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A cost-effective volume miniaturized and microcontroller based cytochrome *c* assay

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ABSTRACT

In this paper, a portable, cost-effective electrochemical assay is presented for rapid, sensitive, and quantitative detection of cytochrome *c* (cyt *c*) release. 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. The acquired biosensor data are processed into digital form by the microcontroller and further transferred to a PC through USB port for analysis. GUI based system implemented here makes the analyzer easy to operate. Under optimal conditions, the electroanalytical behavior of the CcR-CNT-PPy-SPE biosensor linearly responds to the cyt *c* concentration range from 10 nM to 500 μM with a detection limit of 10 nM and a sensitivity of 0.102 ± 0.005 μA μM^{-1} cm^{-2}. The performance of the volume miniaturized SPE based biosensor coupled with the portable microcontroller based instrument was further 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.

1. Introduction

The important role of cardiomyocyte apoptosis is well established in a number of clinical abnormalities including myocardial infarction, myocardial ischemia and heart failure [1,2]. The aberrant increase in cellular reactive oxygen species (ROS) levels as a by-product of oxidative stress can cause mitochondrial dysfunction to cardiomyocytes, which subsequently releases the cytochrome *c* (cyt *c*) from mitochondria to cytosol [3,4]. Consequently, the absolute quantification of cyt *c* release is great important as a preclinical indicator of oxidative stress induced cardiomyocyte apoptosis associated with cardiovascular diseases. At the present time, laboratory assays including ELISA, western blot, HPLC, flow cytometry, and spectrophotometry are widely used to detect the cyt *c* [5–10]. Unfortunately, these analytical methods require expensive instruments, time consuming sample preparation procedures and cannot be used as point-of-care testing. Therefore, the development of inexpensive, portable and easy to use analytical assays for the measurement of cyt *c* has been the focus of intensive research efforts.

Over the past two decades, electrochemical biosensors have gained considerable attention and evolved as an alternative technique for the measurement of cyt *c* [11–13]. These biosensors either based on electrostatic interaction or cyt *c* oxidase, lacking specificity or measure only the non-apoptotic form of cyt *c* (Fe(II)) and hence are not suitable for the measurement of apoptosis. Recently, we have reported a cyt *c* reductase (CcR) based biosensor for the measurement of cyt *c* [14]. It enables to monitor apoptosis by measuring cyt *c* (Fe(III)) release with good selectivity. However, it is highly limited to the biological samples in terms of required volume

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(2 mL), usage of the costly reagents and also an expensive commercial electrochemical workstation restricting the users for clinical and field trials. In addition, it limits the long term implementation in point-of-care clinical applications. Therefore, there is a real need for the miniaturization of volume size and the development of low cost electrochemical biosensor device for the measurement of cyt *c* in biological samples.

Replacement of conventional electrochemical cells by screen printed electrodes (SPE) connected with portable potentiostats is a main trend in the shift of lab electrochemical equipments to handheld field analyzers [15,16]. 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 [17–19]. The most significant advantage of these miniaturized SPE based electrochemical biosensors is their compatibility with the microelectronics that allows the commercialization of laboratory prototypes into potential bioanalytical devices. Many researchers have challenged to develop low cost, standalone and portable potentiostat which can be used in different electrochemical biosensors for the past two decades. Most of the reported potentiostats are mainly based on amperometric techniques [20–25]. However, potentiostats performing cyclic voltammetric technique with high sensitivity for detecting low levels of biomarkers in real samples are limited [26–29]. So, in this approach we have combined the distinct advantages of CcR functionalized SPE and USB based sensing device for the measurement of cyt *c* levels released from the cardiomyocytes.

2. Materials and methods

2.1. Chemicals, reagents and apparatus

CcR from porcine heart, cyt *c* from bovine heart, monoclonal cyt *c* antibodies, bovine serum albumin (BSA), H_2O_2, glutaraldehyde, pyrrole, sodium hydrogen phosphate, disodium hydrogen phosphate and Bradford reagent were purchased from Sigma–Aldrich (St. Louis, USA). Single walled carbon nanotubes (CNT) were purchased from Carbon solutions Inc., CA, USA. Rat cardiomyocytes cell line H9c2 was obtained from American type culture collection (ATCC), USA. Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin 1X solution were obtained from HiMedia, India. All the solutions were prepared with doubly distilled water.

A three electrode type planar screen-printed electrode (SPE) system consisting of a carbon working electrode, a carbon counter electrode, and an Ag/AgCl reference electrode (Zensor R&D, Taiwan) was employed for designing of miniaturized electrochemical biosensor. The structural characterizations of the nanocomposite modified electrodes were obtained by using scanning electron microscope (SEM) equipped with energy dispersive X-ray (EDX) analysis (FEI Co., Netherlands). The phase contrast images were acquired by using an Olympus-1X71 inverted microscope (Olympus Co., USA). The cyclic voltammetric measurements were carried out with a commercial CHI1200B electrochemical workstation (CH Instruments, USA).

2.2. Portable electrochemical device description

Cyclic voltammetry is a very important electrochemical technique used to study the mechanism of charge transfer reaction of redox species, in particular to determine the concentration of biomolecules using its oxidation/reduction reaction pathways. In cyclic voltammetry, the biasing voltage across the working and reference electrodes linearly swept, while the resultant current across the working and counter electrodes measured throughout the electrochemical process in a three electrode type design. We have designed and programmed the portable electrochemical device to perform cyclic voltammetry technique for the cyt *c* analysis.

2.2.1. Circuit design and hardware architecture

The circuit diagram of the microcontroller based portable cyclic voltammetric analyzer is shown in Fig. 1. A miniaturized three-electrode system is connected to a PIC18F4550 microcontroller based data acquisition unit containing home-made potentiostat circuit, voltage output digital to analog converter (DAC), a level shifter, and an op-amp inverter. The necessary voltammetric waveform between the specified voltage ranges to be applied to the electrodes is produced by MAX5154, a 12-bit DAC. The output current generated at the WE as a result of electrochemical reaction is

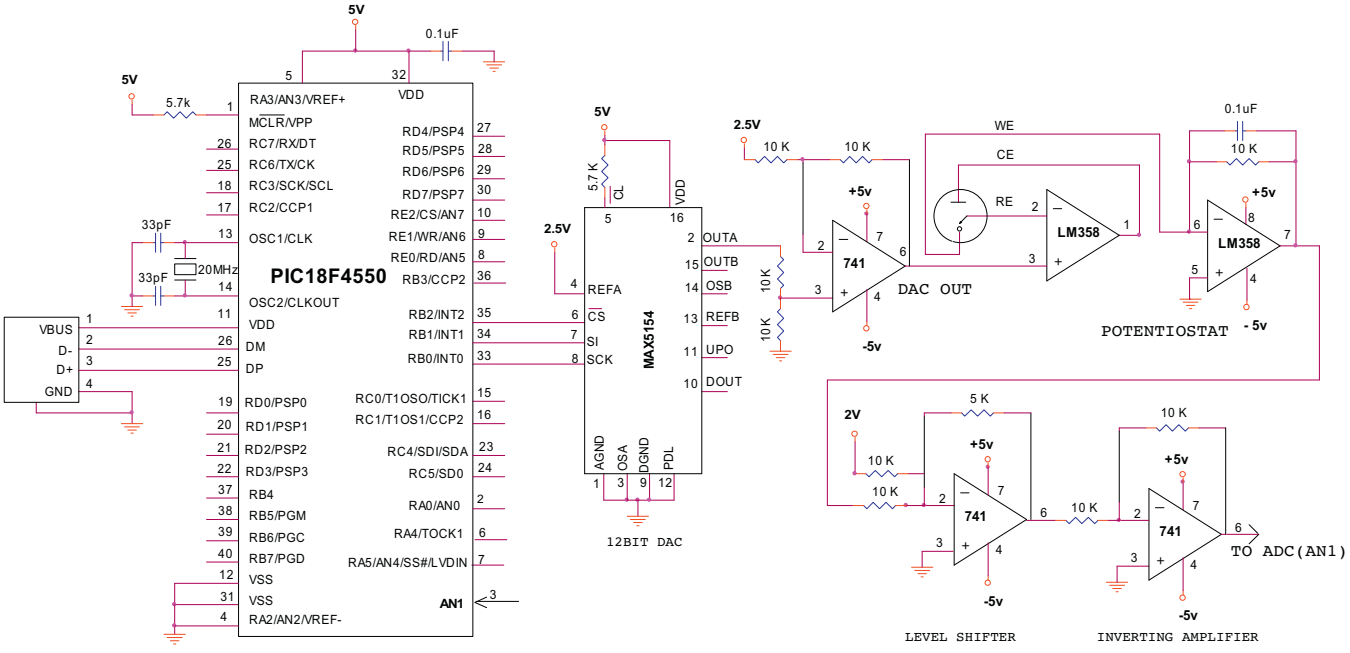

Fig. 1. Block diagram of the portable microcontroller based cyclic voltammetric device.