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COMMUNICATION

A novel immobilization strategy for electrochemical detection of cancer biomarkers: DNA-directed immobilization of aptamer sensors for sensitive detection of prostate specific antigen†

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We report on a novel strategy for DNA aptamer immobilization to develop sensitive electrochemical detection of protein biomarker, with prostate specific antigen (PSA) as a case biomarker. Thiolated single-stranded DNA (ssDNA) was co-immobilized with 3-mercapto-1-propanol on gold electrodes, and used as a scaffold for DNA aptamer attachment through hybridization of the aptamer overhang (so-called “DNA-directed immobilization aptamer sensors”, DDIAS). In the approach, the complementary DNA aptamer against PSA was assembled by the probe ssDNA onto the electrode to detect PSA; or the probe ssDNA directly hybridized with complementary DNA aptamer/PSA complex following their pre-incubation in solution, so-called ‘on-chip’ and ‘in-solution’ method, respectively. A double stranded DNA intercalator with ferrocenyl (Fc) redox marker was synthesized to evaluate the feasibility of the strategy. Results demonstrate that ‘in-solution’ method offers a favourable media (in homogenous solution) for the binding between the aptamer and PSA, which shows to be more efficient than ‘on-chip’ approach. DDIAS show promising analytical performance under optimized conditions, with a limit of detection in the range of fM and low non-specific adsorption.

Cancer is a leading cause of death and accounted for 8.2 million deaths in 2012 worldwide according to the report of World Health Organization (WHO), and prostate cancer is the fifth cause of cancer-related deaths. However, the chance of treating cancer could be increased if it could be diagnosed at an early stage. Prostate specific antigen (PSA) is a biomarker that has played a significant role for the detection of prostate cancer, in particular in clinical serum samples^{1,2}. A PSA level of 4 ng/mL in human serum sample

is usually regarded as the cut-off value for potential diagnosis of prostate cancer³. To this end, it is essential to develop ultra-sensitive and specific biosensors for accurate PSA detection for early cancer diagnosis. Even though PSA is currently not considered as a very reliable biomarker, there is general consensus that future diagnostic devices will need to test for a panel of biomarkers, which includes PSA.

DNA aptamers have proven to be powerful tools in the fields of molecular recognition and biosensors due to their low cost, ease of synthesis and application as electrochemical biosensors because of bearing negative charge⁴⁻⁷. Compared to antibodies, aptamers are stable and able to sustain reversible denaturation⁷. A DNA aptamer against PSA was identified by Savory and co-workers in 2010⁸, and several groups have been using it for PSA sensing⁹⁻¹¹. Signal amplification with nanoparticles is found to improve the limit of detection (LOD) of PSA^{9, 11}. However, the complicated immobilization procedures and facile aggregation of the nanoparticles were observed to limit the utility of this sensor platform.

DNA-directed immobilization (DDI) was firstly proposed by Wacker et al^{12, 13} for multiplexed antigen detection, based on the self-assembly of semi-synthetic DNA-protein conjugates to generate a protein microarray assay. In this strategy, protein tumour marker carcinoembryonic antigen (CEA) was employed as a model system and the detection limit could reach as low as pg/mL range. Chevolut and co-workers¹⁴⁻¹⁶ further developed the DDI method for a glycoarray, which used glycoconjugate molecules that present a DNA sequence for anchoring onto DNA chips through hybridization. The in-solution approach was performed by allowing saccharide/lectin recognition before immobilization by hybridization on the surfaces and subsequent detection. The developed glycoarray using DDI method showed promising analytical performance with detection limit at nM range. However, DDI for both protein microarray and glycoarray required the challenging semi-synthesis of DNA anchoring antibody or carbohydrate and troublesome purification steps.

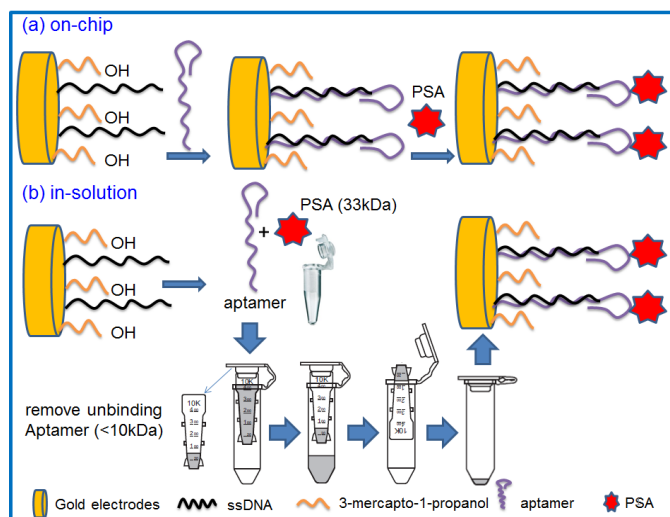
Here, we propose to use a DDI strategy for aptamer immobilization: DNA-directed immobilization of aptamer sensors (DDIAS), which does not require the synthesis of DNA-protein or DNA-carbohydrate complexes. We introduced the aptamer, which serves as the function of protein modified single-stranded DNA

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† Electronic supplementary information (ESI) available: Experimental details. See DOI:xxxx

(ssDNA), as the specific linker to assemble the antigen onto the electrode. A ssDNA probe was firstly co-immobilized with 3-mercaptopropyl-1-propanol (MCP) onto the electrode as a scaffold. The surface density can be effectively controlled to allow efficient hybridization with complementary DNA aptamer¹⁷. The complementary DNA aptamer against PSA could be assembled on the electrode to bind with PSA; or the probe ssDNA directly hybridized with complementary DNA aptamer/PSA complex following their pre-incubation in solution, so-called ‘on-chip’ and ‘in-solution’ method, respectively (Scheme 1). In particular, the ‘in-solution’ method allows the aptamer and PSA binding in a homogenous media, which was expected to be more sensitive for PSA detection. To limit the competition binding with ssDNA probe from “free aptamer” (<10 kDa) and aptamer/PSA complex (>33 kDa), a filtration device was employed to separate them according to their different molecule weight, which further implement the previous DDI approach^{13, 14, 16}. This protocol simplified the elaboration of the biosensor and can be used as a general effective immobilization strategy for the detection of versatile analytes. Additionally, compared to typical direct immobilization of aptamer onto a solid substrate, the linear linkage of DNA will allow sufficient steric space for the active site of aptamer to bind with the protein and prevent the formation of secondary structure of the DNA aptamer. To the best of our knowledge, this is the first time this method with aptamer sensors has been demonstrated to detect cancer biomarker.



Scheme 1. Illustration of the strategy for the elaboration of DNA-directed immobilization aptamer sensors (DDIAS) for PSA detection: (a) ‘on-chip’ method and (b) ‘in-solution’ method, aptamer and PSA were incubated in solution and then the unbinding aptamer was removed with a filtration device.

Fig. 1A presents a typical Nyquist plot of the DDIAS for the detection of PSA at 10 $\mu\text{g/mL}$ with ‘on-chip’ and ‘in-solution’ method. Note that even though the electrodes for ‘on-chip’ and ‘in-solution’ method are different, the initial resistance of the ssDNA/MCP layer on the electrodes is reproducible following the same cleaning and immobilization procedures. Keighley *et al.*¹⁷ optimized the surface density of ssDNA by co-immobilization of 6-mercaptopropyl-1-hexanol (MCH) to get a highly efficient hybridization with complementary strand DNA using a label-free EIS assay. The maximum percentage change of charge transfer resistance upon hybridization with fully complementary target oligonucleotides was obtained from oligonucleotide probes electrodes co-immobilized

with MCH at a DNA mole fraction of 20%. The electrodes in our experiment were designed with the ratio between probe ssDNA and MCP at 1:3 (ssDNA mole fraction at 25%) for high hybridization efficiency. The change of charge transfer resistance (ΔR_{ct}) to the background of ssDNA was proportional to each binding event, such as hybridization of complementary aptamer, detection of PSA using ‘on-chip’ and ‘in-solution’ method. Fig. 1 A shows that both DNA hybridization and PSA binding increased the charge transfer resistance, and ΔR_{ct} shifted by 12.5% for the hybridization of complementary aptamer with probe ssDNA. The detection of PSA either with ‘in-solution’ or ‘on-chip’ method lead to a significant change of ΔR_{ct} , 27.6 % and 98.6 %, respectively. As shown in Fig. 1 B, electrodes functionalized with ssDNA probe were employed to incubate with PSA as control, and only a small shift of charge transfer resistance (2.1 %) was observed, indicating that ssDNA cannot specifically bind PSA and co-immobilization of ssDNA/MCP could play a useful role of limiting the non-specific adsorption of PSA on the electrode surface.

In the EIS measurement, negatively charged ferri/ferrocyanide couple $[\text{Fe}(\text{CN})_6]^{3-/4-}$ was employed as the redox marker for electrochemical characterization, thus the diffusion of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and mass loading of PSA onto the surface of the electrodes have a significant effect on the charge transfer resistance. The electrodes modified with ssDNA are negatively charged, and as more negative charges were introduced onto the surface upon hybridization with aptamer, the redox marker diffusion to the surface of the electrodes is hindered due to repulsive interactions. Thus, charge transfer resistance shifts to a high value upon the aptamer hybridization with ssDNA probe are observed. These results are in agreement with our early report using EIS for DNA detection^{17, 18}.

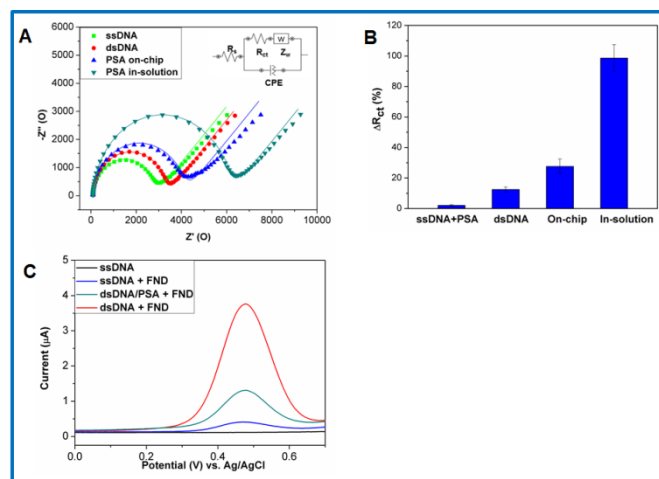


Fig. 1. (A) Typical Nyquist plot of DDIAS for PSA detection, electrodes was functionalized with ssDNA probe, aptamer hybridized with ssDNA probe, PSA binding with aptamer with ‘on-chip’ and ‘in-solution’ method, respectively; (B) change of charge transfer resistance (ΔR_{ct}) of the different layers on electrodes surface interpreted from Fig. 1 A; (C) Differential pulse voltammetry (DPV) measurement of electrodes modified with ssDNA aptamer, ssDNA probe binding with aptamer and ssDNA probe binding with aptamer/PSA complex incubation with FND intercalator, and the ssDNA aptamer without incubation with FND was recorded as blank. The data was recorded from at least 3 individual electrodes.

Additionally, the binding with PSA leads to an increase of charge transfer resistance due to a high mass loading on the surface of the electrodes, which will hinder the redox marker approaching the surface. Interestingly, the ‘in-solution’ method for PSA detection induced a significant shift of charge transfer resistance compared to the ‘on-chip’ method, indicating that the ‘in-solution’ approach is

more efficient than ‘on-chip’ for PSA detection. This was associated with the fact that the ‘in-solution’ method offers a homogeneous binding media for aptamer and PSA; however, the aptamer binding with PSA takes place in a solid-liquid phase for the ‘on-chip’ method. Upon the binding event between ssDNA probe and aptamer/PSA, the increase in mass loading as well as negative charge of the aptamer account for the increase in charge transfer resistance. For the ‘on-chip’ method, the aptamer was firstly assembled by hybridization on the electrodes, leading to steric hindrance for PSA to attach onto the electrode surface. Consequently, the ‘in-solution’ method is promising and effective for PSA detection, and it will be further optimized for highly sensitive detection.

To evaluate the hybridization of the ssDNA probe with DNA aptamer/PSA complex, a ferrocene intercalators (N,N'-((((1,3,6,8-tetraoxo-1,3,6,8-tetrahydrobenzo [*lmn*] [3,8] phenanthroline-2,7-diyl) bis(ethane-2,1-diyl)) bis(oxy)) bis(ethane-2,1-diyl))bis(oxy)) bis(ethane-2,1-diyl)) diferrocenamide, FND) was synthesized (Scheme S1) to bind with dsDNA from aptamer/PSA complex with ssDNA probe. The synthesis protocols are presented in the supporting information. Fig.1 C illustrates the interaction between intercalator and ssDNA aptamer, dsDNA and the dsDNA/PSA complex characterized with Differential pulse voltammetry (DPV) measurement without redox markers in the electrolyte. The current depends on the interaction between intercalator and biomolecules functionalized on the electrodes. As shown in Fig. 1 C, a small current can be measured from ssDNA aptamer and FND. FND is a double stranded DNA intercalator which can bind with the duplex helix structure, and is not able to specifically bind with ssDNA¹⁹. However, the secondary conformation of the anti-PSA aptamer (internal duplex due to the loop structure formed) may be formed after the immobilization on the electrodes⁸. The small current observed from ssDNA is likely due to the binding between the intercalator and the aptamer structure. DNA hybridization increases the current due to more double stranded DNA formed on the electrodes, indicating that ssDNA probe as a scaffold could well direct the assembly of aptamer/PSA complex onto the electrodes. The measured current of dsDNA from hybridization of probe ssDNA with aptamer ssDNA was higher than that from hybridization with aptamer ssDNA/PSA complex. This was associated with less duplex helix structures being generated for aptamer/PSA hybridization due to the steric hindrance of PSA on the electrodes. In conclusion, the DPV results offer evidence that the DNA hybridization could direct the assembly of the aptamer/PSA complex formed in solution onto the electrodes.

The change in charge transfer resistance of the electrodes before and after binding with intercalators was recorded and the results show that the intercalator increases the charge transfer resistance (see Fig. S1). Therefore, the synthesized intercalator could potentially be employed for signal enhancement for our DDIAS. This could be comparable with our recently reported cobalt complex for enhancing impedimetric DNA detection²⁰. For our DDIAS platform, the enhanced charge transfer resistance was achieved after incubation with ferrocene intercalators. As the intercalators are neutral, the impedimetric signal enhancement is likely due to the expansion of the dsDNA and stronger charge barriers on electrodes after incubation with intercalators, leading to the difficulty of ferri/ferrocyanide redox marker to diffuse onto the surface of electrodes. Furthermore, it is possible that the introduction of ferrocene intercalators may help drive the generation of the loop structure of the aptamer, which leads to an increase in the change transfer resistance of the system. A systematic study of the binding between ferrocenyl intercalators and full complementary dsDNA is currently under investigation.

In order to improve the analytical performance of the developed aptamer sensors for the ultrasensitive detection of the real samples, the surface density of immobilized ssDNA probe and the concentration of aptamer were optimized. Various concentrations of aptamer (from 5 μM to 5 nM) were investigated by firstly binding with 50 ng/mL PSA (1.5 nM) in solution, and then the aptamer/PSA complexes were filtrated and hybridized with ssDNA probe on the electrode. As shown in Fig. 2A, the highest ΔR_{ct} value was obtained at 50 nM of aptamer and it shifted to lower values with increasing aptamer concentration. At higher aptamer concentration ranges (from 50 nM to 5 μM), the quantity of aptamer molecules was excessive to that of PSA in solution as the aptamer and PSA were incubated with the equivalent volume. Even though the aptamer/PSA complex was filtrated, it's likely to remain tiny amount of excessive ‘free aptamers’. When the solution was transferred onto electrodes, there may be a competitive binding with ssDNA probe between the excessive aptamer and aptamer/PSA complexes. The same portion of aptamer/PSA complex binding on electrodes, compared to the aptamer, will lead to increased shifts of charge transfer resistance due to the additional high mass load of PSA onto the surface of electrodes meantime. However, the lower concentration of aptamer at 5 nM probably obtain less portion of aptamer/PSA complex in solution, which make the ΔR_{ct} shift to lower value. An equilibrium may exist on the binding between aptamer and PSA, the lower concentration of aptamer reduced the yields of aptamer/PSA complexes. As a result, the aptamer concentration was optimized at 50 nM affording the highest ΔR_{ct} , which allows the sensitive detection of PSA using the ‘in-solution’ method.

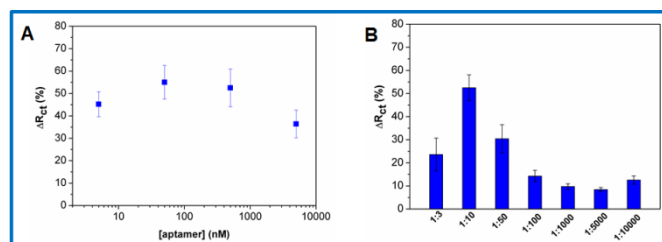


Fig.2. Optimization of ‘in-solution’ method for PSA detection (A) Effects of aptamer concentration on ΔR_{ct} and (B) effect of the molar ratio between probe ssDNA and MCP on ΔR_{ct} of aptamer sensors.

The molar ratio between probe ssDNA and MCP on electrodes is crucial for the DDIAS, as it determines the surface density and hybridization with complementary aptamer or aptamer/PSA complex. More importantly, MCP plays a role in limiting non-specific adsorption, which is particularly essential to be optimized for protein detection. Fig. 2B shows the effect of various mole ratios between ssDNA probe and MCP on the ΔR_{ct} value. The optimal value for the detection of 50 ng/mL PSA was obtained for a 1:10 ratio, which allows a low surface density on the electrodes and keeps high DNA hybridization efficiency. Compared to the optimal ratio (ssDNA: MCP, 1:4) for DNA assay in our early report¹⁷, the fraction of MCP increased in our PSA assay under the optimized condition. The relatively lower density of DNA probe will be easier attached by the aptamer/PSA complex; furthermore, the higher MCP ratio would be more efficient for limiting non-specific adsorption of PSA.

As a result, the surface density and the aptamer concentration were optimized to obtain a high variation on the charge transfer resistance values. The optimized DDIAS is shown to be a promising tool for the detection of PSA, and in particular the ‘in-solution’ method can be potentially used for the detection of other biomolecules as the binding event takes place in solution. Under the optimized conditions, we employed DDIAS to detect various concentrations of PSA to evaluate the analytical performance of the

sensors. As shown in Fig. 3A, ΔR_{ct} increased with increasing PSA concentration, and the detection of 0.5 pg/mL (1.5 fM) of PSA could be reached with a ΔR_{ct} value of 12.1%. This value is higher than the one (ΔR_{ct} 8.2%) obtained for control experiments with 10 μ g/mL BSA as a reference protein for non-specific adsorption. The LOD was estimated to around 0.5 pg/mL (1.5 fM) (3σ) in solution, and a dynamic range was obtained from 0.05 ng/mL to 50 ng/mL ($R^2=0.985$). Chen *et al.*¹⁰ proposed detection PSA using resonance light scattering (RLS) spectral assay based on PSA aptamer modified gold nanoparticles (AuNPs) and could sensitively detect 0.032 ng/mL PSA. Liu *et al.*⁹ developed an aptamer sensing platform of a graphite electrode modified with gold nanoparticles covered with graphitized mesoporous carbon nanoparticles for human PSA detection, based on the signal amplification of the biotin-avidin system. They were able to quantitatively measure PSA concentration range from 0.25 to 200 ng/mL, with a lowest LOD of 0.25 ng/mL. An optimized enzyme-linked immunosorbent assay (ELISA), which is the gold standard and most widely used assay for PSA, was reported to obtain a LOD at ng/mL range¹⁰. Compared to these reports, our DDIAS could reach a LOD at pg/mL range without any signal amplification procedures. Fig. 3B presents the non-specific adsorption evaluation of the developed DDIAS by interaction with 10 μ g/mL BSA and PSA, respectively. For the developed DDIAS, there are changes of charge transfer resistance for BSA detection from both 'on-chip' and 'in-solution' method. For the 'on-chip' method, ΔR_{ct} was 7.5 % and 27.6 % for BSA and PSA detection, respectively. However, the 'in-solution' method is demonstrated to be more effective in obtaining positive signal (ΔR_{ct} from PSA at 98.6 %, BSA at 8.2%) from detection of the same concentration of PSA and BSA. It is suggested that the 'in-solution' method is more specific and favourable for PSA detection, which could be potentially employed for the detection of proteins in complex samples. Moreover, in order to investigate the stability of the customized biosensors, the measurements were performed before and after the incubation of electrodes with 5 M sodium chloride (NaCl) solution, and the results (Fig. S2) show that the signal has no changes from incubation with NaCl. This indicates that our DDIAS is able to withstand salt solution, which will be beneficial for the detection of PSA in complex media.

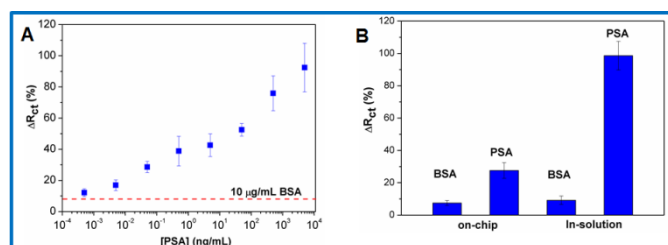


Fig. 3. (A) Effects of different concentration of PSA on the ΔR_{ct} performed 'in-solution' method (B) comparison of non-specific adsorption of DDIAS between 'on-chip' and 'in-solution' method with BSA as control.

In summary, we have demonstrated a novel strategy for aptamer immobilization with DNA-directed immobilization method for cancer biomarker detection with electrochemical impedance spectroscopy. The 'in-solution' and 'on-chip' methods were compared, and we conclude that the 'in-solution' approach is more effective due to the homogenous binding media for aptamer and protein. The feasibility was validated with double stranded DNA intercalators synthesized in-house. We demonstrated that the DDIAS is able to sensitively detect PSA at the fM range under optimized parameters, and favourable for limiting non-specific adsorption. Additionally, the proposed strategy could be further used for a wide range of protein cancer biomarkers detection, such as peptide, protein or even small molecules like drugs assuming that a specific

DNA aptamer candidate is available. We hope to use the effective DDIAS for the detection of the cancer biomarker in real samples, such as serum, urine as well as wastewater for epidemiological studies. The versatile and sensitive biosensors will be beneficial for the application in the fields of healthcare and environmental monitoring.

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