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Recent advances in cytochrome c biosensing technologies

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This review is an attempt, for the first time, to describe advancements in sensing technology for cytochrome c (cyt c) detection, at point-of-care (POC) application. Cyt c, a heme containing metalloprotein is located in the intermembrane space of mitochondria and released into bloodstream during pathological conditions. The release of cyt c from mitochondria is a key initiative step in the activation of cell death pathways. Circulating cyt c levels represents a novel in-vivo marker of mitochondrial injury after resuscitation from heart failure and chemotherapy. Thus, cyt c detection is not only serving as an apoptosis biomarker, but also is of great importance to understand certain diseases at cellular level. Various existing techniques such as enzyme-linked immunosorbent assays (ELISA), Western blot, high performance liquid chromatography (HPLC), spectrophotometry and flow cytometry have been used to estimate cyt c. However, the implementation of these techniques at POC application is limited due to longer analysis time, expensive instruments and expertise needed for operation. To overcome these challenges, significant efforts are being made to develop electrochemical biosensing technologies for fast, accurate, selective, and sensitive detection of cyt c. Presented review describes the cutting edge technologies available in the laboratories to detect cyt c. The recent advancements in designing and development of electrochemical cyt c biosensors for the quantification of cyt c are also discussed. This review also highlights the POC cyt c biosensors developed recently, that would prove of interest to biologist and therapist to get real time informatics needed to evaluate death process, diseases progression, therapeutics and processes related with mitochondrial injury.

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1. Introduction

1.1. Cytochrome c, a brief understanding

Cytochrome c (cyt c), a small (molecular weight: ~12,500 Da, 104 amino acids), highly water-soluble redox active metalloprotein is located in the intermembrane space of mitochondria (Hayashi and Capaldi, 1972; Martinou et al., 2000). It has a highly conserved three dimensional (3D) structure and a covalently attached active heme prosthetic group (Fig. 1). In mitochondrial respiratory chain of energy production, cyt c functions as a single electron carrier between two membrane bound complexes viz. complex III and (bc complex or cytochrome c reductase) and complex IV (cytochrome c oxidase). During the mitochondrial electron transfer reaction, the heme active site of cyt c alternates between a reduced ferrous (Fe2+) and oxidized ferric (Fe3+) states (Mathews, 1985; Skulachev, 1998; Wang et al., 2002). Cyt c is a multi-functional enzyme, involving in both life and death decisions of cell. It participates in electron transfer as a part of the mitochondrial electron transport chain (ETC) and is thus an indispensable part of the energy production process. Its release from mitochondria is an essential step for the formation of the apoptosome and the progression of cell death processes (Hüttemann et al., 2011; Wang, 2001).

1.2. Role of cyt c in mitochondrial electron transport chain

Mitochondria are often referred to as ‘powerhouse of the cell’, because they produce approximately 90% of the required energy in the form of adenosine triphosphate (ATP) through oxidative phosphorylation. Thus, the primary role of oxidative phosphorylation is the production of energy, which drives all cellular processes.

Mitochondrial ATP production occurs through the flow of electrons from nicotinamide adenine dinucleotide (NAD) or flavin adenine dinucleotide (FAD) reducing equivalents. These electrons are passed through a series of respiratory complexes in the inner mitochondrial membrane called electron transport system (Fig. 2). It results in the formation of an electrochemical gradient, which enables the ATP synthase to synthesize the energy rich ATP. The mitochondrial respiratory chain comprises series of five multi-subunit enzyme complexes (complexes I, II, III, IV and V) and two electron carriers (ubiquinone and cyt c). These two electron carriers play critical role in the efficient transfer of electrons in the electron transport chain (Hatefi, 1985; Koopman et al., 2007; Kushnareva et al., 2002).

1.3. Role of cyt c in apoptosis

In addition to its central role as power source, mitochondrial respiratory chain is also the major source for generation of reactive oxygen species (ROS) (Kannan, 2000). Under normal physiological conditions, 1–2% of molecular oxygen consumed by mammalian cells is metabolized to ROS. Consequently, the ROS including superoxide anion radical (O2–), hydrogen peroxide (H2O2), and hydroxyl radicals (OH–) are constantly formed in all aerobic cells. Low levels of endogenous ROS are essential to the cells for regulating various physiological processes such as cell signaling pathways and regular cell proliferations (Burdon et al., 1989). However, an excess production of ROS in cells under various pathological conditions can cause mitochondrial dysfunctions, protein oxidation, DNA mutations and excessive cellular damage, all of which can lead to translocation of cyt c from mitochondria to cytosol. The translocation of cyt c from mitochondria to cytosol is a critical event in the activation of intracellular signaling; it results in the degradation of the intracellular signaling molecules (Kaufman et al., 2002).
in a cascade of caspase activation and leads to programmed cell death—apoptosis. The mitochondrial/DNA damage and apoptosis have been implicated in disease that are linked to oxidative stress/hypoxia such as atherosclerosis, cancer, diabetics, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke, septic shock, aging and other degenerative diseases in humans (Karunakaran et al., 2014; Uttara et al., 2009). Apoptosis or programmed cell death is a regular, highly conserved physiological process, and is an active research topic of biochemical and biomedical sciences. This regulated process is responsible for removal of damaged or infected cells from the cellular population, which links apoptosis to the cell cycle, replication and DNA repair (Kim et al., 2005). Moreover, apoptosis is one of the main mechanisms governing accurate embryonic development and the maintenance of tissue homeostasis. Apoptosis in cells, usually characterized by specific morphological and biochemical changes, viz. nuclear shrinkage, chromatin condensation, DNA fragmentation and membrane blebbing (Culmsee and Landshamer, 2006). The research communities all over the globe predicts that there are two main pathways are involved in apoptotic cell death: (i) the extrinsic or death receptor pathway and (ii) the intrinsic or mitochondrial pathway (Patel et al., 1996; Tait and Green, 2010). In both pathways, translocation of cyt c from mitochondria to cytosol is one of the most important regulatory steps. Once cyt c has been released into the cytosol it interacts with a protein called Apaf-1. This leads to the recruitment of pro-caspase 9 into a multi-protein complex with cyt c and Apaf-1 called the apoptosome. Formation of the apoptosome leads to activation of caspase cascades, which further leads to the activation of apoptosis. Such cyt c release has been reported for apoptosis induced by chemotherapeutic drugs, oxidative stress, UV irradiation, serum, and glucose deprivation (Fig. 3).

1.4. Other important biological functions of cyt c

Current cancer therapy protocols such as chemotherapy, γ-irradiation, or immunotherapy, primarily exerts their anti-tumor effects by inducing apoptosis in cancer cells. This releases the functional apoptosis initiating protein, cyt c into the cytoplasm of cells. Recent reports suggests that cyt c not only released into cytoplasm of cells, but furthermore it leaves the cells and reaches the serum of patients those who undergoes cancer therapy. Thus, the release of cyt c can be used to monitor and evaluate the efficacy of therapy towards cancer treatment. In the last few decades, it is confirmed that cyt c is also released into the blood circulation, following myocardial infarctions, cardiac arrest and resuscitation. In myocardial infarctions, the oxygen supply to regions of the heart becomes interrupted. During such events cells die, release cyt c into the circulating blood. Recent studies suggest that measuring levels of circulating cyt c could also serve as in vivo marker of mitochondrial injury, organ damage and prognosticate survival after resuscitation from cardiac arrest. Some similar observations have been made in patients with a wide variety of critical conditions. For example, Adachi et al. reported rapid rise in serum cyt c concentrations in patients showing systemic inflammatory response syndrome and multi organ dysfunction syndrome, with higher levels observed in patients who did not survive the episode. Likewise, the levels of cyt c inpatients...
2. State-of-the-art of cyt c detection

2.1. Flow cytometry

The techniques available currently at laboratories to measure cyt c release includes flow cytometry, Western blot, and enzyme-linked immunosorbent assay (ELISA). Flow cytometry is a technique for counting, examining and sorting microscopic particles suspended in a stream of fluid. It allows simultaneous multiparametric analysis, physical and/or chemical characteristics of individual cells within a heterogeneous population of cells. It is an automatic, fast, and reproducible process for the detection and quantification of proteins and biomolecules in cell culture. Several authors have demonstrated the detection of cytosolic cyt c released from mitochondria using flow cytometry (Campos et al., 2006; Ng et al., 2012; King et al., 2007). Although flow cytometry technique is sensitive and can be used to detect early stages of apoptosis (Waterhouse and Trapani, 2003a), this technique however, requires procurement of expensive capital instrument which hinders its use for routine clinical diagnosis. Furthermore, this technique requires a highly trained technician to ensure reliable results.

2.2. Western blot

Western blotting (immunoblotting or protein blotting) is one of the important technique for the immunodetection of cyt c (Liu et al., 2012). It involves an electrophoretic sieving (in which proteins are separated by size) followed by an immunoassay. In this technique, first, a mixture of proteins is separated based on molecular weight through gel electrophoresis. These results are then transferred to a membrane producing a band for each protein. The membrane is then incubated with labels antibodies specific to the protein of interest. Western blot technique is routinely implemented for basic research and as a confirmative test for cyt c detection in biological assays and clinical regulatory tests because of high selectivity conferred by using both separation and immunoassay. For example, lung mitochondria and cytosol cyt c protein expression were analyzed using Western blotting technique by Du et al., in a examining the regulatory effect of H2S on lipopolysaccharide (LPS) induced apoptosis. Walker et al., employed Western blot technique to evaluate the applicability of arsenic trioxide as a potential drug for lung cancer treatment by monitoring cyt c release (Walker et al., 2016). Kavathia et al., developed a Western blotting assay to measure total cyt c in serum from a large clinically defined normal group in order to assess the global balance of systemic markers of apoptosis (Kavathia et al., 2009). Wang et al., investigated the cardioprotective effects of berberine against myocardial ischemia/reperfusion injury through attenuating mitochondrial dysfunction and myocardial apoptosis. They measured cyt c expression both in mitochondria and cytosol using western blotting (Wang et al., 2015). Although, useful in deriving accurate results, Western blot technique, still suffers from laborious protocols and time consuming procedures that include electrophoresis of the proteins through a gel and transfer to a membrane, membrane blockage, and incubation with primary, secondary antibodies to detect cyt c. The total western blot procedure usually takes at least 48 h to complete and the results are not very accurate as the bands obtained with western blot are very difficult to quantify. Moreover, the difficulties in miniaturization and low sensitivity makes the Western blot techniques not suitable for POC diagnosis.

2.3. ELISA

The ELISA is another immunological method; like western blot technique, it also utilizes the antibodies for the detection of cyt c. ELISA is the most effective approach for specific detection of cyt c and has been used extensively for disease diagnoses and in biochemical research (Langs-Barlow et al., 2015; Skemiene et al., 2013). Of late, there are two different formats for ELISA methods widely used for cyt c detection. Two strategies namely (i) Sandwich immunoassay and (ii) Competitive immunoassay have investigated to develop efficient ELISA assays for Cyt c detection, as described below.

2.3.1. Sandwich immunoassay

The sandwich immunoassay is the most commonly used commercial assay for the detection of cyt c. In this type of immunoassay, cyt c is “sandwiched” between two antibodies (primary and labeled secondary antibody).

The schematic representation of commonly used protocol adopted for sandwich immunoassay is shown in Fig. 5. The first step in the sandwich immunoassay is coating of a constant amount of primary antibody (or capture antibody) specific to cyt c on to the microplate surface. Following coating, addition of a series of dilutions of the cyt c standards to antibodies immobilized on the plate in the next step. After removing unbound cyt c by washings, addition of, an enzyme-linked secondary antibody specific for cyt c is the next step. Following washings to remove any unbound enzyme labeled secondary antibody, a substrate solution usually added to wells and the color developed in proportion to the amount of cyt c bound to the antibody is monitored. The color development will be stopped using a stop solution and the intensity of the color is measured (Dixit et al., 2012). In sandwich
2.3.2. Competitive immunoassay

Unlike to sandwich type immunoassays, competitive immunoassays for cyt c uses only a single antibody, however it uses a labeled cyt c, which competes with unlabeled cyt c to bind with the coated antibody. Direct competitive immunoassays (Fig. 6) involve the immobilization of specific antibody to a microtiter plate. Unlabeled antigen and enzyme-labeled antigen (labeled with HRP or ALP) are competing for the antibody binding sites. The amount of antibody-bound labeled antigen is quantified colorimetrically, where the color intensity is inversely proportional to the amount of free antigen or un-labeled antigen in the sample.

In a study by Javid et al., ELISA was used to monitor cyt c levels in serum of cancer patients undergoing chemotherapy (Javid et al., 2015). Similarly, Kadam et al., used ELISA technique to evaluate clinical significance and the changes in concentration of cyt c and other components released in serum of breast cancer patients undergoing adjuvant chemotherapy (Kadam and Abhang, 2015). Barczyk et al., modified commercially available cyt c ELISA to monitor cyt c levels in the extracellular medium of apoptotic cells and in the serum of cancer patients using ELISA, reflecting therapy-induced cell death burden. To increase the sensitivity and the reliability of the ELISA, the group replaced the primary (coating) antibody with a more suitable one (Barczyk et al., 2005). In a study by Jemmerson and coworkers, commercially-available sandwich ELISA and Western blot technique were used to quantify cyt c release in the extracellular fluid from death-induced cultures of cell lines (Jemmerson et al., 2002). Although the commercial test kits provides high sensitivity and selectivity, they usually involves time consuming protocols, require test expensive test kits, bulky ELISA readers, and the demand for skilled professionals. This restricts the deployment of ELISA at POC. Devices for POC cyt c detection must not only be sensitive, but also fulfill the following criteria, of robustness, operation simplicity, and overall cost per assay. To be used for clinical diagnosis, the devices must be accurate enough to detect normal as well as elevated levels of the protein concentrations and resistant to false positive and false negative alarms. To design sensitive and cost-effective medical technologies that diagnose clinical abnormalities, researchers all over the globe paying close attention to personnel health-care monitoring systems with the use of biosensors.

2.4. Fluorescent activation image analysis

The release of cyt c from mitochondria to cytosol can also be visualized with an aid of fluorescence imaging tools. Tagging of cyt c with green fluorescent protein (GFP)-tagged cyt c has made it possible to tracking cyt c redistribution within cells in real time. For example, Waterhouse studied the translocation of cyt c from mitochondria by selective permeabilization of the plasma membrane followed by immunocytochemistry and fluorescence microscopy. This assay is based on the idea that permeabilization of cells will allow cytoplasmic cyt c-GFP to diffuse out of the cells. Cells with cytoplasmic cyt c-GFP will therefore have less GFP fluorescence than cells with intact mitochondria. This method, however, is only useful for cells that express GFP-cyt c (Waterhouse and Trapani, 2003b). Similarly, Goldstein et al., studied the mechanism and kinetics of cyt c by using GFP tagged cyt c (Green et al., 2000). In another study, the research group utilized a cyt c fusion that binds fluorescent biarsenical ligands (cyt c-4CYS) as well as cyt c-GFP to track cyt c translocation from mitochondria in different cell types during apoptosis (Goldstein et al., 2005). Chen et al., reported aptamer based nanosensor that realizes the direct fluorescence activation imaging of cyt c translocation from mitochondria (Chen et al., 2015). This strategy relies on spatially selective cytosolic delivery of a nanosensor constructed by assembly of a fluorescently-tagged DNA aptamer on PEGylated graphene nanosheets. The cytosolic release of cyt c is able to dissociate the aptamer from graphene and trigger an activated fluorescence signal. It also enables real-time visualization of the Cyt c release kinetics and direct identification of the regulators for apoptosis. Ohana et al., used yellow fluorescent protein (YFP) fragments to fuse with cyt c to screen the molecular events that happens during mitochondrial apoptosis (Yivgi-Ohana et al., 2011). The YFP system enables to monitor the conformational changes associated with activation of other apoptotic proteins.

3. Biosensor for cyt c detection

A biosensor is an analytical device used for the detection of analytes, consisting of three parts: (i) the bioreceptor, (ii) the transducer or the detector element, and (iii) the reader device. A bioreceptor or biorecognition element is a biomolecule that recognizes the target analyte. The transducer converts the biorecognition event into measurable signal. The reader unit is used to analyze and display the results. Biosensors can be classified based on either (i) biorecognition element used or (ii) the transduction

Fig. 5. Diagrammatic representation of sandwich ELISA.

Fig. 6. Diagrammatic representation of competitive ELISA.
Enzymes, antibodies, and aptamers are the main classes of biorecognition elements widely used in biosensing applications. Enzymes catalyze the conversion of the analyte into a product. Among the great varieties of transduction techniques, electrochemical and optical methodologies have evolved as versatile and powerful techniques for accurate biosensing. The biosensor to be applied for POC detection system should fit in to the listed criteria. **A) Label-free:** The biosensor should be label-free and should not require a labeling process for the detection of target molecules because the labeling process are complicated, costly, and time consuming. **B) Selectivity:** To avoid false positive and false negatives, the receptor should have good selectivity for the target analyte. Selectivity is the most crucial criterion for the reliable operation of a POC system. However, ultra-sensitivity is not required because upon positive diagnosis by the POC system, the analyte should be accurately reanalyzed to determine the exact phase of the disease with cutting edge technologies in a central laboratory. **C) Integration and packaging:** For an inexpensive POC system, the biosensor should be easily integrated and packaged with user-interface electronics to minimize the system size and production cost.

3.1. **Enzymatic biosensor for cytochrome c**

Cyt c exists in an oxidized (ferric) or reduced (ferrous) form. Inter-conversion between the two states is brought about by catalytic reaction with two different membrane bound enzymes, namely, cytochrome c oxidase (CcO) and cytochrome c reductase (CcR). CcO oxidizes reduced form of cyt c while CcR reduces its oxidized form. Although the structures of the two forms of cyt c are similar, the difference in oxidation state make significant difference in binding and biochemical properties. As recently reviewed by Brown and Borutaite, the reduced and oxidized forms of cyt c have different abilities to activate apoptosis (Brown and Borutaite, 2008).

### 3.1.1. Cytochrome c oxidase based biosensor

The CcO is a complex metallo-enzyme containing 13 protein subunits and four metal centers (CuA, heme a, heme a2, and CuB) with a combined molecular weight of ≈2.05,000 Da for the monomeric enzyme (Kadenbach and Hüttemann, 2015; Michel, n.d.). Being the terminal complex (complex IV) of ETC, CcO accepts electrons from reduced cyt c and transfers them to molecular oxygen. This reaction is coupled to the transfer of protons from the matrix to the inter-membrane space by CcO. Although it has 13 subunits, the subunits I and II are essential for the function of enzyme. Subunit I has three of the four metal catalytic sites: heme a, and the heme a2/CuA center, where the oxygen reduction takes place, a process which is coupled to proton translocation. Four electrons from cyt c (one from each cyt c molecule) are subsequently transferred via the CuA center in subunit II and heme a to the oxygen reduction center heme a3/CuB (Fig. 8).

Interesting feature of mitochondrial membrane proteins, especially CcO are its high hydrophobicity and low stability, which makes them very difficult to immobilize on the electrode surface, consequently, use in biosensor applications. Still, a number of biochemical and biophysical studies have challenged the immobilization of CcO on the electrode surface by coating the surface with lipid bilayers. Such bilayer coatings are considered as simple models for biological membranes, mimicking the natural environment of the integral membrane proteins which provide improved stability. The environment maintains the structural and functional integrity of membrane proteins at the electrode surface (Burgess et al., 1998; Cullison et al., 1994; Salamon and Tollin, 1996). The dimensions of these bilayer structures employed were approximately 50 Å (Rhoten et al., 2002; Tsukihara et al., 1995). Hawkridge and his team has developed an approach that couples thiol self-assembly with deoxycholate dialysis, to create an electrode-supported lipid bilayer membranes for the immobilization of CcO (Cullison et al., 1994; Rhoten et al., 2002). A submonolayer of octadecyl mercaptan serves to anchor the bilayer on the electrode surface. This group also demonstrated the potentiometric and amperometric biosensing of reduced form of cyt c using the CcO immobilized electrodes (Lewis et al., 2006). The biosensor showed a linear relationship for the cyt c concentration range from 1 μM to 10 μM with a detection limit of 0.1 μM at a signal to noise ratio of 5. In an another biosensing application, the research group
demonstrated the effect of temperature and pH on the effect of CcO immobilization towards cyt c detection (Rhoten et al., 2000). Octadecanethiolate submonolayer was derivatized on the surface of gold quartz crystal microbalance electrode to anchor the bilayer and immobilize CcO. The sensor system thus designed, amperometrically detected cyt c at a lowest concentration of 0.25 μM, with a signal to noise ratio of approximately 2.5.

Similar biomimetic systems involving histidine (his)-tag technology was also demonstrated for the immobilization of CcO. Friedrich et al., attempted to preserve the catalytic activity of CcO on a gold film electrode by reconstituting the enzyme with a protein-tethered bilayer lipid membrane (Friedrich et al., 2008). However, in this orientation, the cyt c binding site is directed away from the electrode pointing to the outer side of the protein-tethered bilayer lipid membrane architecture (Fig. 9). Ashe et al., have designed a CcO based biosensor for the detection of cyt c levels in serum (Ashe et al., 2007). The biosensor was constructed by immobilizing the enzyme, CcO in didodecyldimethylammonium bromide (DDAB) lipid vesicle dispersion on to a gold electrode. The DDAB vesicles in aqueous solution mimic the environment of inner mitochondrial membrane and provide suitable platform for the effective immobilization of CcO. The vesicles also enables the direct electron transfer between the electrode and the enzyme’s redox centers without using any mediators. Square-wave voltammetry was used to detect cyt c levels in human serum samples. The sensor’s response to cyt c was linear from 0.2 μM to 4 μM. The lowest level of cyt c detected by this sensor (0.2 μM), was much lower than the lowest level of 0.8 μM reported previously for patients with myocardial infarctions.

Covalent immobilization of CcO on the electrode surface has also been reported for the design of cyt c biosensor. Thiol based self-assembled monolayer (SAM) to immobilize the CcO to detect reduced form of cyt c has been reported by few research groups. Li et al., have reported the covalently binding of CcO with SAM of 3-mercaptopropionic acid on a gold electrode by using a carbodiimide coupling (Li et al., 1996). Immobilized CcO exhibits a reversible voltammetric response due to the redox reaction of cyt a3 in the enzyme active site and mediate the redox reaction of cyt c in the solution. The electron transfer rate constant of the CcO based biosensor was depends strongly on the strength and composition of the supporting electrolyte. Recently, Batra et al., fabricated an amperometric biosensor for determination of cyt c by immobilizing CcO onto nickel oxide nanoparticles decorated carboxylated multiwalled carbon nanotubes/polyaniline film electrodeposited gold electrode (Batra et al., 2013). The sensor had a good linear relationship between the current (mA) and cyt c concentration in the range 5 pM to 500 nM with the detection limit was 5 pM (S/N = 3). Serum cyt c was also estimated for POC applications.

Although CcO based biosensors mainly used to measure reduced form of cyt c by oxidizing it, there are some reports showing CcO could also use to detect oxidized form of cyt c. For example, a study by Cullison and Hawkridge showed that CcO can measure either both oxidized or reduced cyt c when an appropriate voltage is applied (Cullison et al., 1994). However, the rate of reduction of oxidized form of cyt c by CcO was less than the oxidation of reduced form of cyt c. Ashe et al., also used to detect oxidized form of cyt c in serum samples using CcO to evaluate its possible application in detecting early myocardial infarctions (Ashe et al., 2007).

Recent discovery by Pasdois et al., suggested that the oxidized cyt c plays an important role in triggering the caspase activation and apoptosis induction (Pasdois et al., 2011). Moreover, in permeabilized cell models, the cytosolic cyt c (Fe3+) is rapidly oxidized to Fe3+ by the mitochondrial CcO (Brown and Borutaite, 2008) thus making the CcO based biosensors difficult to quantify the apoptotic form of cyt c (Fe3+). In addition, upon immobilization, it is reported that the electron transfer is blocked in active centers of the CcO (Hrabakova et al., 2006). Since the analytical applications of CcO based biosensors are limited only to reduced form of cyt c, there is a real need for developing assay for measuring oxidized form of cyt c. Our group has developed an alternate biosensor for the quantification of oxidized form of cyt c by immobilizing CcR onto the nanocomposite modified electrodes. CcR is a complex III of the electron transport system, catalyzing one electron reduction of oxidized cyt c (Fe3+).

3.1.2. Cytochrome c reductase based biosensor

CcR (complex bc1) is also an integral membrane protein of the mitochondrial respiratory chain. The overall reaction catalyzed by CcR involves the reduction of two molecules of cyt c and the 2-electron oxidation of ubiquinol (QH2) to ubiquinone (Q). This process is coupled with transfer of four protons from the mitochondrial matrix to the intermembrane space.

Fig. 9. Schematic representation of immobilization of CcO onto a protein-tethered bilayer lipid membrane (Friedrich et al., 2008) (reproduced with permission from Elsevier Limited).
Ubiquinone + 2 cyt c{Fe^{3+}} + 2H^{+}_{in}
→ ubiquinol + 2 cyt c{Fe^{2+}} + 4H^{+}_{out} \tag{1.1}

Mitochondrial CcR is a homodimer with 11 polypeptide chains, while prokaryotic CcR complexes contain as few as 3 polypeptide chains (Millett et al., 1827). The bc1 complex contains three protein subunits with redox prosthetic groups known as the core subunits: (i) a di-heme cyt b containing both the relatively high-potential \(b_{H}\) heme and the lower potential \(b_{L}\) heme; (ii) cyt c1 with the \(c\)-type heme; and (iii) Rieske protein containing an \([2Fe–2S]\) iron–sulfur cluster \([22–24]\). According to a well-known biochemical knowledge, cyt c \((Fe^{3+})\) acquires electrons from Fe-S-cyt c1 clusters of CcR and gets reduced \((cyt c(Fe^{2+}))\) by following Q cycle mechanism (Crofts et al., 2008) (Fig. 10). Experimental evidence indicates that the peripheral domain of the Rieske iron protein moves back and forth between positions close to cyt b and cyt c1 thus facilitating the electron transfer within the CcR (Kramer et al., 2004).

Our group reported a novel approach for the detection of oxidized form of cyt c \((Fe^{3+})\) for the first time using CcR immobilized on nanoparticles decorated electrodes (Pandiaraj et al., 2014a, 2013a). Two different kinds of nanomaterial decorated biosensor platforms were used for the construction of biosensors: (a) carbon nanotubes (CNT) incorporated polypyrrole (PPy) matrix on Pt electrode and (b) self-assembled monolayer (SAM) functionalized gold nanoparticles (GPN) in PPy-Pt. The incorporation of CNT/GNP into the PPy matrix exhibited nanoporous structures with large effective surface area for the immobilization of CcR and enhanced the enzyme biocatalytic activity for the sensitive determination of cyt c. The CcR-CNT based biosensor achieved a very low detection limit, wide linear range and high sensitivity over the CcR-GNP based biosensor. The schematic representation of cyt c biosensor architectures were illustrated in Fig. 11. Furthermore, the CcR-CNT based cyt c biosensor was applied to quantify the cytosolic cyt c released from the mitochondria of apoptotic human lung carcinoma A549 cells and the results were validated with the standard western blot analysis (Pandiaraj et al., 2013b).

Replacement of conventional electrochemical cells by screen printed electrodes (SPE) connected with portable potentiostats is a main trend in the shift of lab electrochemical equipment to handheld field analyzers. The SPE system can be considered as a disposable electrochemical cell, which reduces the required sample volume, simplifies the apparatus, and makes the point-of-care testing easy to handle and cost effective (Carvajal et al., 2012; Gilbert et al., 2011). In one of the approaches for detecting oxidized form of cyt c levels, our group have combined the distinct advantages of CcR functionalized SPE (Pandiaraj et al., 2014a). The developed cyt c assay consists of two parts: (i) a miniaturized electrochemical biosensor based on cytochrome c reductase (CcR) functionalized screen printed electrodes (SPE); (ii) a microcontroller based data acquisition unit integrated with potentiostat circuit capable of performing cyclic voltammetry technique for the analysis. The working electrode surface of SPE was integrated with polypyrrole (PPy)-carbon nanotubes (CNT) nanocomposite for an enhanced immobilization of the enzyme, CcR (Fig. 12). The performance of the volume miniaturized SPE based biosensor coupled with the portable microcontroller based instrument was evaluated, by applying it for the measurement of mitochondrial cyt c release during cardiomyocytes apoptosis. The results are validated well with the commercial electrochemical analyzer and standard ELISA.

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Fig. 10. Schematic illustration of electron transport through various active sites of mitochondrial complex III.

Fig. 11. Schematic representation of working mechanism of CcR based biosensors (Pandiaraj et al., 2013b) (reproduced with permission from Elsevier Limited).
3.2. Cyt c immunosensors

Immunoassays are quantitative analytical methods that make use of antibodies as immunological reagents. Antibodies are very selective and only bind to their specific targets, even in the presence of a huge range of other materials in a sample. The highly specific binding and large association constants (Kd) of antibodies make them useful for detection and quantitation of analytes in complex sample matrices ranging from culture media to body fluids such as blood, urine, saliva, and sweat. (Li et al., 2008; Pandiaraj et al., 2014a; Rieger et al., 2009). ELISA is an important commercial immunoassay widely employed for protein detection (Lilja et al., 2008; Taufika Islam Williams et al., 2007). However, ELISA is an optical approach, has limitations in colored samples analysis, experimental time, and sample size. Hence, it is difficult to employ ELISA for point-of-care testing (vide supra). In this context, the development of electrochemical immunosensors as an alternative to the conventional immunoassay systems, drawing more attention in a wide range of uses, especially for determination of clinically important analytes. Electrochemical immunosensors can provide a precise and real-time measurement of protein biomarkers owing to their unique combination of exquisite specific antigen-antibody interaction and sensitive electrochemical transduction (Guodong et al., 2007; Viswanathan et al., 2009).

3.2.1. Labeled immunosensors

As the antibody and antigen are intrinsically unable to act as redox partners, most of the immunosensors requires a label that aids in generating an electrochemical signal. The labels are often conjugated to either the antibody (eg. sandwich immunoassay) or antigen (eg., competitive immunoassay). These labels can be enzymes (ELISA), radio isotopes (radio immunoassay (RIA)), fluorescence probes, or chemiluminescent markers. Among these, ELISA has grown a much interest in the recent years because of its simplicity. The enzymes like horse-radish peroxidase (HRP) and alkaline phosphatase (ALP) are mainly used as the enzymatic labels in ELISA.

Commonly, there are two different formats for ELISA is available for antigen detection: sandwich assays and competitive assays (vide supra). In label-free or direct immunoassays, antibodies are immobilized on the sensor surface plate and subjected to the binding interaction with the antigen of interest. On specific molecular recognition of the antigen by the immobilized capture antibody, there will be changes in the interfacial charge, current, capacitance, impedance, mass and thickness at the immunosensor surface, which in turn has a direct effect on the electron transfer reaction of the probe at the immunosensor-electrolyte solution interface. So, the direct electrochemical immunoassay is usually followed the measurement of impedance, capacitance, current, potential changes due to the formation of immunocomplex. In impedimetric immunosensors, the protein levels could be

Fig. 12. Schematic diagram of the miniaturized cyt c biosensor assay (Pandiaraj et al., 2014a) (reproduced with permission from Elsevier Limited).

Fig. 13. Working principle of label or mediator based immunosensors.
detected by monitoring the impedance changes caused by immuno-complex with an aid of electrochemical redox probe ([Fe(CN)]3−/4−) (Fig. 13).

3.2.1.1. Fluorescent labels. In fluorescent biosensors, a fluorescent probe attached receptor (antibody or aptamer) binds to the specific protein and transduce a fluorescent signal which can be readily detected and measured. In some cases fluorescence quenching of labels were used for the detection of cyt c. Various fluorescent probes including metal nanoclusters, quantum dots. Shamsipur et al., reported two different fluorescent metal nanoclusters labels for the detection cyt c (Shamsipur et al., 2016). They are, (i) hemoglobin-stabilized gold nanoclusters, and (ii) aptamer-stabilized silver nanoclusters. A limit of detection in the nanomolar level (about 15 nM) was achieved for Cyt c detection using both AuNCs and AgNCs based sensing platforms. Selective quenching effect of glutathione-capped CdTe quantum dots by cyt c has been reported by Cao et al., for cyt c detection(Cao et al., 2009). This method is selective to cyt c as only cyt c quenched the fluorescence of the GSH-CdTe QDs (at pH > 8.0), and no significant fluorescence changes were observed for hemoglobin or other proteins. The linear range of the calibration curve obtained with this method was from $3.2 \times 10^{-8}$ to $2.4 \times 10^{-6}$ mol L−1 and the detection limit is $3.0 \times 10^{-9}$ mol L−1. Yin et al., tagged cyt c binding aptamer with a fluorescent probe called carbboxy fluorescein and used vandum sulfide nanosheets to absorb to the probe and quench its fluorescence efficiency (Yin et al., 2015). The proposed sensing system shows gives a linear range of 0.75 nM to 50 μM, and a limit of detection of 0.50 nM.

3.2.1.2. Chemiluminescent labels. Chemiluminescence resulting from the reaction of luminol and oxidants (H2O2) has been extensively studied and applied for the determination of protein biomolecules. For example, Robert Feisssner reported a CL procedure for the determination of Cyt c using a variety of luminol-based substrates, giving a linear ranging from 4 to 400 ng (Feisssner et al., 2003). Li et al., used a complex of cyt c and H2O2 to catalyze chemiluminescence reaction of luminol- H2O2 for cyt c detection. The method showed linearity over the concentration of cyt c ranging from 5 to 700 ng ml−1, with a detection limit of 2 ng ml−1 (3σ) (Li et al., 2010). Dong et al., investigated electrogenehemed chemiluminescence behavior of luminol at CdS quantum dots, graphene, and CdS/graphene (CdS/GR) nanocomposites modified gold electrodes. Inhibition of chemiluminescence intensities of the nanocomposite probe in by cyt c was used for cyt c detection (Dong et al., 2016).

3.2.1.3. Electrochemiluminescent labels. Sakaida et al., proposed a sandwich electrochemiluminescence (ECL) immunoassay serum cyt c level measurement using ruthenium-chelate labeled secondary antibodies(Sakaida et al., 2005). An ECL aptasensor for the detection of cyt c was proposed by Bin et al., using CdS:Mn quantum dot-modified TiO2 nanowires as ECL emitter (Bin et al., 2016). Cyt c can be sensitively detected based on the quenching of ECL with a LOD of 9.5 FM. Hu et al., synthesized graphene oxide sheets/polyaniline/CdSe quantum dots nanocomposite for the ECL detection of cyt c (Hu et al., 2013). The detection mechanism is based on the measurement of redox quenching effect of the nanocomposite when it reacts with cyt c. Under the optimized conditions, the ECL intensity decreased linearly with the cyt c concentrations in the range from $5.0 \times 10^{-8}$ to $1.0 \times 10^{-4}$ M with detection limit of $2.0 \times 10^{-8}$ M. Similarly, Wang et al., prepared and ECL nanocomposite probe by combing CdSe quantum dots, graphene oxide, and chitosan (Wang et al., 2012) and employed it for detecting cyt c.

3.2.1.4. Resonance Rayleigh scattering. Resonance Rayleigh scattering (RRS) is a special elastic scattering generated when the wavelength of Rayleigh scattering is located at the molecular absorption bondor close to it. Recently, novel materials, such as nanoparticles in association with the RRS technique, have been successfully utilized for analysis of proteins. For example, Ya et al., demonstrated an RRS assay of cyt c using Glutathione (GSH) capped Ag2Te nanoparticles (Yan et al., 2016). The RRS intensity of nanocomposite optical probe (negative charge) is enhanced greatly when positively charged cyt c mixed. Real time production of cyt c in living cells can be dynamically imaged using plasmon resonance energy transfer (PRET) spectroscopy.

3.2.2. Label-free immunosensors

Although labeled or mediator based immunosensors are widely used for protein detection, however, it was reported that the long term presence of the redox probe influences the activity of the immobilized antibody (Rickert et al., 1996). Label-free direct electrochemical immunosensor thus can be useful to detect proteins possessing metal prosthetic group with reversible redox activity. The direct label-free electrochemical immunosensors for metalloprotein are very attractive, since it requires simple assay procedure that does not require a complicated labeling process to measure the antigen.

For detection of cyt c, the specific cyt c monoclonal anti-cyt c were immobilized on the electrode to capture cyt c, and then the cyt c adsorbed electrode measured through its electrochemical activity due to heme of cyt c(Fe (III)/Fe (II)). This direct redox response was effectively utilized here for the quantitative measurement of cyt c without usage of any redox probe or enzymatic label. Further, the direct electron transfer of cyt c with the electrode surface was enhanced by modifying the electrode surfaces with nanostructures. Our group reported two types of nano-architected immunosensor platforms to improve the direct electroactivity of cyt c (Pandiaraj et al., 2014b). The first configuration involved the SAM modification on GNP in PPy tailored SPE. Second architecture features an effective incorporation of CNT using nano-fion on PPy modified SPE (Fig. 14). However, the overall analytical performance of GNP based immunosensors (detection limit 2 nM; linear range: 2 nM – 150 μM; sensitivity: 154 nA nM−1) was better than the CNT-PPy (detection limit 10 nM; linear range: 10 nM – 50 μM; sensitivity: 122 nA nM−1). The greater analytical performance of the anti-cyt c/SAM/GNP/PPy immunosensor is attributed to the densely and uniformly distributed GNP that greatly increased the active surface area for the formation of SAM of cy steine. The well-organized SAM on GNP/PPy nanocomposite also made it easier to form the stable and covalent immobilization of huge number of anti-cyt c which could further improve the sensitivity and linear range of the immunosensor.

Wen et al., have developed an immunosensor for cyt c by immobilizing anti-cyt c at the interface of gold nanoparticle–polydopamine (AuNP/PDA) composites. This method achieved a linear range (0.1–100 μM) for standard cyt c with a detection limit of 0.03 ± 0.01 μM. EIS and differential pulse voltammetry (DPV) were used to measure cyt c concentrations. The anti-cyt c or anti-caspase-9 functionalized-immunosensor provided a biomimetic interface for immunosensing of cyt c or caspase-9 in Hela cells during apoptosis and the results were validated with flow cytometry analysis (Wen et al., 2014). Intracellular cyt c measurement in a single cell using optical nanobiosensor was demonstrated by Song and coworkers. The optical nanobiosensor composed of a plastic-clad silica fibers with 600-μm and immobilized anti-cyt c, labeled with biotin conjugate. Fluorescent measurement with the help of biotin conjugate solution was used to detect cyt c release during photo induced apoptosis of cancer cells. The results of the labeled nanobiosensor was correlated with standard ELISA
protocols (Joon Myong Song et al., 2004).

3.3. Nucleic acid based cyt c biosensors

3.3.1. DNA based cyt c biosensors

Since each organism has unique DNA sequences, any self-replicating microorganism can be easily identified. Biosensors based on nucleic acid as biorecognition element are simple, rapid, and inexpensive and hence it is widely used in protein detection. Besides enzymes and antibodies, nucleic acids (especially DNA) are also widely used to detect cyt c. The non-enzymatic DNA based cyt c biosensor mainly uses the electrostatic interaction between the cyt c and the DNA to detect cyt c. Cyt c is a positively charged protein and has lysine residues surrounded by its heme prosthetic group.

A sensor for cyt c was developed using a carbon paste electrode (CPE) modified with cellulose-DNA by Lee et al. Since DNA has negative charged phosphate groups, which can electrostatically adsorbed with positively charged cyt c. The magnitude of the redox current observed for adsorbed cyt c was directly proportional to the concentration of cyt c present in the solution. The cellulose-DNA modified CPE results in a linear relationship at a concentration of cyt c between 1 μM and 100 μM (±5% at n=5). The detection limit of cyt c was estimated to be 0.5 μM (S/N = 3). A real sample analysis was carried out for cyt c in a rat mitochondria fraction to evaluate the validation of this method (Lee et al., 2004). Similarly, Liu and coworkers reported a simple approach for detect cyt c using DNA-modified glassy carbon electrode (Liu et al., 2006). The direct electron transfer of horse heart cyt c on DNA-modified glassy carbon electrode was examined using differential pulse voltammetry for the detection of cyt c. In this case a linear relationship was obtained for the cyt c concentration range from $4.0 \times 10^{-6}$ M to $1.2 \times 10^{-5}$ M with a detection limit of $1.0 \times 10^{-6}$ M (S/N = 3).

Later on, Liu and Wei described the detection of cyt c at bio-compatible gold nanoparticle supported bilayer lipid membrane (sBLM) modified with anionic sites (Liu and Wei, 2008). They have deposited Au nanoparticles electrochemically through the sBLM modified with lauric acid to build the anionic sites modified nanoscale electrode array to detect cyt c. The detection limit of this electrode was 50 nM for cyt c (S/N = 3) and the linear range of cyt c concentration spans between $1.0 \times 10^{-7}$ and $3.2 \times 10^{-6}$ M.
Similarly, Zhao et al., have also constructed a self-assembled gold nanoparticles multilayer architecture on a gold electrode based on the recognition between complementary oligo-DNA strands (Zhao et al., 2008). The negatively charged DNA is negatively charged, it is rational that cyt c with positive charge in pH 7.4 buffer can easily bind to HS-ssDNA modified on either the AuNPs or the substrate gold electrode surface via orientated electrostatic interaction. In some cases, researchers studied the direct electrochemistry of cyt c at nanoparticle modified electrode for its detection. Wang et al., 2002 reported the detection of cyt c using its direct electron transfer at CNT modified electrode (Wang et al., 2002). Similarly, the direct electron transfer between GNP and cyt c for the cyt c sensing has been reported by Wang et al., (Wang and Wang, 2004).

These methods, however, suffer from lack of selectivity for the detection of cyt c, especially in tissue or biological samples, due to the fact that the interaction of the recognition elements (negative charge) with the cyt c is only based on electrostatic interaction. Still, these methods are prone to interferences by other positively charged species present in the samples and hence are not applicable to the biological systems.

### 3.3.2. Aptamer based cyt c biosensors

Aptasensors are biosensors that use aptamers as the biospecific recognition element. Aptamers are artificial DNA orRNA oligonucleotides selected in vitro which have the ability to bind to proteins, small molecules or even whole cells, recognizing their target with high affinity and specificity similar to those of antibodies. Ocaña and coworkers reported a label-free aptasensor for the detection of cyt c. Graphite-epoxy composite (GEC) electrode was employed to immobilize the aptamers by simple wet physical adsorption procedure (Ocaña et al., 2014). Electrochemical impedance spectroscopy technique was used to monitor the biosensing event and confirmation of steps performed for building the cyt c aptasensor using [Fe(CN)₆]³⁻/⁴⁻ redox marker. The aptasensor has a good detection range for cyt c between 50 pM and 50 nM, as well as a high sensitivity with a low detection limit of 63.2 pM.

Loo et al., have designed a new type of cyt c aptasensor using bio-barcode (BBC) assay (Loo et al., 2014, 2013). They used an aptamer-based bio-barcode (ABC) to detect a cell death marker, cyt c and its subsequent application to screen anti-cancer drugs. Normally, the BBC assay involves a magnetic microparticle (with antibody to capture the target of interest) and gold nanoparticle (with recognition antibody and thiolated single-stranded barcode DNAs) to form a sandwich around the target. The concentration of target is determined by the amount of barcode DNA released from the nanoparticles. Different from the conventional immunoassays such as ELISA using enzyme to amplify signals, the BBC assay amplifies signals by using PCR. Two types of particles are involved in the assay. The first is a magnetic bead conjugated with polyclonal capturing antibodies. The second is a gold nanoparticle coated with monoclonal recognition antibody for the same target so that an immuno-sandwich is formed with the magnetic bead. The second nanoparticle also carries hundreds of single-stranded DNA molecules of known sequence that hybridizes with its complementary DNA through Watson–Crick base pairings. These hybridized DNA are the barcode markers. After reaction with the analytes, sandwich structures are obtained by magnetic separation and marker DNA are released by melting and serve as templates in PCR amplifications. These marker DNA can be detected easily by standard techniques such as agarose electrophoresis or real-time PCR technique. The aptamer BBC assay is highly sensitive and the whole process can be finished only in 3 h. The efficacy of various drugs with potential anti-cancer activity was also assessed.

In a work by Poturnayova et al., binding response of aptamers specific to cyt c was analyzed by thickness shear mode acoustic transducer. The methods were compared with the electrochemical technique and found that the detection of cyt c by electrochemical transducer was less favorable in comparison with acoustic transducer. Electro-chemiluminescence and fluorescence based cyt c apta sensors has also been reported (vide supra) (Bin et al., 2016; Yin et al., 2015). Stepnova et al., proposed a novel electrochemical aptasensor for detecting cyt c on the base of glassy carbon electrode modified electropolymerized neutral red and decarboxylated pillar[5]arene (P[5]A-COOH) bearing terminal neutral red and aminated aptamer specific to cyt c (Stepanova et al., 2016). Surface-enhanced Raman scattering (SERS) based apta-sensor has also been reported for the detection of cyt (Xia et al., n.d.). The target binding event leads to an enhancement in SERS intensity of SERS-active molecule adsorbed on the gold surface. The detection limit of the sensor is 2 nM with a dynamic range spanning to 1 μM (Table 1).

### 4. Trend towards POC cyt c measurement

POC biosensors allows the detection of wide range of biomarkers at clinical settings. These systems provides the advantages of rapid results and thus ensuring earlier diagnosis and effective treatment. To use in clinical settings and POC diagnosis, the biosensor has to be small, produce rapid results, cost-effective, stable, and should offer continuous/frequent monitoring. The advances in microelectronics and an inherent simplicity afforded by the electrochemical transducers have allowed a novel way to
fabricate miniaturized and low-cost biosensors for routine clinical diagnosis. Our group have recently reported, a portable, cost-effective electrochemical assay for rapid, sensitive, and quantitative detection of cyt c release (Pandaraj et al., 2014a). The fabricated device comprising Ccr-CNT-PPy nanocomposite modified SPE integrated with a low-cost PIC microcontroller based data acquisition unit performing cyclic voltammetry for the measurement of cyt c (Fig. 15). Further, this assay was successfully applied to measure cyt c release from cardiomyocytes and the results were well correlated with standard ELISA. Since the oxidative stress can cause apoptosis to cardiomyocytes through an increased production of H2O2 in cells, we treated cardiomyocytes with H2O2 to mimic the cell death by oxidative stress. Cardiomyocytes cell lines were treated with 50 μM and 200 μM H2O2 for 24 h to induce the cell death. Exposure of cultured cardiomyocytes to H2O2 led to a dose dependent decrease in cell viability, as assessed by MTT assay and miniaturized cyt c biosensor. In the future, the realization of commercial applications will hinge on the continuous improvements in the stability, reproducibility, sensitivity, and specificity of the devices in combination with industrial developments of low-cost processing techniques.

5. Future perspective and conclusions

This review compiles, for the first time, role of cyt c in various biochemical and biomedical process and techniques employed for its detection. The release of cyt c from mitochondria results in activation of cell death pathways and has been demonstrated to play an important role in various neurodegenerative disorders. Studies have that cyt c can also act as an antioxidative enzyme in the mitochondria by removing ROS from mitochondria. The detection of cyt c release is thus critically important as this not only provides valuable information about the nature and extent of apoptosis but serves as a preclinical indicator of various pathologies, medical diagnostics and therapeutic treatment. For example, detection of serum cyt c levels during cancer diagnosis would provide valuable information about prognosis and suggests severity of the disease in cancer patients. However, monitoring of serum cyt c during chemotherapy serve as a sensitive cell death marker to assess the efficacy of cancer therapy and screening of an appropriate anti-cancer drug. Early detection of serum cyt c serves as a preclinical indicator of various pathologies including myocardial infarctions, brain damage, and severe liver damage. Thus early detection and continuous monitoring of cyt c would allow an individual’s more effective patient adjusted therapy with lesser side effects and good clinical outcomes. Researchers have designed novel biosensing technologies that could detect physiological concentration of cyt c. The recent advancements in biosensor technologies have enabled the transition of clinical laboratory cyt c assay to near-patient settings, and provide therapist and biologist with timely diagnostic details to make appropriate decisions regarding diagnosis and treatment. Such developed next generation future sensors would be useful for cyt c related diseases diagnostics and in treatment decision. Enzymatic sensors (both CoO and Ccr based biosensor) offers continuous monitoring of cyt c release while antibody and aptamer based sensors would be suitable for designing a disposable biosensor chip, the one similar to commercial flow immunoassays chip. Successful application of smart nanomaterial to develop miniaturized sensor and further integration to developed portable sensing systems for cyt c detection offers faster, selective, and higher sensitivity than standard ELISA techniques. The progress made in label-free immunosensing and enzymatic biosensing format for cyt c detection enable continuous monitoring of cyt c without the need for any additional reagents.

Finally, this review would serve as a call to promote research related to design of newer biosensing technologies based on various receptor and efficient transducer systems to detect cyt c. We believe that there is a considerable scope to design smart and efficient sensing systems for detecting cyt c at POC application. Such developed POC device would be easy to handle, need low-power to operate, cost-effective, and performing detection in-field/on-site. Such developed system combined with clinical trials correlate cyt c concentration with clinical abnormalities and may provide useful bioinformatics needed to decide therapeutics timely and diseases management for personalized health care.

Conflict of Interest

Authors declare no conflict of interest.

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Fig. 15. Photograph of the USB based electrochemical analyzer showing the interface with cyt c biosensor and laptop (Pandaraj et al., 2014a) (reproduced with permission from Elsevier Limited).


