Microchim Acta (2017) 184:117–125
DOI 10.1007/s00604-016-2001-6

ORIGINAL PAPER

Aptamer-based impedimetric determination of the human blood clotting factor IX in serum using an interdigitated electrode modified with a ZnO nanolayer

Ong Chong Cheen[1,2] · Subash C. B. Gopinath[1,3] · Veeradasan Perumal[1,4] · M. K. Md Arshad[1] · Thangavel Lakshmipriya[1] · Yeng Chen[5,6] · R. Haarindraprasad[1] · Balakrishnan S. Rao[1,7] · Uda Hashim[1] · Kannaiyan Pandian[8]

Received: 2 June 2016 / Accepted: 27 October 2016 / Published online: 5 November 2016

Abstract This article describes a sensitive impedimetric method for the determination of human blood coagulation factor IX protein (FIX) which is present in extremely low concentration in serum. An interdigitated electrode (IDE) whose surface was layered with zinc oxide was modified with two kinds of probes. One is an antibody, the other an aptamer against FIX. A comparative study between anti-FIX aptamer and anti-FIX antibody showed the aptamer to possess higher affinity for FIX. A sandwich aptamer assay was worked out by using the FIX-binding aptamer on the surface of the IDE. It has a detection limit as low as 10 pM which makes it 4 to 30-fold more sensitive than any other method reported for FIX. Moreover, to practice detection in clinical samples, FIX was detected from the human blood serum by spiking. In our perception, the sensitivity of the ZnO-modified IDE presented here makes it a promising tool for sensing clinically relevant analytes that are present in very low (sub-pM) concentrations.

Keywords Coagulation · Hemophilia · Impedance · Resistance analysis · Factor IX · Aptamer · Monoclonal antibody · Clotting

Electronic supplementary material The online version of this article (doi:10.1007/s00604-016-2001-6) contains supplementary material, which is available to authorized users.

✉ Subash C. B. Gopinath
subash@unimap.edu.my

[1] Institute of Nano Electronic Engineering (INEE), Universiti Malaysia Perlis, 01000 Kangar, Perlis, Malaysia

[2] Faculty of Engineering Technology, Universiti Malaysia Perlis, 01000 Kangar, Perlis, Malaysia

[3] School of Bioprocess Engineering, Universiti Malaysia Perlis, 02600 Arau, Perlis, Malaysia

[4] Centre of Innovative Nanostructure & Nanodevices (COINN), Universiti Teknologi PETRONAS, 32610 Bandar Seri Iskandar, Perak Darul Ridzuan, Malaysia

[5] Department of Oral Biology & Craniofacial Sciences, Kuala Lumpur, Malaysia

[6] Oral Cancer Research and Coordinating Center (OCRCC), Faculty of Dentistry, University of Malaya, 50603 Kuala Lumpur, Malaysia

[7] Fakulti Kejuruteraan dan Alam Bina, Universiti Sains Islam Malaysia (USIM), Bandar Baru Nilai, 71800 Nilai, Negeri Sembilan, Malaysia

[8] Department of Inorganic Chemistry, University of Madras, Guindy Campus, Chennai 600 025, Tamil Nadu, India

Introduction

Blood coagulation process is a hemostatic mechanism initiated by forming hemostatic plug to arrest bleeding from the damaged blood vessel. There are two distinct pathways in blood coagulation process, which are intrinsic and extrinsic. Factors involved in these pathways are responsible for different action during clotting [1–3]. Bleeding disease, a so-called 'Haemophilia B' or 'Christmas disease' is caused due to deficiency of one of the clotting pathways. Among all clotting factors in clotting cascade, factor IX protein (FIX) is highly connected to most of the proteins through these two pathways [2, 4]. The concentration of normal level of FIX in human blood serum is 57 μg mL^{-1} (87 nM) [5, 6]. While other proteins such as albumin exists in higher level, which is 35 to 50 mg mL^{-1}. Similarly, another protein globulin is also exist in higher amount in serum (25 to 30 mg mL^{-1}) [7]. Therefore, sensitive and specific biosensor is needed for the selective detection of FIX in blood serum.

Sensitivity of sensor varies with different systems. It is vital to determine the low abundance of target with higher performance, to be a successful sensor [8–13]. Development of useful biosensor requires key characteristics, such as specificity and sensitivity [5]. Further, two other important factors should be taken into consideration, which are correctly orientated immobilized biomolecules on sensing surface and minimized non-specific biofouling [5]. In this study, immobilization of probe on solid surface was performed, because non-specific antigen-capturing efficiency is lower compared to in-solution-based detection. It is due to the random orientations and steric hindrances of the immobilized molecules on solid surfaces [6]. While minimizing non-specific bio-fouling will lead to the improvement in signal to noise ratio (S/N) [5]. In addition, with the right orientation of high-dense biomolecule immobilized on surface of sensor, the level of detection will be improved [6]. A common approach to minimize non-specific biofouling is by addition of right blocking agent on the desired sensing surface.

On the other hand, choosing probe also plays an important role for the specific and sensitive detection. Antibody has been used as probe since the early stage of sensor development in 1967, whereby the first immobilized antibody-solid is proposed by Catt and Niall [6, 14]. Later-on, enzyme-linked immunosorbent assay (ELISA) was discovered followed by the usage of several antibody-sensing strategies on solid surfaces. For antibody to bind and detect the antigen, it needs to be specific, form an antigen-antibody complex for detection [15]. Alternatively, in 1990 there was a substitute for antibody was proposed, known as aptamer or "chemical antibody" [6]. It has similar property as antibody except it has been used to produce mostly in vitro by systematic evolution of ligands by exponential enrichment (SELEX) strategy [16–19]. This selection process involves consecutive steps of complex formation, separation and amplification. Need to repeat these steps for several rounds. After 10–14 iterative rounds, it will yield high affinity molecule (aptamer) [20, 21]. Aptamers are smaller in size, stable, cheaper and ease in modification [22]. Immobilization of aptamer onto a solid surface in a correct manner leads to higher specific binding, as demonstrated against the clotting factor thrombin [23–25]. This is due to aptamer property or functionality, which is highly dependent on the formation of three-dimensional conformation of the aptamer [26]. A potential RNA aptamer was generated and it appears to have higher capability to bind FIX. It also discriminated other structurally similar coagulation factors by ~5,000 fold [27]. Since both aptamer and antibody are represented to complement each other, a combination of antibody and aptamer-sensing strategy was designed [6].

The promising low-cost point-of-care clinical test systems have been attracted in the past, make them preferred cost-effective sensors [23, 24]. In addition, it is expected that the sensor doesn't need labeling, a great approach for developing a good sensor. In this study, we used electrochemical impedance spectroscopy with interdigitated electrodes (IDE) for biosensing purpose [28]. This strategy benefits with ease of automation, batch fabrication, and miniaturization. IDE is made up of series of microband electrodes, whereby alternating microbands are connected [11–13, 29]. The structure of IDE will provide a high sensitivity, fast establishment of steady state, a large aspect and a great signal-to-noise ratios [29]. Using sensitive dielectric method, IDE will detect electrical signal by biomolecular interaction directly. This is through the analysis of resistance and capacitance that occurs at electrode surface layered with oxide material [29]. Zinc oxide (ZnO) is a promising material among other oxide-based materials for the construction of unique electrical and optical based high-performance sensors. ZnO nanowire has been considered widely as a preferable choice of transducer in biosensors. It is due to its higher surface area to volume ratio (associated with elevated sensitivity). In addition, ZnO owned a large binding energy and large bandgap which yield stability to the materials [13, 30]. IDEs have large surface and a short distance between anionic and cationic electrodes, which makes data collection to be effective and yields greater sensitivity [29]. As for the biomolecule pattern on IDE, sandwich strategy is commonly used. It is because of greater specificity and able to minimize background signal. For this type of strategy, two probes are necessarily bind two different sites on an antigen. In this study, FIX was sandwiched between antibody and aptamer as capturing and reporting probes, therefore, complement each other to detect FIX.

Materials and methods

Factor IX, monoclonal anti-FIX antibody produced in mouse, human serum, ethanolamine, and (3-Aminopropyl) triethoxysilane (APTES) were purchased from Sigma-Aldrich, USA (http://www.sigmaaldrich.com/united-states). Aptamer was synthesized commercially. Sensor chip (interdigitated electrode) was purchased from Siltera Malaysia Sdn. Bhd. (http://www.silterra.com). All reagents are stored according to supplier recommendations. On the fabricated sensing surface, microscopic studies and structural analyses were carried out according the guidelines described by Perumal et al. [11].

Surface functionalization on interdigitated electrode

IDE was initially washed with 0.5 % ethanol to clean the surface and then it was dried. To activate amine-functionalized substrate, ethanolic solution of (3-Aminopropyl) triethoxysilane (2 %) was dropped on the bandgaps of the IDE for 3 h at room temperature. After being washed with ethanol and dried, 2.5 % of glutaraldehyde was dropped onto amine-functionalized silicon surface for 2 h at room temperature. It forms an aldehyde-activate surface. Phosphate buffer saline (PBS) with 2 mM $CaCl_2$ was used to

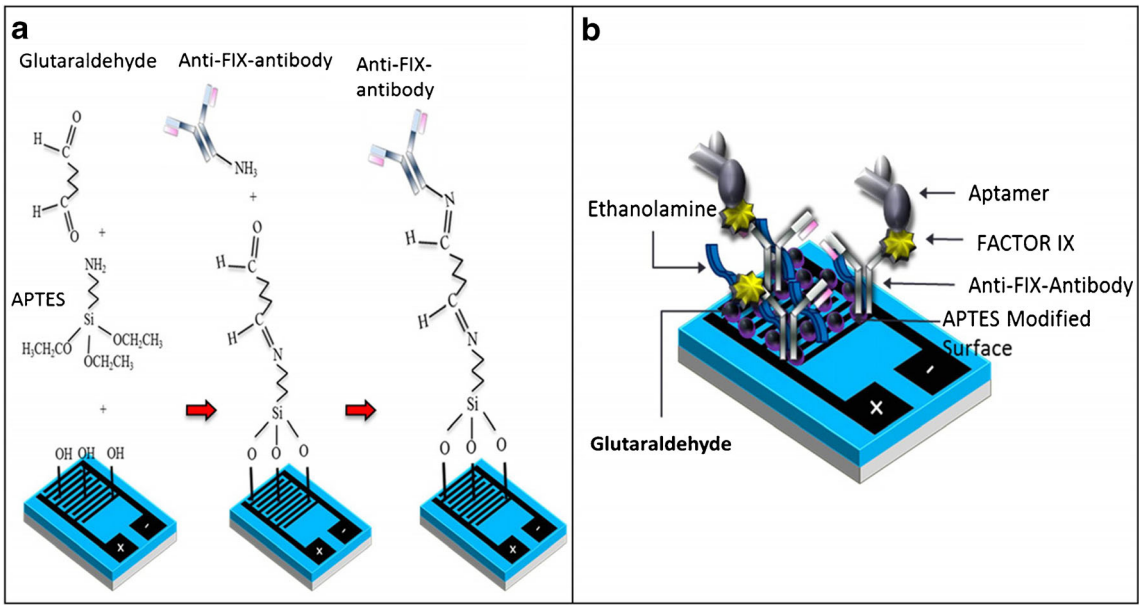

Fig. 1 (**a**) IDE surface react with APTES and glutaraldehyde for attachment of antibody (**b**) Schematic illustration of chemical surface modifications on the sensing plate for FIX detection. Glutaraldehyde was attached to the amine-modified surfaces, followed by anti-FIX-antibody. FIX protein (500 nM) was passed on the surface followed by attachment of aptamer

wash thoroughly the unbound molecules on the surface. Figure 1a shows how the surface of silicon was modified chemically.

Interactive analysis of anti-FIX-antibody and FIX

To analyze interaction between anti-FIX-antibody and FIX, IDE used have bandgaps as small as 130 nm, therefore, it must give a greater sensitivity. ZnO nanowired IDE was chemically modified with APTES and glutaraldehyde as mentioned above. Then, 500 nM of FIX was added as constant for 1 h. After IDE washed by PBS, ethanolamine was passed for 30 min and washed away after immobilized on surface of sensor. Different concentrations of anti-FIX-antibody (10 pM to 100 nM) were applied on FIX-immobilized surfaces. All measurements were done using Electrochemical Impedance Spectroscopy (EIS). PBS was used to clean the IDE before every measurement. All experiments have been performed under room temperature.

Interactive analysis of anti-FIX aptamer and FIX

Initially, IDE undergo same chemical modification as mentioned above. For this study, different concentrations of aptamers (10 pM to 100 nM) were passed onto FIX-immobilized surface (500 nM of FIX). Ethanolamine was injected on surface for 30 min as blocking agents prior to pass the aptamer. For preparation of aptamer to be interacted with FIX, aptamer need to undergo heating up to 90 °C for 2 min and then cooled to room temperature for proper folding. After cooled to room temperature, aptamer was ready for series of dilution with PBS. All measurements were done using impedance spectroscopy and PBS was used to clean the IDE before every measurement [11].

Sandwich assay: antibody-FIX-aptamer

To detect FIX, antibody-FIX-aptamer type of sandwich assay was also performed, where aptamer was passed on the antibody-FIX immobilized surface. To prepare antibody-FIX immobilized surface, 100 nM of anti-FIX-antibody was dropped on glutaraldehyde-modified silica surface for 1 h at room temperature. Remaining anti-FIX-antibody was removed and washed with PBS solution. Blocking agent, 1 M ethanolamine was dropped onto FIX immobilized surface to minimize biofouling and washed after 30 min with PBS. To develop antibody-FIX immobilized surface, 500 nM of FIX was dropped onto antibody immobilized surfaced. FIX bind to antibody and the remaining unbound FIX was washed away with PBS. To complete sandwich assay, denatured aptamer was dropped onto antibody and FIX complexed (antibody-FIX) surface to amplify the response. Figure 1b shows schematic diagram of sandwich assay method.

Limit of detection by sandwich assay

To determine detection limit of FIX by this sandwich assay, we dropped different concentrations of aptamer onto the antibody-FIX immobilized surfaces. Chemical modification on the surface of IDE was carried out as described above. For this titration, FIX concentration was kept constant at 500 nM and ethanolamine at 1 M and titrated using different concentrations of aptamer (10 pM to 100 nM). After attaching aptamer onto the antibody-FIX

immobilized surface, Impedance spectroscopy was used to measure for every concentration to see the difference among them.

Detection of FIX in human serum

Initially, determination of non-specificity (bio-fouling) with different dilution factors in human serum was performed. These dilutions (1:10 to 1:10000) were independently passed onto ethanolamine immobilized surface. Initially, the highest dilution factor (1:10,000) was tested on the glutaraldehyde and ethanolamine modified silica surface, consequently by other lower dilutions. Dilution was halted when there are no changes in electrical properties detected on the IDE. All other surface chemical modifications and detection strategies were similar as described above.

To determine the detection limit of FIX in serum, we titrated with aptamer-serum dilutions on the antibody-FIX immobilized surface. 500 nM of FIX was mixed with human blood serum and passed on the antibody-immobilized silica surfaced. Aptamer-serum was prepared by diluting aptamer in buffer, which was pre-mixed with human serum.

Results and discussion

Choice of materials

The objective of this study is to develop a high sensitive sensor using interdigitated electrode (IDE) and we demonstrated by electrochemical impedance spectroscopy (EIS) measurements. The primary advantages of this sensor are label-free and liable for real-time quantitative measurements. This IDE sensor also have advantages of the smaller dimensional size, reliability, simplicity, and economical. The fabricated IDE is less complex which involves only a single mask and simple fabrication process. The synthesized sensing surface with silver electrode was modified with Zinc oxide (ZnO) nanowires. Silver electrode has been well documented with its multifunctional actions, stability and non-toxic nature, considered to be better than gold-based electrode in our case. Whereas, ZnO is found to have good optical and electrical properties as demonstrated in the sensing applications [11, 13]. ZnO nanowire also contains high isoelectric point and it facilitates the specific surface immobilization of the negatively charged molecules. Further, it is ideal for chemical functionalization and minimized biofouling than gold and polyaniline materials. Nanowires created on this sensing surface facilitate a wide sensing surface to capture the higher numbers of biomolecules. IDEs used were chemically modified by APTES and glutaraldehyde before being immobilized by biomolecules. Blocking agent used was ethanolamine to prevent non-specific biofouling. We wished to investigate the sensitivity that can be obtained from sandwich method to be applied for clinical relevant samples. Human coagulation FIX exists in normal human blood stream in low amount, therefore, chosen as model analyte in this study. A sensitive sensor was demonstrated by antibody-FIX-aptamer sandwich strategy. Aptamer and protein sizes are 10.9 and 150 kDa, respectively, make the size ratio of 1:137 (Aptamer:Antibody). The stability of this 33-mer aptamer (2'Fluoro-modified with cytosine and uracil; Supplementary Figure S3) is quite strong as evidenced by Rusconi et al. [31] and Lakshmipriya et al. [6], with the experiments involve serum samples. The potential of this anti-factor IX aptamer has been evidenced by phase 2 clinical trials [32–34].

Field-emission scanning electron microscopy (FESEM)

Surface morphology of ZnO nanostructure was characterized via FESEM. FESEM image as presented in supplementary figure S1 reveals the synthesized ZnO in a spot like crystal structures spread on the surface of IDE. A higher magnification of FESEM image is shown in Fig. 2 revealed the flower-like ZnO nanostructure. From this analysis, it was obvious that there is a clear grown nanowires on the sensing surface used. Further, these nanostructures are uniform in sizes and distribution and facilitate the larger surface area to capture higher number of biomolecules.

Determination of greater affinity on FIX between antibody and aptamer

To determine which probe has higher affinity, a constant 500 nM of FIX was immobilized on surface of two similar

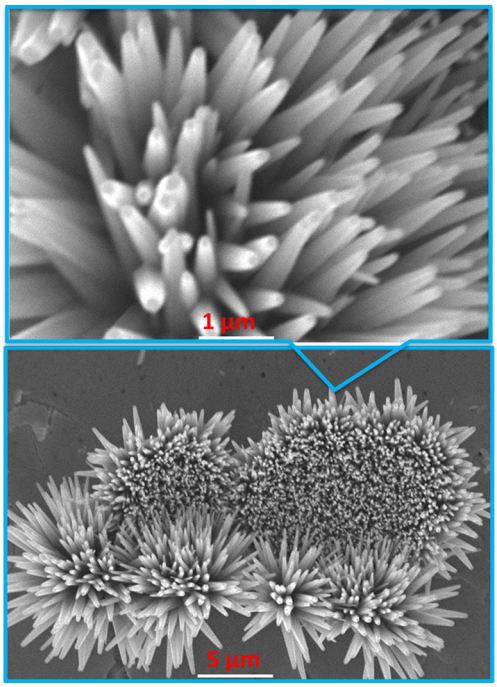

Fig. 2 FESEM image of low and high magnification demonstrate crystal ZnO nanostructure

IDEs. To make sure, there was no non-specific (bio-fouling) present, ethanolamine was used as a blocking agent. On IDE, anti-FIX antibody (100 nM) was passed on FIX immobilized surface and in another case, aptamer was used instead with similar concentration. To ensure specific binding between anti-FIX aptamer and FIX, calcium ion was amended in the PBS as reported elsewhere [31, 35]. Measurements by EIS were taken before and after probe and analyte have been passed on the sensing surface. Nyquist plot of AC impedance spectroscopy spectra obtained were shown in Fig. 3.

The curve shown in the diagram represents interfacial charge transfer resistance, Rct. As shown in Fig. 3a–d, antibody exhibit smaller resistance charge transfer (Rct) value (~2.12Ω) while aptamer exhibit larger Rct value (~5.6Ω). The greater differences show there were more dielectric response changes on aptamer-FIX complexed surface than antibody-FIX complex. Based on this finding, we were able to determine which probe to be used for surface immobilization for sandwich assay. Since anti-FIX-antibody showed less affinity it will be used as surface capturing. While aptamer will be used on top of FIX to amplify (reporter) the reading for greater sensitivity level.

Concentration-dependent analysis – comparison between aptamer and antibody on FIX

To make sure about the above results, serial dilutions (10 pM to 100 nM) of anti-FIX aptamer and antibody were used to analyze on FIX using IDE. Measurements of EIS were taken for every concentration applied on FIX-immobilized surface and PBS with calcium ion was used to clean the surface of IDE before being measured. Figure 3a and b showed Nyquist plots for both anti-FIX-antibody and anti-FIX aptamer on FIX, using IDE. Both figures show that the Rct for anti-FIX-antibody is lower compared to the Rct for anti-FIX-aptamer. As seen also in Fig. 3e the average differences in Rct value (~0.25 Ω) with different concentrations of anti-FIX-antibody were less distinctive compared to Rct value differences (~0.40 Ω) as shown in Fig. 3f (with the concentrations of anti-FIX aptamer), which showed clear distinct reading. This reveals that the aptamer has a better sensitivity. From this result, we concluded that aptamer is suitable to be used on top of FIX as a reporter. Therefore, antibody-FIX-aptamer sandwich strategy was designed. The theory behind is the decrease in resistance upon immobilization when the voltage and current is

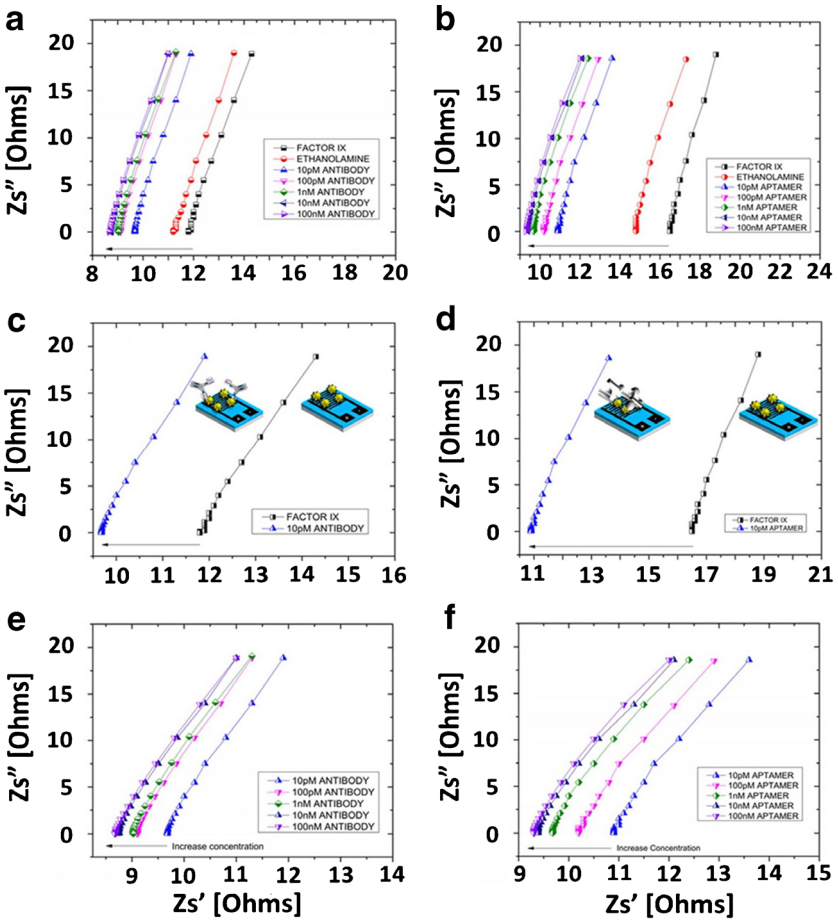

Fig. 3 (**a**) Impedance spectra of FIX with different concentrations of antibody (10 pM-100 nM) (**b**) Impedance spectra of FIX with different concentrations of aptamer (10 pM-100 nM) (**c**) Impedance spectra of FIX and anti-FIX-antibody (**d**) Impedance spectra of FIX and anti-FIX-aptamer (**e**) Impedance spectra on different concentrations of antibody (10 pM-100 nM) on FIX (**f**) Impedance spectra on different concentrations of aptamer (10 pM-100 nM) on FIX

passing on IDE (Fig. 4a). In which electron transfer from one gap to another is through two ways; direct and through the fringes. Upon immobilizations, the electron transfer was at a greater rate since positively charge attitude on protein, which explained the decrease in resistance.

Determination of sensitivity using sandwich assay

Knowing that anti-FIX-antibody will be used for surface immobilization, 100 nM anti-FIX antibody was immobilized on a glutaraldehyde-modified surface and measured by EIS. After anti-FIX-antibody was immobilized, ethanolamine was used to block and make sure there is no biofouling followed by attachment of 500 nM of FIX on anti-FIX-antibody. Then, the lowest concentration (10 pM) of aptamer was attached to the surface. Followed by different concentrations of aptamer were passed on the antibody-FIX complexed surface. As shown in Fig. 4b, the Rct values for FIX and anti-FIX antibody are little compared to the amplification by aptamer. Sandwich assay also showed a better and distinct difference among the concentrations of aptamer (~0.53 Ω) (Fig. 4c and d), proved that sandwich assay yielded a greater sensitivity. Detection limit obtained was down to 10 pM, the lowest concentration of aptamer applied on the IDE. We can relate that the detection limit of FIX was equal to lowest concentration of aptamer, which showed clear and distinct difference in electrical properties upon passed on FIX. Further, the value obtained from the lowest concentration was 3 fold higher than basic value obtained (3σ method). Usually, the limit of detection is controlled by two factors, one is performance of device and another one is due to original affinity between molecules. With FIX and anti-FIX aptamer, previously it was reported that the affinity is in picomolar ranges [5, 31, 35]. The sandwich assay shown by Lakshmipriya et al. [6] has a 38 pM detection limit. With our study, we achieved 4 fold higher than previous report, displayed the success of sandwich method.

Detection of FIX in the presence of human serum by sandwich assay: spiking experiments

For the application of human serum to detect FIX, different dilution factors (1:10 to 1:10,000) of serum were tested. Different dilution factors were injected on ethanolamine blocked IDE surface. To identify the suitable dilution factor, all dilution factors were plotted on Nyquist plot. As shown in

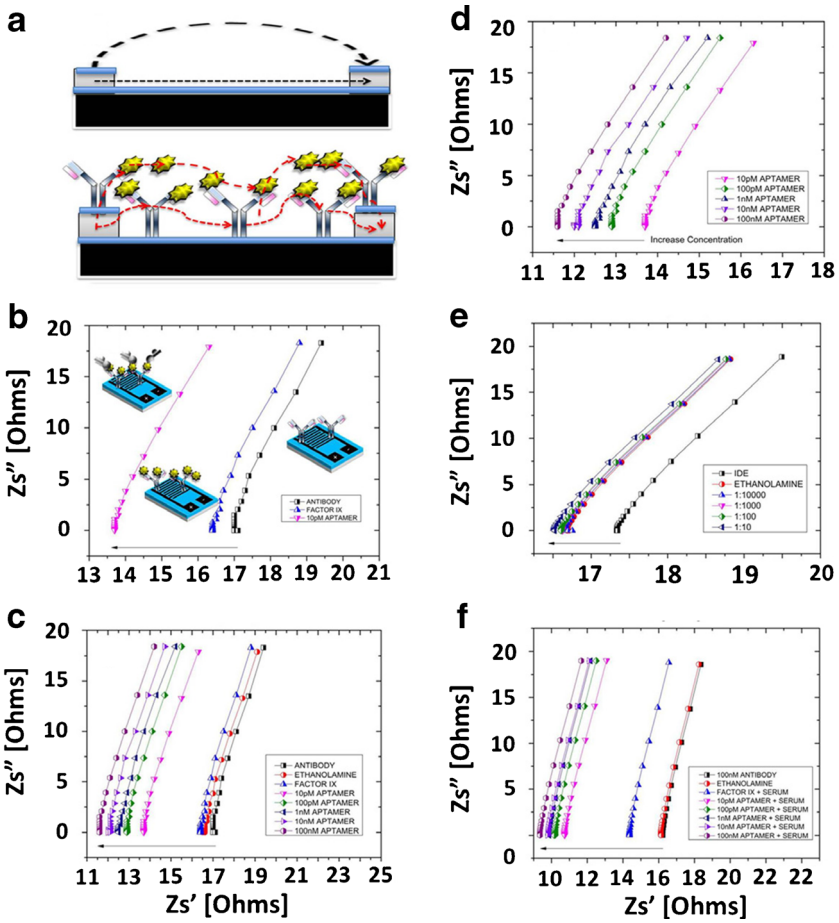

Fig. 4 (**a**) Schematics of IDE without and with immobilization. Impedance spectra on; (**b**) antibody-FIX-aptamer assay, (**c**) antibody-FIX-aptamer sandwich assay whereby series of aptamer dilutions (10 pM-100 pM) were used, (**d**) different concentrations of aptamer (10 pM-100 nM) on antibody-FIX immobilized surface (enlarged), (**e**) different dilutions of human serum on ethanolamine blocked sensing surface, (**f**) antibody-FIX-aptamer sandwich assay whereby FIX and series of aptamer dilutions (10 pM-100 nM) were mixed with human serum

Table 1 Comparison of recently described schemes for detection of factor IX

Sensor type/Method	Surface	Probe	Equilibrium Constant (kD)/Limit of detection (LOD)	Ref.
Radio isotope labeling	Solution	Aptamer	580 pM (kD)	[31]
Waveguide	SiO_2	Aptamer	500 nM (LOD)	[8]
Baicore (SPR)	Gold+polymer	Aptamer	37 pM (kD)	[6]
Biacore (SPR)	Gold+polymer	Antibody	48 nM (kD)	[6]
Biacore (SPR)	Gold+polymer	Aptamer+Antibody	800 fM (LOD)	[6]
Biacore (SPR)	Streptavidin	Aptamer	365 pM (kD)	[35]
BioDVD	Gold	Aptamer	1 nM (LOD) 365 nM (kD)	[9]
Impedance Spectroscopy	Zinc oxide	Aptamer & Antibody	10 pM (LOD)	Current work

Fig. 4e, dilution factor 1:10 showed a distinct change, means there are presence of other interferences in serum when attaching on ethanolamine. In this case, 1:100 was the most suitable dilution factor, since 1:10 dilution factor showed difference in dielectric properties compared to ethanolamine surface. From the finding we obtained, 1 µl of human serum was diluted with 99 µl of 500 nM of FIX is suitable to analyze. Then, FIX-containing human serum was passed on the antibody-immobilized surface IDE. PBS containing serum (1 µL of serum: 99 µl of PBS) and calcium ions (2 mM) were used to dilute aptamer. Figure 4f was showed the attachment of FIX mixed with human serum showed a greater value of Rct (~1.87 Ω) compared to the sample without human serum (~0.7 Ω) (Fig. 4c). Aptamer-serum amplification was also showed much Rct value (~3.63 Ω) compared to the value without serum (~2.7 Ω). This is due to cumulative effect already existing FIX in the serum. This experiment is also anchored that our experimental set-up is stable with serum. This study proved that IDE sensor is applicable for clinical sample and has greater sensitivity. Usually, higher sensitivity is primarily depends on interactive molecules. Our study with sandwich assay, improved further and reached the sensitivity to the level of 10 pM, confirmed the high-performance of IDE sensor. The attainment of this sensitivity is found to be better compared to the previous achievements with other sensors. The current work displays 10–50,000 fold higher sensitivity than others and ~12 times lesser than the sensitivity shown by Lakshmipriya et al. [6] (Table 1). The sensing materials are displayed in Table 1 includes gold and silica. Among these two materials as shown in these studies, gold was assisted by polymer in order to reduce the non-specificity. Whereas, silica is considered as good as ZnO in terms of non-fouling for immobilizing factor IX. These studies are mainly involved the optical sensing systems. Whereas the study shown here

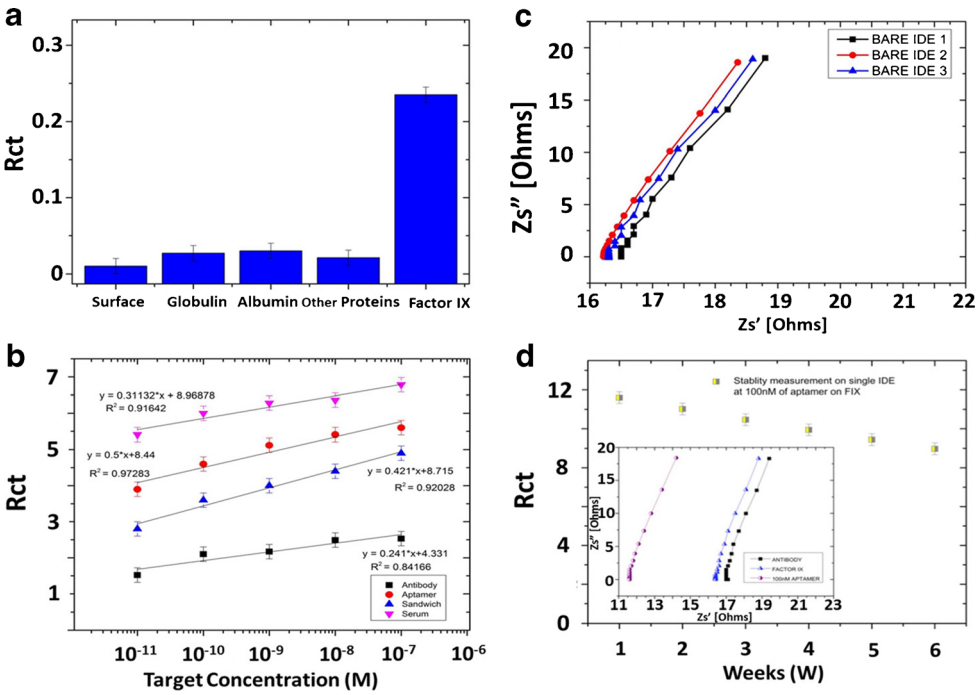

Fig. 5 (**a**) The bar diagram shows selectivity of the IDE sensor towards detection of FIX and other proteins. (**b**) Linear regression curve for different concentrations of FIX with different methods of immobilization. (**c**) The reproducibility curve shown with 3 different measurements under same process conditions. (**d**) Showing the stability of FIX biosensor. Inset shows Nyquist plot of 100 nM aptamer on antibody-FIX complexed surface

involves electrochemical sensor, having several advantages over optical system, such as easy to operate, low sample consumption, ideal sensing surface, suitable for point-of-care and cheaper. Original concentration of FIX in human serum is 57 μg mL^{-1} (87 nM) (with 1:100 dilution, it is equivalent to 870 pM). Current detection limit is 10 pM, so that, FIX in the serum dilution used for present study is 87 times higher than sensitivity attained. It proved the suitability of current sensor for clinical assessments with serum samples. On the other hand, FIX and anti-FIX aptamer interactions, originally was shown with the dissociation constant of 0.65 ± 0.2 nM [31]. Later Gopinath et al. [35] and Lakshmipriya et al. [6] have determined the dissociation constant to be 418 pM and 37 pM, respectively.

Analytical performance on FIX biosensor

Analytical performance of FIX biosensor was tested as shown in Fig. 5a–d. The ΔRct value of FIX at 87 nM was ~0.235 Ω, which was nearly 7.8 times larger than that of albumin (~0.03 Ω) at the same concentration existed in human serum. It is proving that the FIX biosensor has a great specificity towards FIX protein compare to other proteins present in blood (Fig. 5a). Figure 5b showed that FIX biosensor have good linearity and detection limit of 10 pM was predicted using S/N ratio and less than 3σ. The reproducibility graph (Fig. 5c) of FIX biosensor has a relative standard deviation (RSD) of 1.5 % with 3 parallel measurements prepared under similar processing conditions. The stability results (Fig. 5d) showed that prepared dielectrode is very stable and maintain at 77 % of its activity even after 6 weeks.

Conclusion

In this study, using IDE and electrochemical impedance spectroscopy, a sensitive sensor for the detection of FIX protein was demonstrated. The main advantage of the sensor in this study is detection with lower amount of target, highly stable surface and suitable for surface functionalization with appealing analytical high-performances [11, 13, 30]. IDE has advantages over conventional electrode as it displays higher sensitivity and it can be obtained from with similar surface area. A sensitive sensor is shown with the assistance of antibody-FIX-aptamer sandwich pattern of sensing strategy. Results obtained showed a significant increase in sensitivity, whereby the limit of detection was attained to 10 pM, even in the presence of other serum proteins. The strategy proposed in this study using antibody-FIX-aptamer sandwich pattern able to detect specifically FIX in a mixed sample, which will be a great cost-effective biosensor for finding clotting deficiencies in human serum.

Acknowledgments Y. Chen thanks the support from High Impact Research grants - "MoE Grant UM.C/625/1/HIR/MOE/DENT/09" and "UM-MoHE HIR UM.C/625/1/HIR/MOHE/MED/16/5"

Compliance with ethical standards The author(s) declare that they have no competing interests.

References

1. Gopinath SCB, Balasundaresan D, Akitomi J, Mizuno H (2006) An RNA aptamer that discriminates bovine factor IX from human factor IX. J Biochem 140:667–676. doi:10.1093/jb/mvj203
2. Gopinath SCB, Shikamoto Y, Mizuno H, Kumar PKR (2007) Snake-venom-derived factor IX-binding protein specifically blocks the gamma-carboxyglutamic acid-rich-domain-mediated membrane binding of human factors IX and X. Biochem J 405:351–357. doi:10.1042/BJ20061737
3. Gopinath SCB (2008) Anti-coagulant aptamers. Thromb Res 122:838–847. doi:10.1016/j.thromres.2007.10.022
4. Lowe GDO (2001) Factor IX and thrombosis. Br J Haematol 115:507–513. doi:10.1046/j.1365-2141.2001.03186.x
5. Lakshmipriya T, Fujimaki M, Gopinath SCB et al (2013) A high-performance waveguide-mode biosensor for detection of factor IX using PEG-based blocking agents to suppress non-specific binding and improve sensitivity. Analyst 138:2863. doi:10.1039/c3an00298e
6. Lakshmipriya T, Horiguchi Y, Nagasaki Y (2014) Co-immobilized poly(ethylene glycol)-block-polyamines promote sensitivity and restrict biofouling on gold sensor surface for detecting factor IX in human plasma. Analyst 139:3977–3985. doi:10.1039/c4an00168k
7. Busher JT (1990) Serum albumin and globulin. Clin Methods Hist Phys Lab Exam 497–499
8. Gopinath SCB, Awazu K, Fujimaki M et al (2008) Influence of nanometric holes on the sensitivity of a waveguide-mode sensor: label-free nanosensor for the analysis of RNA aptamer-ligand interactions. Anal Chem 80:6602–6609. doi:10.1021/ac800767s
9. Gopinath SCB, Awazu K, Fons P et al (2009) A sensitive multilayered structure suitable for biosensing on the BioDVD platform. Anal Chem 81:4963–4970. doi:10.1021/ac802757z
10. Fujimaki M, Nomura K, Sato K et al (2010) Detection of colored nanomaterials using evanescent field-based waveguide sensors. Opt Express 18:15732–15740. doi:10.1364/OE.18.015732
11. Perumal V, Hashim U, Gopinath SCB et al (2015) "Spotted Nanoflowers": gold-seeded zinc oxide nanohybrid for selective bio-capture. Sci Rep 5:12231. doi:10.1038/srep12231
12. Balakrishnan SR, Hashim U, Gopinath SCB et al (2015) A point-of-care immunosensor for human chorionic gonadotropin in clinical urine samples using a cuneated polysilicon nanogap lab-on-chip. PLoS One 10:e0137891. doi:10.1371/journal.pone.0137891
13. Haarindraprasad R, Hashim U, Gopinath SCB et al (2015) Low temperature annealed zinc oxide nanostructured thin film-based transducers: characterization for sensing applications. PLoS One 10:e0132755. doi:10.1371/journal.pone.0132755
14. Gopinath SCB, Tang T, Citartan M, Chen Y (2014) Current aspects in immunosensors. Biosens Bioelectron 57:292–302. doi:10.1016/j.bios.2014.02.029
15. Wojcik EGC, Cheung WF, Van Den Berg M et al (1998) Identification of residues in the Gla-domain of human factor IX involved in the binding to conformation specific antibodies. Biochim Biophys Acta Protein Struct Mol Enzymol 1382:91–101. doi:10.1016/S0167-4838(97)00149-0
16. Gopinath SCB, Hayashi K, Lee J-B et al (2013) Analysis of compounds that interfere with herpes simplex virus-host receptor

interactions using surface plasmon resonance. Anal Chem 85: 10455–10462. doi:10.1021/ac4025522
17. Gopinath SCB, Hayashi K, Kumar PKR (2012) Aptamer that binds to the gD protein of herpes simplex virus 1 and efficiently inhibits viral entry. J Virol 86:6732–6744. doi:10.1128/JVI.00377-12
18. Gopinath SCB (2007) Methods developed for SELEX. Anal Bioanal Chem 387:171–182. doi:10.1007/s00216-006-0826-2
19. Lakshmipriya T, Fujimaki M, Gopinath SCB, Awazu K (2013) Generation of anti-influenza aptamers using the systematic evolution of ligands by exponential enrichment for sensing applications. Langmuir 29:15107–15115
20. Gopinath SCB, Awazu K, Fujimaki M (2012) Waveguide-mode sensors as aptasensors. Sensors 12:2136–2151. doi:10.3390/s120202136
21. Suenaga E, Kumar PKR (2014) An aptamer that binds efficiently to the hemagglutinins of highly pathogenic avian influenza viruses (H5N1 and H7N7) and inhibits hemagglutinin-glycan interactions. Acta Biomater 10:1314–1323. doi:10.1016/j.actbio.2013.12.034
22. Luo Y, Xu J, Li Y et al (2015) A novel colorimetric aptasensor using cysteamine-stabilized gold nanoparticles as probe for rapid and specific detection of tetracycline in raw milk. Food Control 54:7–15. doi:10.1016/j.foodcont.2015.01.005
23. Zhang L, Li L (2016) Colorimetric thrombin assay using aptamer-functionalized gold nanoparticles acting as a peroxidase mimetic. Microchim Acta 183:485–490. doi:10.1007/s00604-015-1674-6
24. Sui N, Wang L, Xie F et al (2016) Ultrasensitive aptamer-based thrombin assay based on metal enhanced fluorescence resonance energy transfer. Microchim Acta 183:1563–1570. doi:10.1007/s00604-016-1774-y
25. Hao L, Zhao Q (2016) Microplate based assay for thrombin detection using an RNA aptamer as affinity ligand and cleavage of a chromogenic or a fluorogenic peptide substrate. Microchim Acta 183:1891–1898. doi:10.1007/s00604-016-1833-4
26. Park JH, Cho YS, Kang S et al (2014) A colorimetric sandwich-type assay for sensitive thrombin detection based on enzyme-linked aptamer assay. Anal Biochem 462:10–12. doi:10.1016/j.ab.2014.05.015
27. Van Gerwen P, Laureys W, Huyberechts G et al (1997) Nanoscaled interdigitated electrode arrays for biochemical sensors. Proc Int Solid State Sensors Actuators Conf (Transducers '97) 2:73–80. doi:10.1109/SENSOR.1997.635249
28. Rana S, Page RH, McNeil CJ (2011) Impedance spectra analysis to characterize interdigitated electrodes as electrochemical sensors. Electrochim Acta 56:8559–8563. doi:10.1016/j.electacta.2011.07.055
29. Ohno R, Ohnuki H, Wang H et al (2013) Electrochemical impedance spectroscopy biosensor with interdigitated electrode for detection of human immunoglobulin A. Biosens Bioelectron 40:422–426. doi:10.1016/j.bios.2012.07.052
30. Gopinath SCB, Perumal V, Kumaresan R et al (2016) Nanogapped dielectric impedance surface for sensitive detection of Mycobacterium tuberculosis 16 kDa antigen. Microchim Acta
31. Rusconi CP, Scardino E, Layzer J et al (2002) RNA aptamers as reversible antagonists of coagulation factor IXa. Nature 419:90–94. doi:10.1038/nature00963
32. Maier KE, Levy M (2016) From selection hits to clinical leads: progress in aptamer discovery. Mol Ther Methods Clin Dev 5: 16014. doi:10.1038/mtm.2016.14
33. Vavalle JP, Cohen MG (2012) The REG1 anticoagulation system: a novel actively controlled factor IX inhibitor using RNA aptamer technology for treatment of acute coronary syndrome. Futur Cardiol 8:371–382. doi:10.2217/fca.12.5
34. Povsic TJ, Vavalle JP, Aberle LH et al (2013) A Phase 2, randomized, partially blinded, active-controlled study assessing the efficacy and safety of variable anticoagulation reversal using the REG1 system in patients with acute coronary syndromes: results of the RADAR trial. Eur Heart J 34:2481–2489. doi:10.1093/eurheartj/ehs232
35. Gopinath SCB, Shikamoto Y, Mizuno H, Kumar PKR (2006) A potent anti-coagulant RNA aptamer inhibits blood coagulation by specifically blocking the extrinsic clotting pathway. Thromb Haemost 95:767–771. doi:10.1160/Th06-01-0047