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ORIGINAL PAPER

Voltammetric immunoassay for the human blood clotting factor IX by using nanogapped dielectrode junctions modified with gold nanoparticle-conjugated antibody

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Abstract The authors describe an electrochemical method for the determination of the blood clotting factor IX (FIX). A nanogapped dielectrode (with a < 100 nm junction) was modified with antibody against FIX, and the resulting system was characterized by both impedance spectroscopy and voltammetry. In order to attain the improved sensitivity, gold nanoparticles were electrostatically attached to FIX. The current to voltage (I-V) measurement was carried out from 1 V to 5 V, where the entire calibration plot with 5 V was taken. This results in a limit of detection as low of 1 pM, which is much lower compared to the real concentration of FIX (87 nM) in human blood serum. The analytical range extends from 1 pM to 0.1 μM. The electrode is highly specific over other serum proteins.

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Introduction

Healthy human plasma contain 3–5 mg.L^{-1} of FIX [1], required for normal coagulation process and a lower level of FIX leads to a bleeding deficiency [2–4]. FIX deficiency can be detected in human plasma using different methods such as coagulation screening test, coagulation factor assay, and bleeding test [4, 5]. Radioimmunoassay [6], ELISA [7], liquid chromatography [8], gel electrophoresis [9], Surface Plasmon Resonance [10], waveguide mode sensing and BioDVD platform [11] have also been used to quantify the level of FIX in the test sample. Generally antibody and aptamer are used as the probes against FIX in the above methods. To overcome the current challenges, such as usage of minimal sample, simplified system for point-of-care testing, the current study has been launched. For point-of-care testing, it is highly expected that the system better be feasible with both AC and DC currents. In this study, an analytical system has demonstrated to analyze FIX using antibody as the probe. For this analysis, nanogapped system between two electrodes was fabricated with the gap sized below 100 nm as stated before [12] and used for the determination and discrimination of FIX by antibody conjugated gold nanoparticle (GNPs).

GNP mediated sensing proved to enhance the detection of biological elements due to the higher surface area to accommodate a large number of molecules [13, 14]. Additionally, GNPs have good catalytic property, biocompatibility, conductivity and promote the limit of detection [15–18]. Lakshmipriya et al. [1] have detected FIX using aptamer by waveguide mode sensor and improved the detection using

streptavidin-conjugated GNPs. Biomolecules can be easily immobilized on GNP through electrostatic interaction or physical adsorption and also by chemical functionalization. For the current study, the antigen (FIX) was immobilized on the GNPs to improve the detection on the FIX antibody immobilized polysilicon surface having dielectrode. Interactive analyses were performed on the nanogap created between the electrodes with an increment of current proportionally when biomolecules are bound to the surface, similar to other available electric sensors [19]. Our analytical system is suitable for both impedance and current versus voltage (IV) measurements, demonstrated here for its feasibility with the analysis of FIX and its antibody.

Materials and methods

Reagents and biomolecules

Aminopropyl-trimethoxysliane (APTES; http://www.sigmaaldrich.com/catalog/product/aldrich/281778?lang=en&region=MY), Glutaraldehyde solution (50 wt.% in H_2O; http://www.sigmaaldrich.com/catalog/product/sial/340855?lang=en&region=MY&gclid=CPSokJDmo9QCFdOKaAodAQkPUg) and 10 mM Phosphate Buffered Saline (PBS; pH 7.4, at 25 °C) containing NaCl 138 mM; KCl - 2.7 mM (http://www.sigmaaldrich.com/catalog/product/sigma/p3813?lang=en&region=MY&gclid=CNm1lqHmo9QCFUGVaAodlpkEeQ), were purchased from Sigma-Aldrich (USA). Ethanolamine was procured from Fisher Scientific, UK (https://www.fishersci.co.uk/shop/products/ethanolamine-extra-pure-slr-fisher-chemical/10508040). Human FIX was purchased from Abcam (Malaysia; http://www.abcam.com/natural-human-factor-ix-protein-ab62544.html). FIX antibody was purchased from were purchased from Sigma-Aldrich, USA (http://www.sigmaaldrich.com/catalog/product/sigma/f2645?lang=en&region=MY), Gold nanoparticle was purchased from Sigma-Aldrich, USA (http://www.sigmaaldrich.com/catalog/product/aldrich/741957?lang=en&region=MY). The purchased reagents and biomolecules were used without further purification. All chemical and biological reactions on the fabricated active polysilicon surface were done at ambient room temperature. Fabrication of nanogapped dielectric polysilicon surface was carried out as stated before [12].

Chemical functionalization on nanogapped dielectric polysilicon surface

For the molecular assembly on the nanogapped dielectric polysilicon surface, chemical functionalization was performed. Briefly, nanogapped chip was firstly cleaned by deionized water. To establish a self-assembled monolayer of amine terminated groups, 100 μL of 100 mM APTES was dropped on the nanogap and incubated for 1 h at room temperature. The unreacted APTES was removed by rinsing with 5 fold volume by absolute ethanol followed by deionized water. 100 μL of 2.5% Glutaraldehyde was added on amine-modified surface in order to generate a monolayer of aldehyde terminated group and incubated for 30 min. Under this condition, one aldehyde end of glutaraldehyde reacts with amino group of APTES and other end of glutaraldehyde remains available freely. The unreacted glutaraldehyde was removed by rinsing with 5 fold volume of 10 mM PBS buffer.

Biomolecular interactive analysis

After the above surface chemical functionalization, 5 μL of FIX antibody (100 nM), which could cover the active region was dropped on the nanogap having the aldehyde terminated surface and left for 30 min. Unreacted antibody was removed by washing with 10 fold volume of 10 mM PBS buffer (pH 7.4). Then, 10 μL of 1 M Ethanolamine as a masking agent was applied for blocking the aldehyde terminated groups which are not attached by antibodies. After masking, 5 μL of 100 nM of FIX was applied on the chip and kept at room temperature for 30 min and then washed with 10 fold volume of 10 mM PBS buffer (pH 7.4). Readings were taken by impedance and current versus voltage (IV) measurements.

High sensitive interaction of gold-FIX conjugates on nanogapped dielectric junction

To obtain high-sensitive detection, FIX was conjugated electrostatically on GNPs and then added on the FIX antibody bound surface as performed above. For that, the same surface modifications were carried out as in the above case. After immobilize FIX antibody on the glutaraldehyde modified surface, FIX-GNP conjugates were added with FIX concentrations, 1 pM, 10 pM, 100 pM, 1 nM, 10 nM and 100 nM. Samples of FIX-GNP conjugates were prepared by adding 5 μl of GNP into appropriate final concentration of FIX. To check the limit of detection, the resultant data were plotted using OriginPro 9.0. Incubation and washing steps were mandatorily followed as stated above.

Specific detection of FIX

To check the specific detection of FIX, after immobilizing the FIX antibody on glutaraldehyde modified surface, the higher concentration of globulin, albumin, other proteins (mixture of factors VIIa and XI) were independently passed on the antibody modified surfaces. These proteins were tested with the final concentration of 100 nM. Similarly, human blood serum was tested under the condition of spiking FIX (100 nM) and without spiking.

Impedance and IV measurements

All measurements were performed as stated before [12, 20]. Briefly, dielectric impedance spectroscopy measurements were performed using a Novocontrol alpha high-frequency analyzer (Hundsangen, Germany). Amperometric measurements of current to voltage (I-V) were carried out using Keithley 6487 Picoammeter, with 2 wired point-probing to characterize the dielectrode system on the polysilicon surface. The current to voltage (I-V) measurement was carried out from 1 V to 5 V, where the entire calibration plot with 5 V reading was taken. The impedance spectra of the real and imaginary parts of impedance, Zs' and Zs" were received by sweeping the frequency of 1–100 MHz with the applied AC amplitude of 1 V RMS. The 100 Mhz data were selected for the sensitivity calibration plot. Dielectric properties altered by amine and glutaraldehyde functionalization, followed by interaction of antibody with different concentrations of FIX-GNP conjugates were studied by Nyquist plot. Each measurement was done at room temperature in triplicates.

Surface characterizations

The fabricated polysilicon surface was characterized using Scanning Electron Microscopy (SEM, JEOL JSM-6460LA) at 20 kV. Surface chemical functionalization was analyzed by Fourier transform infrared spectroscopy (FTIR, Spectrum 65, Perkin-Elmer). The analyses were performed for the surface with APTES, APTES/glutaraldehyde and APTES/glutaraldehyde/Antibody immobilization. All samples were examined at the wavelength ranges from 0 to 4500 cm^{-1}.

Results and discussion

Hemophilia is the disease, also called 'a Royal disease' caused by the defective level of FIX in human blood, leads to a clotting deficiency. In the human blood clotting cascade, FIX involves both extrinsic and intrinsic clotting pathways and with the deficiency in these pathways clotting is not proceed successfully [4]. A threshold quantitative detection of FIX helps to identify the disease for the proper treatment. In the past, quantification of FIX has been demonstrated with different analytical and non-analytical sensing systems, which include Surface Plasmon Resonance, waveguide mode sensing, BioDVD platform [1, 4, 5, 21]. Herein, additional evidence was introduced to support the detection of FIX by its antibody with the help of nanogapped dielectric polysilicon substrate, suitable to operate with DC and AC currents. To attain a high-performance with this analytical system, antibody-GNP conjugates were used and specificity analysis was done with proteins other than FIX, reside in human blood serum.

Surface characterizations

Figure 1 **a, b** displays the surface image of the nanogapped dielectric system under scanning electron microscopy (SEM) observation. The measurement with SEM shows the gap between two electrodes to be <100 nm. Due to the lesser nanogap, it has already been proven its efficient detection of biomolecules with this chip [12]. This nanogapped polysilicon surface was functionalized chemically by APTES followed by glutaraldehyde in order to capture FIX antibody. The steps involved in the complete surface chemical modifications and biomolecular assembly processes are indicated in the Fig. 1 **c-g**. These modifications are confirmed by Fourier transform infrared spectroscopy (FTIR) spectral profiles. FTIR results clearly displayed the changes in the spectral arrangements for APTES, APTES-glutaraldehyde and APTES-glutaraldehyde-antibody modified surfaces.

Impedance measurements for molecular assembly

The molecular assembly/interaction during the experiments steps were measured by both impedance and IV. Figure 2 infers that there is a change on the surface with the assembly of molecules. It can be obviously seen from the semicircle that indicate the conformational changes on the surface upon step by step process with increasing overall charge transfer resistance (Rct) of the analytical system. The bare polysilicon showed the lowest impedance and after introduce APTES on the chip; the resistance was increased to the value of 1.8×10^5 Zs' ohms. This result confirms the proper modification of amine on the nanogapped dielectrode polysilicon surface. Then with glutaraldehyde immobilization, there was an increment with the Rct to 3.7×10^5 Zs' ohms. Antibody and ethanolamine attachments were confirmed by increasing the Rct to 4.6×10^5 and 4.8×10^5 Zs' ohms, respectively. Since antibody covered most of the aldehyde tethered surface, ethanolamine shows only a small variation in the changes. Finally, FIX was added on the FIX antibody modified surface with the concentration of 100 nM and the spectrum shows the clear increase in the Rct to 5.9×10^5 Zs' ohms.

Current vs Voltage (IV) measurements for molecular assembly

The above-mentioned immobilization and assembly processes were also monitored by a Picoammeter, with 2 wire point-probing systems IV measurement. As shown in Fig. 3, the bare surface without any surface modification shows the current flow at the lower level and after attaches the APTES, current flow was increased to 1.0×10^{-4} V, it confirms the proper modification of the surface with amine. With further modifications, the current flows were decreased by each attachment. After attaching glutaraldehyde, the current

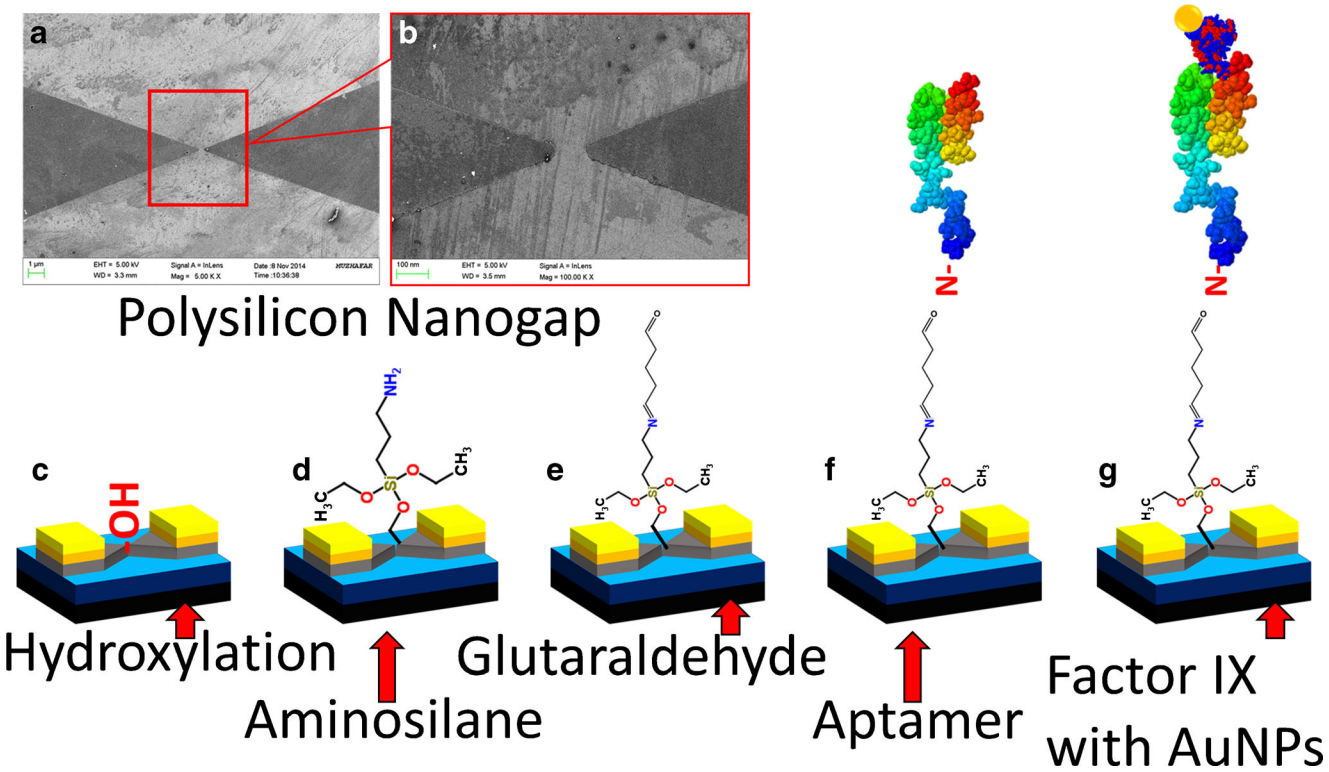

Fig. 1 Surface characterization on the nanogapped dielectric-polysilicon surface. Scanning Electron Microscopy observation (a) and enlarged view (b). Surface functionalization shown with bare (c); APTES immobilized (d); Glutaraldehyde modified (e); antibody immobilized (f) and FIX complexed antibody (g)

decrement was to 8.0×10^{-5} V. Then, the current was changed to 5.0×10^{-5} V after immobilizing FIX antibody. It clearly confirms that the proper antibody binding on the glutaraldehyde modified surface. After the antibody attachment, the remaining places of glutaraldehyde were blocked by ethanolamine, it lead to the current flow with small variation due to the higher attachment of FIX antibody bound to the aldehyde-modified surface. This step is exactly imitating the above impedance measurement, where also the higher immobilization of antibody was evidenced. Finally, FIX protein was passed on the FIX antibody modified surface and it was found that the current flow has a great difference with decrement upon the addition of FIX, and the changes were from 5.0×10^{-5} to 2.3×10^{-5} V.

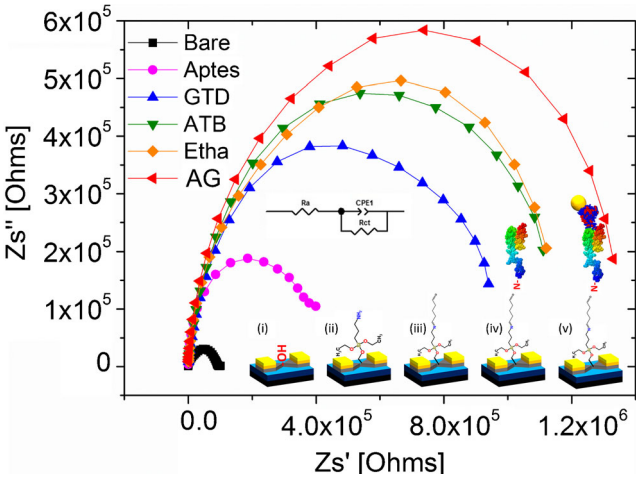

Fig. 2 Impedance analysis on the surface modifications. The impedance spectra of the real and imaginary parts of impedance, Zs' and Zs'', were got by sweeping the frequency of 1–100 MHz with applied AC amplitude of 1 V RMS and plotted using Nyquist plot. Figure inset shows the modification steps and equivalent circuit

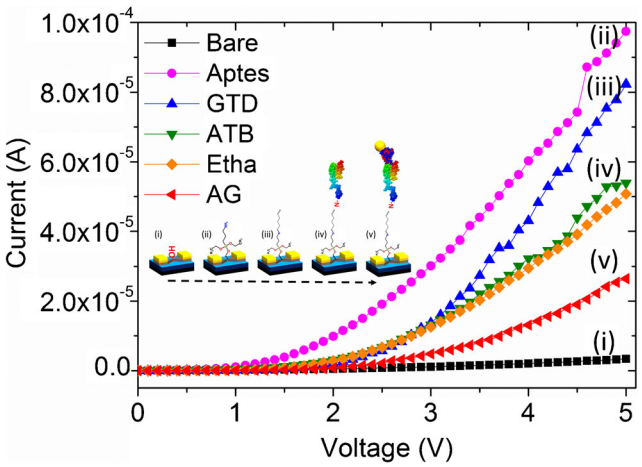

Fig. 3 Current versus voltage (IV) measurements on the surface modifications. Amperometric measurements of current to voltage (I–V) were carried out. A linear sweep voltage of 0–1 V at 0.05 V step voltage was used throughout the analysis. Figure inset shows the modification steps

Enhancing detection by FIX-GNP conjugates

To enhance the level of FIX detection and to demonstrate the high-performance detection, herein analyzed the interaction of FIX-GNPs against FIX antibody. GNP can binds with the protein through electrostatic interaction or ionic bonding or chemical modification [12, 22, 23]. Smaller sized biomolecule can be easily modified by chemical means using thiol-group to attach on GNP, but the bigger sized molecules (protein or antibody) with a larger molecular weight can conjugate GNP without a specific chemical modification due to more attraction between amines on the protein and GNPs [23]. In this study, FIX protein with 55 kDa was bound to the GNPs by mixing. Previously, Gopinath et al. [23] have demonstrated the stable binding of FIX on the GNP surface electrostatically even under the high salt concentration. Other biomolecules, such as nucleic acid is also found to interact with GNP electrostatically [24]. In this study, prepared the FIX-GNP conjugates and titrated from 1 pM to 1 nM of FIX and detected on the FIX antibody modified surfaces. Figure 4 shows clear changes in charge transfer resistance with the concomitant increase with FIX concentrations. The high concentration (1 nM) of FIX yielded the Rct to the level of 3.7×10^6 Zs' ohms. With the lowest concentration of 1 pM, the Rct level shows the background level and considered as not significant. FIX with 10 and 100 pM gives the Rct of 0.9×10^6 and 1.5×10^6 Zs' ohms, respectively. With 1 nM of FIX the current flow was noticed to be with the Rct of 1.8×10^6 Zs' ohms. The higher concentrations of FIX, 10 and 100 nM displayed with 2.9×10^6 and 3.7×10^6 Zs' ohms, respectively. These results indicated clearly that the lower detection of FIX (1 pM) was achieved. An overview on the reported nanomaterial-based methods for the determination of factor IX is displayed in the Table 1. Previously, Lakshmipriya et al. [1] have detected the FIX by its aptamer on the waveguide surface with the limit of detection of 100 f. as the highest sensitivity achieved with the assistance of polymer conjugated GNPs, and they also detected the FIX protein using the sandwich pattern with aptamer and antibody until the limit of 100 pM [10]. The current study with the sensitivity level of 1 pM may consider being the better after the methods designed by Lakshmipriya et al. [1, 10]. Other applied methods stated in the Table 1 with different substrates, the sensitivities were attained in the range of picomolar [4, 5, 21, 25]. Since healthy human plasma contains FIX around 3–5 μg/ml, the limit of detection attained in the present study is also well suited to detect the FIX in human plasma. On the other hand, the signal enhancement with FIX-GNP conjugates was ~100 folds higher compared to the level without GNPs, brought the sensitivity to 1 pM. The main appealing characteristics of the current work compared to other established methods are relatively cheaper, convenient, consumes lesser sample and with battery operating feasibility for point-of-care testing.

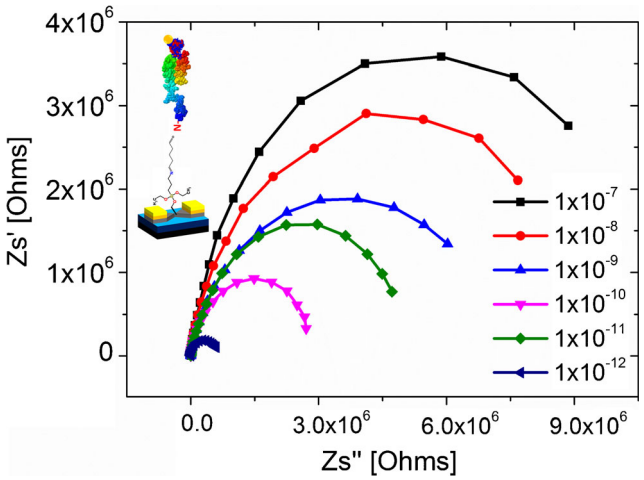

Fig. 4 Impedance analysis for the interaction of FIX-GNP conjugates. The impedance spectra of the real and imaginary parts of impedance, Zs' and Zs", were got by sweeping the frequency of 1–100 MHz with applied AC amplitude of 1 V RMS and plotted using Nyquist plot. The concentrations of FIX measured are from (i) to (vi) which represents from 100 nM to 1 pM. Figure inset shows the molecular assembly

High-analytical performance by nanogapped dielectrode system

The sensitivity was calculated based on the experimental values and a linear correlation of the differences in the charge transfer resistance, $\Delta Rct = Rct_{FIXantibody} - Rct_{FIXantigen}$ with respect to the logarithm of FIX concentrations are shown in Fig. 5 **a**. The sensitivity of the reported analytical assay was calculated using the equation below,

$$Sensitivity = \frac{Slope\ of\ calibration\ plot,\ \ m\left(\mu A\ \mu M^{-1}\right)}{Active\ Surface\ Area,\ \ A\ (cm^2)}$$

The slope, $m = 7.5 \times 10^{-7}$ μA μM − 1 was got from the Fig. 5 **a** and area; A is the maximum detection spot on the active surface ($0.00262 cm^2$). Therefore, the sensitivity of the system calculated was 286.26 μA μM^{-1} cm^{-2}. It was observed that ΔRct increases linearly with increasing FIX concentrations from 1 pM to 0.1 μM. The value (ΔRct) difference between Rct on antibody immobilized surface and antigen interacted surface was found to be well proportional to the natural logarithm of FIX concentration with a linear equation of ΔRct = 1.534E6 + 1.986E7x, (R^2 = 0.98906). The limit of detection was estimated to be 1 pM using the signal to noise ratio of more than 3σ (where σ is the standard deviation of the blank solution, n = 5). The detection limit is lower than previous report using another probe (aptamer) interacted with FIX, the dissociation constant (Kd) determined by Rusconi et al. [10] was 580 pM using radioisotope labeled aptamer. Similarly, Gopinath et al. [3] reached the Kd value of 365 and

Table 1 Comparison of the currently available detection systems for factor IX

Method applied	Material used	Immobilized molecule	Limit of detection	Reference
Radio isotope	Solution	Aptamer	580 pM	[5]
Waveguide	Silica & Polymer	Aptamer	100 pM	[1]
Waveguide	Gold & Polymer	Aptamer	100 fM$	[1]
SPR	Gold & Polymer	Aptamer	37 pM	[10]
SPR	Gold & Polymer	Antibody	48 nM	[10]
SPR	Gold & Polymer	Aptamer & Antibody Sandwich	800 fM	[10]
SPR	Streptavidin	Aptamer	365 pM	[4]
BioDVD	Gold	Aptamer	1 nM	[21]
Interdigitated Electrode	Zinc oxide	Aptamer & Antibody Sandwich	10 pM	[25]
Waveguide	Silica	Aptamer	500 nM	[26]
Voltammetry	Polysilicon	Antibody	1 pM	Current work

*Dissociation Constant; $ Represents the best method demonstrated for determining the factor IX at the lowest concentration

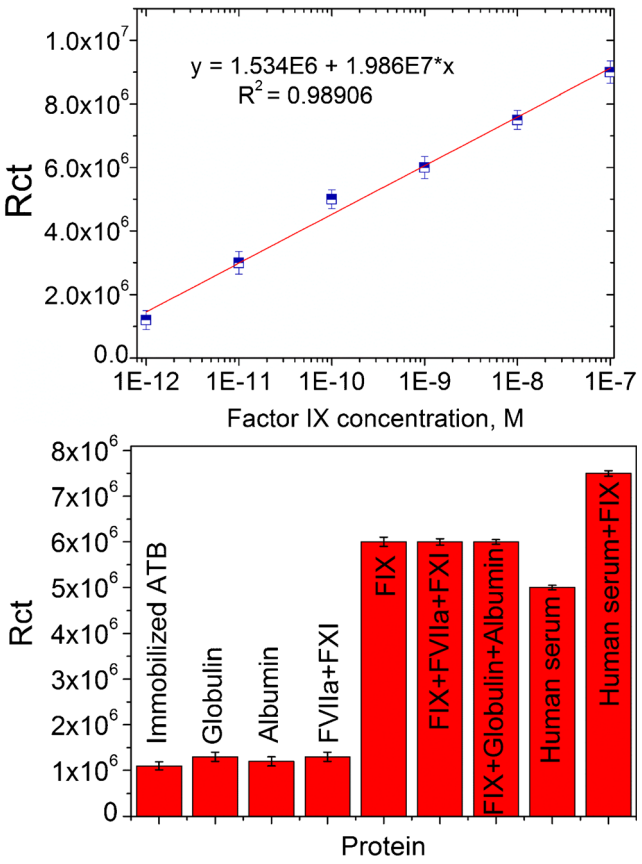

Fig. 5 High-performance analysis on the polysilicon surface. (a) Linear regression analysis. Results are expressed as mean value of three independent determinations. Limit of detection was calculated based on signal-to-noise ratio. The current to voltage (I-V) measurement was carried out from 1 V to 5 V, where the entire calibration plot with 5 V reading was taken. (b) Specificity analysis. Specific binding of FIX antibody was analyzed against different proteins from human blood serum. Results are expressed as mean value of three determinations

418 pM with aptamer against FIXa and FIX, respectively, determined by surface plasmon resonance biosensor.

To check the specificity of antibody used in this study to detect FIX, different proteins which are abundant in the human serum were evaluated by passing them independently on FIX antibody immobilized surface. The normal level of albumin in the human serum was reported to be 45 $g.L^{-1}$, similarly the level of other factors FVIIa is 3.6 $mg.L^{-1}$ and FXI is 3–6 $g.L^{-1}$ [1]. For the specificity analyses, different samples were used, namely albumin, globulin, mixture of factors (factors VIIa, FXI). In which, the current analytical system displays ~5 folds higher detection by FIX antibody against FIX compared to other tested proteins from the serum (Fig. 5 **b**). We also analyzed with real human serum alone, 100 nM of FIX-spiked human serum and mixture of FIX (100 nM) and the above negative proteins. There were no significant differences between FIX alone and FIX-spiked samples. However, with only human serum there was a reduction in the signal compared to the FIX alone. But, FIX spiked human serum has shown an increment, this might be due to abundance of FIX in the human serum.

Conclusions

Detection and quantification of FIX are the mandatory events to know the level of disease and help to treat. Here nanogapped dielectric analytical system was used to detect FIX (conjugated to GNPs) against its antibody by impedance and IV measurements. With these analyses, could achieve the picomolar level of detection with a higher specificity by discriminating other proteins and the human serum. This detection level is suitable for detecting the FIX abundance in real human serum and comparable with other currently available methods, even though the voltammetry measurement shown

here is not the best. This method is favorable to diagnose clotting deficiency, feasible with AC and DC currents for point-of-care testing. Compared to the currently demonstrated sensing systems for detecting factor IX, this analytical system has positive characteristics such as, convenient to handle without prior experiences and consumes the lower volume of samples with the feasibility for bed-side analysis.

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

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