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Electrochemical aptasensor for human osteopontin detection using a DNA aptamer selected by SELEX

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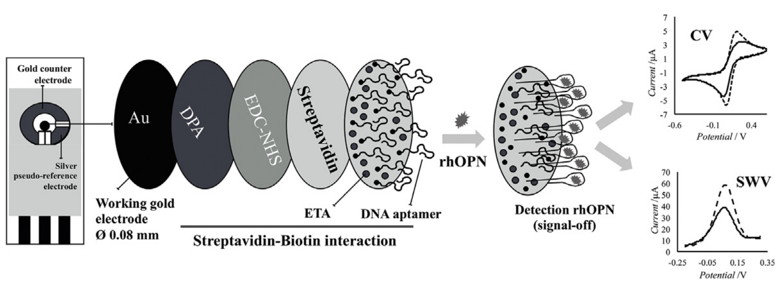
HIGHLIGHTS

- A DNA aptamer against OPN, a tumor biomarker, selected by SELEX iterative *in vitro* process is reported for the first time.
- An electrochemical (square wave voltammetry) signal-off DNA aptasensor was designed exhibiting affinity towards OPN.
- The DNA aptasensor had satisfactory sensitivity and selectivity towards OPN, with low signal interferences from other proteins.
- The DNA aptasensor had a LOD of 1.3 ± 0.1 nM (synthetic human plasma) within OPN levels found breast cancer patients.
- Preliminary results showed that DNA aptasensor can detect OPN in real human plasma, similarly to the standard ELISA method.

GRAPHICAL ABSTRACT



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ABSTRACT

A DNA aptamer with affinity and specificity for human osteopontin (OPN), a potential breast cancer biomarker, was selected using the SELEX process, considering its homology rate and the stability of its secondary structures. This aptamer exhibited a satisfactory affinity towards OPN, showing dissociation constants lower than 2.5 nM. It was further used to develop a simple, label-free electrochemical aptasensor against OPN. The aptasensor showed good sensitivity towards OPN in standard solutions, being the square wave voltammetry (SWV), compared to the cyclic voltammetry, the most sensitive technique with detection and quantification limits of 1.4 ± 0.4 nM and 4.2 ± 1.1 nM, respectively. It showed good reproducibility and acceptable selectivity, exhibiting low signal interferences from other proteins, as thrombin, with 2.6–10 times lower current signals-off than for OPN. The aptasensor also successfully detected OPN in spiked synthetic human plasma. Using SWV, detection and quantification limits (1.3 ± 0.1 and 3.9 ± 0.4 nM) within the OPN plasma levels reported for patients with breast cancer (0.4–4.5 nM) or with metastatic or recurrent breast cancer (0.9–8.4 nM) were found. Moreover, preliminary assays, using a sample of human plasma, showed that the aptasensor and the standard ELISA method

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quantified similar OPN levels (2.2 ± 0.7 and 1.7 ± 0.1 nM, respectively). Thus, our aptasensor coupled with SWV represents a promising alternative for the detection of relevant breast cancer biomarkers.

1. Introduction

Osteopontin (OPN) is a matricellular protein, expressed by a variety of cell types and found in several biological fluids and tumor tissues [1,2]. The OPN contains around 314 amino acid residues subject to multiple post-translational modifications, thus resulting in a molecular weight range between 41 and 75 kDa [1,3,4]. The OPN is rich in aspartate, glutamate and serine residues. Besides, it contains several functional domains such as arginine-glycine-aspartate (RGD), serine-valine-valine-tyrosine-glutamate-leucine-arginine (SVVYGLR) and the thrombin cleavage site (RSK) [4–7]. High levels of OPN in serum or plasma have been detected in people suffering from cancer and may be associated with tumor progression, aggressiveness and metastasis [8–14]. Indeed, human OPN has been pointed as a potential biomarker in a number of cancers including breast, ovarian, prostate, lung, liver and colon [3,11,13–19]. Moreover, OPN has been considered as a possible therapeutic target for blocking tumor growth and subsequent metastasis [20–22]. Therefore, the detection of OPN can be used to monitor the disease progression and provide useful information about its prognosis [23]. Commonly, OPN detection in plasma samples is performed through enzyme-linked immunosorbent assay (ELISA). Bramwell et al. [11] quantified human OPN in plasma samples of patients with breast cancer using ELISA. The average OPN concentration determined was 46 ng mL^{-1} (~0.7 nM)[1] ranging from 22.6 ng mL^{-1} (~0.4 nM)[1] to 290 ng mL^{-1} (~4.5 nM)[1]. For patients with metastatic breast cancer or with recurrent breast cancer, higher mean OPN levels were found 60.7 ng mL^{-1} (~0.9 nM)[1], ranging from 23.3 ng mL^{-1} (~0.4 nM)[1] to 543 ng mL^{-1} (~8.4 nM)[1] Despite the usefulness of ELISA, alternative methods ought to be developed for the detection of OPN at the lower concentrations that are usually found in early stages of the disease. Advances in the biosensors field, namely the possibility of designing new and extremely specific bioreceptors (e.g. aptamers), make them very promising for the detection of low levels of protein biomarkers, such as OPN, in plasma or blood samples [24–29].

Aptamers are short single-strand DNA (ssDNA) or RNA oligonucleotides (20–100 nucleotides) that can fold into unique tertiary structures [30,31]. It is possible to isolate aptamers, from an oligonucleotide library, with high affinity and specificity to a wide variety of targets, ranging from small molecules to large proteins, using the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) methodology [32,33]. Usually 5 to 15 cycles of selection are necessary to isolate one or few aptamers (RNA or DNA) that possess the highest affinity and specificity to the desired target [34]. Overall the use of aptamers as bioreceptors in biosensors is gaining an increased interest, especially as alternatives to antibodies, mainly due to their unique features. Aptamers are easy to produce and synthesize; present a good stability over a wide range of pH, temperature and/or storage conditions; are resistant to denaturation and degradation; are amenable to chemical modifications, thus enabling their immobilization in several surfaces; and can be labelled with fluorophores, and other tags facilitating their applications as bioreceptors in biosensors [25,35–37].

Mi et al. [20] described the first RNA aptamer against OPN that was used by Cao et al. [38] to develop an electrochemical aptasensor using a pyrolytic graphite disk electrode (3.0 mm diameter), functionalized with AuNPs. The human OPN detection limit achieved by SWV was 10.7 ng mL^{-1} (~0.2 nM).[2] The aptasensor high sensitivity could be attributed to the use of nanoparticles that increase the superficial area available for aptamers immobilization [39]. However, these strategies increase the biosensor cost and can affect its reproducibility and performance when complex samples are used [40]. Recently, a voltammetric aptasensor, based on the same RNA aptamer, was used to detect human OPN with satisfactory performance, reproducibility and stability but with a greater detection limit (3.7 ± 0.6 nM) [41]. Also, the RNA aptasensor was sensitive to thrombin, a protein that is also present in human blood and serum, which could be a drawback when real samples are used. Hence, herein it is reported for the first time the isolation and characterization of a high affinity DNA aptamer for human OPN, through SELEX. The binding affinity of the selected DNA aptamer was determined by fluorescence assays. Moreover, this aptamer was used as bioreceptor for the development of a label-free electrochemical DNA aptasensor to detect human OPN. The aptasensor was tested by cyclic voltammetry (CV) and SWV and its performance was evaluated using standard solutions prepared in PBS buffer (pH 7.4), synthetic and real human plasma samples.

2. Experimental section

2.1. Material and reagents

Nitrocellulose membranes (0.45 μm) were purchased from Whatman. dNTP mix (containing dATP, dCTP, dGTP, and dTTP (4 mM each)) and Taq DNA polymerase (5 U μL^{-1}) were obtained from New England Biolabs. TOPO TA cloning Kit was acquired from Invitrogen. GRS Plasmid Purification Kit was purchased from Grisp Research Solutions. Kieselgur, sodium acetate, urea, 3,3-dithiodipropionic acid (DPA), N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), ethanolamine (ETA), sulfuric acid (purity = 99.999%) and synthetic human plasma (P9523) were obtained from Sigma-Aldrich. Potassium hexacyanoferrate (III) [$K_3Fe(CN)_6$] and potassium hexacyanoferrate (II) [$K_4Fe(CN)_6$] were obtained from Acros Organics and potassium dihydrogen phosphate (KH_2PO_4) from Merck. Sodium chloride (NaCl), potassium chloride (KCl), sodium hydrogen phosphate (Na_2HPO_4) and Ethanol were acquired from Panreac. Recombinant human osteopontin (rhOPN, 65 kDa, isoelectric point (p*I*) = 3.5), recombinant bovine osteopontin (rbOPN, 60 kDa, p*I* = 3.59–4.46) and Quantikine ELISA kit as well as the Quantikine Immunoassay Control Set 565 for Human Osteopontin were purchased from R&D Systems. Thrombin from human plasma (THR, 37.4 kDa, p*I* 7–7.6), bovine serum albumin (BSA, 66 kDa, p*I* = 4.7), lysozyme from chicken egg white (LYS, 14.3 kDa, p*I* = 10–11) and streptavidin were obtained from Sigma-Aldrich. A real human

[1] Reported OPN levels in ng mL^{-1} were converted to OPN levels in nM, assuming an OPN molecular weight of 65 kDa.

[2] Reported OPN levels in ng mL^{-1} were converted to OPN levels in nM, assuming an OPN molecular weight of 65 kDa.