxyl, and epoxy groups. GQDs can bind to ssDNA through π-π interactions, but it has no such effect on double-stranded DNA. Park et al. used GQDs as electrical signal substances to detect the Hg2+ concentration by measuring the current generated during the electrochemical reduction of GQDs [89].

4.3.3 Metal–organic frameworks

Metal–organic frameworks (MOFs) are crystalline materials with an infinitely regular and infinitely expanding periodic network structure formed by the self-assembly of metal ions and organic ligands through coordination bonds, covalent bonds, and weak intermolecular bonds (π-π stacking, van der Waals forces, hydrogen bonding, and other electrostatic interactions, etc.) [90]. MOFs are nanomaterials with good stability, large porosity, and specific surface area that are of great interest in gas storage, drug delivery, and sensors. Due to the intrinsic peroxidase

catalytic activity, MOFs can also be used in electrochemical biosensors. Xu et al. constructed a Pb^{2+} electrochemical biosensor based on the MOFs prepared based on Fe [91], and AgPt nanoparticles are employed to increase its electrical conductivity and electrocatalytic activity, and the obtained sensitivity approaches 0.032 pmol/L. However, even though MOFs have enzymatic activity to improve sensitivity, their synthesis process is very complicated, and the characterization of the modification process is also very critical, so it is not suitable for routine use.

5. Conclusion

Investigation and evaluation of dyes play a vital role in the process of introduction novel labels and their corresponding sensing methods, which signify opportunities for the development of biosensors. This chapter highlights the utilization of dyes as biosensing labels and some most important sensing mechanisms for biological, biotechnological, and biomedical applications. These designs and applications have been much attracted for in vivo and in vitro analysis due to their high sensitivity and selectivity, fast response, biocompatibility, etc. Further developments in novel synthetic approaches of functional nanomaterials and sensing strategies will accelerate the discovery of unique properties of dyes, which will further improve their applications towards future biosensing platforms.

Acknowledgements

The authors are grateful to ÅForsk Foundation (grant number, 20-280), Formas (grant number, 2019-01583), STINT (grant number, IB2020-8594) and I Bergh scholarship. Qilu young scholar program of Shandong University (grant number, 11500082063141) is also acknowledged for the financial support.

Conflict of interest

The authors declare no conflict of interest.

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