

Nanobiosensors and fluorescence based biosensors: An overview

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Received 25 April 2018; revised 18 July 2018; accepted 29 August 2018; available online 15 November 2018

Abstract

A biosensor can sense biological elements after interaction with the recognition element. The signal produced due to interaction of the analyte with its biochemical element is transduced by a transducer and detected by appropriate modes. The miniaturization of these biosensors at the nano level using nanostructures as a platform for sensing the analyte or its detection is called a nanobiosensor. Several biological elements can be detected like nucleic acids, enzymes, antibodies, microorganisms, toxins, cells etc. with high specificity. This mini review focuses on the different types of nanobiosensors based on the type of analyte and the type of transducer used for detection. The different types of fluorescence based design of biosensors are also discussed along with the metal enhanced fluorescence based nanobiosensors. The application of biosensors towards the diagnosis of various diseases, targeted drug delivery and imaging is also discussed.

Keywords: *Bionanotechnology; Biosensors; Fluorescence; Nanobiosensors; Nanostructures.*

How to cite this article

Girigoswami K, Akhtar N. Nanobiosensors and fluorescence based biosensors: An overview. *Int. J. Nano Dimens.*, 2019; 10 (1): 1-17.

INTRODUCTION

On the basis of field of application biosensor can be defined in different ways like- "a biosensor is a chemical sensing device in which a biologically derived recognition entity is coupled to a transducer, to allow the quantitative development of some complex biochemical parameter" and also: "a biosensor is an analytical device incorporating a deliberate and intimate combination of a specific biological element (that creates a recognition event) and a physical element (that transduces the recognition event)" [1, 2].

Leland C. Clark was the father of biosensors for the development of enzyme electrodes in the year 1962 [3]. After this invention, scientists from different fields like Very Large Scale Integration (VLSI), Chemistry, Materials Science and Physics have contributed by developing highly reliable and sophisticated biosensor based devices in medicine, diagnostics, biotechnology and agriculture. The applications of biosensors were also extended for detection and prevention of

bioterrorism in military [4-6]. Aptamer based detection of mycotoxins in food samples have also revolutionized the safety aspect of food industry [7]. Harmful algal blooms (HABs), which consist of six major biotoxins, in oceans are posing threats to human beings. Biosensors have developed to detect such biotoxins produced from algae and other microorganisms in the harvested fish/shellfish from oceans [8]. Metal oxide core shell nanoparticle (WO_3) based gas sensor for sensing acetone in diabetic patients who exhale acetone in their breath as a result of ketosis is also been developed [9].

A biosensor is a device comprising of two components: (i) a bio-element, and (ii) a sensor-element. The following figure (Fig. 1) illustrates the operation of a biosensor.

The working of any biosensor can be explained with the following example. In a biosensor, a particular "bio" element can recognize a particular analyte followed by the "sensor" element which can transduce the change of the biomolecule into

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a measurable signal in the form of electrical, optical, piezoelectric, calorimetric etc. signal. The bio element used in the sensor should be highly specific and sensitive to the analyte which is to be detected and should not detect any other analyte. Biosensors can be classified as follows depending on the mechanism of transduction: (i) Optical biosensors, (ii) Calorimetric biosensors, (iii) Ion-Sensitive Field Effect Transistor (ISFET) biosensors, (iv) Resonant biosensors, (v) Electrochemical and (vi) Reagentless biosensors. Electrochemical biosensors can be further classified into (a) Conductometric, (b) Amperometric, and (c) Potentiometric [10]. Acquiring high market

potential, the blood glucose sensing is the major application so far [11]. The applications of biosensors are versatile including biomedical industry, military, environmental pollution monitoring etc. as shown in Fig. 2.

TYPES OF BIO-ELEMENTS AND SENSOR ELEMENTS

The basic component of a biosensor is the bio-element and the sensor-element (Fig.1). There are different types of bio-elements and sensing element being used in biosensing [12-17] and some of them are listed in Fig. 3.

Different combinations of the bio-elements and sensor-elements can constitute different

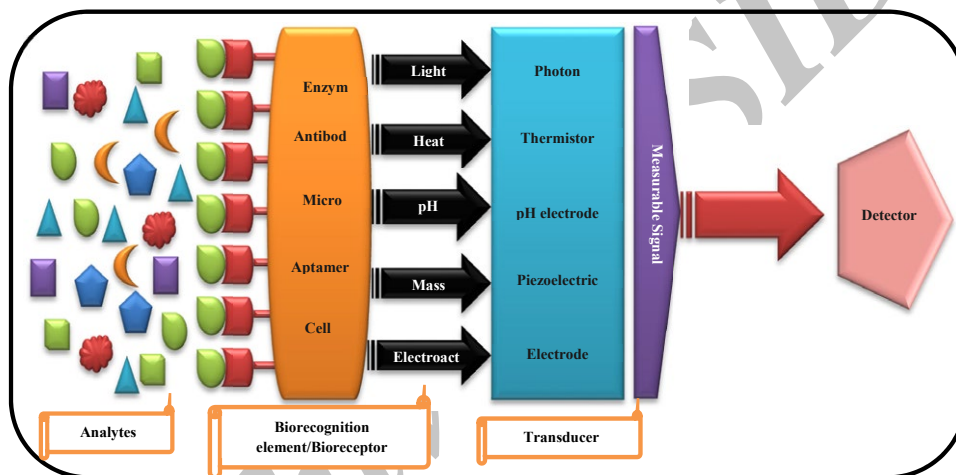


Fig. 1: Scheme showing a representative biosensor.

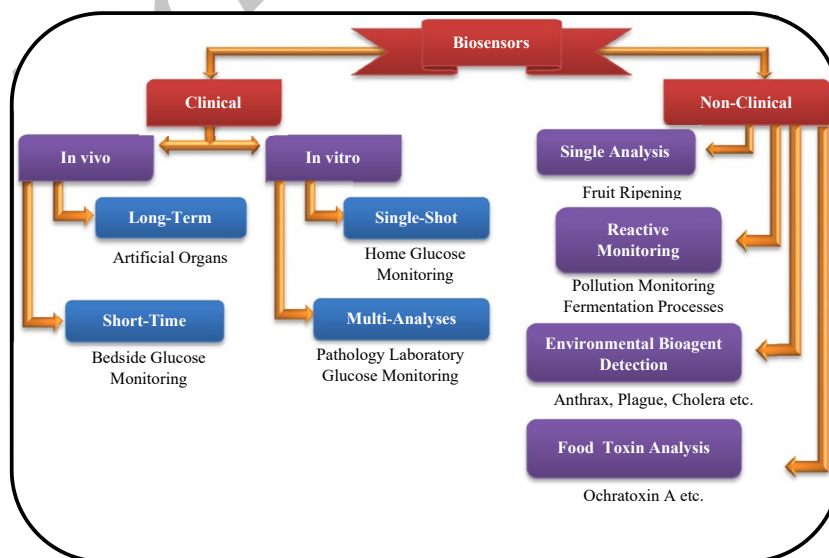


Fig. 2: Different applications of biosensors.

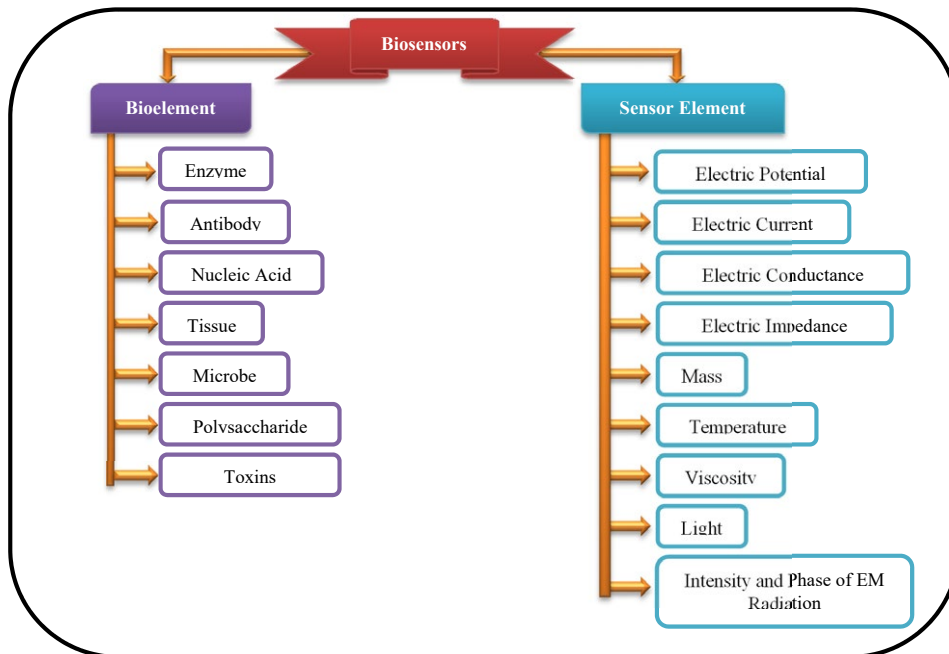


Fig. 3: Components and elements of biosensors.

types of biosensors depending upon a variety of applications. There are four ways of combining the “bio” and the “sensor” element: Membrane Entrapment, Matrix Entrapment, Physical Adsorption, and Covalent Bonding. For membrane entrapment, the analyte and the bioelement are separated by a semi permeable membrane, and the sensor is attached with the bioelement. For porous entrapment, there is an encapsulation matrix with high porosity which is formed around the biological material. This matrix also helps in binding it to the sensor. For physical adsorption, the surface of the sensor serves as an adsorption platform for the biomaterial though different weak interactions like Van der Waals forces, hydrogen bonds, hydrophobic forces, and ionic forces. For covalent bonding the sensor surface is treated with a reactive group to which the biological materials can bind [18].

Based on the bioanalyte to be detected biosensors are of different types. Enzyme based biosensors are available in the medical field like detection of glucose, urea, cholesterol etc. There is also development of enzyme based biosensors that can painlessly monitor the body metabolites [19]. Single protein molecule catalysis can be also detected using single-walled carbon nanotube based transistors. This hybrid device can monitor the real time protein activity

up to single molecule based on fluorescence. Klenow fragment (KF) of DNA polymerase I based nanocircuits are also used to monitor polymerization of DNA with sensitivity up to a single base addition [20]. Many nucleic acid based biosensors are also available especially the oligonucleotide based biosensors which can detect specifically the complementary nucleic acids like micro RNAs. Synthetic oligonucleotide like peptide nucleic acid (PNA) or locked nucleic acid (LNA) based biosensors are also used to detect analytes adding new perspective in the area of molecular biology [21]. Single nucleotide polymorphism in the Korean-specific *BRCA* mutation site was detected by investigators using PNA based microarray analysis with high specificity [22]. As point of care diagnostics, antibody based biosensors have come into field in recent times. As an ideal biorecognition element, antibodies provide high specificity and sensitivity. Monoclonal antibodies and antibodies produced by recombination can be immobilized for specific detection [23]. In some cases antibodies often fail in their activity when they are immobilized due to restriction in the binding site spatial structure. As an alternative there are several non-antibody-binding proteins available which can be used for electrical and micromechanical biosensor designing [24].

TYPES OF BIOSENSORS BASED ON PRINCIPLE OF DETECTION

Resonant biosensors

In resonant biosensor, the antibody (bio-element) is coupled with an acoustic wave transducer and when the antigen (or analyte molecule) gets attached to the membrane there is an alteration in the membrane mass. Whenever there is a change in mass of the membrane there is a subsequent change in the transducer's frequency of resonance, which can be measured [25]. Acoustic biosensors are sometimes miniaturized by incorporation of microfluidics and electronics, giving rise to faster detection time, handling many samples at a time and a low signal to noise ratio. Among the several kinds of acoustic biosensors, bulk acoustic wave sensors, surface acoustic wave sensors, and micro/nano-electromechanical system (MEMS/NEMS) sensors are some of them [26].

Optical biosensors

Light is measured as the output transduced signal for this type of biosensor, as an outcome of either electrochemiluminescence or optical diffraction. Optical biosensors widely use the principle of surface plasmon resonance (SPR), bioluminescent optical fiber, evanescent wave fluorescence, interferometry, ellipsometry and reflectometric interference spectroscopy and surface-enhanced Raman scattering. These biosensors can detect sensitively and selectively a versatile range of analytes like tumour markers, antibodies, toxins, drugs and tumour cells [27]. Typically, in devices based on optical diffraction, a protein is immobilized via covalent bonds over a silicon wafer. The wafer is then given Ultra Violet (UV) light exposure on the top of a photo-mask resulting in the inactivation of the antibodies attached in the regions of exposure. Then, the wafer which is diced is incubated with the analyte forming antigen-antibody binding in the active regions. This creates a diffraction grating and when a light beam, say laser, falls on this grating, a diffraction signal is produced. The diffracted signal can be measured directly or can be measured after amplification yielding an improved sensitivity [28].

The luminescence that is produced in solutions because of electrochemical reactions is called Electrochemiluminescence (ECL). In ECL the intermediates that are generated electrochemically make highly exergonic reactions which give rise to an electronically excited state.

When this intermediate relaxes to a lower level state it eventually emits light with wavelength corresponding to the energy gap between the two states [29]. Among the different biosensor applications of ECL, gold nanoparticle based detection of cell surface protein [30] and detection of organophosphate pesticides [31] are recently being studied.

Calorimetric biosensors

Calorimetric biosensors are based on either heat absorption or heat production, which can further change the temperature of the medium of the reaction mixture. The construction of calorimetric biosensors is usually done by immobilizing enzyme molecules that are tagged with temperature sensors. When there is a reaction taking place by binding of the analyte and the enzyme, heat is produced which is measured and is calibrated against the analyte concentration. The overall heat produced or absorbed is directly proportional to the total number of molecules participated in the reaction and molar enthalpy. The temperature is measured via a thermistor, known as enzyme thermistors, which are highly sensitive. Thermal biosensors do not need to be recalibrated frequently and are usually not sensitive to the electrochemical and optical properties of the sample used. Some of the examples of thermal biosensors are the pesticides detection and pathogenic bacteria detection [32].

Ion-sensitive biosensors

Ion sensitive biosensors are constructed with an ion-sensitive surface over semiconductor Field Effect Transistors (FET). Whenever the ions interact with the semiconductor, there is a change in the surface potential which is measured. The construction of Ion Sensitive Field Effect Transistor (ISFET) is done by covering the sensor electrode with an ion selective polymer layer. The diffusion of ions through the layer of polymer changes the surface potential of the FET, which can be monitored. ENFET (Enzyme Field Effect Transistor) is a kind of ion sensitive biosensor which is usually applied for pH detection [33]. Silicon nanowire-based ISFETs (SiNWFETs) are used for the label free detection of DNA, drug screening etc [34].

Electrochemical biosensors

Electrochemical biosensors are based on the principle that many chemical reactions can

produce or take up electrons and changes the electrical properties of the reaction solution. This change can be measured based on the electrical parameters. Electrochemical sensors can be classified as: (1) Conductometric, (2) Amperometric and (3) Potentiometric. The DNA-binding drugs detection, detection of hybridized DNA or glucose concentration determination etc. are the few applications of electrochemical biosensors. There are 2D nanomaterials based on graphene oxide and reduced graphene oxide that can be used as a platform for electrochemical biosensing of biomarkers for cancer like nucleic acids, small molecules and proteins [35].

Conductometric

In this type of biosensor, the electrical conductance or electrical resistance of the solution is measured. There will be a change in the resistivity or conductivity of the solution if ions or electrons are produced during electrochemical reactions, which can be measured and calibrated. The sensitivity of the sensor is low if conductance is measured compared to resistance measurements. A sinusoidal voltage i.e., AC is used to generate electric field to minimize the losses due to Faradaic processes, and also helps in double layer charging and concentration polarization [36].

Amperometric

In case of amperometric biosensors the parameter is current which is measured. In biological test sample certain electroactive species are present that can be detected with amperometric biosensors. If the biological test sample does not have intrinsic electroactivity, then enzymes are employed to catalyze the production of electroactive species [37]. Detection of glucose using amperometric biosensor is very common and in some cases biosensors are also developed based on electrochemical impedance spectroscopy [38].

Potentiometric

Whenever there is an oxidation-reduction taking place in an electrochemical reaction a potential is developed. When the electrode in the solution is subjected to a ramp voltage there is a flow of current generated due to the electrochemical reactions. The voltages corresponding to the reactions are the hallmark of a specific reaction and specific species [39].

REAGENTLESS BIOSENSORS

“Reagentless” is the term that implies that other than the biosensor, no other reagent is added to the system and the output is being studied with fluorescence as the readout. In other biosensors, like a coupled enzyme assay, various other components may need to be present [40]. Reagentless biosensors have two essential elements - a recognition element and a reporter. The recognition element selectively senses the target analyte from a group of similar molecules. It can be a small molecule that can bind to the analyte, a macromolecule like nucleic acid or protein or an enzyme that can react with the analyte to yield the product. On the other hand, the reporter can produce a signal whenever the recognition element interacts with the analyte. This signal can be electrons or light that is measured without any disturbance from the rest of the assay [41]. The example of a reagentless biosensor is inorganic phosphate biosensor [42]. The element of this sensor is a phosphate binding protein that can selectively bind P_i . A coumarin fluorophore is covalently bound to a single cysteine on the protein surface which enhances the fluorescent intensity nearly ten-fold when phosphate binds. Upon binding with phosphate the protein conformation changes which further changes the fluorophore environment, thereby changing its fluorescence. In this kind of biosensor the protein itself acts as a transducer and hence no separate transducing element is required. Quenchbody is a kind of reagentless fluorescence antibody based biosensor which acts on the principle of quenching of the attached dye and its antigen dependent release to the environment [43]. A protein nanochip based reagentless biosensor was developed by changing the antibody sequence without changing its activity which yielded fluorescence upon binding with its specific antigen. mAbD1.3 antibody which was directed against hen egg white lysozyme, was modified at a fragment scFv and an environment sensitive fluorophore was engineered which could detect egg lysozyme at nanomolar range [44]. Upconverting glasses (UCG), made by doping of fluorohafnate glasses with Tm^{3+} Yb^{3+} can exhibit near-infrared excitation and emit visible light. This property was used to design a reagentless biosensor for glucose determination which modifies the fluorescence of the enzyme glucose oxidase-fluorescein (GOX-FS) after enzymatic reaction with glucose when combined with UCG.

The detection limit for glucose was 1.9 mM and can be used to detect glucose in fruit juices [45]. For the continuous analysis of biochemical data related to clinical, industrial, environmental and food samples optical continuous monitoring systems (OCMS) were developed. Any protein or enzyme can act as biochemical receptor and the fluorescence signal change even with a single biochemical reaction can be monitored [46]. Thus, in reagentless biosensor the main advantage is the use of a single component for the system causing minimal modification or interference [40].

FLUORESCENCE BIOSENSORS

Fluorescent sensors have less response time with high selectivity and sensitivity and has profound applications in clinical diagnostics, microscope based analysis, environmental monitoring, etc. On the other hand, polymeric materials have been synthesized with tunable optical properties, well-controlled architecture, and different functionalities. These polymeric materials can be manipulated with various sensing mechanisms and have made remarkable changes in the design and functioning of different fluorescent based sensors [47].

In recent years, fluorescence spectroscopy has been extensively used for the detection of transition and heavy metal ions with high sensitivity and simplicity [48-49]. The limitation of such chemosensors is that they have only single signal for detection, i.e., the fluorescence intensity, which could easily be perturbed by the environmental and instrumental conditions [50]. To overcome this situation ratiometric chemosensors has been introduced that can reduce or eliminate the effects of such factors

by the self-calibration of the two fluorescent emission bands. Fluorescent Resonance Energy Transfer (FRET) based ratiometric sensors have been designed recently [51-52]. There are two methods for designing ratiometric sensors (i) ICT (intermolecular charge transfer) and (ii) FRET (Fluorescence Resonance Energy Transfer). In many ICT based ion sensors, the ratio between two relatively broad signal emissions becomes difficult to determine. The advantage of FRET over ICT is that, in case of FRET, the ratio between two fluorescence intensities is independent of the external factors, like- fluctuation of the source of excitation and sensor concentration. Recently, the most effective method for the detection of ions in environment is FRET based sensors [53].

Conjugated polymers are also used as macromolecules for fluorescent sensors with both light harvesting and electron delocalizing properties. This property makes it suitable for optical sensing yielding amplified output signal through electron and energy transfer mechanisms. The photophysical phenomenon of aggregation-induced emission provides new avenues to design sensors involving macromolecules that have weak fluorescence emission in solution but have a significant increment in fluorescence at its aggregated or solid state [47].

Basic physics of fluorescence

Principle

Fluorescence and luminescence are the two terminologies that should be defined at the beginning. The phenomenon by which light can be produced through excitation by any method without increasing the temperature is called Luminescence. The different modes of luminescence phenomena

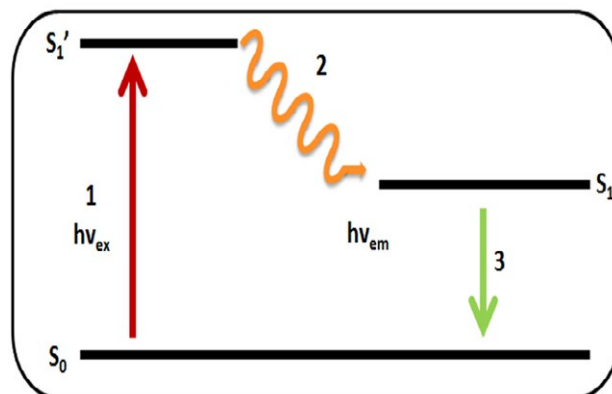


Fig. 4: Jablonski diagram depicting a fluorescence event.

are chemiluminescence (done by chemical means), electroluminescence (done by electrical discharges), or triboluminescence (done by crushing). Fluorescence is defined as 'a short-lived type of luminescence created by electromagnetic excitation which is generated when a substance absorbs light energy at a short (higher energy) wavelength and then emits light energy at a longer (lower energy) wavelength' [54]. In fluorescence, the time interval between absorption and emission is very short, usually at the order of 10^{-9} to 10^{-8} seconds [55]. The illustration of a single fluorescence event can be depicted by Jablonski Diagram [56], named for the Ukrainian born physicist Aleksander Jablonski (Fig. 4).

In Stage 1, a photon is supplied from an outside source, having energy $h\nu_{\text{ex}}$ (say). Lying in the ground energy state S_0 , the fluorescent molecule absorbs the energy supplied by the photon and creates an excited electronic singlet state S_1' that lasts for a finite time, usually 1 to 10 nanoseconds (sec^{-9}). Within this duration there is a conformational change in the fluorescent molecule and it is subjected to myriad potential interactions with its molecular environment. At the beginning of Stage 2 there is a partial dissipation of the energy absorbed by the fluorophore which creates a relaxed singlet state S_1 . Further, the fluorophore moves to the second phase after the emission of energy, $h\nu_{\text{em}}$ and finally, in Stage 3, the fluorophore returns to its ground state, S_0 . The term fluorescence was coined from the mineral fluorspar (calcium fluoride) which would emit visible light (fluoresce) when it is exposed to electromagnetic radiation in the UV wavelength as observed by Sir George G. Stokes in 1852. The studies of fluorescent substances by Stokes' proceeded towards the

formulation of Stokes' Law, which states that 'the wavelength of fluorescent light is always greater than that of the exciting radiation. Thus, for any fluorescent molecule the wavelength of emission is always longer than the wavelength of absorption [57].

Fluorescence spectra and Stokes shift

Fluorophores or fluorochromes are the molecules that can display fluorescence. In molecular biology, the most commonly used group of fluorophores have planar conformation and are heterocyclic molecules such as fluorescein (aka FAM), Coumarin, Cy3, Thioflavin T etc. These fluorophores have a characteristic absorbance and emission spectrum. Peak absorbance is defined as the specific wavelength at which there will be an efficient absorption of energy by one of these molecules and peak emission is the specific wavelength at which they will emit the energy most efficiently. The scheme showing absorbance peak and emission peak is shown in Fig. 5. The value of wavelength obtained by taking difference between the peak absorbance and the peak emission is termed as the Stokes Shift after Sir George Stokes [58].

Fluorescence Resonance Energy Transfer (FRET)

The phenomenon of FRET takes place by the direct excitation of an acceptor fluorophore by the energy donated by a donor fluorophore followed by the donor excitation by electromagnetic radiation in the appropriate wavelength (Fig. 6). Fluorophores emit energy characteristically in the form of light, but some fluorophores can emit in the form of heat. The molecules that dissipate the absorbed energy in the form of heat are known as

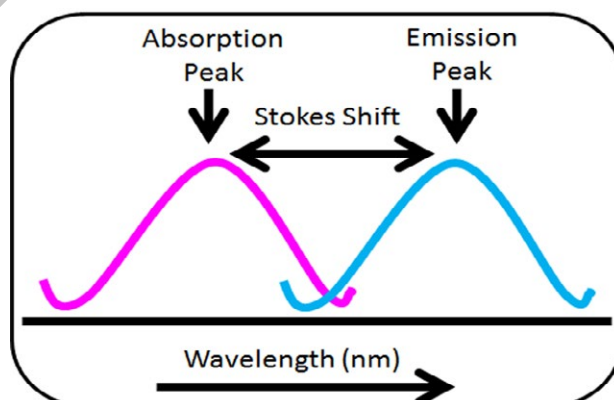


Fig. 5: The absorbance peak and emission peak (in nanometers) of the fluorophore showing the Stokes Shift.

quenchers. The quenchers are very useful because they can absorb energy over a wide range of wavelengths but can also dissipate this absorbed energy in the form of heat and they remain dark. Due to these properties; quenchers can be very useful molecules for energy acceptors in FRET pairs.

The relationship between the donor acceptor proximity is very critical for the FRET phenomenon. The overlap between donor fluorescence and acceptor absorbance is shown in Fig. 7.

Design and principles of fluorescent biosensors

To couple the target recognition with a change in fluorescence of the reporter is the critical step in biosensor development. Depending on the nature of sensing element different designs of fluorescent biosensors can be developed.

Single fluorophore

In single fluorophore based biosensor, the change in fluorescence intensity of a single fluorescent reporter which is sensitive to the microenvironment and is attached to the recognition element is monitored. As the target molecule binds to the recognition element there is a change in conformation of the recognition element which further changes the microenvironment of the fluorophore (Fig. 8a). This change in fluorescence intensity can happen due to various mechanisms such as the accessibility of the solvent is changed and thereby causing dynamic quenching or a change in surrounding environment polarity, or a change of fluorophore protein interactions. The change in fluorophore protein interaction is used for the phosphate biosensor [59]. Based on the periplasmic binding

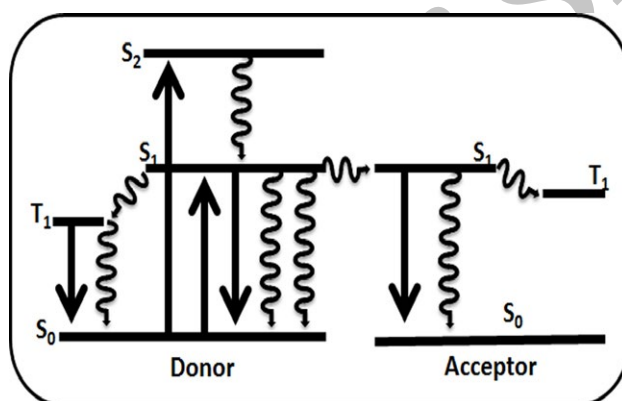


Fig. 6: Jablonski Diagram illustrating fluorescence resonance energy transfer, FRET. The energy corresponding to the excitation and the emission of the donor molecule is similar to that model shown in Fig. 4. The emitted energy of the donor in a FRET pair excites the acceptor molecule. The resonance energy is emitted as in Fig. 4 but at a much higher wavelength than would be emitted by the donor molecule alone.

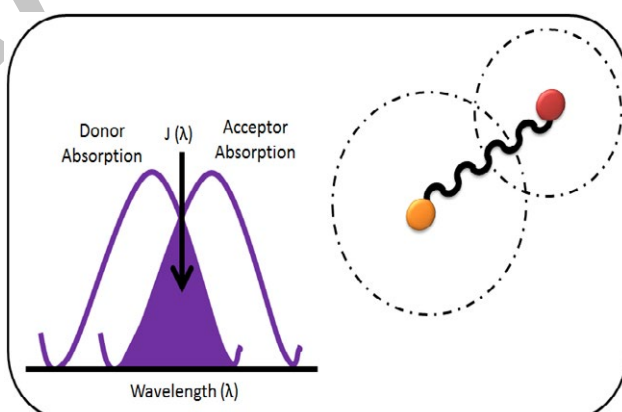


Fig. 7: Illustration of the compatibility and proximity of the donor and acceptor fluorophore pair in FRET. The co-relationship between the absorbance spectra and emission spectra of the fluorophores of the FRET pair is shown on the left and the proximity of the Förster radii of the fluorophores for a FRET pair which is acceptable for FRET is shown in the right.

proteins similar to PBP, example of this type exist, where there is an induction of a huge movement of the two subdomains with respect to each other upon ligand binding that closes the ligand-binding cleft [42, 60].

On the other hand, there can be a change in fluorescence intensity when the fluorophore directly interacts with the bound target molecule (Fig. 8b). For example, in a single stranded specific DNA biosensor, based on the single-stranded DNA-binding protein (SSB), whenever DNA binds, there is an enhancement in the fluorescence of the fluorophore (diethylaminocoumarin) (single, environment-sensitive) in the absence of a change in recognized conformation [61].

Two fluorophores

The biosensors involving two fluorophores are designed based on the principle of FRET (Fig. 8c). In such biosensors, fluorophore pairs,

particularly a FRET donor and an acceptor, are fused with the recognition element. Whenever, there is a ligand-induced change in conformation that can alter the distance or relative orientation of the fluorophores of the FRET pair, a change in FRET is observed. Compared to the single fluorophore probes, the signal provided by FRET is ratiometric, based on the ratio of the acceptor to donor emission. This enables us for the quantitative measurements even in a complex environment and the ratio is independent of biosensor concentration. However, there are limitations in a FRET based biosensor due to nonspecific labeling. But among the genetically encoded biosensors such as for Ca^{2+} , cAMP and cGMP, sugars, phosphate and ATP, FRET-based mechanisms are commonly used [40]. The other way of fluorescence emission enhancement is by breaking the stack of two identical fluorescent dyes (Fig. 8 d).

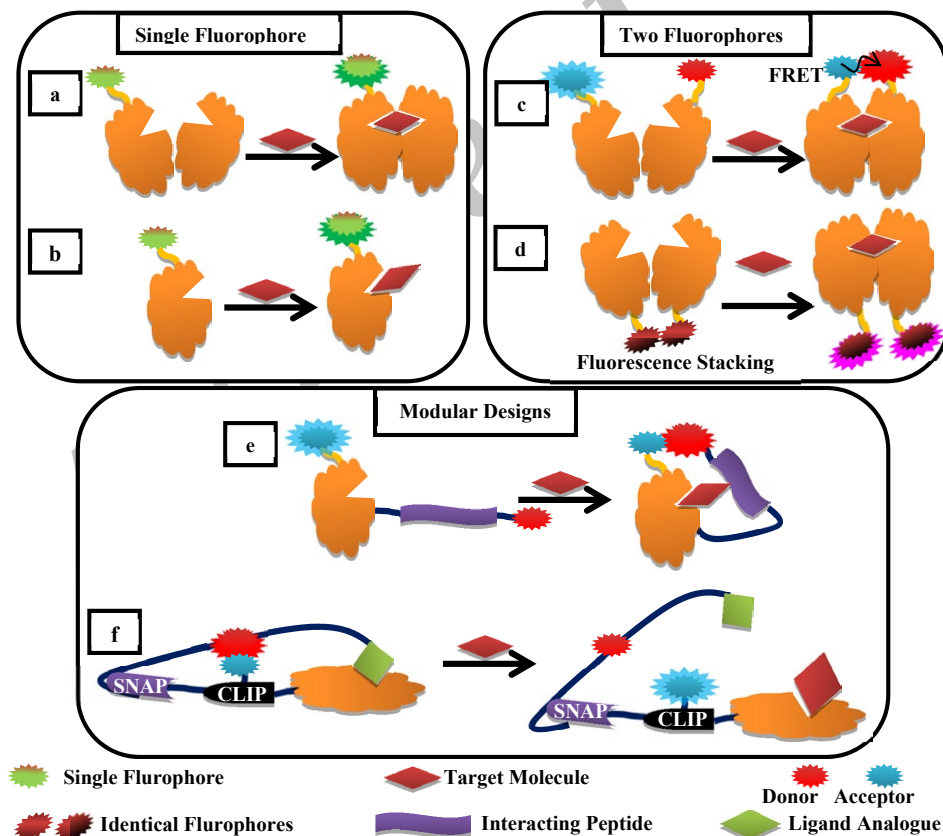


Fig. 8: Different schemes of fluorescent reagentless protein based biosensors. Single-fluorophore based biosensors: Change in conformation (a) or target interaction (b) changes the environment of fluorophore. Two-fluorophore based biosensors: In between two different fluorophores FRET is recorded (fluorescent proteins) (c), or by breaking the stack of two fluorescent dyes which are identical (d). Modular design based biosensors: a part in the merged system with the recognition element can interact with either the target bound (e) or the target-free state (f), so that when the target binds the signal is transduced.

Modular designs

In all the above type of biosensor, the recognition of the target and signal transduction both is present in a single nucleic acid or protein recognition element. In modular design, multiple transducing elements are incorporated into the same molecule to improve the mechanism of signal transduction. The scheme shown in Fig. 8e shows the domain of a peptide or protein that can bind to the recognition element, only when it is bound to a ligand. The recognition domain and the domain of interaction come in close vicinity when the target molecule binds which yields a fluorescence signal, through a FRET pair. This principle is extensively used for a cluster of genetically encoded, Ca^{2+} biosensors by using FRET pairs like BFP/GFP or CFP/YFP [62-64].

One of another example of modular biosensor design has been described in Fig-8f, where an intramolecular ligand analogue is used with the recognition domain [65]. In the absence of a target, the ligand analogue occupies the binding site of the recognition element giving the biosensor a closed conformation.

Advantages and limitations of fluorescence biosensor

The advantages and limitations of fluorescent biosensors are given in Table 1.

NANOSTRUCTURE BASED ENHANCED - FLUORESCENCE

The sample size to be detected at the cellular level is usually in the range of nanoliters or even femtolitres. For detection of such samples using conventional spectroscopy and microscopy, the concentration of the fluorophore used is extremely low thereby yielding poor fluorescence signal. This limitation opens an avenue for the development

of better fluorophores, especially with enhanced photostability. A lot of progress has been made in the last decade in the area of new fluorescent probes for biological studies like cyanine dyes, Alexa Fluors, and the large range of indicators proposed by Molecular Probes Inc. and different other companies to yield a better fluorescent signal [66]. But these fluorescent probes are not cost effective. Another way to overcome this limitation is the use of light amplification from fluorophores by coupling them to metal nanostructures, termed as Metal Enhanced Fluorescence (MEF). MEF has been extensively studied and provides a promising strategy for image contrast enhancement and improving the detection sensitivity [67]. The principle of MEF involves the localized SPR excitation in metallic nanoparticles which gives rise to the confinement of electromagnetic fields at nanoscale in proximity of the metal surface and enhances significantly the local density of the optical states. Extensive applications of these are used to study the interactions between the plasmons and the fluorophores, like fluorescence enhancement as well as the realization of a nanolaser [68-72]. The enhancement mechanisms behind the plasmon-enhanced fluorescence are of two types: excitation enhancement and emission enhancement. However, its application has been limited due to higher costs of material and fabrication and susceptibility to fluorescent quenching during certain conditions [73].

The alternative which is comparable to MEF is proposed to be the use of nanostructured zinc oxide as it is non-toxic, abundant and cost effective metal oxide exhibiting fascinating plasmonic properties [74, 75]. The capability of ZnO nanorods exhibiting strong fluorescence enhancement has been revealed by several

Table 1: The advantages, disadvantages and limitations of fluorescent biosensors.

Technique used	Advantages	Disadvantages & Limitations
Fluorescent Intensity	<ul style="list-style-type: none"> ➤ It is compatible for the fluorogenic assay. ➤ Easily miniaturized. 	<ul style="list-style-type: none"> ➤ Very less information about quality control ➤ Sensitive to the inner filter and interference by auto-fluorescence
Fluorescence Polarization /Anisotropy	<ul style="list-style-type: none"> ➤ Simple and reasonably predictive. ➤ Not sensitive to effects of inner filter. ➤ Technique is ratiometric. ➤ Quality control is improved. ➤ Even small ligands (<15KDa) are detectable. 	<ul style="list-style-type: none"> ➤ Effects of local motion. ➤ Limitations come for suitability due to dye-ligand size lifetime and change in molecular weight. ➤ Dynamic range limited. ➤ Can suffer from auto-fluorescence.
FRET	<ul style="list-style-type: none"> ➤ Simple method and reasonably predictable. ➤ Appropriate for short inter/intramolecular distance(<5 nm). ➤ A huge number of donors and acceptors are available. 	<ul style="list-style-type: none"> ➤ Requires multiple labels. ➤ Sensitive to inner-filter and interference by auto-fluorescence. ➤ To get high changes in signal it needs very short distance. between donor and acceptor ➤ Most of the dyes monitor the quenching of the donor only.

researchers; but Dorfman *et al.*, have reported the utility of ZnO at nano-scale as a promising fluorescence detection platform [76]. The capacity of randomly oriented and aligned nano-rods, nano-scaled ZnO film to increase fluorescence has been reported by researchers yielding up to a 20-fold increase in fluorescence intensity [77, 78]. Recently, a nanostructure of ZnO named, ZnO nanorod (NR) array has been used as fluorescence enhancement (FE) platform for ultrasensitive detection of biomolecules because of its capacity to detect DNA molecules, cancer biomarkers or anti IgG at a concentration as low as a few femtomolar or pico-gmL⁻¹ level [77, 79, 80]. The factors that primarily contribute to the enhancement include interfacial electron transfer, large surface area of ZnO NRs as well as their waveguide structures in which the hexagonal structures of the ZnO NRs can be considered as Fabry-Perot Resonators (FPRs) with the end faces acting as reflecting mirrors [81]. This wave-guiding property has made ZnO NR arrays suitable for its application in a variety of photonic devices, such as nanowire lasers and optical sensors [78].

Investigators have explored the waveguide modes of inorganic nanostructures both experimentally and theoretically [82-85]. The studies suggested that the fluorescence signal from the fluorophores near NRs can eventually be captured and guided by waveguide structures [86]. The intensity of local field within the proximity of NRs which is increased by the resonance of the guided fundamental mode [82-83] and the evanescent field associated with the high-order modes [84-85] can reinforce the excitation of the fluorophores near and absorbed onto the NR surface quite significantly, thereby increasing the fluorescent intensity. One of the important factors that determine the waveguide properties of Fabry-Perot resonators is the reflectance

of the reflecting mirrors. Till date the ZnO nanostructure based Fluorescence Enhancement platforms reported were fabricated on either glass slides or Si wafers [77, 79]. However, for both the substrates Fluorescence Enhancement was restricted because Si has a much higher refractive index compared to ZnO along with strong light absorption and glass is completely transparent to the visible and near-infrared (NIR) light. To overcome this difficulty, researchers have developed a ZnO nanoflower based nanobiosensor for the detection of amyloids grown on various substrates and compared the efficacy. The ThT dye which specifically detects beta amyloids was adsorbed over ZnO nanoflower grown over a nano Ag thin film coated glass surface. The model amyloid used for detection was insulin amyloids and the fluorescence detection was made by the fabricated nanobiosensor. The detection signal was nearly 10 fold higher than the signal which was obtained on bare glass. The dual effect of Ag thin film as a reflecting mirror and metal enhanced fluorescence (MEF) as well as large surface area of ZnO nanoflower petals contributed in fluorescence enhancement. The linearity of the sensor is also verified using different concentrations of the amyloid and yielded a cost effective, faster and sensitive amyloid biosensor [87].

APPLICATIONS OF FLUORESCENCE BASED BIOSENSORS IN VARIOUS DISORDERS

Nanobiosensors are an outcome of a combination of interdisciplinary technologies imparted from chemistry, nanotechnology, biology and medical science. The detection of various diseases using biosensors require high specificity of the biomarker related to the disease, a non invasive or less invasive protocol and a control to discriminate between the markers related to different diseases. The importance of

Table 2: The different nanoparticles used for targeted drug delivery and theranostics.

Structure	Size	Role in drug delivery
Magnetic nanoparticles entrapped in dendrimers	40-50 nm	Targeted drug delivery, destruction of cancer cells and imaging [112]
Cadmium sulfide quantum dots	100 nm	Reduces <i>E.coli</i> growth by delaying the lag phase [113]
Liposomal nanoformulation of Rhodamine 6G	100-120 nm	Multidrug resistant gram negative bacteria inactivation for sewage treatment [114]
Liposomal nanoformulation of 5 fluorouracil and doxorubicin	100-130 nm	Efficient reduction in ascitic tumor size compared to bare drug [115]
Biologically synthesized silver nanoparticle	10-40 nm	Efficiently kills HeLa cells compared to its chemical counterpart [116]
Engineered iron oxide nanoparticles	100-130 nm	Targeted drug delivery, sorting of cancer cells and imaging [117]
Silver nanoparticles	34 nm	DLA tumour cell death [118]

biosensors for disease detection is to rapidly know about the health status, the disease onset and a quick intervention for the affected individual. Fluorescence based nanobiosensors are very much useful and are developed recently for several medical applications. Biosensors for the detection of cardiovascular diseases, diabetes and cancer are very widely explored [88]. Interstitial measurement of glucose in subcutaneous fat based glucose sensors are also developed by researchers [89]. There are some disadvantages for interruption with low molecular weight substances that diffuse through the polyurethane layer of the sensor outer membrane. This problem can be overcome by coupling microdialysis or ultracentrifugation with the glucose sensor. The recent glucose sensor is based on electrochemical transducer where the sensors are inserted in between the interstitial space through invasive method [90]. In a non-invasive approach to detect glucose; infra red spectrometry, glucose level in excreted body fluids (sweat, tears, saliva and urine), enzyme based electrodes, microcalorimetry, optical sensors, extraction of glucose directly from the skin mediated by sonophoresis and iontophoresis are also deployed [91-93]. Although there are some anomalies found between the glucose level in the excreted fluids and that in the blood because there are differential effect on glucose level in urine due to diet and exercise [94]. Glucose oxidase based several biosensors have been developed so far immobilized over nano platform [95, 96]. There are also many fluorescence based biosensors which can continuously monitor the blood glucose of diabetic patients [97].

At ideal conditions, any fluorescent probe should be not only stable and bright but also easily permeable to all cell types, non toxic, non-immunogenic and does not affect the individual. Practically these conditions are difficult to achieve using a single fluorophore based biosensor to be delivered into the cell. Engineered biosensors formulated with nanocarriers are used in such instances for targeted drug delivery, prolonged circulation in the system and long retention in the tumors. Fluorescent biosensors are now becoming a promising field for personalized medicine as it can detect various pathogen based diseases, viral infections, cancer etc. by human eye; whereas biopsy extraction is needed for *ex vivo* detection of such disorders [98]. An 800nm organic heptamethine indocyanin green

(ICG) based dye is a near infrared probe used for visualizing the angiogenic structures using albumin as a nanocarrier by highlighting the sentinel lymph nodes [99]. Tumour imaging by targeting cell surface receptors like the integrins and GLUT receptors conjugated with near infrared probes like cyclic arginine-glycine-aspartate (RGD) based peptide (c(RGDyK)) and 2-deoxyglucose respectively [100, 101] is also been achieved. To visualize the peripheral nerves during surgery and avoiding substantial damage to the neurons, fluorescent peptide tracers are being used [102]. The cancer microenvironment is also exploited for targeting the probe molecules specifically to the cancer cells. The metalloproteases secreted by the cancer cells cleave the target sequences which normally cannot enter the cells, thereby making a cell penetrating sequence that enters the cancer cells as 'smart probes' [103-105]. The acidic microenvironment of cancer tumours are applied for theranostics with pH-activable fluorescent moieties attached with cancer antibodies [106] and pH-activable cell penetrating peptides conjugated to a fluorescent probe (PHLIPS) [107]. Fluorescent probes which is quenched under normal conditions but are activated near cancer tumors due to the release of tumor specific gamma glutamyl transferases (GGT) is also used to visualize localized cancer, surface lesions and metastasis [108]. The detection of cancer utilizing the difference in the level of biomarkers like Bcr-Abl kinase activity in chronic myeloid leukemia is employed by Forster resonance energy transfer (FRET) technology. FRET based biosensor was taken for assessing the drug efficacy and HTS/HCS screening [109, 110]. The different types of nanomaterials and their dimensions are very important for their use in cancer research [111]. The characteristics of nanoparticles used for drug delivery and theranostics are as follows:

Carbon nano tubes (CNTs) enable electron transfer, highly stable and is a strong nanomaterial used for detection of cancer biomarkers like prostate specific antigen (PSA), osteopontin, phosphoprotein, miRNA 21 etc. for prostate cancer, carcinoembryonic antigen for cervical cancer, carcinoma antigen -125 for ovarian cancer and other disease biomarkers [119]. The single walled carbon nano tube (SWCNT), multi-walled carbon nano tube (MWCNT), graphene sheets are used to anchor the detection molecules and fabricate the sensor.

Apart from cancer, cardiovascular disease (CVD) is another threat to human mankind. There are many CVD biomarkers that can be detected using fluorescence based biosensors [120]. CRP, Mb and cTnI can be detected through a microfluidic based immunosensor [121]. Cytokines like TNF- α , IL-1, IL-6 and IL-8 related to CVD can be detected using sandwich ELISA coupled with fluorescence microsphere immunoarray platform leading to point of care diagnostics [122]. Fluorescence dye labeled core shell silica nanoparticle based biosensor using sandwich fluoroimmunoassay for the detection of IL-6 has also been developed [123]. Enhancement of fluorescence signal using nanostructures for the detection of TNF- α was achieved by photonic crystals attached with fluorescent quantum dots [124]. Other developments in CVD nanobiosensors include aptamer based sandwich assay for CRP detection [125], CRP detection without using any antibody [126], antibody tagged fluoro microbead chip attached with microchannels for the detection of cTn1 [127] based on gold functional surfaces to conduct five tests simultaneously.

Graphene based biosensors have gained huge interest in the detection of various disease because of their layered structure, large specific area, electronic transportation properties, ease in functionalization, good adsorption capability, biocompatibility and resistance to many oxidizing agents. It acts as an excellent biosensing platform for the detection of disease biomarkers based on fluorescence as an output signal like specific proteins, immunoassay for pathogen detection, thrombin detection which is responsible for tumour growth, metastasis and angiogenesis [128]. Detection of pathogens using chimeric fluorescent protein bullets have made the rapid detection of *E. coli*, *E. Faecalis* and *clostridium perfringens* in drinking water [129]. Nanobiosensors for the detection of disease biomarkers related to neurological disorders (ND) like mad cow disease etc. has opened an avenue for the early treatment strategy of the individuals suffering from ND [130]. Ligand binding domain (LBD) based biosensors for detection of digoxin and progesterone is developed by fusing LBD with either a fluorescent protein or a transcriptional activator and is stabilized by mutation. This resultant fused product only gets accumulated in cells which contain the target ligand [131]. Disease detection at the level of single fluorescent molecule with

the help of single molecule microscopy and spectroscopy is also been explored for the past decade based on nanobiosensors. *In vitro* and *in vivo* fluorescent labeling of the target biomolecule is done with high sensitivity and is detected through fluorescence microscopy or fluorescence spectrometry. DNA, proteins, miRNA, intact cells, enzymes that are specific as disease biomarkers are detected by single molecule counting [132]. Heat inactivated 16 kDa heat shock protein of *Mycobacterium Tuberculosis* was detected using different llama antibodies by researchers [133, 134] further leading to biosensors for the detection of tuberculosis [135]. Kidney diseases are related to higher urea concentration in the urine of the affected individual. Estimation of pH and urea in the urine samples was estimated in urine by using polymeric fluorophore and enzyme based biosensors with high accuracy and sensitivity without any interference. The combination of fluorescent dye and polymer used was FITC-dextran/ and FITC-dextran/RuBpy for pH detection from 4-8 and urea detection up to a limit of 0-50 mM [136]. There are many nanomaterials used for the designing of biosensors for the diagnosis of various diseases [137] and other applications [138].

CONCLUSION

Biosensors have versatile applications in the field of medicine, engineering, biomedical engineering, toxicology, ecotoxicology etc. and their miniaturization becomes useful for a cost effective, rapid and sensitive detection. Nanobiosensors have now captured the field of biosensors as the different nanostructures at ultra low size exhibit novel properties which are not shown by their bulk counterparts. Various transducing mechanisms are discussed in this review which opens an avenue for the manipulation of these nanostructures to detect the signals obtained after transduction. Fluorescence is a unique principle which can be detected as an outcome of a bioanalyte and its recognition element combination with high specificity. The nanobiosensors can be tagged with different fluorophores to enhance the fluorescence signal utilizing the nanostructure characteristics. This review enlightens the basics of different types of biosensors and their applications giving emphasis on fluorescent biosensors.

The applications of nanobiosensors also

become important apart from its designing. Among the different applications, this review discusses about the applications of biosensors in disease diagnosis. The different disease detection like, diabetes, CVD, neurodegenerative disorders, cancer, pathogenic infections, tuberculosis, kidney disorders etc. are been elaborately discussed. The different biomarkers for the detection of these diseases based on nanobiosensors and fluorescence based biosensors are also reviewed.

ACKNOWLEDGEMENT

The authors are grateful to Chettinad Academy of Research and Education for their infrastructural support.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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