

Electroanalysis on an Interdigitated Electrode for High-Affinity Cardiac Troponin I Biomarker Detection by Aptamer–Gold Conjugates

Jintao Zhang, Thangavel Lakshmi priya, and Subash C. B. Gopinath*



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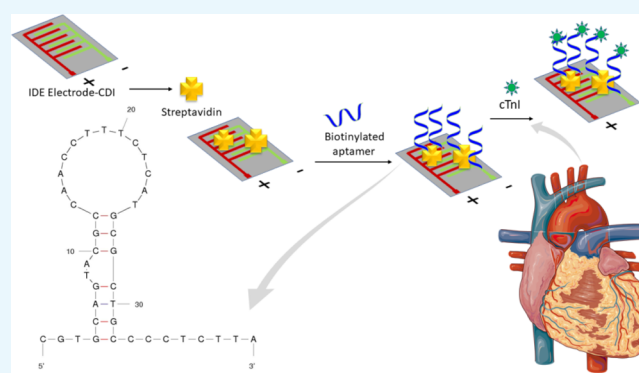


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ABSTRACT: The primary reasons for myocardial infarction (MI) are pericarditis, arrhythmia, and heart failure, causing predominant deaths worldwide. Patients need a potential diagnostic system and treatment before cardiomyocyte damage. Cardiac biomarkers are released from myocytes immediately after a heart attack. Troponin is an efficient biomarker released from dead cells within a few hours. Aptamers are artificial antibodies used effectively in the biosensor field for biomarker detection. Along with aptamers, the application of nanomaterials is also expected to enhance the detection limits of biosensors. In this investigation, selected aptamers against cardiac troponin I (cTnI) were conjugated with gold nanoparticles (GNPs) to diagnose MI and compared with an aptamer-only control group on an interdigitated electrode surface. Based on electroanalysis, cTnI was detected at concentrations as low as 1 fM, and the detection limit improved to 100 aM when the aptamer was conjugated with GNP. In addition, aptamer–GNP conjugates increased the current level at the tested concentrations of cTnI. Control experiments with noncomplementary aptamers and relevant proteins did not result in notable changes in the current, demonstrating the selective detection of cTnI.



1. INTRODUCTION

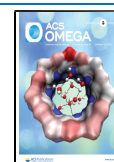
Cardiovascular disease is a major life-threatening global disease with a high morbidity and mortality worldwide.¹ Myocardial infarction (MI) is commonly defined as a heart attack due to the reduction of blood flow to the heart.² According to the WHO, it is expected that ~23 million people will die annually from MI by 2030. MI can be identified by electrocardiogram (ECG); however, it lacks sensitivity. ECG needs supporting evidence from blood-based cardiac biomarker analysis for the identification of cardiac-related diseases. MI is due to cell death in the heart; ultimately, myocytes cannot regenerate, which affects the cardiac function. During cell death, biomarkers, such as myoglobin, creatine-kinase MB, cardiac troponin, and C-reactive protein, will be released to the cytoplasm more rapidly from these dead cells than from normal cells. Cardiac troponin I (cTnI) is the subunit of troponin and has been found to be an efficient biomarker to diagnose MI.³ Cardiac troponin T (cTnT) and cTnI are specific and sensitive and are considered as standard biomarkers for MI diagnosis. These proteins are released from dead cells within 2–4 and 3–4 h upon MI onset. Their concentration reaches a peak after 1–2 days, and they remain in the bloodstream even after 10 days, which facilitates the diagnosis of MI.^{4,5} Various sensing methods, such as surface plasmon resonance, optical sensing, enzyme-linked

immunosorbent assay, and electrochemistry, have been used to detect cTnI.^{6–9} Detection probes include antibodies, aptamers, DNA, and RNA, which are used to quantify the level of cTnI.^{10–12} Among these probes, aptamers have received great attention from researchers due to their selective binding affinity to their targets. In this research, an aptamer specifically binding cTnI was utilized to determine the level of cTnI by using an interdigitated electrode (IDE) sensor. IDE is desired for use in this study due to its advantage in improving the performance of the sensor by adjusting the gap and finger sizes. In addition, IDE tolerates a wide range of metals, which are used as both the substrate and the electrodes, is inexpensive, and easy to manufacture. Furthermore, because the fabricated substrate is a metal oxide material, it is highly suitable for surface chemical functionalization to capture a high number of biomolecules. This sensor is suitable for applications with a wide range of clinically relevant biomarkers.

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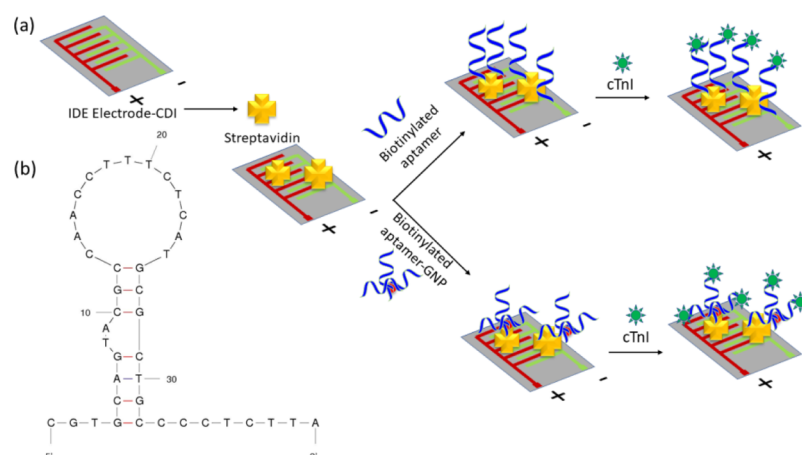


Figure 1. (a) Schematic illustration of the detection of cTnI. Detection by aptamer or aptamer–GNP conjugates on IDE sensing surfaces. The aptamer was immobilized on the sensing surface through a chemical linker followed by a biotin–streptavidin strategy and detected by cTnI. (b) Predicted secondary structure of the aptamer by mfold software showed a stem-loop structure.

An aptamer is a synthesized RNA/DNA oligonucleotide sequence selected from the randomized library through the steps of binding, partitioning, and amplification.^{13–15} Since the generated aptamers offer advantages over antibodies, they can be utilized for multiple applications. In particular, aptamers are more attractive as biosensors because of their unique features, such as their high performance to bind to targets.⁹ In this research, an aptamer generated against cTnI was used as the detection probe to diagnose MI on the IDE surface.

Along with aptamers as suitable probes, nanomaterials applied in the field of biosensors can improve the detection of targets.¹⁶ In particular, DNA can be easily conjugated with various nanomaterials and be used to detect diseases in an efficient way. Among different materials, gold is a well-established material and is utilized to immobilize biomolecules on sensing surfaces to improve detection. Due to the optical properties of gold, it is a widely accepted material in the construction of optical-based biosensors.^{17,18} Moreover, utilizing gold in piezoelectric and electrochemical sensors is attractive because of its excellent conductivity, high surface-area-to-volume ratio, biocompatibility, and catalytic properties. In this research, electroanalysis was conducted using aptamers conjugated to gold nanoparticles (GNPs), which were used as the capture probe to detect cTnI and compared with aptamer-only immobilized IDE surfaces without GNPs.

2. RESULTS AND DISCUSSION

Early diagnosis of MI is a challenging task due to the lack of an efficient sensing system suitable for a wide range of applications. Current ECG and other diagnostic systems are not advantageous. In addition, it is required to diagnose MI in a short period of time. Circulating biomarkers have recently gained great attention for diagnosing MI efficiently.¹⁹ Troponin is a sensitive cardiac biomarker, which helps to diagnose MI immediately after its release from the cells.^{20,21} Researchers are focused on identifying a suitable effective diagnostic system to quantify the level of troponin. In this research, cardiac biomarker (cTnI) detection by its aptamer and aptamer–GNP was carried out on the IDE surface. Figure 1a shows the schematic illustration for the cTnI detection on aptamer- and aptamer–GNP-modified IDE surfaces. The aptamer exhibits strong stem regions with two loops, facilitating the high-affinity binding of cTnI. To attach

aptamers on the IDE surface, a biotin–streptavidin pattern was utilized. Since it was indicated that biotin–streptavidin has a high binding affinity, it can be used to improve the immobilization process and conjugate other biomolecules onto the sensing surfaces.^{13,22–25} Streptavidin was attached to the IDE surface through a 1,1'-carbonyldiimidazole (CDI) chemical linker, and a biotinylated aptamer was attached to the streptavidin-modified IDE surfaces. CDI has been widely used for conjugating amino acids during peptide synthesis and aromatic amines to form amides. Similarly, this strategy was used to link the amine groups in streptavidin to the hydroxyl groups of the sensing surface.²⁶ In this way, more aptamers can bind on the sensing surface, which can improve the detection of cTnI. In addition, to enhance the detection of cTnI, the aptamer for cTnI was complexed with GNPs. Gold-complexed biomolecules are stable, and on the surface of GNPs, a large number of biomolecules can bind and attract more target molecules.

The sensitive and selective detection mechanism is outlined in Figure 2 in which the current sensing system operates based

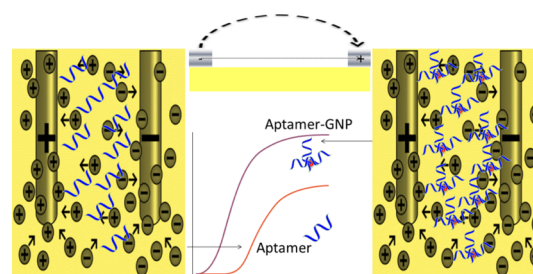


Figure 2. Dipole mechanism between two electrodes. Charge movements are between positive and negative electrodes. A graphical explanation with the aptamer and aptamer–GNP is shown.

on the dipole moment between the positive and negative electrodes in the presence of an electrical field. This electrical field is enhanced in the presence of molecular crowding, and it showed an apparent difference depending on whether aptamer–GNP or aptamer-only surfaces were present. The secondary folding pattern of the aptamer was predicted by mfold online software, which revealed the formation of stem and loop structures (Figure 1b).

In this research, the aptamer and GNP combined strategy reached a lower limit of cTnI detection on the IDE sensing surface to detect MI. After the fabrication process, the surface uniformity of the IDE surface with gap and finger regions was confirmed by a 3D nanop profiler (Figure 3a,b).

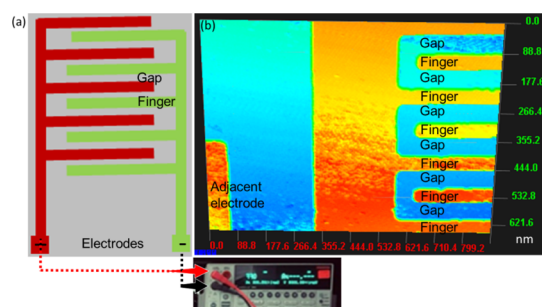


Figure 3. (a) Structure of the IDE. (b) 3D nanoprofile image. The measurement system by an ammeter is displayed. Gap and finger regions are shown. The dimensions were of width 4000 μm ; length 7000 μm ; gap size 10 μm ; 16 finger-gap pairs; electrode size 10 μm ; finger length 3500 μm ; and electrode thickness 45 nm.

2.1. Comparison of Aptamer-Only and Aptamer–GNP Immobilization on IDE Surfaces. Figure 4 shows the surface immobilization of aptamer and aptamer–GNP on CDI–streptavidin IDE surfaces. The current level was recorded for each modified surface to confirm the molecular assembly. The CDI-modified bare surface displayed a current level of 1.85×10^{-5} A; when streptavidin was attached to the surface, the current increased to 3.81×10^{-5} A. This enhancement in the current clearly indicated the immobilization of streptavidin on the CDI-modified surfaces. Before adding the biotinylated aptamer, the remaining surfaces were masked by ethanolamine to eliminate a biofouling effect originating from other molecules (Figure 4a). On these streptavidin-modified surfaces, the binding of aptamer and aptamer–GNP was individually carried out, and the alterations in current were compared. As stated elsewhere, single-stranded oligonucleotides/aptamers can attract gold by covalent binding, electrostatic attraction, and hydrophobic absorption.¹⁸ Due to these phenomena, more aptamers are attached to the GNPs. With only the aptamer, the current level was altered from 6.32×10^{-5} to 2.5×10^{-5} A; in the case of the aptamer–GNP surface, the current level changed to 2.09×10^{-5} A (Figure 4b). The difference in these changes might be due to the higher number of biotinylated aptamers attached to the

surface of the GNPs, leading to a higher number of binding sites for the streptavidin-immobilized surface.

2.2. Comparative Detection of cTnI on Aptamer- and Aptamer–GNP-Modified IDE Surfaces: Electroanalysis. Since it was proven that aptamer–GNP resulted in a more pronounced change in the current on the IDE surface, it was expected that binding of cTnI onto this surface would result in a higher binding affinity compared to that of the surface modified by an aptamer only. Figure 5a,b shows the comparison of 10 pM of cTnI detection on aptamer- and aptamer–GNP-immobilized IDE surfaces. As shown in Figure 5a, after adding 10 pM cTnI, the current level increased from 2.5×10^{-5} to 6.18×10^{-5} A. The variation was found to be 3.68×10^{-5} . When 10 pM cTnI interacted with the aptamer–GNP-modified IDE surface, the current level increased from 2.09×10^{-5} to 6.5×10^{-5} A. Compared with the aptamer-modified surface, cTnI binding to the aptamer–GNP-modified surface resulted in a greater current change at a similar concentration (Figure 5b). This may be due to more aptamer attachment on the surface when using GNP and aptamer conjugates, leading to more interactions of cTnI with the surface.

2.3. Comparison of the Detection Limit of cTnI on Aptamer- and Aptamer–GNP-Modified IDE Surfaces. The detection limits of cTnI on aptamer and aptamer–GNP surfaces were determined and compared by titrating different concentrations of cTnI. The diluted concentrations ranging from attomolar to picomolar were dropped individually on the aptamer- and aptamer–GNP-modified surfaces. The current alterations were noted for each experiment after washing the surface with PBS buffer. Figure 6a shows the binding affinity of cTnI from 100 aM to 10 pM on the aptamer-modified surface. As shown in Figure 6a, when 100 aM cTnI was added to the aptamer surface, no noticeable changes in the current were recorded. When increasing the concentration to 1 fM, the current level increased from 2.5×10^{-5} to 2.69×10^{-5} . Upon further increasing the concentration to 1 fM, 10 fM, 100 fM, 1 pM, and 10 pM, the current increased gradually to 2.85, 3.23, 4.02, 4.89, and 6.18×10^{-5} , respectively. This result clearly indicated a proper binding of cTnI at the different concentrations to the specific aptamer-immobilized IDE surface. After this confirmation, similar concentrations of cTnI were dropped individually onto the aptamer–GNP-modified IDE surfaces for comparison. As shown in Figure 6b, when 100 aM cTnI was added, an increase in the current from 2.05×10^{-5} to 4.56×10^{-5} was observed. This result confirms

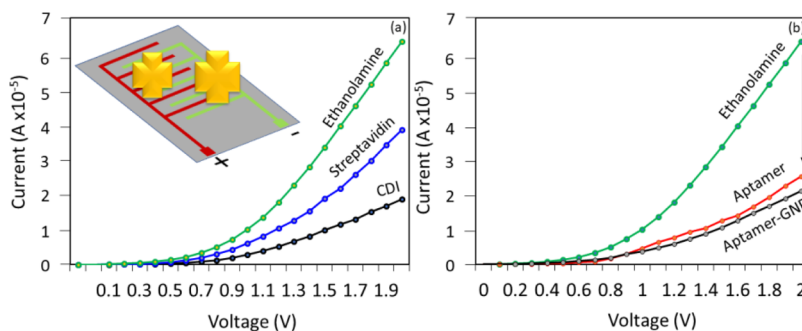


Figure 4. (a) Surface functionalization process for aptamer immobilization on the IDE sensing surface. For each immobilization, the current changes were clearly noticed. The figure inset shows a diagrammatic illustration. (b) Comparison of aptamer and aptamer–GNP immobilization on a streptavidin-modified IDE surface. The figure inset shows the difference in changes. The arrow indicates the direction of the current changes.

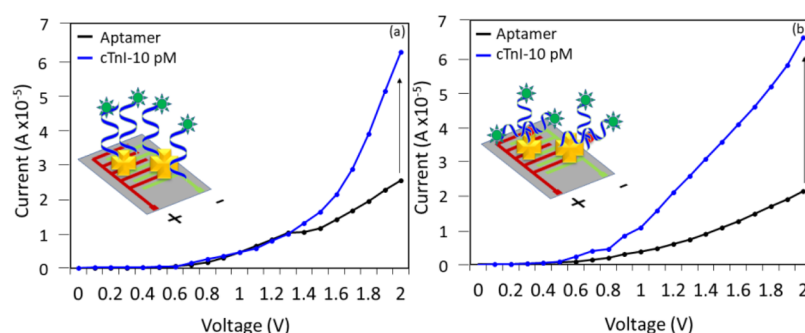


Figure 5. Comparative detection of cTnI at 10 pM on the (a) aptamer-modified surface and the (b) aptamer–GNP-modified surface. Figure insets show the diagrammatic illustrations. The arrows indicate the direction of the current changes.

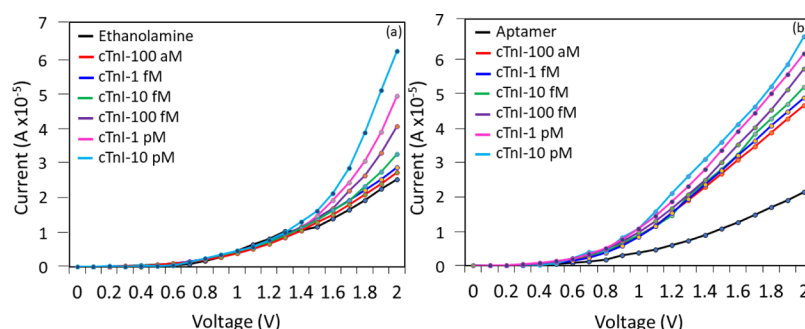


Figure 6. Comparison of the limit of detection of cTnI at concentrations from 100 aM to 10 pM, which were individually dropped on the (a) aptamer-modified surface and the (b) aptamer–GNP-modified surface.

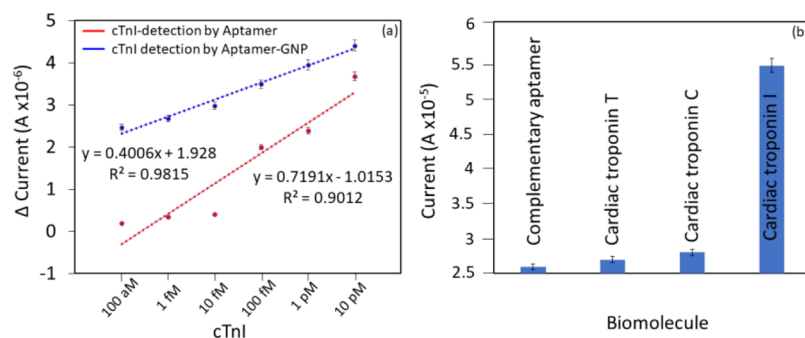


Figure 7. (a) Dynamic range analysis. The current differences for different concentrations of cTnI on aptamer- and aptamer–GNP-modified surfaces are shown. A linear regression analysis was performed by 3σ estimation. The limit of detection (LOD) was estimated from the calibration line at low concentrations and compared with the background signal ($S/N = 3:1$). $LOD = \text{standard deviation of the baseline} + 3\sigma$. (b) Specificity analysis. Control experiments were performed with complementary aptamer sequences and control proteins. Different types of troponin biomarkers released in the heart were chosen. The values are averaged from triplicates ($n = 3$). Experiments were conducted on different chips.

the successful binding of cTnI at this low concentration to the aptamer–GNP-modified surface. Furthermore, with an increase in the concentration to 1 fM, 10 fM, 100 fM, 1 pM, and 10 pM, the current changes increased gradually to 4.77 , 5.07 , 5.58 , 6.01 , and 6.5×10^{-5} , respectively. Compared with the previous experiment (aptamer-only), cTnI binding to the aptamer–GNP-conjugated surface was high, and a more pronounced increase in the current for cTnI detection was observed. The difference in the current changes was plotted for these two experiments, and the detection limit was calculated by 3σ estimation. As shown in Figure 7a, the cTnI detection limits were 1 fM and 100 aM on the aptamer and aptamer–GNP surfaces, respectively. These values correspond to a 10-fold lower detection limit of cTnI on the aptamer–GNP surface. This experiment showed that the aptamer-conjugated GNP could improve the overall detection of cTnI. Previously,

different research experiments have proven that gold nanomaterial-conjugated biomolecules could improve the detection of targets due to their higher stability and proper attachment of biomolecules on the sensing surfaces.^{27,28} In this research, when the aptamer was conjugated with GNP, it formed a stable complex, and proper arrangement on the IDE sensing surfaces was achieved to attract more cTnI molecules and enhance the current level.

2.4. Control Experiments for Selective Detection of cTnI. Three control experiments were conducted to evaluate the selective detection of cTnI on aptamer- and aptamer–GNP-modified surfaces. In the first control experiment, instead of aptamer, complementary aptamer sequence was used, while the others used control proteins (cTnT and cTnC) instead of cTnI. As shown in Figure 7b, there were no noticeable changes in the current in these control experiments, confirming the

Table 1. Comparison of Cardiac Troponin I Biomarker Detection with Current Biosensors

sensing system	probe	limit of detection	advantage	references
surface plasmon resonance	monoclonal antibody	250 ng/mL	improved sensitivity	29
chemi-luminescence	antibody	27 ng/mL	fast detection	30
colorimetry	monoclonal antibody	10 ng/mL	low-cost, fast detection	31
silicon-FET	monoclonal antibody	92 pg/mL	high selectivity, sensitivity, anti-interference	32
graphene-FET	monoclonal antibody	0.1 pg/mL	higher sensitivity, label-free	33
aptasensor	aptamer	1.2 ng/mL	fast detection	34
zinc oxide-FET	monoclonal antibody	3.24 pg/mL	high selectivity; sensitivity, low-cost deposition, low-complexity	35
interdigitated electrode	aptamer	24 pg/mL	cheaper, higher sensitivity, simple fabrication	current work
interdigitated electrode	aptamer–gold	2.4 pg/mL	cheaper, high-performance, simple fabrication	current work

specific interaction of aptamer with cTnI. Between specific (nonfouling) and nonspecific (biofouling) surfaces, large differences were found. Overall, the sensor displayed a high performance compared to the currently available sensors (Table 1).

3. CONCLUSIONS

Cardiovascular disease, a serious death-causing disease, occurs globally. MI is basically defined as heart failure. Diagnosing MI within a shorter period of time after mild heart failure is crucial for saving the life of a patient. cTnI is widely used as an efficient biomarker to diagnose MI. This research focused on quantifying the level of cTnI through its specific aptamer on an electroanalysis-based IDE sensing surface. Furthermore, a GNP-complexed aptamer was utilized to increase the detection limit of cTnI. At a 1 fM concentration, cTnI was efficiently detected by the aptamer on the IDE sensor. The detection of cTnI improved to 100 aM when aptamer–GNP conjugates were used. Control experiments with complementary aptamer sequences and control proteins did not show significant alterations in current, indicating the specific interaction of cTnI and aptamer on the IDE sensing surface. The major limitations of the sensor shown here are the short circuit with nanogaps (<100 nm) between the fingers and biofouling with nonmetal oxide substrates.

4. MATERIALS AND METHODS

4.1. Reagents and Biomolecules. CDI, cardiac troponin proteins (cTnI; cTnT; cTnC), phosphate-buffered saline (PBS), and GNPs (15 nm) were purchased from Sigma-Aldrich (USA). Ethanolamine was ordered from Fisher Scientific (UK). The biotinylated aptamer for cTnI was synthesized and received from a local supplier (Apical Scientific Oligo, Malaysia). The sequence of the specific aptamer was adapted from the study by Jo et al. (2015).¹⁹ The complementary aptamer sequence for the control experiment was also prepared. One micromolar of the diluted aptamer was mixed with one optical density of GNPs and kept at room temperature (RT) for 30 min. The unbound GNPs were separated by centrifugation at a speed of 10,000g for 10 min, and the pellet was washed with PBS buffer. The conjugated capture probe with GNP was kept at 4 °C until further use. Mfold online software was utilized to predict the secondary structure of aptamers with stems and loops.

4.2. Interdigitated Surface Fabrication: Wet-Etching Process. First, the silica wafer was cleaned by RCA2 and RCA1 solutions to eliminate unnecessary deposits. Then, thermal oxidation was performed for 1 h at 500 °C to form a layer of silicon dioxide (SiO₂). After that, aluminum (Al) was coated on the SiO₂ layer by using Al coil in a thermal

evaporator. The electrode pattern was formed on an Al substrate by using the conventional photolithography technique. This process was performed by coating the positive Al-modified substrate with a spin coater and soft baking at 90 °C (1 min) to eliminate moisture. Then, the IDE pattern was transferred to the substrate by using an ultraviolet (UV) light exposer. The unexposed area was removed by dipping the wafer in a photoresist developer. The developed IDE electrode was hard baked (at 110 °C for 1 min) to remove the moisture content and to enhance the adhesion between the SiO₂ and the Al layers. Finally, the unexposed Al layer on the IDE was etched by immersing the wafer in etchant solution containing Al. The fabricated IDE was washed with acetone and distilled water and placed in the dry cabinet until further use. The structure of the fabricated IDE was observed under a 3D nanoprofiler.

4.3. Immobilization of Aptamer or Aptamer–GNP on the IDE Sensing Surface through Chemical Modification. The aptamer for cTnI was attached to the IDE sensing surface through biotin–streptavidin coupling. Initially, streptavidin was attached to the sensing surface through CDI as the chemical linker. A CDI concentration of 0.5 M (diluted in 30%) was initially coated onto the IDE surface and kept for 1 h at RT. After 1 h, the unbound CDI was removed by washing the surface thoroughly with distilled water. Streptavidin (200 nM, diluted in PBS) was dropped onto the CDI-modified surface and kept for 1 h at RT. The unattached CDI surface was further blocked with 1 M diluted ethanolamine. Finally, 1 μM biotinylated aptamer–GNP (1:10) was added to the surface to allow for interactions between streptavidin and biotin. The current changes were recorded during all immobilization steps to confirm the immobilization process. A similar procedure was utilized to attach the aptamer–GNP on the IDE sensing surface.

4.4. Comparative Electroanalysis of cTnI on Aptamer- and Aptamer–GNP-Modified IDE. The detection of cTnI was compared on aptamer- and aptamer–GNP-modified surfaces. For this purpose, 200 nM cTnI was dropped onto the aptamer- and aptamer–GNP-modified surfaces, which were kept for 30 min at RT. Then, the surface was washed with PBS buffer five times to remove unbound cTnI. The current changes were recorded during all interaction steps (cTnI with aptamer and aptamer–GNP).

4.5. Comparison of the Detection Limit of cTnI on Aptamer-Modified Surfaces. To compare the detection limit of cTnI on the aptamer and aptamer–GNP surfaces, different cTnI concentrations ranging from attomolar to picomolar were titrated individually by 10 order dilutions onto the modified IDE surfaces. After each washing step, the current changes were recorded for comparison.

4.6. Selective Detection of cTnI by Its Specific Aptamer.

The selective detection of cTnI was confirmed via three control experiments, namely, cTnI binding with complementary aptamer sequences and binding of specific aptamer sequences with control proteins (cardiac troponin C and cTnT). The current changes in these experiments were compared with those resulting from the specific interaction of the desired aptamer sequence and the target cTnI. All molecular assembly and interaction experiments on the IDE surface were performed under wet conditions at RT, and thorough washing was performed between each step unless otherwise stated. The current measurements (A) were performed in the presence of PBS (10 mM; pH 7.4) as the electrolyte using Keithley 6487 with a linear sweep from 0 to 2 V recorded the values at steps of 0.1 V (at 20 s).

AUTHOR INFORMATION

Corresponding Author

Subash C. B. Gopinath – Institute of Nano Electronic Engineering, Universiti Malaysia Perlis, Kangar, Perlis 01000, Malaysia; Faculty of Chemical Engineering Technology, Universiti Malaysia Perlis, Arau, Perlis 02600, Malaysia; orcid.org/0000-0002-8347-4687; Email: subash@unimap.edu.my

Authors

Jintao Zhang – Department of Children's Heart Center, Fuwai Central China Cardiovascular Hospital, Henan Provincial People's Hospital, Zhengzhou City, Henan Province 450000, China

Thangavel Lakshmipriya – Institute of Nano Electronic Engineering, Universiti Malaysia Perlis, Kangar, Perlis 01000, Malaysia

Complete contact information is available at:

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Notes

The authors declare no competing financial interest.

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