Chapter 1

Introduction

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1.1. Biosensor

In 1992, International Union of Pure and Applied Chemistry (IUPAC) defined biosensor as *'an analytical device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals'* [1].

Typically, it is made by integrating a biological element with a physicochemical component, to provide quantitative analytical information of a biological response in terms of an electrical signal. The physicochemical component can be a transducer or the detector element to detect an analyte by converting the biological response into an appropriate signal after a specific interaction. The signal generated is converted into a measurable electrical parameter such as a current or voltage through a suitable transducer, which is processed and displayed in read-out unit. Biosensors consist of three parts: a component that recognizes the analyte and produces a signal, a signal transducer, and a reader device [2]. A typical biosensing device is illustrated in Figure 1.1.

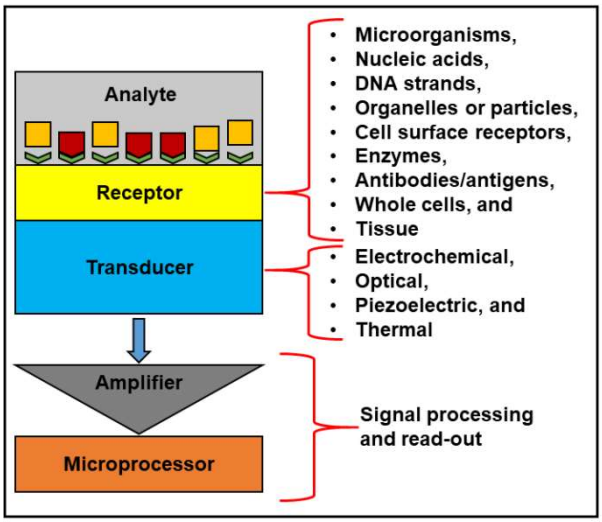

Figure 1.1: Illustration of biosensor.

In 1962, Leland C. Clark and his group carried out his first experiment to detect oxygen using platinum (Pt) electrodes [3], which is marked as the origin of the biosensor. In the reported work, they trapped glucose oxidase (GOD) enzyme to surface of Pt electrode through a piece of dialysis membrane. He explained the hypothesis that the concentration of oxygen is modified, when glucose comes into contact with the enzyme immobilized Pt electrode due to the enzymatic activity (Figure 1.2). The oxygen consumption is more, when the glucose concentration is high, and vice versa. This indicated that increase or decrease in hydrogen peroxide and oxygen can be used to measure glucose concentration [4]. Since then, the biosensors have been widely explored and extensively utilized in pharmaceutical, environment, food and medical field [5].

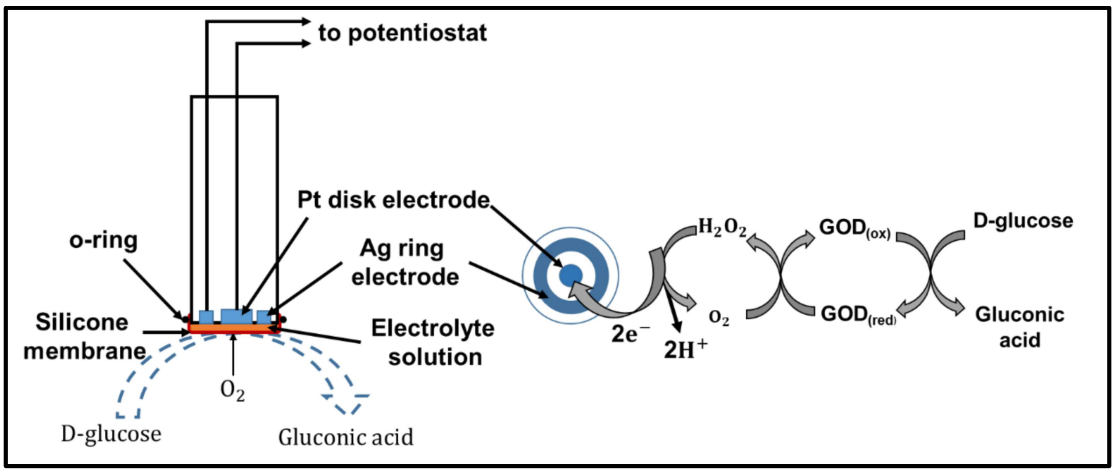

Figure 1.2: The biosensor concept proposed by L.C Clark [6].

Amongst the various classes of biosensors developed, cell-based biosensors find potential application in comprehending fundamental biological processes with reference to a whole cell or a group of cells [7].

1.2. Cell-based biosensor

Cell is the basic building block of all organisms, which governs all functions of the complex heterogeneous biological system. Any abnormality in a single cell affects the entire characteristic behaviour of tissues, organs and organism [8]. Therefore, it is necessary to understand the complex biological system at their cellular level appropriately to prevent and treat any complex diseases [9].

Traditional (label-based) techniques used to monitor cellular behaviour include fluorescence microscopy, flow cytometry and single endpoint assays such as WST-1 assay, XTT, MTT assay and BrdU assay [10]. The traditional methods of monitoring cellular transformation are either qualitative or semi-quantitative with extensive labour-intensive protocols and expensive equipment. Moreover, the label based techniques (cell-based assays (CBA)) greatly loses the consistency and reproducibility of the experiments, and they also disturb the normal physiological state of the cells resulting to destruction [11]. Therefore, to precisely monitor the cellular behaviours to external stimuli, a real-time, non-invasive and label-free method is desirable.

The cell-based biosensors (CBBs) directly use the living cells, which offer exceptional opportunities for bio-detection and drug discovery. The biochemical effect of the direct spatial contact of living cells is converted into quantitative electrical signals by sensors or transducers, thus bridging the gap between electronics and biology. CBBs are endowed with certain advantages compared to molecule-based approaches such as non-invasive long-term recordings, label-free detection, reduced response time, etc [12] Hence, CBBs are extensively used to determine phenotypic changes of cells especially cell growth and apoptosis, wound healing, and found their potential application in *in vitro* toxicological studies, drug screening, and etc [13]–[15]. A cell-based biosensor is an analytical device that uses biological component (i.e., living cells as an analyte) as an

active element with a physicochemical component (i.e., transducer or the detecting or sensing element) to monitor the change in the cell physiology electronically in an *in vitro* culture [6], [16]. The transducer is connected to electronic circuitry to process and display the read-outs. The signal comes to the transducer due to various phenomena of the cell such as change in the ion transportation, metabolic pathway, membrane potential, barrier resistance, and morphology of the cells [6], [17]–[20]. A typical cell-based biosensing device is illustrated in Figure 1.3**.**

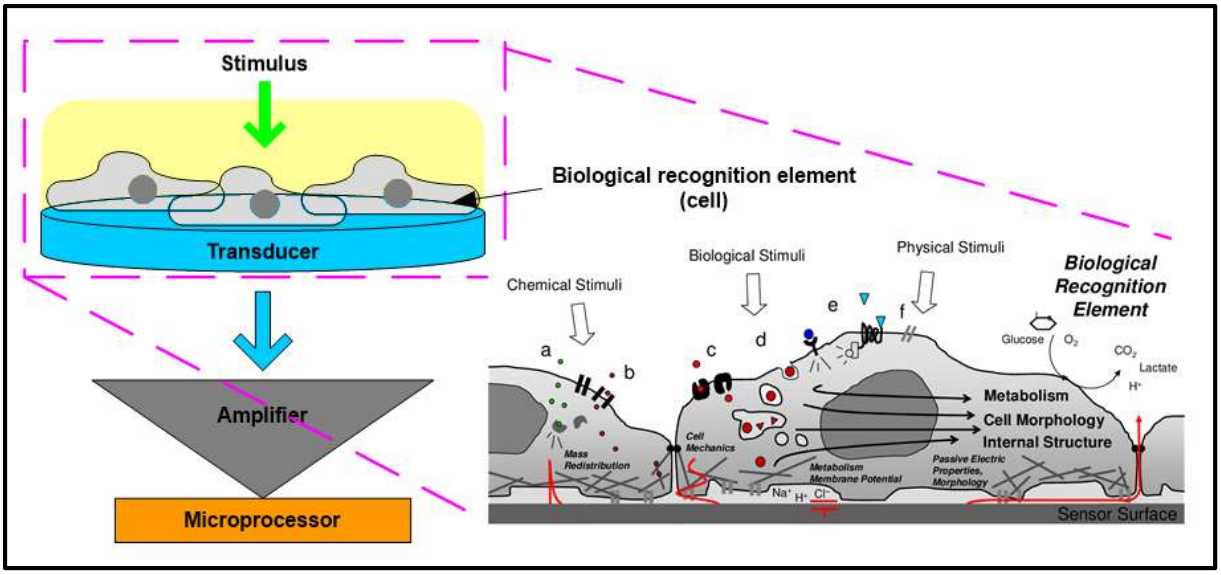

Figure 1.3: Cell-based biosensing device and zoomed images showing the influence of various stimuli namely chemical, biological, and physical on the cells to cause cellular functions and the changes is being converted into a measurable electric signal by the transducer element. The image is adapted from [21] with some modifications.

Although CBB cannot mimic a whole organism's total response upon a stimulus, but they provide a native biological environment at a cellular level that mimic the natural physiological microenvironment.

1.3. Classification of cell-based biosensor

Based on the biosensing transducing element, CBB can be broadly classified into three types; such as electrochemical, optical, and piezoelectric transduction.

1.3.1. Electrochemical biosensor for CBB

The electrochemical biosensor detects the signal as a change in voltage or current, in real-time by coupling a chemically sensitive layer (the recognition element) [22]. The electrochemical biosensor built up of either a two or three-electrode system as shown in Figure 1.4.

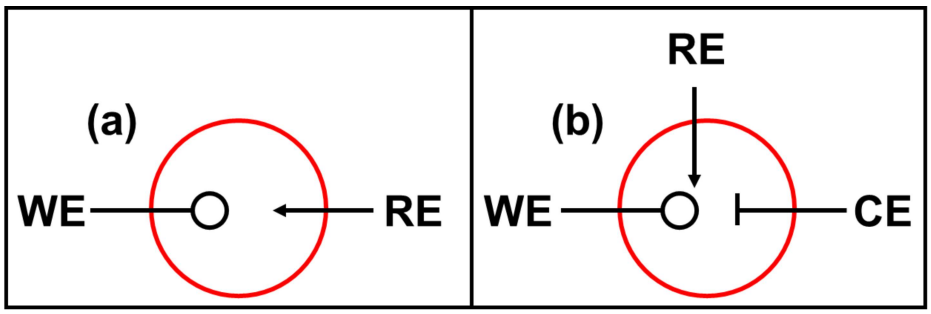

Figure 1.4: Electrodes of electrochemical biosensor (a) two-electrode system, and (b) three-electrode system.

The two-electrode system has a working electrode (WE) with a reference electrode (RE), while the three-electrode system is usually housed with a counter electrode (CE) in addition to a two-electrode system configuration [23]. The stability of the system is determined by choice of electrode material used. Platinum (Pt), gold (Au), graphite, and silicon-based materials are the most commonly preferred bio-recognition electrode, but it also depends on the analyte of interest, to divulge the changes in potential and current precisely [24].

The change in cellular biochemical parameters, including concentrations of inorganic ions (such as H^+, K^+, Na^+, Ca^{2+}, Cl^-, etc.), morphology, membrane potentials and redox potentials can be detected electrochemically [25]. These changes are observed as a

redox (reduction-oxidation) reactions due to changes in ionic concentration in the living cell [26]. The electrochemical sensors for CBB usually involve with the use of a pair of electrodes, namely the working (sensing) electrode, and the reference electrode [27], to divulge the changes in various cellular functions such as cell adhesion, spreading, proliferation and differentiation. Depending on the principle of detection employed, the electrochemical biosensors can be further grouped into amperometric, potentiometric, and impedance. However, electrical cell-impedance sensing (ECIS), and potentiometric transduction techniques have been widely used to study the cellular changes using electrochemical principle [28].

1.3.1.1. Amperometric biosensor

An amperometric biosensor measures a change in electric current resulting from either oxidation or reduction reaction of an electroactive biological element to provide quantitative information of the analyte [29]. The most commonly used sensor is the Clark-type amperometric oxygen sensor, wherein the current produced is directly proportional to the concentration of the target analyte. Figure 1.2 shows schematic representation of Clark-type amperometric oxygen sensor. The analyte solution of the sensor is separated from an electrochemical cell which is immersed in the KCl electrolytic solution. The diffusion of oxygen molecules across the membrane results into a decrease in the applied constant potential across the Pt working electrode, which increases the measured current. Hence, a decrease in current is directly related to dissolve oxygen (DO) concentration in that solution. This method also determines the biological oxygen demand (BOD) by monitoring the blood and tissue oxygen tensions [30], [31].

1.3.1.2. Potentiometric biosensor

The potentiometric biosensor measures the potential difference across the working electrode (WE) with respect to the reference electrode (RE), which uses ion-selective membrane/material as an interface to transduce the biological reaction into an electrical signal. The response due to biochemical activity is directly correlated with the concentration of analyte to the RE. Further the use of a highly stable and accurate RE, make potentiometric biosensors more sensitivity and selectivity. The schematic layout of different types of potentiometric biosensors namely ion-selective field-effect transistor (ISFET), electrolytic-insulator-semiconductor (EIS) capacitive sensor, and light-addressable potentiometric device are shown in Figure 1.5.

All the potentiometric sensors have the same EIS structure, wherein the interfacing layer is an insulating material, which serves as the sensing layer. Therefore, the insulating material should be in contact with the electrolytic solution [32]. Moreover, the structure of the ISFET, EIS capacitive sensor and the LAPS devices are similar to that of the metal-insulator-semiconductor (MIS) diode, which has a metal electrode on the insulator surface and an ohmic contact on the back surface of the semiconducting substrate [33], [34].

To construct an ISFETs, the metal gate of metal-insulator-semiconductor/metal-metal oxide-semiconductor field-effect transistors (MISFETs/MOSFETs) has to be replaced by a reference electrode, and an electrolyte solution made to stay on the top of gate insulating layer [35] as shown in Figure 1.5a. The ISFET sensor is in-contact with the electrolytic solution to be measured, a double layer is formed on the ion-sensitive gate dielectric membrane and the potential at the surface varies with the pH or the concentration of the target species (ions or molecules) in the solution. In response to the

change of the potential at the sensing surface, the carrier distribution in the semiconductor is modified by the electric field effect [32]. The field-effect changes the channel's width under the gate insulator and, therefore, the conductance between the source and drain electrodes. Therefore, the change in gate voltage directly correlates with the ionic concentration of the analyte used [35].

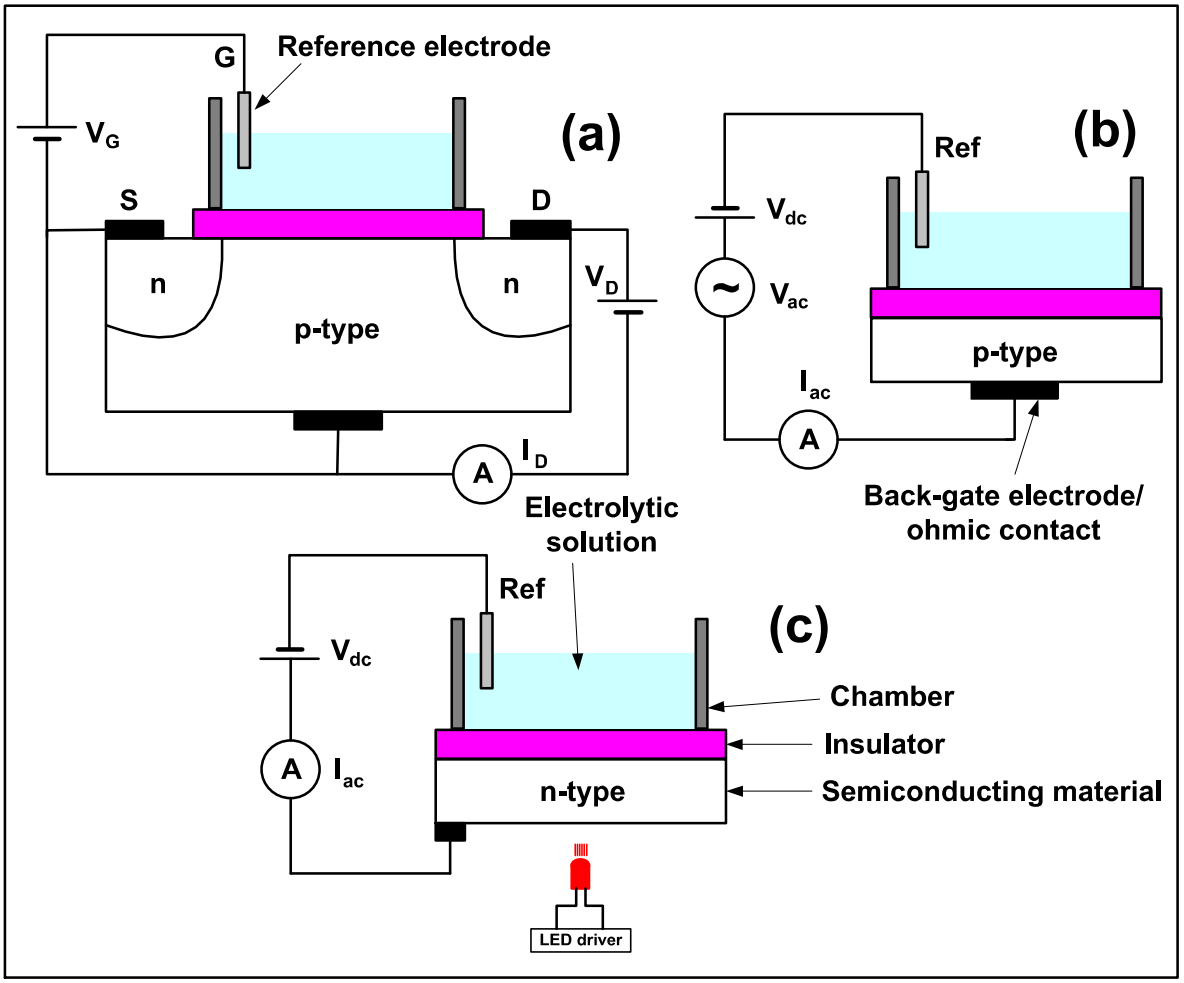

Figure 1.5: Types of potentiometric sensors used (a) ISFET, (b) EIS capacitive sensor, and (c) LAPS devices.

The EIS capacitive sensor has only two terminals (as shown in Figure 1.5b) instead of the four terminals (as in other FET devices), and the operation of working is similar to that of the metal–oxide–semiconductor (MOS) capacitor. When the EIS sensor is in-

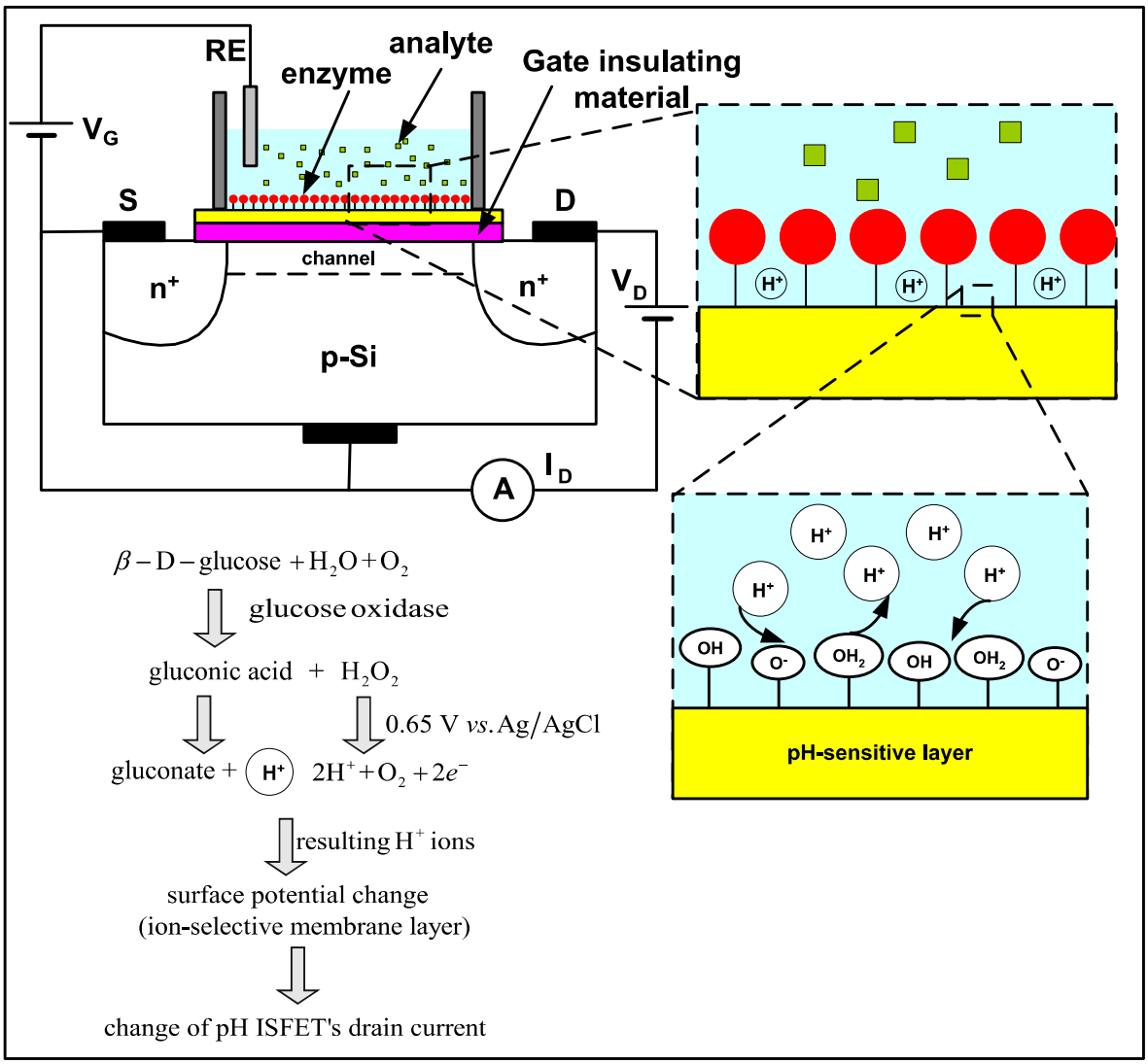

Figure 1.6: EnFET's structure with its principle of operation.

contact with the electrolytic solution to be measured, a double layer is formed on the ion-sensitive gate dielectric membrane's surface and the potential at the surface varies with the pH or the concentration of the target species (ions or molecules) in the solution. In response to the change of the potential at the sensing surface, the carrier distribution in the semiconductor is modified by the electric field effect [32]. The field-effect changes the width and the capacitance of the depletion layer at the insulator–semiconductor interface induced by a negative dc bias voltage but it is superposed by applying a small amplitude of ac voltage, to measure the change in the EIS sensor's

capacitance. Further, the EIS device's sensitivity is typically associated with the H^+ ions adsorption capability of the ion-sensitive membrane layer used. Therefore, gate dielectric material choice is crucial to obtain high sensitivities up to 59.6 mV/pH Nernst value [36].

In LAPS, the field-effect changes the width and capacitance of the depletion layer at the insulator–semiconductor interface induced by a negative dc bias voltage but the induced negative voltage is superposed by using the modulating light in order to illuminate the semiconductor substrate (instead of applying the ac voltage). The response is measured as the change in amplitude of the ac photocurrent as output. A bias voltage is applied to create a depletion layer at the insulator-semiconductor interface junction. It triggers an interaction between sensitive surfaces with the specific analyte, and generates a new potential, causing the width of the depletion layer to get changed at that position with respect to the modulated light source as shown in Figure 1.5c. Hence, measuring this locally generated photocurrent allows us to determine the chemical interactions of that particular region on the sensor surface [37].

The capability of ISFET devices is not limited to detect only H^+ ions. However, by introducing additional or modifying the ion-sensitive layer, it is possible to determine other ionic species like Na^+, K^+, Ca^{2+}, and Cl^- (which are related to cellular metabolic activity) and various other metal ions such as Ag^+, Cd^{2+}, Cu^{2+}, Hg^{2+}, Pb^{2+}, etc. Hence, using an appropriate ion-selective layer in the ISFET, it is possible to determine the adherent cell's physiological event. Typically, adherent cells' response to the external stimuli can be studied by measuring the acidification of living cells and also by the change in the concentration of other inorganic ions. Similarly, by functionalizing enzyme on an ISFETs, the typical structure of an ISFETs is simply modified to detect the analyte concentration, hence, the ISFET is renamed as enzymatic ISFET (EnFET) as

shown in Figure 1.6. The surface potential is determined by measuring the change in drain current (I_D) between source and drain at the interface region, when the gate potential (V_G) is kept constant. Any change in the ionic concentration is due to enzyme-analyte interaction that influenced the surface potential to change, causing a change in source-drain (I_{DS}) current. Therefore, the gate voltage change directly correlates with the concentration of the analyte use [28].

1.3.1.3. Impedimetric biosensor

Impedimetric biosensor or bio-impedance spectroscopy measures the change in current by applying a small amplitude sinusoidal excitation signal to an electrode immersed into an electrolyte, which is used to describe the dielectric (resistive and capacitive) properties, by investigating the bulk and interfacial electrical properties of electrode systems over the function of frequency [38]. The frequency-domain response divulges the physicochemical changes that occur when an analyte binds to a bioreceptor immobilized on an electrode. It provides information about mechanisms on the surface, i.e., the charge transfer processes from the solution to the electrode surface, solution resistance, diffusion transport of species to and from the bulk solution, and double layer capacitance formation [39], [40]. In bioengineering, based on the source of production, bio-impedance can be classified into active and passive type.

1. If the biological medium itself acts as a source of signal generation in determining its bioelectrical properties, then this type of response is termed as "*Active type bio-impedance*" [41]. Example, Biological signal (EMG, EOG, ECG, EEG, etc.) and source of signal generation is "*ionic activities of the cells*" as shown in Figure 1.7a [42], [43].

2. If an external voltage controlled current source (VCCS) is used to excite and determine the bioelectrical properties such as electrical resistivity, conductivity, and permittivity of any biological cells/tissues, then this type of response is termed as "*Passive type bio-impedance*" [44].

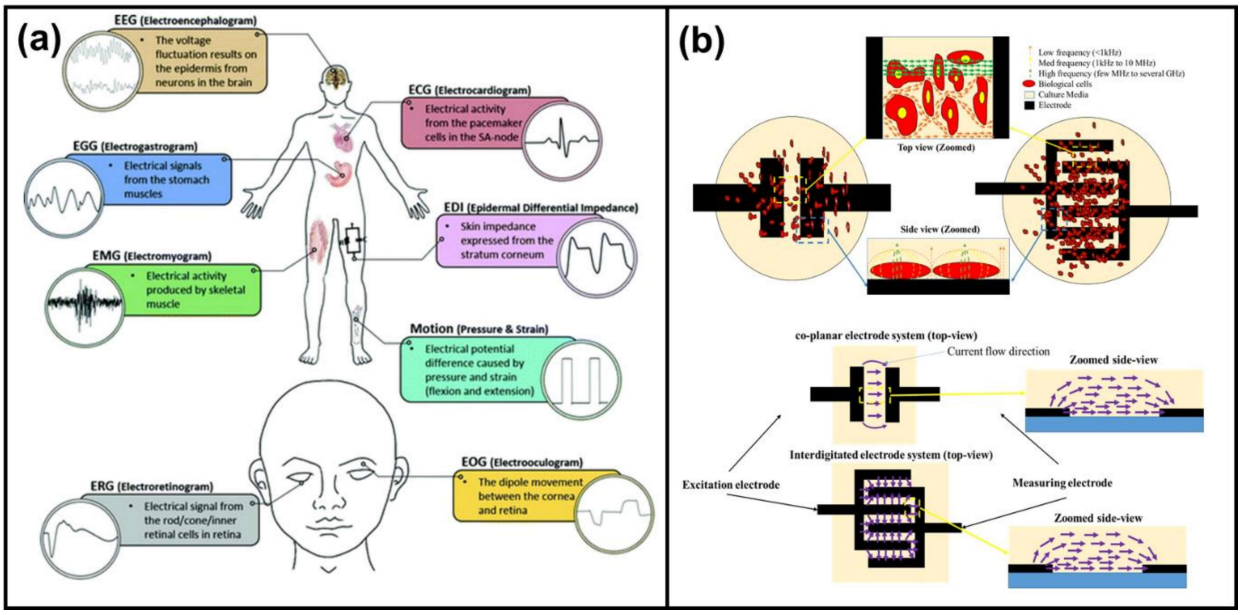

Figure 1.7: Bio-impedance technique based on the type; (a) active, and (b) passive type bio-impedance. (re-used with slight modifications with permission from Springer Nature copyright [43]).

Generally, two types of electrochemical impedance processes take place at electrode namely Faradaic, and non-Faradaic processes. Faradaic processes involve the use of an electrochemical redox reaction through which involves transfer of charges (i.e., electrons or ions) across the electrode-electrolyte interface obeying faraday's first law to govern these charge transfer. While non-Faradaic processes also occur at the electrode-electrolyte interface, but they do not involve any transfer of charge across the interface. It adsorb/desorb the ions and molecules on the electrode surface, which alters the structure of the electrode-electrolytic interface, changes the interfacial resistance to transfer the charge and therefore changes the potential or solution composition.

Although the transfer of charge does not occur, externally current is used to flow (at least transiently) across the electrode area or solution composition to change [45].

Non-faradaic bio-impedance is a well-known technique used to determine the physiological condition of any biological medium by measuring the electrochemical interactions between intracellular and extracellular cell-membrane of the system [46]. The electrical impedance of any two terminal electrode system is the measure of impedance offered to resist the flow of an alternating current through that medium as shown in Figure 1.7b.

Many biological parameters and processes can be sensed and monitored using its impedance as marker, with the advantage of being a non-destructive, label-free method to provide fundamental information about the activity and metabolic state of a cell using microelectrode based cell impedance sensor [47], [48]. Among impedance spectroscopy (IS) techniques, electrical cell-substrate impedance spectroscopy (ECIS), based on two-electrode setups, allows the measure of the electrical properties of the biological cells and provide us an insight into its physiological, morphological and pathological conditions, depending on the frequency of the electrical signal applied [49]. The investigation of the dynamic behaviour of mammalian cells including attachment, proliferation and spreading on a flat surface is first reported by Giaever and Keese 1984 that relies on measuring the change in electrical impedance by applying a constant current across the active sensing area, where the cells are allowed to form a cell monolayer after cell attachment as shown in Figure 1.8. ECIS method seems to be a promising tool that presents a sensitive and continuous monitoring system of cellular parameters that vary along the entire life span of adherent cells in an *in vitro* culture [50] as illustrated in Figure 1.9. In addition, impedance analysis based system provides a quantitative information about the cellular parameters such as cell attachment,

proliferation, cellular micromotion, migration, cell differentiation, and apoptosis as shown in Figure 1.8 [26].

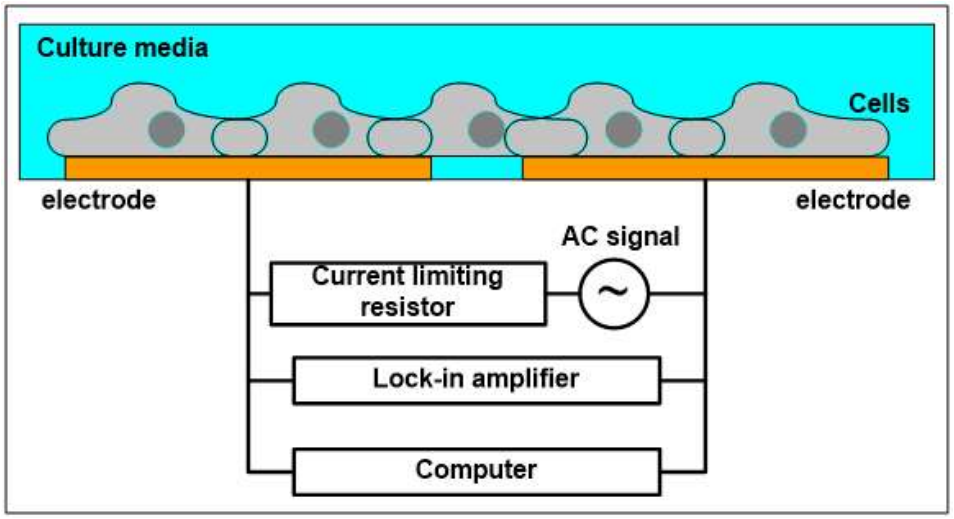

Figure 1.8: ECIS system having a pair of co-planar electrode, signal generator, current limiting resistor, and lock-in amplifier.

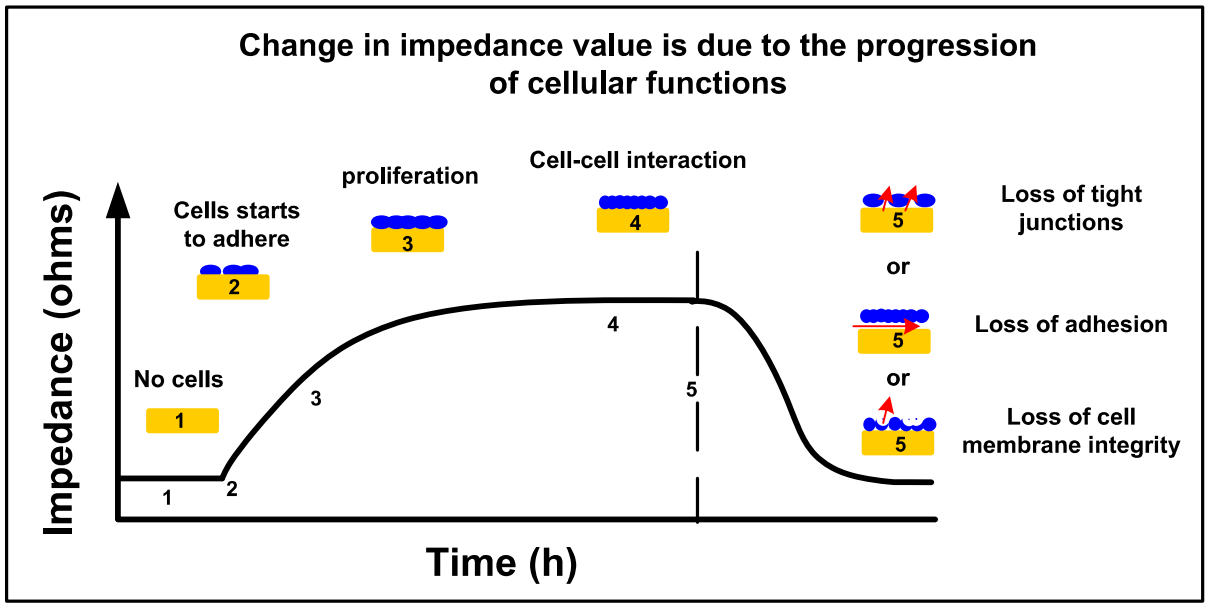

Figure 1.9: Adherent cells growing on an electrode system with its corresponding change in impedance value due to progression of cellular functions; where (1) when no cells is inoculated; an increase in the impedance value is due to (2) cells starts to adhere on the surface, (3) cellular proliferation, (4) cell-cell interaction on the surface; and decrease in the impedance value due to (5) loss of tight junction or adhesion or cell membrane integrity.

1.3.2. Optical biosensor

Optical biosensors utilize the interaction of optical fields (i.e., the electric field) with the analyte to detect a specific bio-species of interest and dynamically monitors the biomolecular interactions by integrating the biorecognition element with a transducer unit [51], [52], the emitted optical signal is directly proportional to that of the analyte concentration. Optical biosensor can perform as a label-free and label-based manner. Label-free optical biosensor involves the interaction of the analyte with the transducer for signal detection while label-based optical biosensor requires specific molecule to label the analyte, and the optical signal generated is measured by colorimetric, fluorescent or luminescent methods [53].

Colorimetric biosensor uses the resulting coloured reaction product (resulted from either heat absorption or heat production phenomena) to detect a particular analyte, which can be visualized *via* the naked eyes or through an optical detector [54], [55].

Reagent-free fluorescent biosensor monitors a particular analyte present in a complex mixture without using any additional reagent. Therefore, to monitor a fluorescent molecule readily immobilized on that platform. It reacts and interacts with the target analyte by which a change in its fluorescence properties is observed [53], [55].

Electrogenerated chemiluminescence (or) electrochemiluminescence (ECL) is, in general, the light emission generated from molecular species i.e., luminophores *via* an electron transfer process [56]. The ECL is triggered by an electrochemical reaction of the luminophores on an electrode surface, when a potential is applied onto an electrode [57]–[59].

Besides this, **transmission light microscopic technique** is considered to be a crucial non-invasive tool, which is generally based on the visual evaluation of the cell

morphology. Additional well-established methods also exist to characterize the cell based on the optical contrast-enhancing technique, such as phase-contrast microscopy, digital interference contrast (DIC) microscopy, and digital holographic microscopy (DHM).

Almost all other optical biosensors for cell study makes use of refractive index (RI) changes at the sensor surface to characterize the cells, which is due to cell-substrate interactions and surface binding phenomena. The RI-based devices, uses the light coupled at the sensor/liquid interface to analyze the change by determining the extent of modulation in RI that affects the properties of the light reflected. Various types of RI-based signal transduction employed in CBAs are interferometry, ring resonators, waveguides, resonant mirrors, photonic crystals, surface plasmon resonance (SPR), and resonant waveguide grating (RWG). Amongst these, SPR and RWG are commonly used. In contrast, luminescence-based optical chemical sensing (OCS) is an another sensor normally used for analyzing the cells. The luminescent probe is immobilized on a planar sensor matrix, and the measurement is based on the intensity of light emitted due to the chemical reaction in the receptor system.

1.3.2.1. Surface plasmon resonance (SPR)

SPR relies on employing a longitudinal electron density oscillation, reflecting the refractive index (RI) in the evanescent field at the interface between a metal and a biological recognition element (cell) immobilized, to probe interactions between an analyte in solution and a biological recognition element [60]–[62]. Therefore, the change in resonance angle (RA) of SPR is proportional to the density of biological molecules in the evanescent field (<500 nm) at the interface. Wherein, most of the incident photons are either absorbed or scattered at the interface, so that a consequent

reflected light gets attenuated greatly. The resonance wavelength and angle of incidence depend upon the permittivity of the interface [60]. Hence, the SPR is able to detect molecular and cell-substrate interactions and structural changes of adherent cells on a surface gold film in a real-time manner without any labelling as shown in Figure 1.10 [61]. SPR is also referred to as dynamic mass redistribution (DMR). The SPR/DMR sensors are generally used to monitor the time-resolved change that happens due to structural and morphological changes during cell adhesion and proliferation processes and also relies on the extra- or intracellular stimuli. Based on this phenomenon, SPR is now being integrated with a microscopic imaging technique to investigate cell-substrate interaction [45].

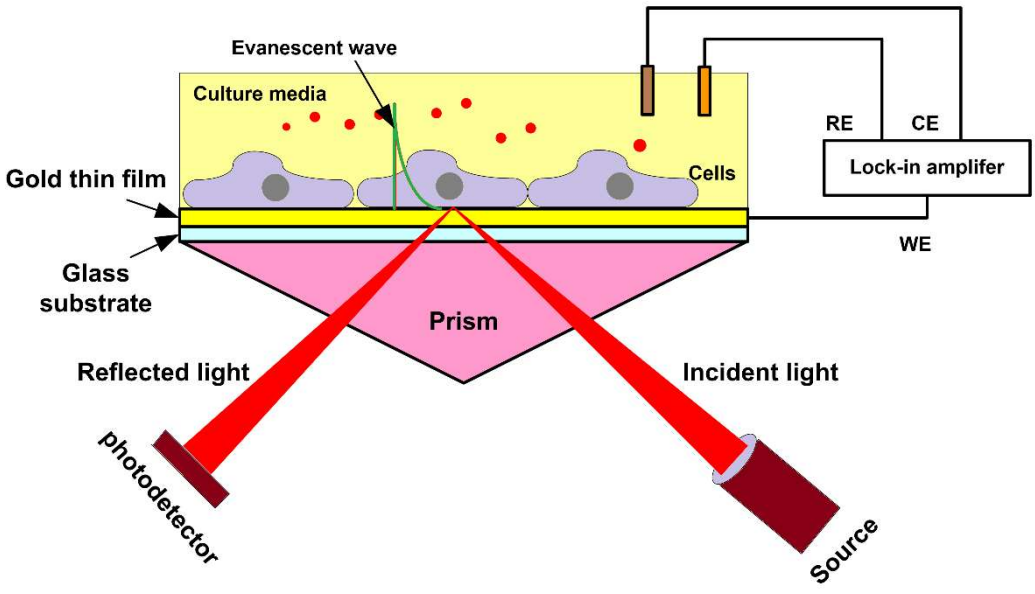

Figure 1.10: Surface plasmon resonance (SPR) in Kretschmann configuration for CBB.

1.3.2.2. Resonant waveguide grating (RWG)

Resonant waveguide grating (RWG) biosensor employs a nano-grating waveguide sensor to measure changes in the refractive index of light that interacts with a surface to transforms a cellular response into an integrative kinetic response, based on stimulus-induced dynamic mass redistribution in live cells as shown in Figure 1.11 [63], [64].

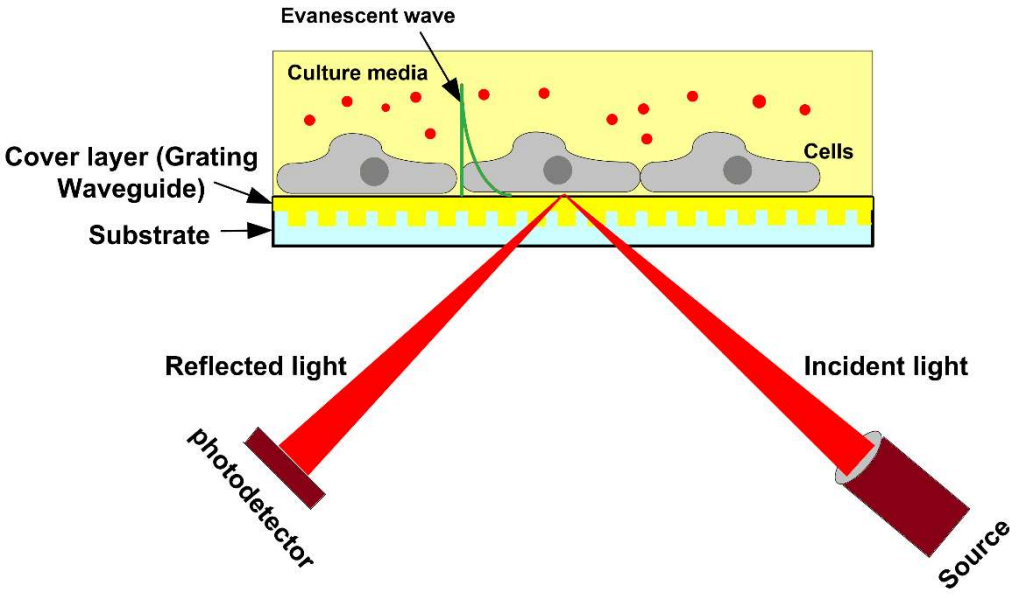

Figure 1.11: Resonant waveguide grating (RWG) for CBB.

1.3.2.3. Luminescence-based optical chemical sensing (OCS)

Luminescence-based optical chemical sensor (OCS) is used directly to measure the change in an intrinsic optical property occurred due to a chemical reaction in the receptor system. Further, it is possible to use the device for indirectly measuring the chemo-sensitive change of optical quantities due to the metabolic or physiologically relevant species in the CBAs *via* a probing method. The fluorescence-based probe owns higher sensitivity in response to a change. The probe is immobilized on a planar sensor matrix to prevent direct contact with cells. A change in the intrinsic optical property, i.e., intensity, anisotropy, lifetime of the fluorescent molecule is observed in proportion to the concentration of the target analyte as shown in Figure 1.12.

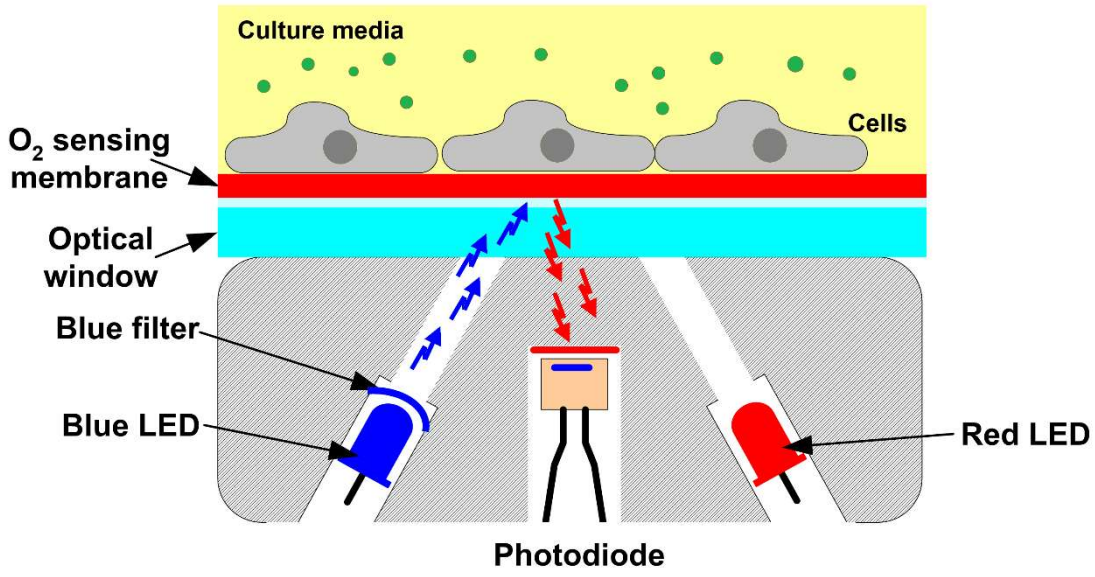

Figure 1.12: Luminescence-based OCS for CBB.

1.3.3. Piezoelectric biosensor

Electroacoustic devices used in biosensors are based on the detection of a change of mass density, elastic, viscoelastic, electric, or dielectric properties of a membrane made of chemically interactive materials in contact with a piezoelectric material. Based on the source of wave production, the piezoelectricity effect, the dipole orientation in the material, and the propagation, one can distinguish the piezoelectric sensor as flexural-plate-wave, surface acoustic wave (SAW), and bulk acoustic wave (BAW).

Generally, the quartz crystal microbalance (QCM) or bulk acoustic wave (BAW) is preferred to use in CBB applications. When an AC potential is applied the thickness-shear mode (TSM) resonating quartz crystal (or piezoelectric material), the crystal excites and produces some shear displacement, which therefore observed as a mechanical oscillation/vibrations. The vibration generated travels in the form of an acoustic wave and decays exponentially into the surrounding medium at its surfaces. Any mechanical alteration occurring within the acoustic wave's decay length in the adjacent medium can be sensitively recorded by a change in the oscillation properties of

the crystal as shown in Figure 1.13. The QCM sensor can detect changes in the viscoelastic properties of the quartz crystal loading by the damping of the oscillation amplitude. Thus, this phenomenon is being applied in various biomolecular interaction studies such as virus detection, protein adsorption, immunoassays, membrane formation and interaction, etc. and also in CBBs. While QCM based CBB provides an essential understanding of the cell-substrate interactions at the micro-level. Moreover, adherent electrogenic cells' viscoelastic properties under the influence of stimuli is studied using the QCM sensor.

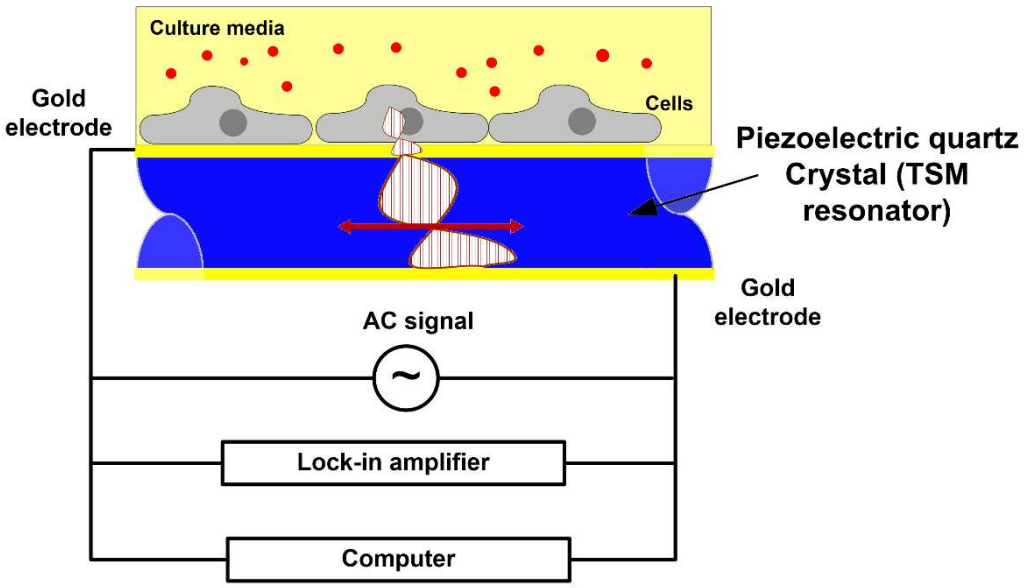

Figure 1.13: Quartz crystal microbalance (QCM) for CBB.

1.4. Strategies for the design and characterization of CBB

The CBB provides meaningful high-content information in a physiological context, which is not accessible by other CBAs available. Therefore, it is considered as an essential tool to investigate pathogen, toxin detection, drug discovery, etc. For example, the CBBs helps to screen the appropriate drug candidate and concentrations in drug discovery research to minimize the number of animal tests, which is desirable for both ethical and cost reasons. In order to make the CBB more efficient, it is necessary to

have an *in vitro* cell culture platform that mimic the natural microenvironment in a spatial and temporal manner.

1.4.1. Design for fabricating the CBB

The choice of transduction material and the structure of CBB plays a crucial role in constructing a CBB, as it involves the direct use of living cells on the sensing surface.

1.4.1.1. Choice of sensing material

The *in vitro* cell-surface interaction is influenced by the substrate's physicochemical and structural properties [65]–[67]. The adherent cell establishes an active communication with extracellular microenvironment *via* the membrane proteins, i.e., the cell membrane-bound receptor, especially integrins, which specifically interact with extracellular adhesive proteins [68], [69]. The membrane-bound receptors establishing a mechanical link not only between the cell membrane and the extracellular matrix (ECM) but also between the ECM and the actin cytoskeleton to progress the predefined basic cell functions such as cell adhesion and cell-cell interactions; resulting in the formation of a cellular monolayer on the surface of an artificial substrate [70]. Further, these membrane-bound receptors play an essential role in the progression of various (other) critical physiological processes, which include cytoskeleton rearrangements, tissue formation, gene expression and cell-mediated immune reactions [71]. The adhesive proteins required to promote the cell adhesion process, in order to trigger various other signalling pathways to enhance cell proliferation and differentiation, is largely dependent on physicochemical and structural properties of the substrate [72]–[74]. Therefore, it is essential to understand the physicochemical and topographical properties of the choice of nanomaterial interested in interfacing with the adherent cells. Moreover, it is also desirous to use biocompatible, electrochemically and thermally

stable material with the other characteristics such as reliable, accurate, selectivity (or specificity), response time, and sensitivity, while considering material for the signal transduction purpose.

1.4.1.2. Structure for fabricating the CBB

Detection sensitivity is a crucial criterion in fabricating CBBs. It depends on sensor configuration such as electrode dimension and the distance between the electrodes [75], [76].

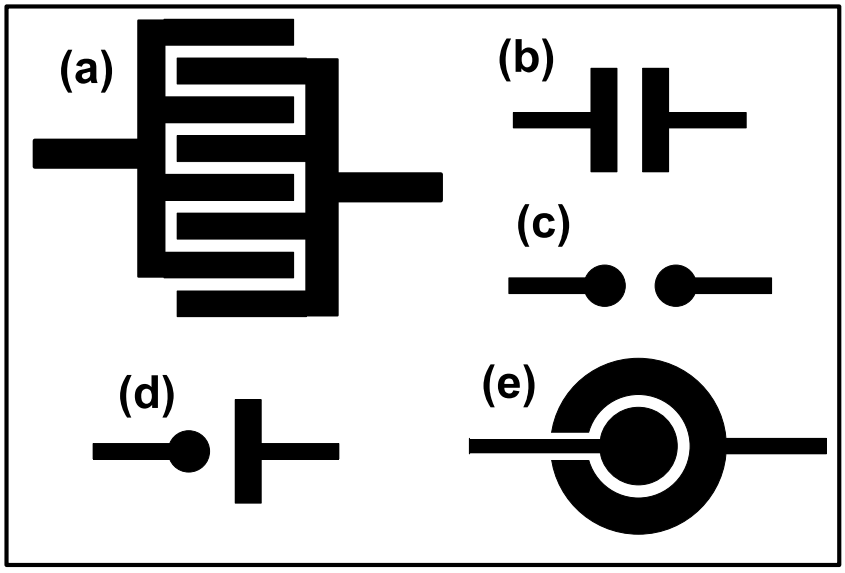

Figure 1.14: Types of co-planar electrode arrangement used; (a) Interdigitated electrode (IDE), and (b)-(e) dissimilar & similar-sized electrodes.

Figure 1.14 illustrates different types of commonly used co-planar electrode arrangement. The conduction takes place through the shortest possible path. It is known that the current (and voltage) flows through all the possible electrically conductive paths but at most, current will flow the low resistance path (i.e. the shortest path). Hence, when an excitation pulse is applied to the excitation electrode or working electrode, the current will flow through the ionic solution (i.e., culture media) surrounded by the microelectrode system in order to complete the circuit. When the cells are inoculated, it drifts downward and starts adhere to the surface of the sensing layer (silver electrode in

case of ECIS system, while metal-oxide thin film for other cases), which alters the available effective area for the current flow causing the change in the impedance of the ECIS system.

1.4.1.3. Thin film deposition techniques for fabricating the CBB

To obtain thin film of metal electrodes, semiconductors or insulators on the substrate, various thin film deposition method are employed. The most common deposition techniques are spin coating, thermal evaporator, and electron beam evaporator.

The spin-coating method uses homogeneous solution, which has to be deposited on the substrate. Usually in this method, a homogeneous solution is prepared by adding a precursor material to an appropriate solvent. Then, a small amount (drop) of this homogeneous solution is applied on the center of the substrate and rotated at speed (up to 10,000 rpm) in order to spread the homogenous solution layer to form a coating (thin film) *via* applied centrifugal force. This creates a uniform thin film onto flat substrate. The machine used for spin coating is called a spin coater. The sol-gel spin coating process is illustrated in Figure 1.15. On the other hand, thermal evaporator and electron beam evaporator are physical vapour based deposition techniques. Usually in this method, vapour of the desire material is evaporated inside the vacuum chamber to deposit on the specific substrates. The fundamental difference between thermal and electron beam evaporator is their heating mechanism. Resistive heating is used in the thermal evaporator whereas electron beam induced heating is used in the electron beam evaporator. Thermal deposition is generally used for the material having low melting point. Most of the metals are preferably deposited using thermal deposition. In contrary to the thermal deposition, electron beam evaporator is used for the material having high melting point. Most of the semiconductors and insulators are deposited using electron

beam evaporator. The block diagram and the evaporation process in the thermal and electron beam evaporator are illustrated in Figure 1.16.

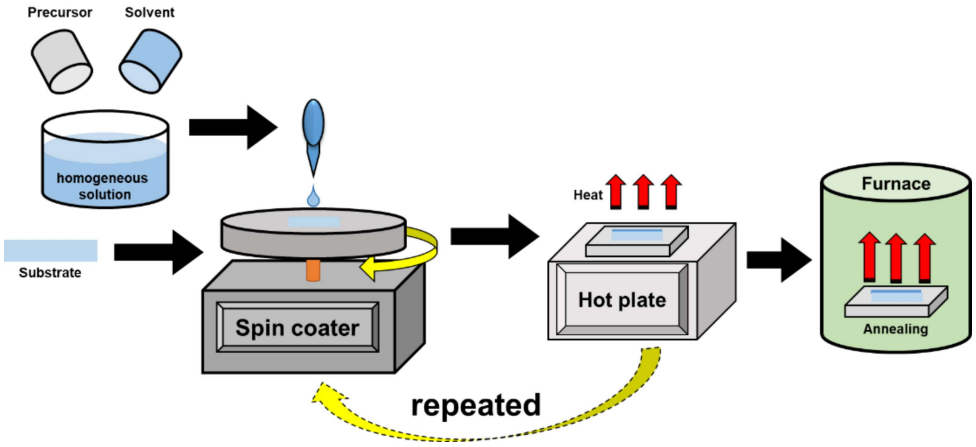

Figure 1.15: Sol–gel spin coating process [77].

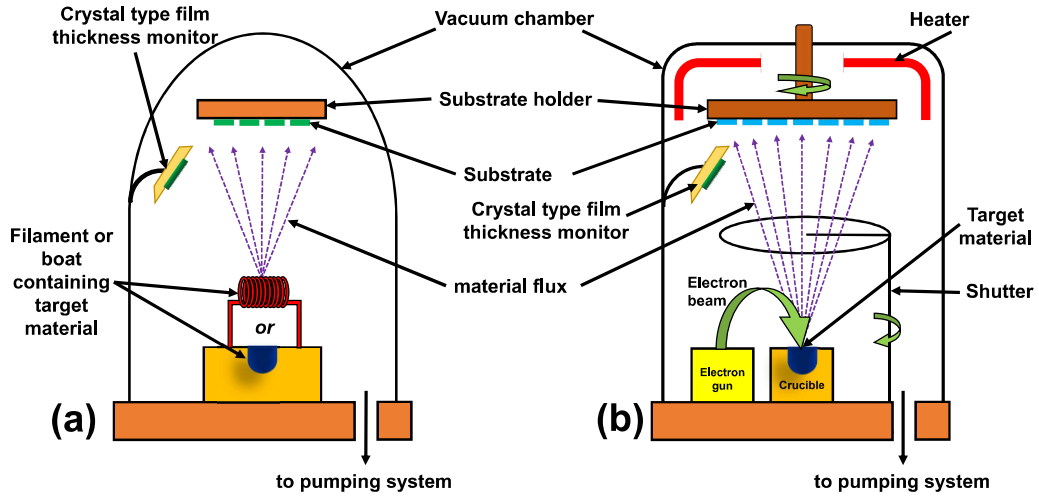

Figure 1.16: (a) Thermal, and (b) Electron beam vacuum deposition method.

1.4.2. Characterisation techniques for the fabricated CBB

The human eye requires contrast to perceive details of objects. Several ingenious methods of improving the contrast of microscopy images have been designed, and each of them opened new applications of optical microscopy in biology. Microscopes provide magnification that allows people to characterise and analyse individual cells and unicellular organisms such as bacteria and other microorganisms. Typically, to

assess the dynamic behaviour of the adherent mammalian cells such as cell adhesion, proliferation, migration, and differentiation [4], fluorescence microscope is commonly used. The block diagram of inverted fluorescence microscope (Make: Nikon, Model: Ti-U) is illustrated in the Figure 1.17a**.**

Typically, a cell-induced change in the characteristic electrical properties of thin film based devices is measured using semiconductor parameter analyser (SPA). The two terminal biosensing devices such as MIM, MSM, heterojucntion diodes are characterized using a two source and measuring units (SMUs). The SPA is used to measure the current-voltage (*I-V*), capacitance-voltage (*C-V*), capacitance-frequency plot, Nyquist plot, magnitude and phase of impedance plot for the biosensing devices. The Pictograph of the SPA (Make: Keysight, Model: B1500A) is shown in Figure 1.17b.

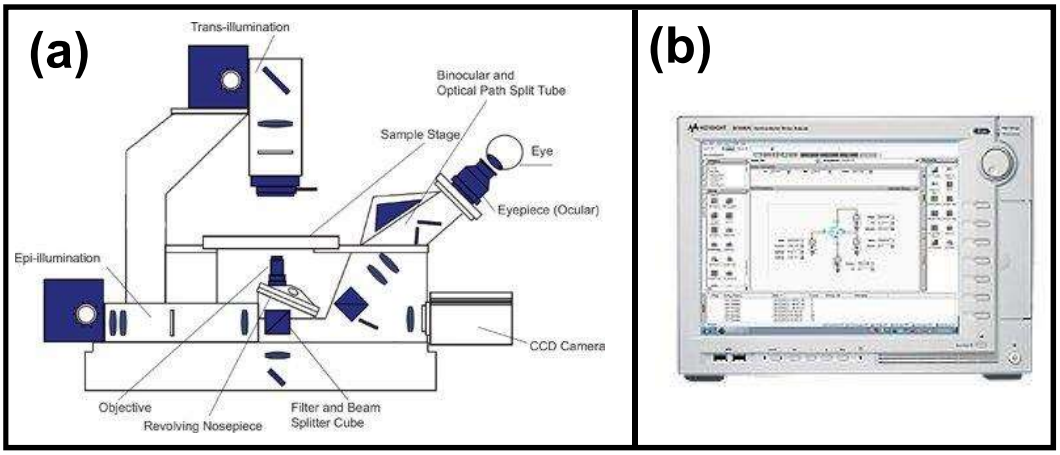

Figure 1.17: (a) A schematic diagram of an inverted fluorescence microscope, and (b) Pictograph of the semiconductor parameter analyser (SPA).

1.4.3. Fundamental behaviour of cells in electric field

The cell membrane has cytoplasm enclosed within it, which has ‘n’ number of proteins and nucleic acids (i.e., biomolecules), and this membrane also serves as a protective layer by isolating/separating the cell from the surrounding environment. The cell

membrane is generally made up of double-layered phospholipids (which are considered to be amphiphilic). The term “Amphiphilic” defines that the molecule will have both lipophilic structures/group (i.e., hydrocarbon) and hydrophilic polar functional groups (which may be either ionic or neural). Naturally, the amphiphilic molecules are arranged or positioned in their polar groups outside (in order to separate the surrounding aqueous medium from) and lipophilic chains towards inside the polar group resulting formation of the bilayer (i.e., phospholipids bilayer). Thus, the phospholipid bilayer within this membrane helps/prevents the movement of ions/molecules in and out of the cell (by forming channels and the pumps selective towards the movement of particular ion or molecule). The membrane surface also has some receptor proteins that allow the cell to monitor/respond to external signalling molecules (such as hormones). The potential difference across the cell is maintained due to the various physiological processes exhibited across the cell membrane to govern and perform the biological cell’s basic functionality. Where, each cell has a characteristic potential difference across the membrane, which is denoted as transmembrane potential.

The behaviour of cells in electric fields can be ascribed to polarization effects that occur at the surface of the cell membrane. So, when the cells is brought into a homogenous electric field, they behave like the insulating particles, due to presence of ion-gated channel-based selective permeability of the cell membrane that specifically induces potential difference across the membrane of about 40 to 70 mV, which is metabolically maintained by active membrane transport (which is referred as natural resting potential). The induced membrane potential superimposes the natural resting potential of the membrane, which attributed to either electrophoretic or electroosmotic effects that change the lateral distribution of mobile membrane receptors, carriers, and the channels within the lipid matrix to cause a change in concentration of intracellular gradients.

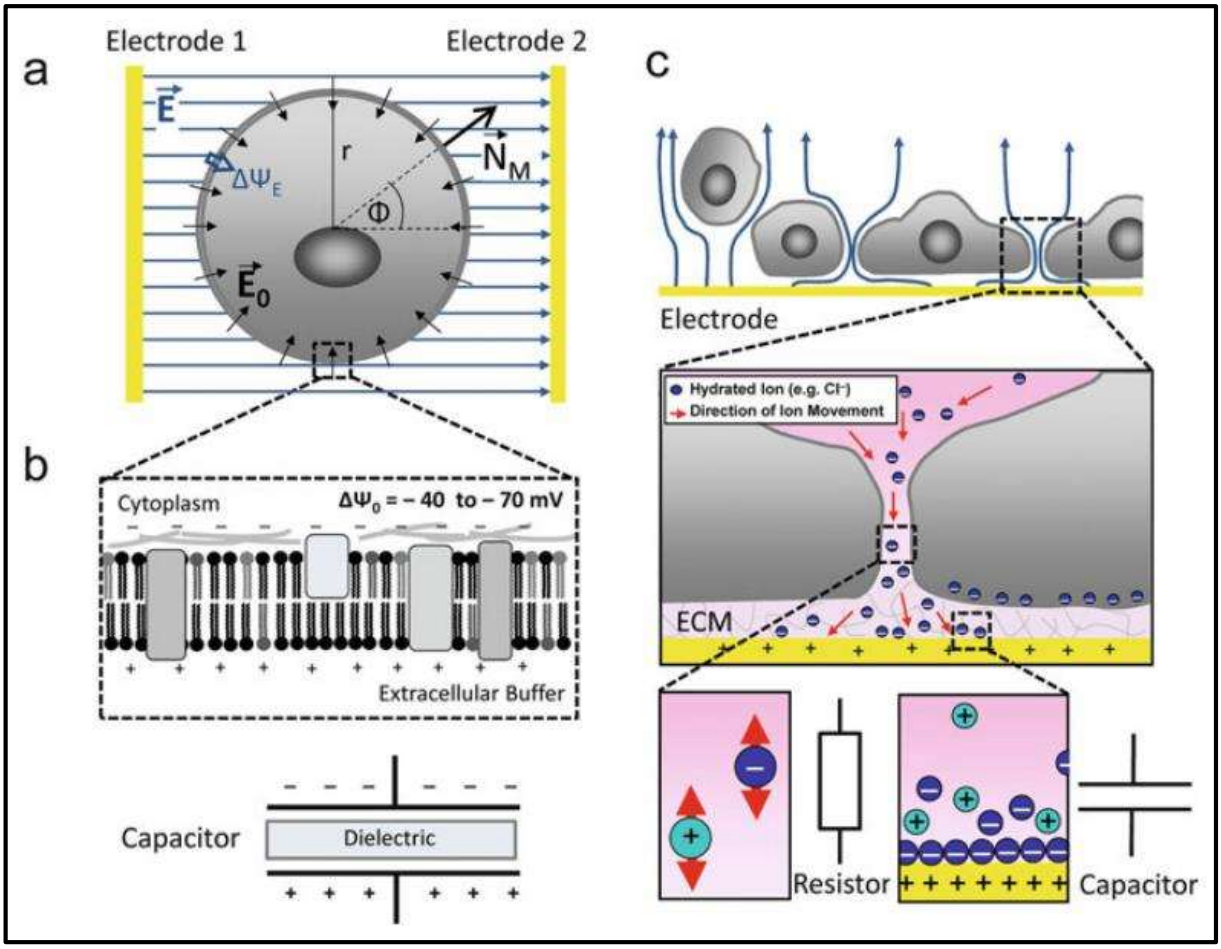

Figure 1.18: Biological cells in electric field, while (a) cell placed in an electric field., (b) cell membrane is represented by as a capacitor due to its hydrophobic nature, (c) adherent cells grown on a conducting surface (metal) [78].

As a whole, the cell can be constructed as resistor-capacitor circuit (RC circuit) using the passive electrical components. The cell membrane is considered as the capacitor, which holds the charges. The membrane potential difference provides the input voltage with ion channels as resistors. The ion permeability of the membrane for sodium, potassium, and other ions is taken into account in terms of a sodium, potassium, and leakage ion channel conductance. The source of endogenous electric field ($\vec{E}$) is shown in Figure 1.18. Further, the electrical properties of the biological cells are considered to be varied as a function of frequency. At a lower frequency (< 1 kHz) the depth of penetration of an AC signal is very less across the cell-membrane; hence the more

current flows in the extracellular region than that of the intracellular region of the biological cells as a function of frequency and it is termed as the 'α' dispersion, which is associated phenomena of ion diffusion exhibited across the membrane. At mid-frequency (1 kHz – 100 kHz), the depth of penetration of an AC signal is more than the 'α' dispersion; hence the more current flows in the extracellular region while comparatively less current flows through the cell membrane into the intracellular region due to which a polarization effect or Maxwell-Wagner phenomena developed across the cell-membrane as a function of frequency and it is termed as the 'β' dispersion. At higher frequency (>100 kHz), the water molecules present in that biological medium gets polarized due to which more current flows in the intracellular region than of the extracellular region of the biological cells as a function of frequency and it is termed as the 'γ' dispersion [8], [79]. Thus, one can analyze the frequency dominated changes – by analysing the change in capacitance, the magnitude and phase of impedance with respect to the applied frequency.

1.5. Literature review

The objective of the present thesis is to assess the functional behaviour of adherent mammalian cells by constructing a low-cost ECIS device as well as by fabricating thin-film nanomaterials based biosensors. In order to outline the scope of the thesis, we will now review some important literature on fabrication and implementation of the cell-based biosensor.

1.5.1. Review on ECIS system for CBB

It has been reported that the impedance of microelectrodes of various sizes and structures provides useful information about the behavioural changes of cells in non-destructive and label-free manner. In 1984, Giaever and Keese have first reported that the inoculated cells attach and spread on a small gold electrode surface, they alter the effective area available for current flow causing an increase in the impedance of the system, when subjected to an alternating electric field at 4 kHz [80]. At the same time, fluctuations in the characteristic impedance is correlated with cell motion [81], due to changes in the membrane structure [82]. Later, Lind *et al.* [83] used this method to record extracellular action potential measurements of the cultured neuronal cells. Wegener *et al*. [84] used the same method to monitor the kinetics of cell spreading on the artificial surfaces using epithelial cells. Ehret *et al.* [85] used an interdigital electrodes (IDEs) to monitor the spreading, attachment and morphology of fibroblasts in culture. Zhang *et al.* [86] have reported fabrication of co-planar gold metal electrode on the stretchable biocompatible polydimethylsiloxane (PDMS) substrate, to analyze the cellular growth and proliferation of bovine aortic endothelial cells (BAECs) under the influence of cyclic mechanical stretch. Overall, the impedance analysis provides information about the physiological, morphological and pathological conditions of the cells, which includes cell adhesion [84], [87], proliferation [14], [88]–[91], micro-motion [81], [92], toxicity [93]–[99], apoptosis [100]–[103], differentiation [44], [104]–[107], when the cells are grown on the specific substrate [81], [84], [94], [108], [109]. However, the traditional ECIS sensors are expensive, because the gold electrode as the transducing element is generally recommend to use[110]–[113].

Hence, researchers started to look for other alternatives based on impedance analysis. Yu *et al.* [114] used an electrolyte–insulator–semiconductor (EIS) structure sensors to

monitor cell adhesion by interfacing it to light addressable impedance analyser. Nabovati *et al.* [115] have reported the use of CMOS capacitive sensor array for continuous adherent cell growth monitoring. Stolwijk *et al*. [116] have reported the use of ECIS sensor for *in situ* electroporation, to determine behavioural aspects of adherent cells during intracellular manipulations non-invasively. Jeong *et al.* [117] used the electrochemical biosensor to analyse three-dimensional (3D) cell culture in order to determine the functional behaviour of cells.

Further, it is desirous to use biocompatible and non-toxic materials as electrode material for the purpose of cell impedance measuring system [118]. Some other commonly used electrode materials are platinum (Pt) [119]–[121], indium tin oxide (ITO) [87], [122]–[126] and other organic electrode material such as poly (3, 4-ethylenedioxythiophene): poly (styrene sulfonate) (PEDOT: PSS) [127]–[129], reduced graphene oxide (rGO) [130]–[135], polyaniline (PANI) [136]–[138], etc. because of their biocompatibility and stable electrochemical properties. On the other hand, the use of such promising substrate materials significantly increases the cost and time of fabrication [87], [89].

1.5.2. Review on thin film based electronic devices for CBB

The revolutions in nanotechnology have enabled to develop new techniques using the nanomaterials as a probe to study the cellular activities [6], [139]. The metal-oxide nanomaterials have shown attractive physicochemical properties, such as conductivity modulation, suitable dielectric, biocompatible etc. The uniqueness in physiochemical and structural properties of the transparent metal-oxide (TMO) thin film nanomaterials have been explored not only in the field of opto-electronics [140], [141] and energy storage applications [142], [143], but also in various other biomedical applications [144]–[148], Most commonly used n-type TMO thin films are zinc oxide (ZnO) [149],

silicon oxide (SiO_2) [150], indium tin oxide (ITO) [91], titanium dioxide (TiO_2) [151], aluminium oxide (Al_2O_3) [152]–[154], cadmium oxide (CdO) [155]–[157], stannic oxide (SnO_2) [158]–[160]. The TMO thin films possess unique electron charge-transfer characteristics, when the surface of the nanomaterial exposed to any particular external environment, and the variation will be either or in the form of change in resistance, capacitance due to change their dielectric properties (i.e., conductivity). These electron transportation across the surface and in the nanomaterial is due to its bulk electron mobility, grain boundaries, bulk oxide material, etc. [161]–[171]. Metal-oxide based biomaterials is used for regeneration and therapeutic applications. However, it has not been used as a transduction element for CBB.

1.5.3. Major observation from the literature survey

It has been reported that ECIS method is a promising tool that presents a sensitive and continuous monitoring system of cellular parameters that vary during transformation. In addition, impedance analysis based system may provide quantitative information about the cellular parameters. However, the impedance measuring devices available in the market for cell study, are expensive and moreover, they are not portable [87], [89]

- ✓ There is a huge demand on the need of portable system for point-of-care applications especially in the field of bioengineering, healthcare and environmental science.
- ✓ Similarly, the traditional ECIS sensor is fabricated using gold metal electrode [110]–[113]. Low-cost ECIS biosensor is also an essential need which can effectively replace the traditional gold coated ECIS biosensor.
- ✓ Metal–insulator–metal (MIM) thin film can be operated bi-directionally and it can be used as a non–linear rectifier. Metal–semiconductor–metal (MSM) has

been used as a photodetector, and widely utilized for gas sensing applications. However, MSM for CBB has not been reported yet.

- ✓ Larger area heterojunction sensor is commonly used in solar cell, and photodetector applications. Yet, large area heterojunction has not been applied to detect the biological changes *in vitro.*

1.6. Research objective

The objective of present thesis is to analyze functional behaviour of the living cells in a label-free and non-invasive manner using cell-based biosensors. Traditionally for this purpose, ECIS system have been extensively used to detect the behavioural changes of biological cells *in vitro* culture. However, the cost of the sensor and device is expensive. Likewise, the lacuna in the design of cell-based biosensing platforms motivated us to design novel biosensors to analyze behavioural changes of various cells *in vitro* culture using electronic devices. For this purpose, we made an attempt to constructed various structures for biosensing platforms to analyze behavioural changes of various cells *in vitro* culture electronically. Attempt has also been made to improve the cell-substrate interaction by using biocompatible sensing material (sensing element).

Further, the challenge includes eliminating electrode fouling, bubble formation, etc. which occurs due to electrolysis and redox reactions between the cell culture media and the electrode. In addition, we have focused on the cellular microenvironments for the improved detection of various cellular functions. The phenotypic changes of the biological cells using a low-cost insulating and semiconducting nanomaterial thin film based electronic devices are considered. Furthermore, it is believed that uniqueness in the physicochemical and structural properties of metal-oxide nanomaterial will play a crucial role in cell-substrate and cell-cell interaction studies, and these functional

behaviour will induce some alternation in the characteristic change of the thin film biosensing devices.

1.7. Scope of the Thesis

The present thesis deals with the fabrication and characterization of thin-film nanomaterials based biosensing devices for assessing dynamic behaviour of adherent mammalian cells. The thesis consists of SIX chapters including the present chapter. The contents of the remaining FIVE chapters are briefly described in the following:

Chapter-2 reports the investigation of functional behaviour of myoblast (C2C12) cells using a thermally coated co-planar silver metal electrode system integrated to a low-cost ECIS system. The constructed metal-insulator-metal (MIM) structured ECIS sensor has an active layer's dimensions of 1.5 mm wide and 4 mm length. At the same time, to develop a low-cost ECIS system, we used the electronic components locally available at the laboratory. Optimized and used as a platform to study the phenotypic change of adherent mammalian cells under the influence of applied electric field.

Chapter 3 describes the fabrication and characterization of e-beam deposited aluminium oxide (Al_2O_3) metal-oxide thin film nanomaterials based biosensing devices for assessing dynamic behaviour of adherent mammalian cells. Typically, the Chapter 3 examines the effect induced by cellular functional behaviour on the characteristic electrical properties of the thin film nanomaterial-based metal-insulator-metal (MIM) device. Further, to improve the cell adhesion process, the MIM device is coated with 2% gelatin protein and used as a platform to study the phenotypic change of adherent mammalian cells under the influence of applied electric field.

Similarly, the **Chapter 4** reports the effect induced by cellular functional behaviour on the characteristic electrical properties of the sol-gel synthesized spin coated zinc oxide

(ZnO) thin film nanomaterial-based metal-semiconductor-metal (MSM) device. The MSM device having ZnO thin film of ~100 nm thickness is used as the active sensing interface and the MSM device is also coated with 2% gelatin protein. The gelatin-coated MSM device is used as a platform to study the phenotypic change of adherent mammalian cells under the influence of applied electric field.

Chapter 5 reports the ability of sol-gel synthesized spin coated zinc oxide (ZnO) thin film nanomaterial-based larger area heterojunction device to support the electrogenic cell's cellular adhesion, and other cellular functionality. Further, the fabricated heterojunction device is functionalized with poly-L-lysine and the poly-L-lysine functionalized ZnO thin film based extended larger area heterojunction device is used to characterized the cell-induced electrical characteristic property change.

Finally, **Chapter 6** summarizes the major objectives and concludes the major findings of the present thesis. This chapter also outlines some future scope of works related to this thesis.