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Gold nano-urchin integrated label-free amperometric aptasensing human blood clotting factor IX: A prognosticative approach for “Royal disease”

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ABSTRACT

This article is clearly presenting the development of a biosensor for human factor IX (FIX) to diagnose the blood clotting deficiency, a so-called ‘Royal disease’ using an interdigitated electrode (IDE) with the zinc oxide surface modification. Gold nano-urchins (GNUs) with 60 nm in diameter was integrated into a streptavidin-biotinylated aptamer strategy to enhance the active surface area. Two different comparative studies have been done to validate the system to be practiced in the current work holds with a higher capability for the high-performance sense. Whereby, the presence and absence of GNUs in the aptasensing system for FIX interaction were investigated using the amperometric measurement, using a linear sweep voltage of 0–2 V at 0.01 V step voltage. The detection limit was 6 pM based on 3σ calculation when GNUs integrated aptamer assay was utilized for FIX detection, which shows 8 folds sensitivity enhancement comparing the condition in the absence of GNU and 50 folds higher than sensitive radio-isotope and surface plasmon resonance assays. Albeit, the surface and molecular characterizations were well demonstrated by scanning electron microscopy, atomic force microscopy, 3D nano-profilometry and further supports were rendered by UV–Vis spectroscopy and Enzyme-linked apta-sorbent assay (ELASA). Furthermore, the spiking experiment was done by FIX-spikes in human blood serum in order to demonstrate the stability with a higher non-fouling.

1. Introduction

'Christmas disease' or 'Royal disease' is commonly engaged with the deficiency of blood clotting factors, such as factor IX (FIX) in the liver. Human blood clotting mechanism is branched into two pathways, namely intrinsic and extrinsic. The intrinsic pathway is primarily occurred due to the blood platelet activation, a significant plasma protein for the initiation of the coagulation cascade. There are several factors involved in this pathway which include XII, XI, IX, and VIII. Despite, the extrinsic pathway involves factor VII, gets activated when the tissue factor is exposed during the injury of the endothelial cell. Both pathways activate the factor X which acts as a prothrombin activator, where it converts prothrombin in the blood into thrombin. Later, thrombin catalyzes the conversion of fibrinogen into fibrin (Gopinath et al., 2006, 2007; Gopinath, 2008). This will obviously contribute to the mesh of fibrin network which manages to plug the gap in rupture area. Albeit, the insufficient concentration or decrease in the activity of several clotting factors may cause hemophilia, especially with the early clotting stage FIX. Herein, we conducted a study based on FIX, as the lacking of FIX in human blood can cause hemophilia B, also known as ‘Christmas disease’. (Cheen et al., 2017) have reported that in human blood FIX exists as lower amount compared to other proteins in blood plasma. Engage to this clinical matter, a precise biosensor for the accurate detection of the low amount of clinical sample is highly demanded. Generally, factor IX (FIX) deficiency can be categorized as a genetic disorder caused by the missing or mutated FIX, a blood clotting protein. Even though, the disease is inherited from parents to the children around one-third of the cases are caused by the changes in the gene and a spontaneous mutation. In addition, defective clotting mechanism is also influencing FIX deficiency to emerge in human blood clotting extrinsic and intrinsic pathways. So the problem occurs in either the clotting pathway will shut-down the activation of prothrombin, which

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will cause an interruption in the clotting process. Herein, a better detection strategy is highly recommended for the accurate and early clinical results.

A better biosensor for the high-performance sensing activity needs several key points to be emphasized. The high sensitivity of biosensor could reveal an awesome outcome for the detection of the least amount of the target in the clinical sample. For this several points are preferred, including anchorage strategies on the sensor surface; minimize the non-specific biofouling, appropriate probe choice and integration of gold particles to increase the surface-volume ratio (Ding et al., 2017; Ong et al., 2018). In this study, the multi-anchoring system was performed whereby the carbonyldiimidazole functionalized surface was immobilized with streptavidin which has four sites for the biotin binding; create a chance for the molecular assembly, whereby it can increase the sensitive detection. Biotin could link with the tailed-aptamer by complementary oligonucleotide pairing, which was utilized to capture the target, FIX. This multi anchoring system has a huge ability and expected to increase the sensitivity of FIX detection.

Selecting aptamer as a probe for clinical research is more recommendable instead of antibody (Gopinath et al., 2016). An aptamer can be artificially generated using Systemic Evolution of Ligands (SELEX) technique, which is branched into three basic footsteps, namely randomized oligonucleotide incubation with the target, segregation of the bound and unbound nucleic acid ligands, and amplification (Blind and Blank, 2015; Citartan et al., 2012). The application of aptamer is widely grown in the diagnostic study due to its appealing characteristics (Ding at al., 2017; Ong et al., 2018). The aptamer is smaller in size compared to an antibody, able to reach the intracellular targets and also less immunogenic (Toh et al., 2014). Furthermore, an aptamer can be easily produced by the chemical synthesis, which is inexpensive and low-time to consume for the production. The contrasting antibody will undergo conformational changes at high or room temperature, while an aptamer can return to their original conformation when restored in optimal temperature. Those outstanding behaviors of aptamer upgrade the usage of aptamer in various fields. More than a few studies were conducted the utilization of aptamer that can improve the sensitivity of sensing performance to several-folds compared to the antibody (Lakshmipriya et al., 2013; Cheen et al., 2017).

Albeit practicing various advancements in the process of clinical sample detection, a faster, accurate and portable methods are still in demand (Sposito et al., 2018). Nanotechnology owing to a great responsibility in revealing a better outcome to provide an early warning before the disease overwhelmed the patient's condition. Hence, for an awesome performance of biosensor nanotechnology branches into several nanomaterials and nanoparticles. Whereby among nanomaterials, gold nano urchins (GNUs) holds unique properties like high conductivity, sensitivity and biocompatibility. Moreover, these properties endorsed to the urchin-like structure implies having spikes turning out from GNUs surface, which can increase the surface to volume proportion causing more active surface zone for biomolecules attachment (Chiwunze et al., 2017). The current work clearly describes the integration of GNUs in electrical amperometric detection for FIX. We utilized biotinylated aptamer-streptavidin conjugation to form a complex covalent binding with aptamer for FIX detection. This complex molecular assembly can increase the sensitivity due to the high affinity between them. Nonetheless, streptavidin can be conjugated with GNUs to improve the detection technique. Coping with that, GNUs owes the greatest property in the current work by enhancing the sensing performance using a small amount of the target in the samples.

2. Material and methods

2.1. Materials and reagents

1,1′-Carbonyldiimidazole (CDI), 16-mercaptohexadecanoic acid (16-MDA) and streptavidin were obtained from Sigma-Aldrich (USA). Biotin was from Wako Chemicals, Japan. Factor IX was acquired from American Diagnostica Inc. (Stamford, CT, USA). Ethanolamine was bought from Fisher Scientific (UK). A stable 3′ poly-A (A24) tailed aptamer with 2-fluoro modification was prepared as stated before (Gopinath et al., 2006). 5′ Biotinylated oligonucleotide with 20-thymines was prepared commercially. Phosphate Buffer Solution (PBS) was procured Sigma Aldrich, USA. Gold nano-urchin was obtained from Sigma Aldrich, USA to improve the limit of detection. Human serum was purchased from Sigma Aldrich, USA for the specificity analysis. N-Hydroxysuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were from GE Healthcare, utilized as the cross-linking and stabilizing agents. 5X ELISA coating buffer was from Biolend (Japan) and TMB-substrate for Horse Radish Peroxidase (HRP) was acquired from Promega, Malaysia. ELISA plates were from Becton Dickinson (France), while Tween-20 was from R & M Chemicals (U.K.).

2.2. Chemical modification on interdigitated electrode

The IDE was fabricated and simplified as the previously described (Cheen et al., 2017). Initially, the IDE was cleaned with 70% ethanol and then dried followed by 0.5 M of CDI was passed on the bandgaps of IDE and incubated for 1 h at ambient temperature. CDI was dissolved in 30% of absolute ethanol. The surface was being washed with 10 mM PBS after each incubation period to remove the unbound molecules on the surface.

2.3. Enzyme-linked apta-sorbent assay (ELASA)

ELASA was carried out to endorse the FIX detection using biosensor at a lower limit of detection compared to the conventional technique. At first, different concentrations of FIX (3 pM, 6 pM, 12 pM, 25 pM, 50 pM, 100 pM, 125 pM, 1 nM) were prepared using 1 × coating buffer and directly coated onto polystyrene wells and incubated overnight at 4 °C. In addition, 1 × coating buffer is from the commercial source, used to stabilize the tertiary structure of the antigen and maximize the adsorption onto ELASA surface. Furthermore, it is also allowing the better antigen-antibody binding event by preserving the antigen recognition site of the antibody.

Then, after washing wells were dropped with two percent BSA for 1 h for the blocking purpose. In ELASA, overnight incubation was only done for different FIX concentration which is the first step in ELASA. The complex of biotinylated anti-FIX aptamer with 10 nM concentration was immobilized onto each well for 30 min after the blocking step. Aptamers have to undergo heating at 90 °C for 2 min prior to the immobilization and then cooled to the room temperature. This heating process is to ensure the proper folding of the aptamer to form a secondary structure. Finally, streptavidin-HRP with 1:1000 dilution was coated on all the wells and allow to incubate for 30 min at ambient temperature. The substrate was added to examine the coupling event between anti-FIX aptamer and FIX. Washing steps were carried out three times for 10 min between each steps using the washing buffer. The optical density (OD) measurements were done using a spectrophotometer at 405 nm and photographs were taken. A control experiment was performed with the FIX and specificity was determined by coating other blood-related protein instead of FIX, which is C-reactive protein.

2.4. Surface morphological analysis

Surface morphology of the fabricated device was initially analyzed by scanning electron microscope (SEM) and Field-emission Scanning Electron Microscopy under 100 μm scale to monitor the electrode size and 200 μm scale for the sensing surface characterization. Surface characterization was further supported by an atomic force microscope (AFM) analysis. In addition, 3D nano-profilometry analysis was done to obtain a clear-cut image of bandgap between the electrodes. After

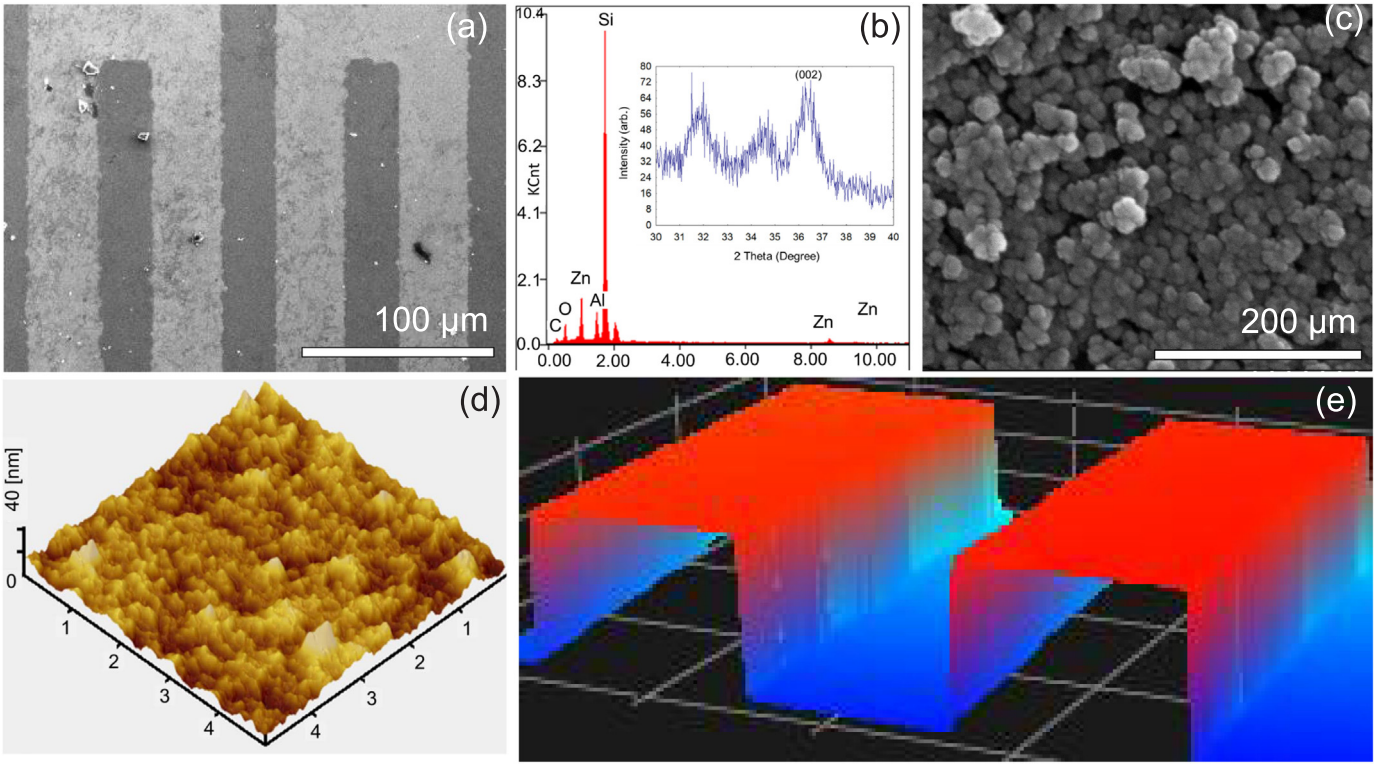

Fig. 1. Surface morphological examination on the electrode bandgaps sensing zone. Scanning Electron Microscopy (SEM) observation (a). Prior to ZnO deposition; EDX profile of sensing zone after ZnO deposition (b). Presence of zinc was confirmed by X-ray diffraction analysis; and the close view of sensing zone using Field-emission Scanning Electron Microscopy (c); Atomic Force Microscopy (d); 3D Surface (Nano) Profilometer (e).

surface functionalization, these three analyses were performed again to validate the attachment of the linker in order to form a strong binding between the sensing surface and the biomolecules. X-ray diffraction analysis was followed as before to confirm the presence of ZnO.

2.5. Biotin-streptavidin conjugation system

This assay was performed with the addition of 1 µM of streptavidin for 1 h on the CDI-modified IDE surface as described above. To avoid the non-specific binding ethanolamine was injected on the surface for 30 min after the streptavidin immobilization. Different concentrations of biotinylated oligonucleotide (dT20) (10 pM, 100 fM, 10 fM, 1 fM) were utilized in this assay. Titration of biotinylated dT20 was carried out from the lowest to highest concentrations. All the electrical measurements were carried out using Keithley 6487 picoammeter.

2.6. GNUs integrated biotin-streptavidin system and interactive analysis of anti-FIX aptamer and FIX

On the chemically functionalized IDE surface, different concentrations of FIX were passed after the constantly applied aptamer surface. Herein, CDI modified electrode gaps was passed on with streptavidin which is pre-mixed with 16-MDA modified GNUs, stabilized by NHS and EDC. Ethanolamine was dropped for 30 min as a blocking agent prior to biotinylated-oligo immobilization. A fixed concentration (1 µM) of biotinylated oligo was applied on the IDE surface followed by the aptamer immobilization. To achieve an outstanding binding event, the heating process needs to undergo by aptamer at 90 °C for 2 min then cooled to ambient temperature for proper folding. Furthermore, PBS buffer was amended with the calcium ion for the specific binding between aptamer and FIX. Electrical measurements were done using Keithley 6487 picoammeter. Different concentrations of FIX (3 pM, 6 pM, 50 pM, 100 pM, 125 pM) were diluted using 10 mM of PBS. PBS containing calcium ion was utilized to wash the IDE surface between each measurement.

2.7. Specific FIX detection in human serum

Evaluation for non-specific binding using blood-related proteins (C-reactive protein and serum albumin) was carried on aptamer modified IDE surface independently. The highest concentration (100 nM) of C-reactive protein was utilized for the sensitivity testing. In addition, the specificity test was done using a human serum with 1:1000 dilution was dropped on the aptamer immobilized IDE surface. Whereby, also followed using 3 pM and 6 pM FIX, were spiked in human serum separately and passed on the IDE surface.

3. Results and discussion

The biotin-streptavidin strategy has been utilized in this study in order to capture more analytes using the suitable aptamer was linked. In conjunction to promote the sensing performance, the maximum binding of biomolecules must be the suitable criteria and plays the predominant role. Due to the concrete affinity between biotin and streptavidin (Lakshmipriya et al., 2016a, 2016b), a huge amount of analytes can be captured. This is attributed because of the tetrameric structure of streptavidin, whereby each streptavidin monomer is able to bind one biotin molecule; ultimately 4 biotin molecules bind to the tetrameric streptavidin. On the other hand, integration of nanomaterial application in biosensor does improve the sensing sensitivity. Despite other nanomaterials, gold having a good characteristic like stable and biocompatible. Gold nano urchin (GNU) having an outstanding structure leading to a high surface to volume ratio, which is able to assist more streptavidin binding. GNU conjugated streptavidin was used for better analyte detection. Herein, we compared the usage of streptavidin alone and streptavidin conjugated GNUs for the detection of FIX. For specificity analysis, blood-related proteins other than FIX were used.

3.1. Surface characterization

3.1.1. IDE surface

IDE used here is a type of design for a biosensor with the interlock like fingers of electrodes. IDE comprises of a couple of electrodes, which are arranged in comb-like structure to form the bandgaps. This configuration permits two electrodes with narrow gaps, able to connect easily during the analysis and allows faster ion diffusion and enhances the power density and better capability. Different techniques were performed for the surface topographical analysis to study the sensing surface in term of size and material present to enhance the detection sensitivity. SEM analysis (Fig. 1a) was carried out and clear-cut image of the electrodes can be seen visibly. Energy dispersive X-ray spectroscopy profile (Fig. 1b) proved that the Zinc oxide (ZnO) deposition on IDE for the better sensing performance (Ding et al., 2017). Further support was rendered by X-ray diffraction analysis was followed as before to confirm the presence of ZnO to confirm the presence of ZnO (Fig. 1b; inset). ZnO particles were observed apparently by higher magnification as presented (Fig. 1c). The AFM image (Fig. 1d) clearly illustrate the morphology between the electrodes bandgap, which shows fairly uniform deposition of ZnO material on the sensing surface (Krishnan et al., 2018). Likewise, this result was supported further by the image obtained from 3D nano profiler (Fig. 1e). This IDE was functionalized with an appropriate linker, CDI in order to create better bonding of upcoming biomolecules to be immobilized on the surface (Fig. 2a). These modifications were confirmed with 3D nano profiler (Fig. 2b), where it clearly spiked out from the gap of the electrode. Fig. 2c displays AFM images which show the nearby molecules were connected by CDI to form a better linkage and the obtained result was supported by SEM images (Fig. 2d).

3.1.2. Gold-nanourchins (GNUs)

The surface morphology study for GNUs was also done using the different microscopic techniques. Fig. 3a represents the SEM image for GNUs using a focused beam of the high energy electrons. Moreover, for the better resolution and higher magnification TEM analysis was performed. Fig. 3b displays the TEM image of GNUs whereby the spiked structure from the surface can be clearly depicted. The size of GNUs can be revealed through this analysis, which is 60 nm and the figure inset present the higher magnification of TEM to strongly support the result obtained. The spiking out like structure has a promising effect on increasing the surface area for the huge binding of biomolecules thus can improve the sensitivity for FIX detection (Zeng et al., 2011). AFM image (Fig. 3c) shows the outstanding size distribution of GNUs which could assist for surface area increment.

3.2. Validation of molecular properties

3.2.1. Spectral analysis

According to López-Muñoz et al. (2012) as the diameter of the gold particle increases there is a concomitant increment with the absorption. Albeit, spectral analysis was carried out for the standard gold nanoparticle with the similar size of GNU and the peak was centered at ~540 nm [Fig. 4a (i)]. While, the similar analysis for GNUs with 60 nm showed the peak maximum was between 550 and 600 nm [Fig. 4a (ii)]. In both cases, using UV–vis spectrophotometer an apparent peak with the shoulder (~420 nm) was obtained. GNUs revealed a higher wavelength position compared to the standard gold nanoparticles, might be due to the spiky surface on GNUs. This comparative analysis validates, GNUs holds a different optical characteristic compared to the standard gold nanoparticles.

3.2.2. Enzyme-linked apta-sorbent assay (ELASA)

In the last decade, enzyme-linked immunosorbent assay (ELISA) technique was blooming drastically among researchers in the widespread applications (Poongodi et al., 2002; Lakshmipriya et al., 2016a, 2016b). The emergence of the aptamer, an alternative to antibodies in ELISA must be a huge sensation for developing a new research arena (Li et al., 2016; Jing et al., 2018). In the current study, we practice ELASA technique due to the outstanding key characteristics like less immunogenicity, low molecular weight, and easy modification (Toh et al., 2014). Initially, different concentrations of FIX (3 pM, 6 pM, 12 pM, 25 pM, 50 pM, 100 pM, 125 pM, 1 nM) were coated independently onto the separate well, in addition a negative antigen (CRP) and the well

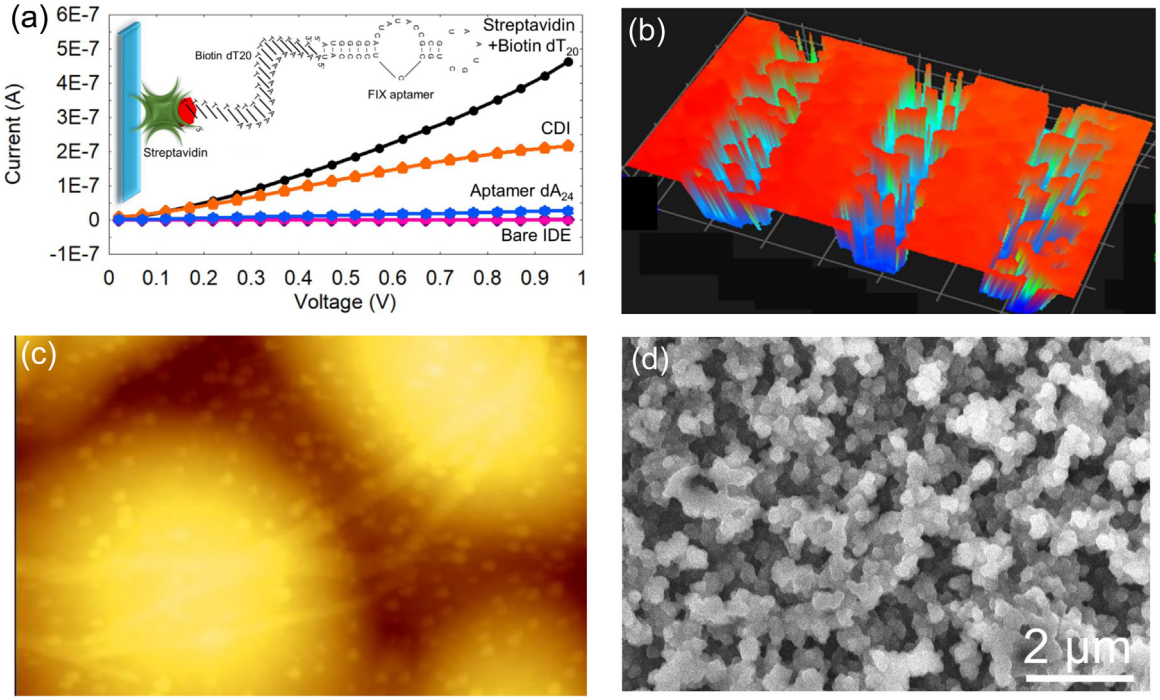

Fig. 2. Surface characterization after the attachment of CDI. I-V measurements (a); 3D Surface (Nano) Profilometer (b); Atomic Force Microscopy (c); Scanning Electron Microscopy (SEM) (d).

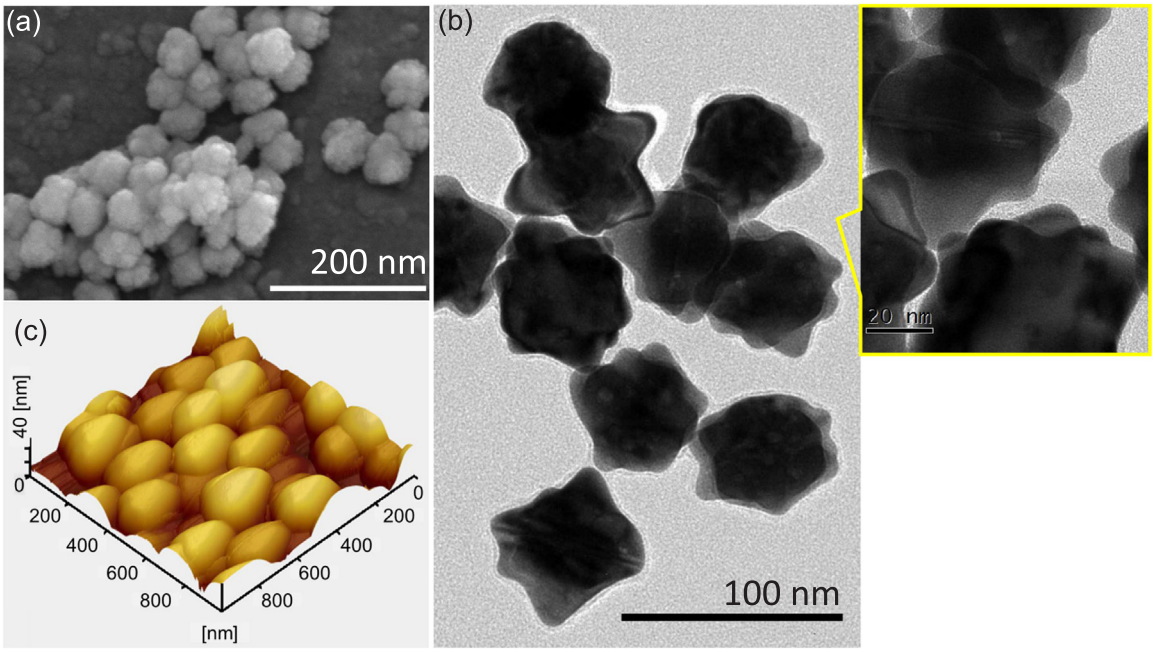

Fig. 3. Surface topography analysis on gold nano-urchin. Scanning Electron Microscopy (a); Transmission Electron Microscopy (b) and figure inset present the enlarged view; Atomic Force Microscopy (c).

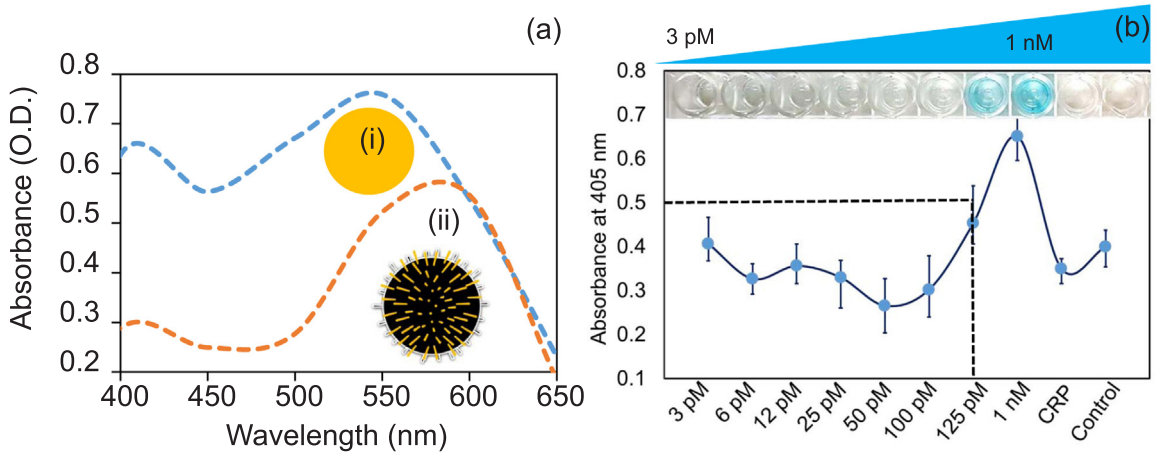

Fig. 4. UV–Vis measurements (a); gold-nanoparticle (i), and gold nano-urchin (ii). Shapes were displayed as figure insets; Enzyme-linked apta-sorbent assay (b). The colour changes after the substrate addition were shown by a photograph. The colour changes occurred at highest concentration. First well starting from 3 pM, 6 pM, 12 pM, 25 pM, 50 pM, 100 pM, 125 pM, 1 nM then non-specific protein which is (CRP) followed by only coating buffer, both act as the control.

absence with the FIX were considered as the controls. After the overnight incubation, anti-FIX aptamer was immobilized. The colour change was observed after the substrate addition to the well with 1 nM and 125 pM FIX concentrations, whereby the control wells does not shows any colour change. The results obtained was photographed and absorbance for each concentration was recorded. While, no colour change in other wells defines that absence of the enzymatic reaction. The highest peak was protruding at 1 nM FIX concentration under UV–vis spectrophotometer (Fig. 4b). Though, utilizing this result further experiment was carried out to monitor the interaction between FIX and anti-FIX-aptamer using label-free electrical detection with IDE sensor.

3.3. Current vs Voltage (I-V) measurements for surface modifications

As previously mentioned the surface functionalization and biomolecules immobilization steps were carried out using Keithley 6487 picoammeter which has two-point probing system I-V measurement. As displayed in (Fig. 2a) bare IDE without surface modification was revealed the current flow at the lowest level. Though, after the CDI attachment the current flow was increased to 2.0×10^{-7}A which validate the good surface modification on IDE. Streptavidin followed by biotinylated-oligo was passed on the IDE surface and the current respond was 4.5×10^{-7}A, which shows further increment from the CDI attachment. Thus, it shows streptavidin and biotin forms a perfect bonding on the sensing surface. Then, anti-FIX-aptamer was immobilized while the current flow decreased to 0.25×10^{-7}A due to the charge variation.

3.4. Electrical analysis for streptavidin-biotin conjugation

Generally, streptavidin-biotin has the strongest non-covalent interaction among others in nature, this strategy is valid for a broad range of biological applications. Additionally, streptavidin holds outstanding features as femtomolar affinity and high specificity which can contribute more to high-affinity protein-ligand interaction study. The current study is about comparing streptavidin-biotin interaction with

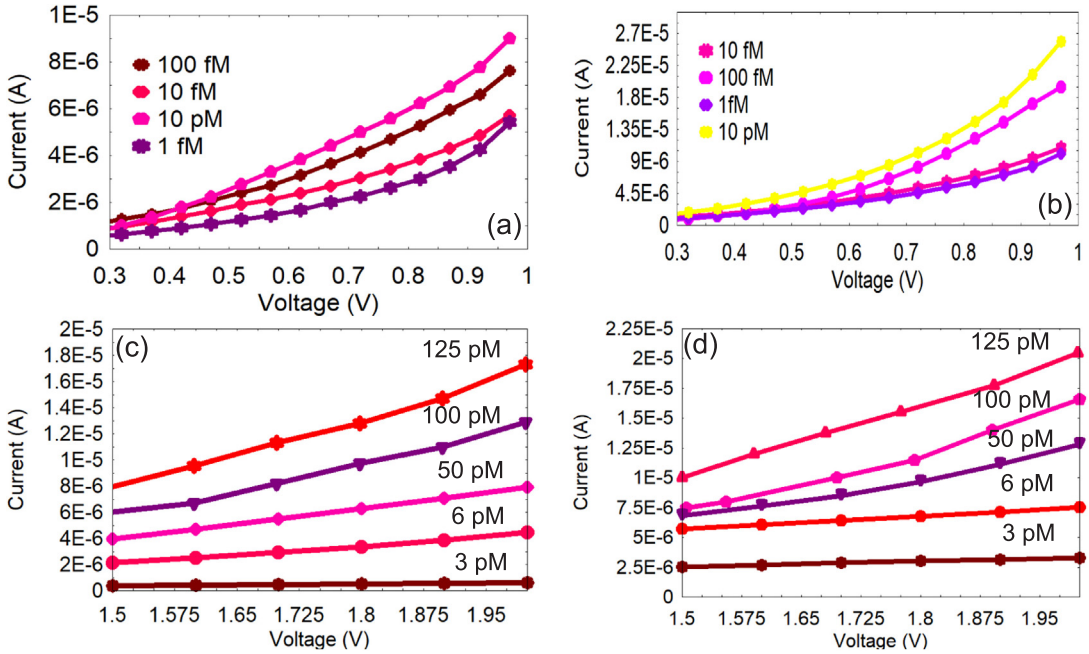

Fig. 5. The current versus voltage analysis using 0–2 V at 0.1 V step voltage. Streptavidin-biotin conjugation assay using different concentrations of biotin without GNUs (a) and with GNUs (b); Interactive analysis for FIX and anti-FIX aptamer with streptavidin-biotin strategy (c) with GNUs (d).

and without GNUs conjugation. Fig. 5a shows the current flow for different biotin concentrations without GNUs conjugated streptavidin. Initially, femtomolar range biotin concentration was passed on streptavidin modified sensing surface independently (1 fM, 10 fM, 100 fM) and the current flows were 5.0×10^{-6} A, 5.25×10^{-6} A and 7.5×10^{-6} A, respectively. Then, 10 pM of biotin was dropped and the current flow was revealed about 9.0×10^{-6} A. On the other hand, Fig. 5b displays the current flow for different biotin concentrations with GNUs conjugated streptavidin. Similarly, begins from the lowest to highest femtomolar biotin concentrations (1 fM, 10 fM, 100 fM) were injected on the streptavidin immobilized IDE surface and the current responds were 9.0×10^{-6} A, 9.15×10^{-6}A and 1.95×10^{-5}A. Finally, 10 pM of biotin concentration was dropped on the IDE surface and the current respond was 2.55×10^{-5}A. This measurement shows that GNUs integrated streptavidin apparently shows the enhancement in sensing performance. Owing to these results, we continued by utilizing streptavidin with and without the integration of GNUs in the interaction study between anti-FIX aptamer and FIX.

3.5. Analysis of FIX and anti-FIX aptamer with the integration of streptavidin-biotin

Due to the emerging nanobiosensor technology which plays a most significant role in the field of medical diagnosis and a few key points need to be highlighted to improve the sensitivity and specificity. The foremost important feature was a correct alignment of immobilized biomolecules on the surface of the device. In the current study, surface treatment and modification were the same as mentioned previously. Streptavidin immobilization followed by the fixed biotinylated oligo concentration by adding on the IDE surface prior to ethanolamine injection, allowing to form a strong affinity for the specific analyte capturing. Then on IDE, poly-A (A24) anti-FIX aptamer was immobilized followed by different concentrations of FIX. The complementary between poly-A and dT20 oligos showed the spacer with 4 adenines (A4) to facilitate the earlier complementation. Furthermore, to make sure the specific interaction between FIX and anti-FIX aptamer, PBS buffer used was modified with calcium ions and utilized to rinse the IDE surface before each and every measurement. Current versus voltage graph was plotted using Dplot software.

Different FIX concentrations were titrated on IDE modified anti-FIX aptamer from the lowest to highest picomolar range concentrations (3 pM, 6 pM, 50 pM, 100 pM, 125 pM). To study the interactive analysis between anti-FIX aptamer and FIX, 3 pM of FIX was passed on the IDE surface and the current flow noted to be 1.0×10^{-6} A. Later, followed by 6 pM, 50 pM and 100 pM were titrated separately onto the anti-FIX aptamer modified surface and the current responses were 4.0×10^{-6} A, 7.0×10^{-6} A and 1.3×10^{-5} A respectively (Fig. 5c). Then, the highest picomolar concentration was dropped on the surface consequently, the current flow for 125 pM was 1.8×10^{-5}. The current increases as the concentration increases which leading to more active interaction occur between anti-FIX aptamer and FIX. Nevertheless, the current versus voltage response among different concentrations proceeding almost to the sensitivity level of 50 pM based on 3σ calculation. As shown in the Fig. 5a & b, from femtomolar until the picomolar concentrations of biotinylated oligo have not shown the significant current differences among them. In addition, upon attaching the gold nano urchin on the sensing surface, it enhances the surface and apparently more aptamer as the probe to be needed. To overcome these issues, we referred the previously reported method for the commercially well-approved Biocore-based surface plasmon resonance surface (Gopinath et al., 2006) and followed to use 1 μM of the biotinylated oligo in order to create a higher aptamer capturing. Even though, Gopinath et al. (2006) used 5 μM of biotinylated oligo in their optical system, we immobilized 1 μM biotinylated oligo due to the sensitive response with the electrical system.

3.6. Analysis of FIX and anti-FIX aptamer with GNUs complexed streptavidin-biotin

Insertion of GNUs in streptavidin-biotin strategy prior to anti-FIX aptamer immobilization can enhance the sensitivity and high-performance FIX detection. GNUs can attach to the protein through various interactions such as ionic bonding, chemical modification and electrostatic interaction. Typically, the smaller sized biomolecules can be modified easily using thiol-group to bind on GNUs. In current work, GNUs was mixed with 16-MHA followed by with EDC and NHS together. Then, the mixture was added with streptavidin and allowed to form strong bonding. Moreover, EDC and NHS are well known cross-

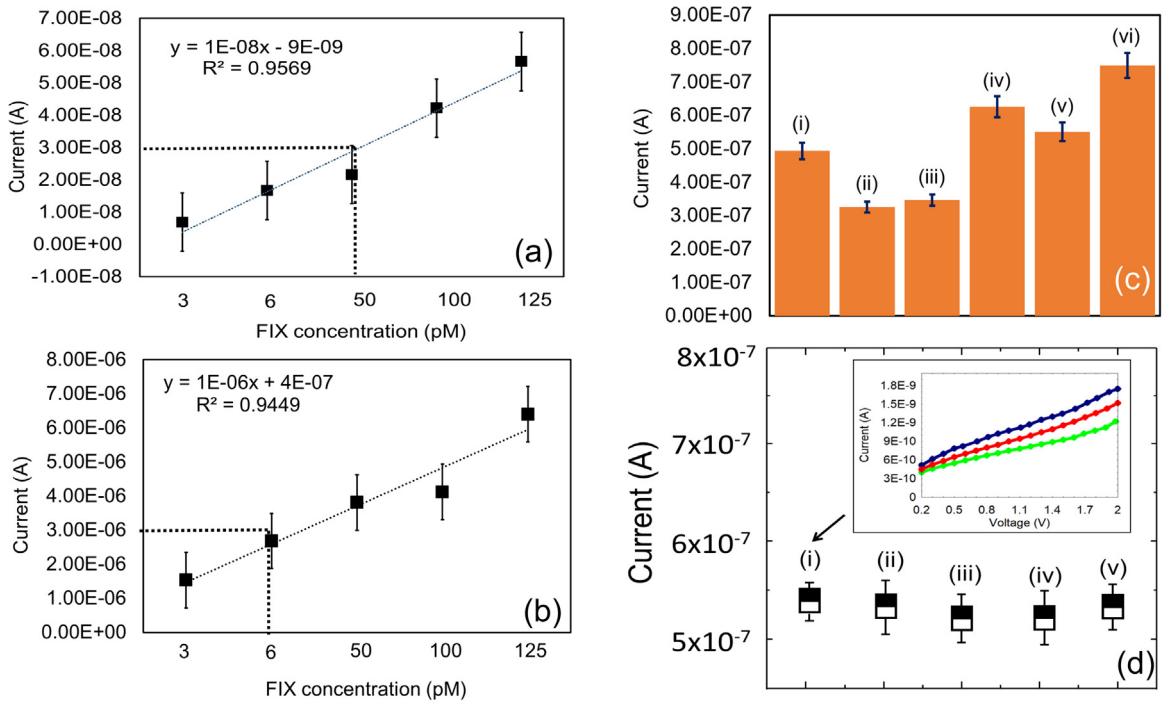

Fig. 6. Analytical high-performance analysis. Linear regression curve for the interaction of FIX and anti-FIX aptamer (a); Linear regression curve for the interaction of GNUs integrated FIX and anti-FIX aptamer (b); Limit of detection was determined based on 3σ calculation. Specificity analysis (c); Immobilized aptamer (i); Serum albumin (ii); C-reactive protein (iii); Human serum (iv); Human serum spiked 3 pM of Factor IX (v); Human serum spiked 6 pM of Factor IX (vi). The capability of FIX to bind with different proteins available in human serum was monitored. Stability of the sensor (d). Bare device (i) Reproducibility analysis was presented by figure inset where three different measurements by the bare device; CDI (ii); Streptavidin (iii); Ethanolamine (iii); Biotin (iv); anti-FIX aptamer (v).

Table 1
Comparison between different electrochemical sensing strategies for FIX detection.

Method	Material	Detection method	Probe	Detection limit (pM)	Reference
Voltammetry	Gold	Label-free	Antibody	1 pM	(Gopinath et al., 2017)
Voltammetry	Zinc oxide	Label-free	Aptamer	10 pM	(Cheen et al., 2017)
Voltammetry	Gold	Label-free	Aptamer	6 pM	Current work

linking agent for carboxyl with 16-MHA on GNUs and amine on the protein. As mentioned above, the similar concentration was titrated independently and the current flow was recorded for every concentration applied on IDE surface. As shown in Fig. 5d, the current reading for 3 pM of FIX concentration was 2.5×10^{-6} A, then, 6 pM was applied on the modified IDE surface and the current flow was 6.25×10^{-6} A. After that, 50 pM was dropped on the sensing surface followed by 100 pM separately and the current response was 1.25×10^{-5} A and 1.62×10^{-5} A respectively. The highest picomolar concentration was applied and the current flow for 125 pM was noted to be 2.12×10^{-5} A. The current increment depends on the concentration reveals the correct and more binding of FIX and anti-FIX aptamer occurred. The interaction analysis of FIX and anti-FIX aptamer with the presence of streptavidin conjugated GNUs protruding the sensitivity level towards 6 pM using 3σ calculation, which is higher than the calculation made in the absence of GNU (Fig. 6a & b). It displays 8 folds outstanding enhancement in current response due to the presence of streptavidin conjugated GNUs compared to without GNUs. This sensitivity was found to be ~50 folds higher than other conventional and sensitive methods such as radioisotope and surface plasmon resonance (Table 1; Gopinath et al., 2006).

3.7. Analytical high-performance

Fig. 6c & d displays the analytical performances for FIX biosensor demonstrated here. In this work, the specificity test was performed using the blood-related proteins, which are noticed to be presence abundantly in human blood serum other than FIX. Herein, 10 nM of CRP and albumin were utilized to monitor the specificity against FIX. Initially, serum albumin was applied on the anti-FIX aptamer functionalized IDE surface and the current measurements were recorded. Likewise, CRP was dropped on the sensing surface and the current flow was monitored. According to Lakshmipriya et al. (2013) human serum contains 45 mg/mL of albumin while CRP is about 0.8 mg/L in healthy individuals (Zubiate et al., 2017). Fig. 6c shows there is a minor difference in current flow between CRP and serum albumin from the immobilized aptamer. The current flow for CRP reveals out about 3.2×10^{-7}A while for serum albumin the current reading was 3.1×10^{-7}A. These current readings show the least difference between these two proteins compared to FIX interaction. It is validating that current sensor shows the least specificity towards other proteins but there is an obvious high current response for FIX.

FIX exists in human blood serum about (~3–5 μg/mL) (Lakshmipriya et al., 2013), herein, we analyzed the existing of FIX in human serum followed by FIX-spiked human serum at the concentration of 3 pM and 6 pM On IDE, 1:1000 dilution of human serum was passed on and the current flow was noted. The current flow shows an increment from anti-FIX aptamer immobilization which means human serum contains FIX protein potential to make specific interaction with anti-FIX aptamer on the IDE surface. Later, FIX with 3 pM and 6 pM concentration was spiked into diluted humans serum. Then, prepared FIX-spiked human serum with 3 pM was applied on the modified IDE

surface and the current reading was 5.4×10^{-7}A followed by 6 pM with the current reading of 7.8×10^{-7}A. It shows a huge increment in current response due to the liberal amount of FIX presence in the human blood. It is validating that this biosensor is extremely precise for FIX detection as presented in Fig. 6c. Stability analysis shows that the sensor utilized in current work is highly stable as a recently available high-performance sensor (Fig. 6d).

4. Conclusion

We have developed a biosensing strategy for the accurate and rapid detection of FIX by using GNUs integrated streptavidin-biotinylated specific aptamer for FIX. The integration of nanotechnology in biosensing application gave a huge impact on this work. GNUs holds outstanding features which can increase the surface area for more streptavidin binding whereby able to capture more biotin molecules. Herein, streptavidin and biotin form strong noncovalent binding with a high affinity which can enhance the interaction between anti-FIX aptamer and FIX. Furthermore, the integration of these strategies in our work improved the sensing performance for FIX. In addition, we practiced using biosensor in this experiment to obtain a good limit of detection and stable sensing surface for better detection of FIX. Thus, results obtained concluded that using GNUs, streptavidin-biotinylated aptamer strategy the limit of detection is 6 pM while without GNUs the limit of detection was 50 pM Hence, it displays a tremendous increment in the sensing activity. This biosensor can be utilized to test human serum because it shows drastic current change after the anti-FIX aptamer immobilization although there is an existing of other blood-related proteins. The strategy developed in this work to detect FIX is highly recommendable for early diagnosis of clotting deficiency in human serum, a so-called 'Royal disease'.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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