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Novel sensitive immunosensor for the selective detection of Engrailed 2

urinary prostate cancer biomarker

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Abstract

Engrailed 2 (EN2) is a homeodomain-containing transcription factor expressed in prostate cancer (PCa) cell lines and is secreted into the urines. It is nowadays considered as a promising non-invasive biomarker for PCa early diagnosis. Herein, we report the design of an electrochemical immunosensor for EN2 detection. The biosensor fabrication involved a covalent immobilization of anti-EN2 antibodies onto a poly para amino benzoic acid (PABA) film electropolymerized on a gold electrode. Square wave voltammetry was investigated for EN2 detection in a phosphate buffer solution in a concentration range of 10^{-5} ng/mL to 1 μ g/mL. The limit of detection of the designed sensor was equal to 10^{-5} ng/mL and the sensitivity was of order of 0.02 mL/ng. The dissociation constant K_d of the "complex" EN2/anti-EN2, estimated from a Hill model, was of order of (0.9 ± 0.2) fM. Experimental results revealed that the immunosensor enabled selective detection of EN2 in a mixture of three proteins which can be found in men' urine: human serum albumin (HSA), prostate specific antigen (PSA) and immunoglobulin G (IgG). Tests in an artificial urine, with an ionic strength of 0.18 M, have been done and results were found comparable to those obtained in PBS (0.16 M).

These encouraging results show a potential promising future for the development of an electrochemical biosensor for robust and accurate urinary biomarkers detection.

Key words: Engrailed 2, prostate cancer biomarker, immunosensor, electrochemical detection, artificial urine.

1. Introduction

Prostate cancer (PCa) is the second most frequently diagnosed malignancy in men worldwide and is the second major cause of cancer-related death in men in 2020 (Globocan 2020; Ikwu et al. 2021). Although PCa is treatable at the early stages, it becomes castration-resistant at the advanced stages and difficult to treat. Hence, a rapid and sensitive diagnosis is essential before the cancer is spread out to the other body organs. PCa is traditionally diagnosed by digital rectal examination (DRE), prostate-specific antigen (PSA) dosage in blood, and by transrectal ultrasound guided biopsy (Descotes 2019). However, PSA is not always reliable because of its low specificity and low positive predictive value (Neeli et al. 2021). In fact, PSA concentration may sometimes increase with prostate benign diseases (such as benign prostate hyperplasia or prostatitis) or with manipulations (like digital rectal examination and catheterization) (Neeli et al. 2021). This led the French High Authority for Health (Web1) and the Canadian Task Force on Preventive Health Care (Bell et al. 2014; Care 2014, Web2) to not recommend its systematic use for prostate cancer screening.

Because of PSA limitations, intensive efforts are currently directed towards a search for alternative PCa biomarkers. Engrailed-2 (EN2) protein is considered as a useful biomarker, as it is expressed in prostate cancer and not by normal prostatic tissue (Killick et al. 2013; Morgan et al. 2011) (Velonas et al. 2013). EN2 is in fact highly predictive of PCa, with a sensitivity of 66% and a specificity of 88.2%, (Morgan et al. 2011) and is expressed at all stages of prostate cancer (T stages 1 - 4) as well as in metastatic disease. EN2 presents several other advantages, mainly: i) non-invasive sampling (Mokni et al. 2021); ii) stability at room temperature which facilitates sample collection, and iii) non-requirement to a DRE, which improves its acceptability to men and reduces clinical costs.

Interestingly, and to the very best of our knowledge, only three studies reported the design of biosensors for EN2 detection. Lee et al. (Lee et al. 2015) grafted gold nanoparticles on the surface of a gold electrode prior to DNA anchoring and EN2 detection in the range 10 fM to 1 nM. The calculated limit of detection (LOD) was equal to 5.62 fM; and depending on the length of the probes, the dissociation constants K_d values of the DNA probes / EN2 complex vary between 66.33 and 98.84 nM. In Settu et al. (Settu et al. 2017) study, the DNA probes were covalently attached to carboxyl-enriched screen-printed carbon-graphene electrodes prior to EN2 detection. The designed aptamer biosensor detected EN2 protein from 35 to 185 nM. Its LOD was of order of 38.5 nM, and the K_d value of the aptamer / EN2 complex was equal to 66 nM. In both cases, the investigations were only done in a phosphate buffer solution.

In this study, we choose to realize an immunosensor for the selective detection of EN2. One of the critical steps affecting antibody immobilization on the sensor surface, and thus the biosensor performance, is the functionalization method (Blél et al. 2017; Sandhyarani 2019; Vashist and Luong 2018; Zhan et al. 2022). Various methods were reported in literature for this task, including physical and chemical adsorption, entrapment, covalent attachment, micro encapsulation and cross linking. Here, 4-aminobenzoic acid (4-ABA) was electropolymerized on the surface of a gold electrode to form a thin film of poly-ABA (PABA). PABA offers several advantages, mainly i) interesting conductive and self-doping properties (Sriwichai et al. 2010), stability and compatibility with biological molecules (Guerrero et al. 2018, 2019), and iii) possibility of presenting activated carboxyl groups which can interact with biomolecules' functional groups (Ferreira et al. 2015; Mattos et al. 2013; Santos et al. 2019). In this work, anti-EN2 antibodies were covalently anchored due to the formation of an amide bond with the activated carboxyl groups of the PABA film.

Reducing the non-specific adsorption (NSA) of the protein of interest, on the surface of the transducer, is another critical parameter in designing a reliable immunosensor. NSA is in fact responsible for the decrease in sensitivity, specificity, and reproducibility for a given sensor. Different strategies were reported in the literature including the adsorption of blocker proteins, anti-fouling treatments, or production of share forces (Lichtenberg et al. 2019). Engrailed 2 protein has however, a particularity that makes it different from other proteins: its isoelectric point is equal to 9.1 (Gasteiger et al. 2003; Kozlowski 2017). That means EN2 is positively charged at physiological pH (7 to 7.4), whereas almost all other proteins are negatively charged. The idea was therefore to use a positively charged blocker: histamine molecules.

Square wave voltammetry was performed to follow up the response of the immunosensor towards EN2 detection in phosphate buffer saline (PBS) solution, prior to selectivity and competitive tests. To evaluate the accuracy of the designed sensor, EN2 concentration quantifications were then conducted in artificial urine media.

2. Material and Methods

2.1. Reagents

Potassium hexacyanoferrate(III) ($K_3Fe(CN)_6$), potassium hexacyanoferrate(II) trihydrate ($K_4Fe(CN)_6$), potassium chloride (KCl), 4-aminobenzoic acid (4-ABA), histamine, and phosphate buffer saline (PBS) were purchased from Sigma Aldrich (Lyon, France) and used as received. 1-ethyl-3-[3-dimethylaminopropyl] carbodiimidehydrochloride (EDC) and N-

hydroxysuccinimide (NHS) were from ThermoFisher (France). Human serum albumin (HSA), prostate specific antigen (PSA), immunoglobulins G (IgG) were purchased from Bio-Rad (France). Engrailed 2 protein (EN2) and anti-Engrailed 2 antibodies were from Abcam.

2.2. Electrochemical measurements

Cyclic voltammetry (CV) and square wave voltammetry (SWV) measurements were performed with a CHI 650 electrochemical workstation (CH Instrument Inc., IJ Cambria Scientific Ltd., Llwynhendy, UK) using gold, platinum and (Ag/AgCl) as the working ($S = 3.14 \text{ mm}^2$), the auxiliary and the reference electrodes, respectively. All electrochemical measurements were done at room temperature and in triplicate.

2.3. Functionalization strategy

2.3.1. Gold electrodes cleaning steps

Before each electrochemical measurement, gold electrodes were first polished by 1, then by 0.3 and finally by 0.05 microns' alumina powders during 1 min for each step. They were after that copiously rinsed with deionized double distilled (DI) water, prior to sonication in ethanol and DI water for 3 min to remove residual alumina particles. The electrodes were finally dried under ambient air.

2.3.2. Gold electrodes modifications steps

The PABA thin film was achieved by cyclic voltammetry (at a potential range of -0.8 to 0.9 V, and a scan rate of 50 mV/s) from a solution of 1 mM 4-ABA in a 0.1 M KCl solution.

The corresponding cyclic voltammogram (Fig. S1a) and electropolymerization mechanism (Fig. S1b) are reported in the supporting information.

PABA thickness was optimized by CV and square wave voltammetry (SWV) measurements. Results presented in Fig. S2 indicate that 6 is the optimum number of cycles as it permits a wide coverage of the gold surface. A higher number of cycles would have led to a blocking of the surface of the working electrode.

The carboxyl groups of the synthesized PABA film were after that activated by dropping 10 μL of EDC / NHS solution (0.15 M / 0.75 M prepared in a PBS buffer at pH 7.3) on the PABA-coated electrodes, which were maintained at 4°C for 20 minutes. This step was followed by a rinse with PBS and further incubation of 10 μL of anti EN2 antibodies (with a concentration of 10 $\mu\text{g}/\text{mL}$ at pH 7.3) for 45 minutes at 4°C.

In immunosensors developing protocols, reducing nonspecific adsorption of the target on the surface is a primary step to obtain a robust and reliable sensor. In this work, we have chosen to use histamine as a blocking molecule and we have adjusted the pH of the medium to 5. In fact, at this pH, both the protein and the blocker (Table. S1) are positively charged, which would prevent non-specific adsorption of EN2 on the pre-functionalized electrodes due to electrostatic repulsion (Fig. S3). In practice, we immersed the pre-functionalized electrode in a histamine solution (0.1 M) at pH 5 for 1 hour at room temperature. The electrodes were after that rinsed with PBS and conserved at 4°C.

Each functionalization step was characterized by cyclic voltammetry before proceeding to the next to ensure successful implementation of the immunosensor (Fig. 1).

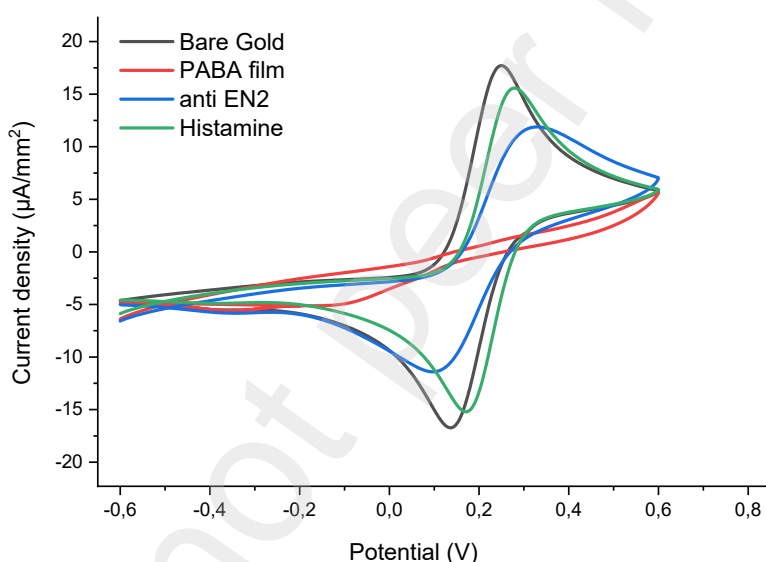


Fig. 1. Electrochemical characterization, by cyclic voltammetry, of the successive functionalization steps starting from the initial bare gold surface, to PABA electropolymerization, antibody grafting and non-specific sites blocking with histamine. Characterizations were made in a PBS buffer 0.1 M containing 5 mM of $[\text{Fe}(\text{CN})_6]^{3-/4-}$

For the bare electrode, the calculated ratio of cathode current peak (I_{cp}) to anode current peak (I_{ap}) was equal to 1 which reveals an electrochemically reversible process (Marken et al. 2010). The current density decrease recorded after Poly-ABA electro polymerization can be attributed to the negative charge of the carboxylic groups (COO^-) at pH 7.4, as it minimizes the charge transfer to the gold surface (Adamczyk et al. 2012; Tekin-Celebi et al. 2012). The covalent

immobilization of anti-EN2 antibodies (Ab) onto the PABA film increased by 63% the current density. This may be attributed to the positive charge of the amino groups (NH_3^+) of the anti-EN2 at pH 7.4. The further blocking step with histamine leads to a current density increase by about 20% due to its positive charge.

3. Results and discussion

3.1. Engrafted 2 immunosensor analytical performances

3.1.1. Sensor's calibration curve

Square wave voltammetry was investigated to follow up the response of the designed immunosensor towards increasing EN2 concentrations ranging from 10^{-5} ng/mL to 1 $\mu\text{g/mL}$. This protocol did not require the use of redox probes, like the common used hexacyanoferrate (II)/(III), as EN2 proteins are electroactive and present a characteristic oxidation peak at 0,1 V (Fig. 2a), which can be probably attributed to the oxidation of the redox-active amino acid residues (cysteine, tryptophan and tyrosine) (Chiku et al. 2008; Enache and Oliveira-Brett 2017; Mazouz et al. 2020).

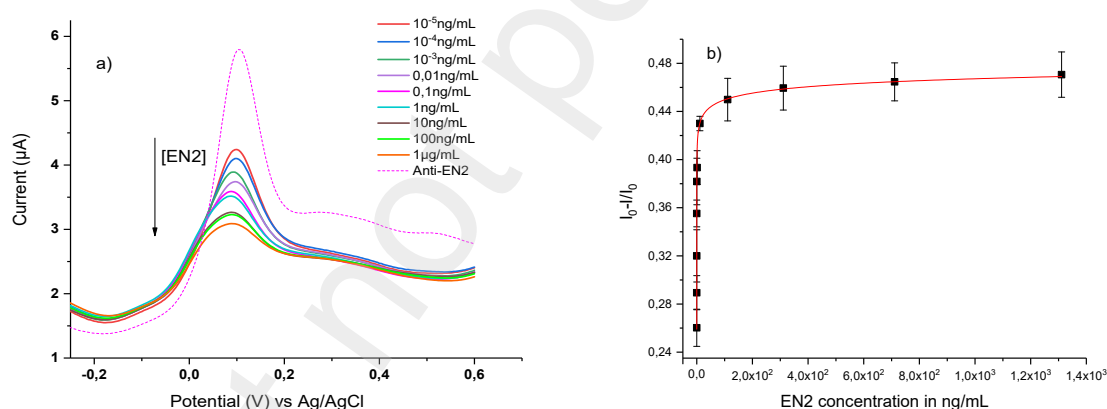


Fig. 2. a) Square wave voltammograms of the EN2 detection; b) Calibration curve of the EN2 immunosensor fitted by Hill's model. Measurements were done in phosphate buffer solution (pH = 7.4)

Voltammograms presented in Fig.2a. show a decrease of the current intensity with increasing concentrations of EN2. This may be attributed to a progressive reduction of the charge transfer. As relevant parameter quantifying the immunosensor response, we considered the relative variation of intensity, $(I_0 - I)/I_0$, where I_0 is the current measured after antibodies grafting and I is the current value obtained after incubation of each EN2 concentration. The corresponding

calibration curve $((I_0-I)/I_0)$ versus cumulative concentrations of EN2) is plotted in Fig. 2b. The sensitivity value, estimated from the slope at the origin, was equal to 20 $\mu\text{L}/\text{ng}$. The limit of detection (LOD) of the designed immunosensor was equal to 10^{-5} ng/mL (0.32 fM), largely lower than those reported in the previous works of Settu et al (38.5 nM) (Settu et al. 2017) and Lee et al (5.62 fM) (Lee et al. 2015).

The dissociation constant K_d is one of the most important parameters in every recognition process. In the case of an immunosensor, it provides information on the affinity between the antibody grafted on the transducers' surface and its antigen. Here, the K_d value was estimated from the fitting of the experimental data with a Hill model (eq. 1):

$$S(C) = \frac{A \times C^\alpha}{K_d^\alpha + C^\alpha} \quad (\text{eq. 1})$$

Where S is the sensor output signal expressed in normalized current variation I_0-I/I_0 , K_d is the dissociation constant, A is an empirical constant, and α is the Hill coefficient.

The Hill coefficient was estimated at 0.010 ± 3.10^{-3} and the dissociation constant was of order of $(3.2 \pm 0.7) \times 10^{-11}$ mg/mL or (0.9 ± 0.2) fM. This very low value is characteristic of strong recognition systems as it is the case for antigen-antibodies. It is also largely inferior to the values reported in the literature and involving EN2 detection with aptasensors (Lee et al. 2015; Settu et al. 2017). These findings clearly show that the affinity of EN2 immunosensors is superior to that of aptasensors.

3.1.2 Selectivity and competitive tests

To evaluate the selectivity of the designed immunosensors, we tested three interferent proteins: HSA, PSA and IgG. For each interferent, we investigated the response of the sensor over a concentration range of 10^{-5} ng/mL to 1 $\mu\text{g}/\text{mL}$. The corresponding detection curves, represented in Fig. 3a, indicate that the electrochemical response of EN2 is twice superior to the other proteins meaning that the designed sensor is selective. Moreover, and besides the fact that the selective response is perfectly differentiable from the others, from a purely quantitative point of view, the response obtained for an EN2 concentration of 0.1 $\mu\text{g}/\text{mL}$ is superior to that obtained for an interfering molecules concentration as high as 1 $\mu\text{g}/\text{mL}$.

Despite their interest, selectivity studies don't provide information on the ability of detecting EN2 within a mixture of proteins. Competitive studies were thus investigated to track the electrochemical response of EN2 in a proteins mixture containing 300 ng/mL of PSA, 50 μ g/mL of HSA and 50 μ g/mL of IgG. These concentrations were not chosen randomly but within the orders of magnitude of those found in the urine of patients with various autoimmune, inflammatory, and cancerous diseases. As shown in Fig. 3b, the response in the protein mix is similar to that recorded in PBS buffer, within uncertainties, confirming thus the ability of the designed sensor to selectively detect EN2 protein in simple and complex media.

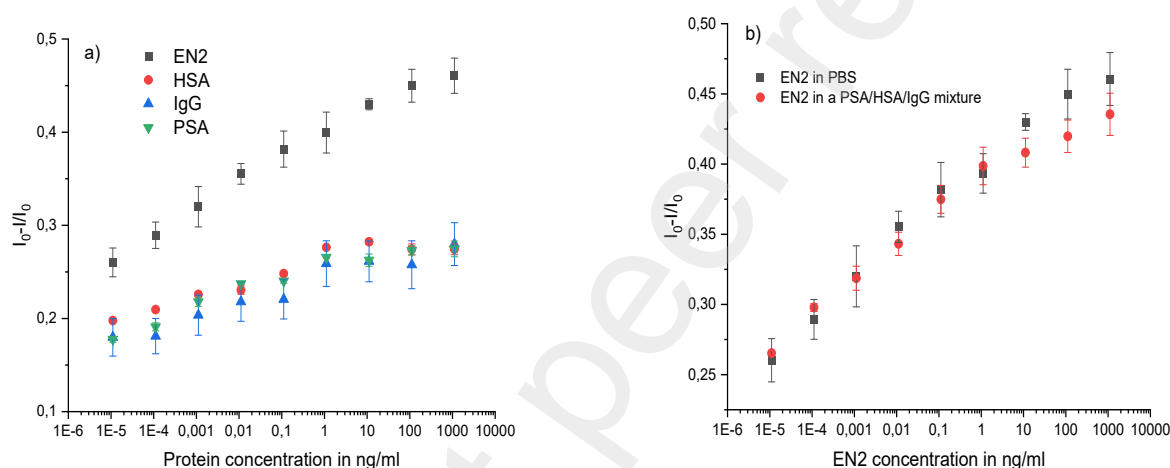


Fig. 3: (a) Selectivity test of EN2 immunosensor (b) Competitive test of EN2 immunosensor

3.3.3. Application in analysis of artificial urine

As urine is a very complex medium, it was essential to test the reliability and accuracy of the calibration curve and the adopted model. The next step was therefore to investigate the matrix effect on sensor's performance. For this purpose, we have prepared an artificial urine medium (AUM) according to Chutipongtanate et al. (Chutipongtanate and Thongboonkerd 2010) formula. The ionic strength was adjusted to 0.18 M close to that of PBS (0.16 M). The detection of increasing concentrations of EN2 protein was performed in AUM and the obtained results are gathered in Fig. 4. This latter clearly shows that the AUM calibration curve is relatively close to that of PBS. Besides in both cases, the experimental data can be fitted by the same Hill model, and what is remarkable is that the dissociation constant K_d is practically the same. Indeed, the relative difference between the K_d values is of about 6%, which is largely negligible compared to the various uncertainties, including that of the modelling.

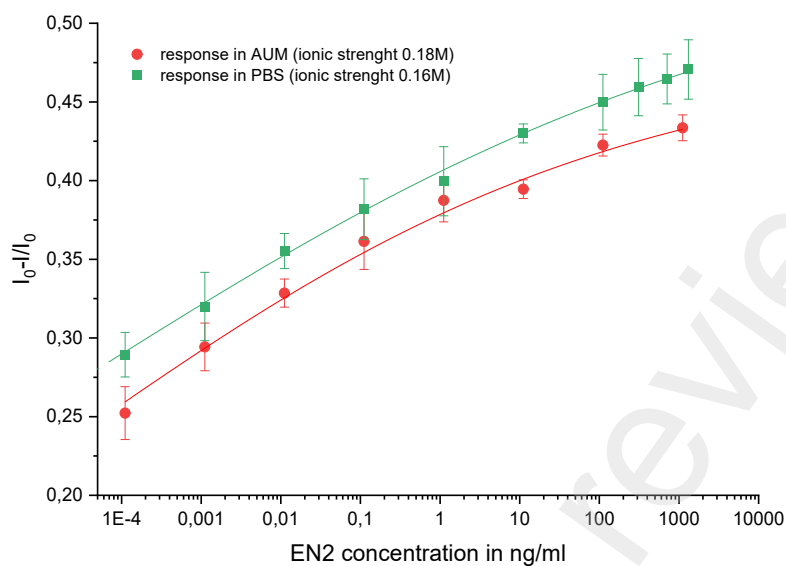


Fig. 4: Calibration curves in PBS (0.16 M) and AUM (0.18M)

These results are very encouraging because they highlight the fact that the immunosensor response is not altered in a complex medium, which is not always the case for other sensors and analytical devices including ELISA. Indeed, Chatziharalambous et al. (Chatziharalambous et al. 2016) showed that among commercially available ELISA tests for urinary cancer markers, only 3 out of 11 checked assays passed the accuracy thresholds and were considered suitable for further applications in markers quantifications. These findings reflect the difficulties on developing robust ELISA assays for biomarkers quantification in urine

The designed immunosensor can be an alternative to ELISA assays and paves the way for an accurate determination of biomarkers concentrations in urine as the detection is not influenced by the complexity of the media.

4. Conclusions

An electrochemical immunosensor was designed for the selective detection of Engrailed 2 protein in simple (PBS) and complex (artificial urine medium). Electrochemical results indicate that the immunosensor exhibits improved analytical performances, compared to previously reported aptasensors, namely very low values of detection limit and equilibrium dissociation constant. The selectivity and competitive tests were investigated with three interfering urinary proteins that can be found in abundant amounts in severe diseases: PSA, HSA and IgG. Results

indicate that the designed immunosensor is able to distinguish the response of EN2 from those of other proteins. Tests in artificial urine show that the dissociation constants are quite comparable to that obtained in PBS, which would allow the use of this immunosensor for the reliable detection of EN2 concentrations in patients' urines. Disposing of a simple, reliable, and robust analytical tool paves the way for clinical use of EN2 as a biomarker for prostate cancer.

Conflicts of interest

The authors report no conflicts of interest in this work.

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Author contribution

Investigation: MM, AT, and SK. Visualization: GA. Writing - Original Draft: MM. AT and GA. Writing - Review & Editing: CZ, AO, AO, and NF. Supervision: NF and CZ. Funding acquisition: AB, AO, AO and NF.

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