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Disposable Printed Lateral Flow Electrochemical Immunosensors for Human Cardiac Troponin T

Eithne Dempsey and Dhanraj Rathod

Abstract— **Here we report an electrochemical ELISA approach for cardiac Troponin T (cTnT) determination based on a lateral flow membrane with underlying screen printed electrodes (<100 Ω/cm^2). The thick film transducer was modified with the anti-cardiac Troponin T antibody via physisorption and the electrochemical performance of the immunosensor was evaluated using cyclic voltammetry. The capture antibody coated immunoelectrode employed for electrochemical determination of Troponin T antigen used a sandwich assay format with horseradish peroxidase conjugated signaling antibodies held in the track of a lateral flow sensor strip. A simple two step procedure realized signal acquisition within <20 min (total assay time). The lateral flow electrochemical immunosensor response resulted in a calibration curve with linear response (0-700 ng/ml cTnT) with limit of detection of 0.15 ng/ml.**

Index Terms— **Immunosensor, Troponin T, lateral flow immunoassay.**

I. Introduction

CARDIOVASCULAR disease (CVD) causes nearly half of all deaths worldwide and includes myocardial infarction (MI) which is one of the most significant forms of ischemic heart disease, with necrosis of the myocardium leading to a reduction in blood flow [1]. In recent years, electrocardiographic investigation has been used as the main method for establishing a MI diagnosis, supported by access to rapid and reliable blood biomarker test data, assisting in diagnosis and appropriate therapy. Clinical investigation of heart diseases has developed in two main directions namely; detection of new cardiac biomarkers and implementation of new devices for point of care (POC) testing with enhanced sensitivity and selectivity. In view of this, detection and quantification of defined cardiac markers is now of primary importance in diagnosis.

There are several high throughput automated systems employed in clinics for disease diagnosis. At present, commercially available bench-top analyzers and hand-held devices are used for cardiac biomarker determination and include; Stratus®CS STAT (Dade Behring, Inc.), i-STAT®(Abbott), Triage®Cardiac Panel (Biosite), Cardiac Reader TM (Roche), RAMP®(Response Biomedical Corp), and PATHFAST®(Mitsubishi Chemical Europe GmbH) [2], [3]. Some such systems can be costly, laboratory confined and require skilled operators.

The most widespread acute myocardial infarction (AMI) biomarkers are troponin I [4], troponin T [5], creatine kinase-myoglobin (CK-MB) [6] and myoglobin (Mb) [7]. All of these cardiac markers differ in terms of their kinetics, cardiac-specificity, and prognostic value. Troponins are regulatory proteins which play a fundamental role in the contraction of cardiac muscle cells [8] and are positioned at regular intervals along the actin muscle strands. They are composed of three subunits, referred to as cardiac troponin T (cTnT), cardiac troponin I (cTnI), and cardiac troponin C (cTnC) [9]. The letters T, I, and C relate to the function of each member of the complex, where troponin-C is a calcium (Ca^{2+}) binding-subunit, troponin-I is an inhibitor, and troponin-T is a tropomyosin binding subunit. These troponin subunits regulate the Ca^{2+} dependent muscle contraction in all muscle cells. Thus, the binding of Ca^{2+} to cTnC results in increased affinity for troponin I which then releases its inhibitory function on actomyosin adenosine triphosphate, and leads to adenosine triphosphate hydrolysis and muscle contraction.

Troponin T is an important and highly specific marker, with a prolonged circulating lifetime in blood after MI [10], [11]. It originates exclusively from the myocardium, and has a molecular weight of 39.7 kiloDaltons (kDa). Initial elevation of cardiac troponin T takes 4-6 hrs, with peak release time registered at 12-24 hrs [10], [12], [13] However, it remains elevated for 7-21 days following myocardial injury, and thereby has replaced less specific markers such as CK, CK-MB, aspartate aminotransferase [14], and lactate dehydrogenase isoenzymes, all of which have previously been used as confirmation of heart injury. During MI, Troponin T is released slightly earlier than troponin I. Therefore, troponin T is considered a valuable indicator in the diagnosis of myocardial damage [15], [16].

Troponin T antibodies which are used to develop immunoassays are highly specific and do not cross-react with skeletal muscle troponin isoforms, such that troponin released from skeletal muscle cannot be detected [17], [18]. Therefore, the cut-off concentrations for cardiac troponin I and T can be reliably set at low levels <0.1 ng/ml. Hence, patients with skeletal muscle injury are unlikely to have elevated circulating levels of cTnT or cTnI (>0.1 ng/ml) [19]. Compared to the previous 'gold standard' creatine kinase MB (CK-MB),

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cTnT is reported to be a highly specific marker for the detection of cardiac injury. An increase in blood concentration of troponin T can also occur in other clinical conditions such as congestive heart failure, cardiomyopathy, myocarditis, heart contusion, renal failure and left ventricular dysfunction in septic shock [18], [20].

Clinical diagnosis, especially those of MI and heart injury, requires reliable test systems. Rapid quantitative determination of cTnT has been introduced into routine clinical usage based on an electrochemiluminescence based immunoassay [20] and immunoassay tests that can be used to monitor the levels of troponin in less than 20 min [13], [20]. Many biochemical methods for AMI diagnosis are based on enzyme-linked immunosorbent assay (ELISA) [22]. However, the ELISA test requires additional chemicals (washing steps), skilled personel to carry out the test, and it cannot be used outside hospital laboratories [22].

Screen printed carbon electrodes (SPE) have been widely used in the design of disposable electrochemical immunosensors for clinical analysis [23]–[25]. Screen printing microfabrication technology is well established in the production of thick film electrochemical transducers [26] and allows for mass production of reproducible yet inexpensive and mechanically robust strip solid electrodes [27]. Other important features of electrodes include miniaturisation of the corresponding device, along with ease of handling, and a disposable format [28], [29]. These electrodes can be constructed from both graphite powder and an epoxy resin [30], resulting in a composite that can act not only as a transducer for electrochemical signal generation, but also permits incorporation of different substances such as mediators, enzymes, antibody and antigen etc.

The first electrochemical immunosensors used for cardiac markers based on a SP allowed detection of Mb over a wide concentration range (0.01 to 10 μg ml^{-1}) [31] while a disposable immunosenssor for human cardiac troponin T based on a streptavidin-microsphere modified screen printed electrode resulted in linear response range between 0.1 and 10 ng/mL cTnT and a detection limit of 0.2 ng/mL [23]. Gomes-Filho *et al*. [32] reported a carbon nanotube based electrochemical immunosensor over the relevant clinical range. Limitations to these and related works include sensor complexity and the requirement for multiple steps in the assay format which restricts on-site use by minimally trained personnel at the point of use.

A lateral flow assay (LFA) is a powerful tool which permits a one step, rapid and low cost method of analysis [31] having well established roots in pregnancy testing and semi-qualitative optical detection [33]. Here we present the combination of an easily constructed antibody modified thick film electrochemical transducer with a lateral flow membrane which offers advantages of convenience, on-board reagents and sample handling in a simple single use operation. Immobilisation of an anti-Troponin T antibody via "wet" and "dry" chemical methods was proven to be successful in immunoassay fabrication. Horseradish peroxidase (HRP) conjugated secondary antibody reagent provided redox signal generation via o-phenylenediamine (OPD) enzymatic conversion to the diiamine with subsequent cathodic redox signal related to troponin T levels.

II. Experimental

A. Materials

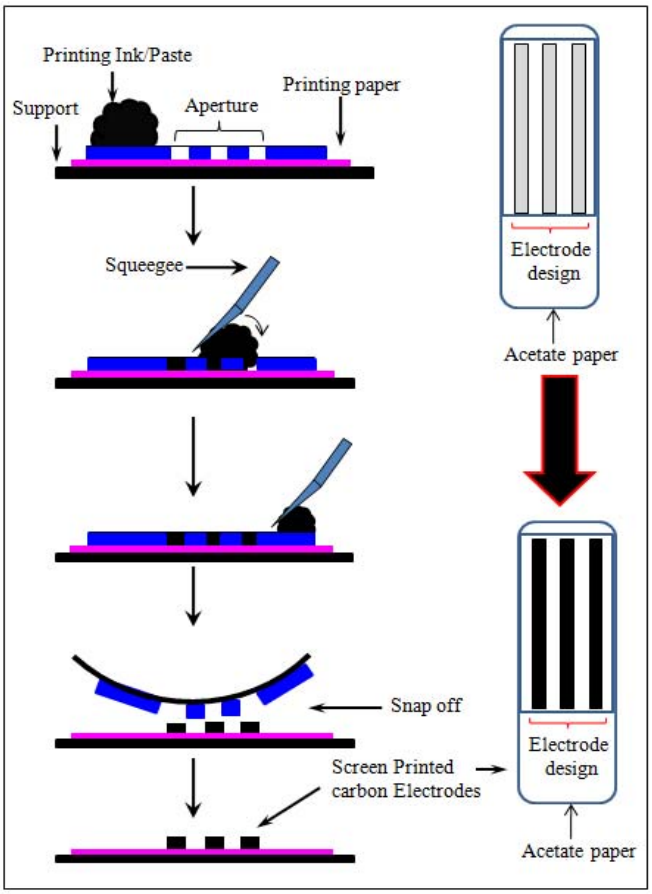
Carbon sensor paste (Gwent UK), silver/silver chloride paste (Gwent UK), acetate paper (Xerox Ireland), insulating tape (Sellotape UK), potassium chloride, KH_2PO_4, K_2HPO_4, hydrogen peroxide (H_2O_2), phosphoric acid (H_3PO_4), o-phenylenediamine, Tween 20 (Aldrich). Bovine serum albumin (BSA) (Aldrich), anti-cardiac troponin T antibody [Ab-cTnT], cardiac troponin T protein [cTnT] and anti-cardiac troponin T antibody-HRP [Ab-HRP] were purchased from Abcam UK. Deionised water of 18 MΩ was produced using Millipore water system and all reagents were used as received.

B. Equipment

An electrochemical work station (CH Instruments Inc. 660) was employed for electrochemical characterisation of electrodes using cyclic voltammetry (CV). Experiments were performed in a conventional three-electrode system using a carbon conducting track working electrode vs. Ag/AgCl (aq.) reference electrode and a carbon counter electrode. Screen printed electrodes (SPE) were fabricated *in house* using a DEK 255 screen printer.

C. Electrochemical Immunosensor Fabrication

1) Immunosensor fabrication using in-house screen printed electrodes: The electrode design (2×35 mm) was printed onto commercial acetate paper using a HP inkjet printer. Transparent single sided adhesive tape was placed on the designed portion, creating an aperture. The screen ink was deposited by a stencil printing process; using a squeegee (a rubber blade used to print the ink through the screen on the substrate). The tape was then removed slowly (snap-off) to ensure that the electrode was not disturbed. Electrodes were then cured at 60°C for 2 hrs and the resistance of each electrode was measured individually. If measured resistance was >100 Ω/cm^2, devices were re-fabricated until this value was achieved. The reference electrode (RE) was formed by depositing a layer of a silver/silver chloride paste on the surface of the central carbon track. The electrode printing process is schematically presented in Scheme 1. Following curing, the middle portion of the electrode was covered with insulating tape maintaining a 5 mm active open area with working electrode (WE), reference electrode (RE) and counter electrode (CE) (Fig.1(A)). The capture antibody raised against the cardiac troponin T was physisorbed by a "wet" chemical process. This was achieved by adding 5 μl of 200 μg ml^{-1} antibody to the electrode surface followed by incubation at 4°C overnight. The capture antibody-coated electrodes were then washed three times with phosphate buffer – Tween (PBS-T) (5 min per wash), pH 7.4 in order to remove loosely attached antibody over the electrode surface. This process created a monolayer of randomly oriented capture antibody at the electrode surface. The electrodes were then air dried at room temperature for 30 min, and maintained at



Scheme 1. Schematic showing the stages involved in the electrode printing process.

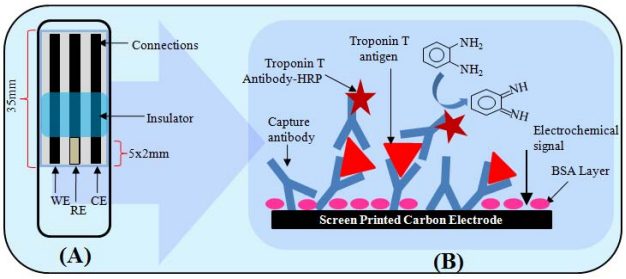
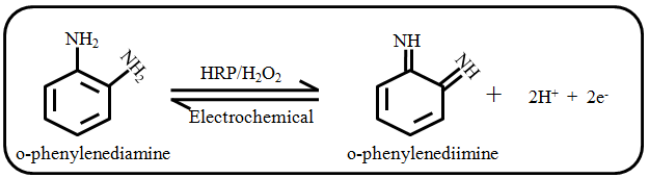


Fig 1. (A) Screen printed carbon working electrode (WE) Ag/AgCl reference (RE) and carbon counter electrode (CE). (B) Working principle of the electrochemical immunosensor.

4°C for 24 hr in order to strengthen the antibody binding to the carbon surface.

The capture antibody coated immunoelectrode then underwent a blocking procedure with 5% (w/v) BSA for 1 hr followed by washing with phosphate buffer – Tween (PBS-T) three times (5 min per wash). The electrode was then employed for the detection of troponin T over a range of concentration (100-1000 ng ml^{-1}) at 25°C using a 20 min binding period. The electrodes were then washed three times with PBS-T (5 min per wash) followed by exposure to the conjugated antibody (Ab-HRP) (100 μl of 10 μg ml^{-1}) for 1 hr. Further washing with phosphate buffer – Tween (PBS-T) followed and then a cyclic voltammogram was recorded in



Scheme 2. Oxidation of o-phenylenediamine to o-phenylenediimine by HRP enzyme (forward reaction) and electrochemical reduction of o-phenylenediimine at the immunoelectrode surface by CV (backward reaction).

1 mM o-phenylenediamine (OPD)/hydrogen peroxide (H_2O_2) in PBS, pH 6 over the potential window +0.2 to −0.2 V at 100 mVs^{-1} vs. Ag/AgCl reference electrode. A schematic representation of the capture antibody-antigen and horseradish peroxidase antibody complex on the electrode surface is presented in Fig. 1. The conjugate antibodies at the electrode surface converted o-phenylenediamine to o-phenylenediimine (Scheme 2). The o-phenylenediimine intermediate was then electrochemically reduced via potential scanning over the potential window +0.1 to −0.1 V at 100 mVs^{-1} vs. Ag/AgCl. Electrochemical cycling was initiated from +0.1 V in order to observe the reduction wave of the diimine molecule which correlated with the concentration of troponin T bound to the capture antibody.

2) Lateral flow Immunosensor Based on Screen Printed Electrode: In house screen printed electrodes were fabricated as described above. The primary antibody raised against troponin T (capture antibody) was immobilised onto the surface of the electrode by adding 5 μl of 200 μg ml^{-1} onto the active area (5×2 mm^2) and incubating at 4°C overnight. Following this process, electrodes were washed as above and the electrode was then air dried at room temperature for 30 min and placed at 4°C for 24 hr. A Whatman filter paper (membrane, 5×50 mm^2) was then placed directly over the SPE area using double sided adhesive tape (70 μm), as shown in Fig. 2 B&C. Following this, the HRP conjugate antibody (5 μl of 200 μg ml^{-1}) was placed on the membrane at a distance of 30 mm from the working electrode, and the sample containing troponin T antigen (cTnT) was immediately added at a distance of 50 mm from working electrode. During this process, the antigen traveled down the strip towards the anti-troponin binding site and formed a complex with the conjugated antibody in the flow path. Subsequent forward migration towards the working electrode realized sandwich formation at the capture antibody zone. This process was allowed to proceed for 20 min, followed by washing with PBS-T three times (1 min per wash). During washing, the PBS-T solution was applied to one end and collected on an adsorbent pad. The washing step assisted in the removal of free antibody conjugate and in this way served to reduce non specific binding (interference), and miminise background signals. Once the washing step was completed, 50 μl of 1 mM OPD/H_2O_2 in PBS, pH 6 was applied directly onto the electrode and the electrochemical signal was measured immediately. The current response obtained from the electrochemistry of o-phenylenediamine was shown to be proportional to the concentration of troponin T applied to the lateral flow sensor.

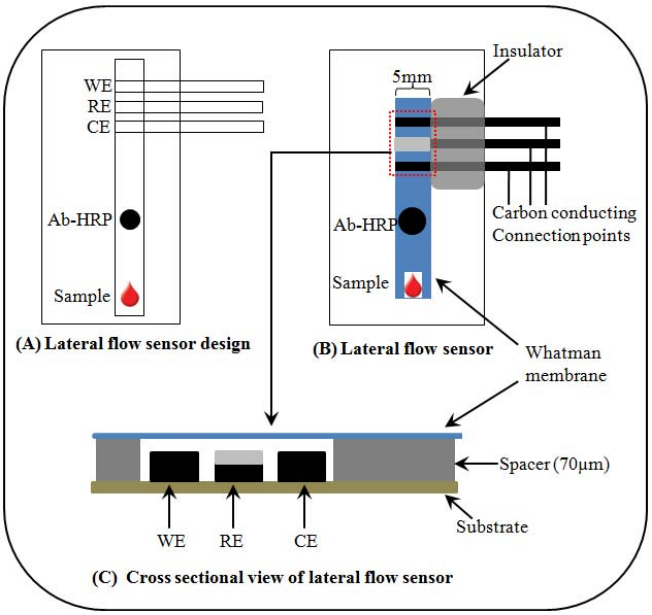


Fig 2. Lateral flow immunosensor fabricated using screen printed carbon electrodes.

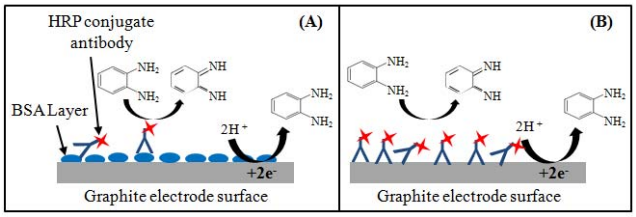


Fig 3. Shows the adsorption of horseradish peroxidase conjugate antibody over the BSA coated (A) and uncoated electrode surface (B).

Experiments were repeated over a range of concentration of troponin T from 100-700 ng ml^{-1}. All standard test solution measurements were repeated five times (inter electrode, n=5) in order to determine the reproducibility and precision of the immunosensor.

III. Results and Discussion

A. BSA Blocking Effect on HRP-Conjugate Adsorption

Non-specific adsorption is a common problem associated with signaling antibodies during immunosensor fabrication, and can be reduced using surface blocking proteins (gelatin, casein, milk protein and bovine serum albumin - BSA). In the current study, bovine serum albumin was used as a surface blocking protein due to its adsorption properties on carbon surfaces [29]. Here we examined the non-specific adsorption of signaling antibody on a BSA-coated and uncoated SPE surface. The adsorption of the signaling antibody in the presence and absence of a BSA layer on the electrode surface is depicted schematically in Fig. 3.

The non-specific adsorption of horseradish peroxidase-conjugate antibody under immunoassay conditions was revealed by peroxidase activity measurements recorded on a BSA coated and uncoated electrode surface. The voltammetric features for the peroxidase formed o-phenylenediimine

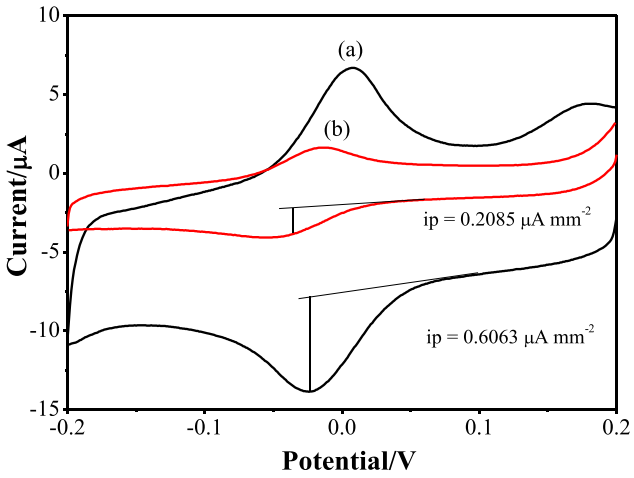


Fig 4. Cyclic voltammogram of in-house screen printed carbon electrode (a) SPE/HRP-Ab, (b) SPE/BSA/HRP-Ab in 1 mM o-phenylenediamine/H_2O_2 in phosphate buffer, pH 6 at 100 mVs^{-1} vs. Ag/AgCl reference electrode.

include; a redox couple between +0.2 V to −0.2 V and an increase in peak current observed (Fig. 4). The adsorption of a high concentration of conjugate at the BSA coated electrode was investigated with respect to the uncoated electrode surface. The influence of BSA immobilisation on the diimine signal at thick film carbon transducers showed a diminished electrochemical signal for diimine reduction, indicating less bound peroxidase conjugate.

The diimine reduction current for the BSA coated electrode was found to be 0.21 μA mm^{-2} (Fig. 4b), while in the absence of BSA (Fig. 4a) the diimine reduction current was elevated to 0.61 μA mm^{-2}. This represents a three-fold increase in response over the BSA coated electrode surface. In both cases the reduction and oxidation of o-phenylenediimine occurred over the potential window +0.1 to −0.1 V. An additional anodic peak appeared at $E_p = 0.135$ V vs. Ag/AgCl, which may be due to further oxidation and dimerisation of the diamine molecule which commenced at a higher potential. This experiment confirmed the significant role of BSA coating in immunoassay fabrication.

B. Detection of Troponin-T at Modified Screen Printed Electrode

Fig. 5a shows the electrochemical response of the capture antibody-coated BSA electrode which appeared from the non-specific adsorption of the horseradish peroxidase-conjugate antibody in the absence of the troponin T antigen. This indicated that the capture antibody-coated BSA electrode was porous enough to allow exchange of electrons at the electrode solution interface. The diimine reduction current of background response was found to be 0.304 μA mm^{-2} due to non-specific adsorption of the conjugated antibody on the capture antibody coated electrode surface (SPE/Ab/BSA/Ab-HRP). The electrochemical signal elevation in the presence of antigen at 100 ng ml^{-1} (SPE/Ab/BSA/cTnT/Ab-HRP) shows a signal of 0.39 μA mm^{-2} due to reduction of o-phenylenediimine. This indicated that the antigen formed a complex with the antibody (Fig. 5b) and elevation in the electrochemical signal of diimine reduction with the antigen

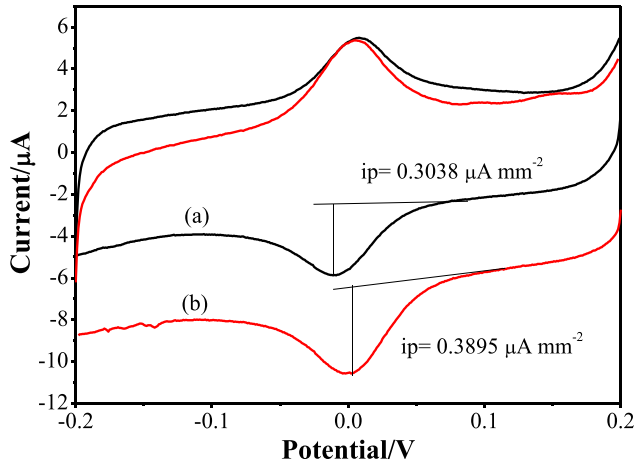


Fig 5. Cyclic voltammogram of modified SPE in 1 mM o-phenylenediamine /H_2O_2 in phosphate buffer pH 6 vs. Ag/AgCl reference electrode at 100 mVs^{-1}, **(a)** SPE/Ab/BSA/Ab-HRP, and (b) SPE/Ab/BSA/cTnT (1000 ng ml^{-1})/Ab-HRP.

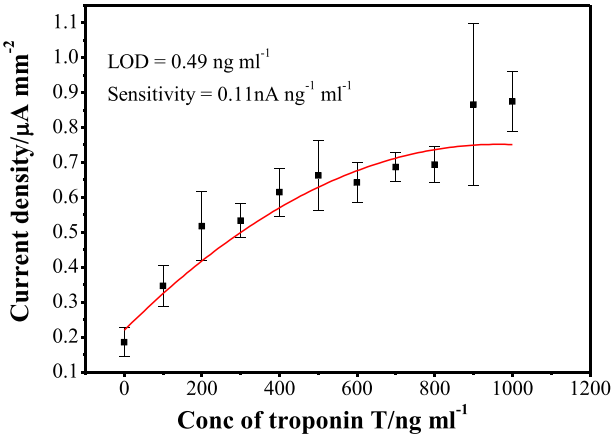


Fig 6. Calibration plots shows current density vs. concentration of troponin T detected at the SPE surface (n=5). Limit of detection = 0.49 ng ml^{-1}.

level over a concentration range of 100-1000 ng ml^{-1} was achieved. Additionally, the increase in the diimine reduction current above the background signal indicated that the capture antibody was exposed to the active antigenic sites for the specific antigen.

A calibration plot for the detection of troponin T over a wide concentration range based on the electrochemical signal of diimine showed proportionality with the antigen concentration as shown in Fig. 6. Initially, increases in the electrochemical signal for o-phenylenediimine showed a linear response for troponin T from 100-500 ng ml^{-1}, and a steady current response appeared from 600-1000 ng ml^{-1}. The immunosensor showed a sensitivity of 0.11 nA ng^{-1} ml^{-1} with a lower limit of 0.49 ng ml^{-1}. The steady response obtained suggested that the capture antibody surface was saturated with antigen, and no further antigenic sites were available. This effect served to limit the use of the immunoelectrode at a working concentration range for antigen detection. Additionally, a limiting current of 874 nA mm^{-2}, at higher concentration indicated that all antigenic sites on the capture antibody were involved in binding.

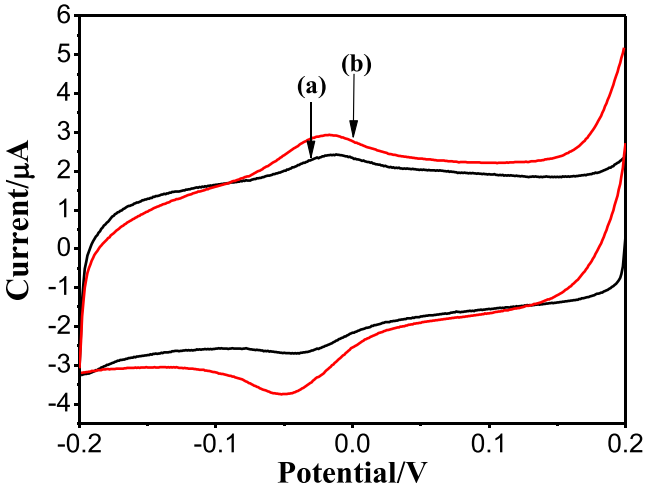


Fig 7. CV of the immunoelectrode in the absence of antigen (a) [SPE/Ab/BSA/Ab-HRP], (b) detection of troponin T antigen (100 ng ml^{-1}) at capture antibody coated BSA electrode [SPE/Ab/BSA/cTnT/Ab-HRP] in 1 mM o-phenylenediamine /H_2O_2 in phosphate buffer, pH 6 at 100 mVs^{-1} vs. Ag/AgCl reference electrode.

All concentrations of antigen were analysed using five replicates (n=5) in order to illustrate the reproducibility and precision for target antigen detection. Statistical analysis of the diimine electrochemical signal arising over this range of antigen concentration resulted in average coefficient of variation of 13.8%. This imprecision may be due to variation in the diimine signal at the electrode surface due to screen printed carbon surface inter-electrode reproducibility and/or the surface blocking effect of the BSA protein. A random orientation of capture antibody at the electrode surface also may be a reason, however, at some concentration points, the coefficient of variation was <10%. In conclusion, the capture antibody-coated BSA electrode was capable of detecting and quantifying the target specific antigen over the range examined.

C. Detection of Troponin T by Lateral Flow Immunosensor

Following the proof of concept study for troponin T detection on a screen printed modified electrode, fabrication of a facile electrochemical lateral flow device was achieved via placement of a Whatman membrane onto the capture antibody-coated BSA electrode, using a spacer of 70 μm thickness. This membrane created a 40 μm gap between the electrode and membrane surface which facilitated Ab-Ag binding. The conjugated antibody (5 μl of 200 μg ml^{-1}) was present at the middle portion of the membrane at a distance of 3 cm from the electrode surface. A 150 μl aliquot of sample containing troponin T antigen over a concentration range of 100-700 ng ml^{-1} was added and allowed to bind for a period of 20 minutes. During this time the Troponin T traveled from one end of the electonic lateral flow device to the detection cell via capillary action. A complex with HRP conjugated antibody was formed en-route and capture occurred at the antibody-coated screen printed carbon electrode surface.

The specific target antigen was measured via the electrochemical reduction of diimine (Fig. 7), over the range 100-700 ng ml^{-1}. A resulting calibration plot showed linearity

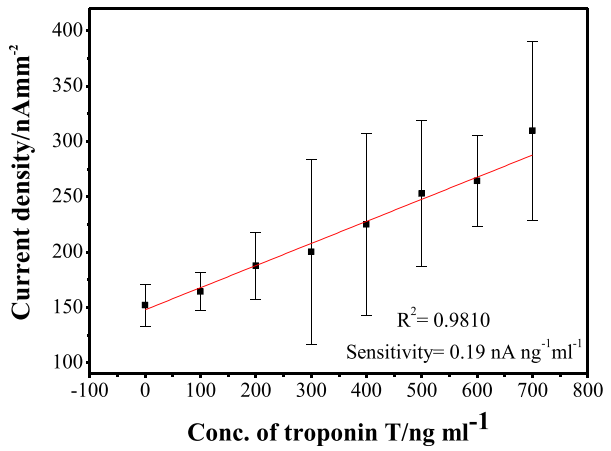


Fig 8. Calibration curve for Troponin T using the electrochemical lateral flow immunosensor, over a concentration range of 100-700 ng ml^{-1} (n=5 devices).

TABLE I

COMPARATIVE PERFORMANCE CHARACTERISTICS OF IMMUNOSENSORS FOR DETECTION OF VARIOUS CARDIAC MARKERS

Detected Marker	Biosensor Type	Sensitivity (ng ml^{-1})	Assay Time (min)	Detection Level (ng ml^{-1})	Reference
cTnI	Potentiometric	1	120	1-500	[34]
	Fluorescent intensity	2	240	0-30	[35]
	Chemiluminiscence	0.027	30	0.1-50	[36]
	SPR	1.4	10	1-10	[37]
	Potentiometric	0.1	20	0.1-100	[38]
	Voltammetric	0.01	100	0.01-50	[39]
	Voltammetric	0.14	20	0.2-10,000	[40]
cTnT	Fluorescent	0.025	240	0.02-0.25	[34]
	FRET	0.7	25	0-10	[41]
	Amperometric	0.2	120	0.1-10	[24]
	SPR	0.01	20-25	0.2-7	[42]
CK-MB	Fluorescence	3	240	3-16	[35]
	Amperometric	13	--	0-300	[43]
cTnT	*Voltammetric/ lateral flow*	0.41 *0.49* *0.15*	120 *20* *20*	0.1-10* *0-1000* *0-700*	*This work* **Graphite rod electrode*

with a detection limit of 0.15 ng ml^{-1}, and sensitivity of 0.2 nA ng^{-1} ml^{-1} (Fig. 8). Here, the Whatman membrane played an important role in rapidly capturing the antigen-conjugate antibody complex on the electrode, generating a more sensitive signal, reducing reagent addition steps and assay time to result. Therefore, this type of lateral flow sensor can contribute towards both the qualitative and quantitative determination of troponin T with the potential for extension to a dual Troponin I and T assay format. The coefficient of variation ranged from 7.3-16.5% for n=5 lateral flow electrochemical devices. Additional design improvements are required to minimize membrane porosity variations and flow rate effects inherent to lateral flow dynamics.

Table 1 gives a comparison of analytical performance characteristics for recent reports regarding cardiac markers based on modified electrodes. The biosensor developed in this work compares well with respect to time to result, convenience of use/minimal reagent handling errors and is one of the few reports in the literature of lateral flow membrane technology integrated with screen printed electrode immunosensors for Troponin T quantitation.

IV. CONCLUSION

Here, we have successfully fabricated a single use disposable electrochemical immunosensor, based on low cost materials - screen printed carbon electrode and lateral flow membrane, for the determination of human cardiac troponin T. Troponin T was detected at concentrations ranging from 100-1000 ng ml^{-1} at a modified screen printed electrode or 100-700 ng ml^{-1} using an electrochemical lateral flow device. A linear relationship was shown to exist between the electrochemical signal from the reduction of o-phenylenediimine and antigen over the range examined, with sensitivity values of 0.11 nA ng^{-1} ml^{-1} and 0.19 nA ng^{-1} ml^{-1}, respectively, within a 20 min assay time. This sensor was easy to fabricate, suitable for mass production, cost effective and provided proof of principle for detection and quantification of a globally relevant biomarker of MI, with the potential to contribute to its early detection at the point of care with the ultimate benefit of reducing patient mortality.

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