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Proof-of-Concept Electrochemiluminescent Assay for the Aptamer-Based Detection of Small Molecules †

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Abstract: This work presents a proof-of-concept assay for the quantification of testosterone based on aptamer recognition and an electrochemiluminescence (ECL) readout. The TESS.1 aptamer was used to demonstrate the novel methodology. The analytical capability of the ECL array was studied using different incubation protocols. To improve the analytical performance, the optimal concentration of the TESS.1 aptamer was investigated. Subsequently, the selectivity of the array was assessed by performing negative control experiments with a randomized ssDNA sequence and two other steroids, *i.e.* deoxycholic acid and hydrocortisone. Complementary analytical techniques were employed to confirm the suggested mechanism, determine the stoichiometry of binding, characterize the aptamer-target interactions; and to elucidate the dissociation constant.

Keywords: electrochemiluminescence; aptamer; small molecules; testosterone; dissociation constant

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1. Introduction

Aptamers are synthetic single stranded (ss) DNA or RNA oligonucleotides which were first described in 1990 after the development of the Systematic Evolution of Ligands by Exponential enrichment (SELEX) method and proposed to be a promising alternative for commercially available antibodies [1,2]. The greatest advantages are: *i)* high stability (temperature and pH); *ii)* synthetically produced, eliminating the use of cell lines or animals [3]; *iii)* affordability; and *iv)* easy to modify [1]. Compared to antibodies, aptamers can recover their native conformation after re-annealing [4,5]. The three dimensional structure of aptamers is necessary for, and may be induced by, the binding with the target [6]. In fact, aptamers often experience significant conformational changes upon target binding which offers great flexibility in the design of novel biosensors [4]. Despite all, only a few aptamers-based sensors are commercialized for diagnostics and therapeutics [1].

Aptamers have been reported for the recognition of numerous targets from proteins [7] to small molecules [8]. Small molecules are characterized by a low molecular weight (<1000 daltons) playing important roles in regulatory biological pathways (*e.g.* vitamins, hormones, messenger molecules and cofactors), which represent a large class of biomarkers of interest [6]. Nevertheless, their detection is challenging due to their small size and low concentrations (frequently in the nanomolar range). Today, they are commonly detected via chromatographic techniques, such as HPLC and GC [1,6]. However, these

methods often require a large amount of (more expensive) organic solvents, a regular maintenance, trained personnel and long-time of analysis. Therefore, the use of aptamer-based biosensors, applicable on-site, is a promising alternative for the detection and monitoring of small molecules [1].

In the last decades, electrochemical methods using aptamers as biorecognition elements for the detection of different small molecules have been developed [1,9]. Nevertheless, electrochemiluminescence (ECL), a type of chemiluminescence reaction triggered by electrochemical methods, is gaining momentum [4,10,11] due to: *i)* absence of a background optical signal; *ii)* precise control of reaction kinetics offered by controlling the applied potential; *iii)* compatibility with solution-phase and thin-film configurations; *iv)* separation of excitation source (electronics) and detection readout (optical) improving the sensitivity, and *v)* possibility of integrating nanomaterials for enhancing the signal. Together, these characteristics make ECL attractive as a highly sensitive and selective analytical methodology [12].

Hence, ECL aptasensors are promising because they combine the advantages of both electrochemical and chemiluminescence biosensors, *i.e.* high sensitivity, low background, cost-effectiveness, and ease of control [3,4]. The technology of this type of assay, employing a 96-well plate with electrochemical capabilities and ECL reader, enables the multiple detection of biomarkers in different formats. To the best of our knowledge, the combination of such devices with aptamers for the ECL detection of small molecules has not been reported yet. Therefore, herein, we present a proof-of-concept ECL assay for the high-affinity detection of testosterone using the testosterone-binding aptamer (TESS.1). This unique approach opens new perspectives and insights in the use of aptamers coupled with ECL sensing for the accurate and highly sensitive detection of a wide range of small molecules simultaneously.

2. Materials and Methods

2.1. Reagents

The initial testosterone (≥99.0% Sigma Aldrich), deoxycholic acid (98.5% Acros Organics) and hydrocortisone (>98% TCI) stocks were prepared in absolute ethanol (≥99.8%) from Fisher Scientific in a concentration of 50, 50 and 30 mM, respectively and the following dilutions of the target were performed using hybridization buffer (10 mM phosphate buffer, 150 mM sodium chloride and 100 mM magnesium chloride, pH adjusted to 7.0).

2.2. DNA Sequences

All DNA sequences were purchased from Eurogentec (Belgium). The randomized DNA sequence was derived from TESS.1 and designed using the OligoAnalyzer Tool of Integrated DNA Technologies. The part complementary to the capture probe was preserved while the other nucleotides of the TESS.1 aptamer were scrambled (**Table 1**). DNA sequences without modifications were used for the ITC and native nESI-MS experiments, while the oligonucleotides carried some modification for the ECL experiments.

Table 1. DNA sequences utilized in this work.

	Sequence of oligonucleotides	**Modifications**
Capture probe	5′- GTC TGC CCG AGA G -3′	Biotin on 3′
Aptamer TESS.1	5′- CTC TCG GGA CGA CGG GAT GTC CGG GGT ACG GTG GTT GCA GTT CGT CGT CCC -3′	DIG on 3′
Randomized TESS.1	5′- CTC TCG GGA CGA CTG ACG GGC ACT CAG TTG TGT TGG GGT CTC GCC CGG TGG -3′	DIG on 3′
TESS.1short	5′- GGG ATG TCC GGG GTA CGG TGG TTG CAG TTC -3′	-

* The bases underlined in the randomized TESS.1 are those whose position is different when compared to TESS.1.

2.3. Electrochemiluminescence Measurements

The difference between the protocols tested lies in the order of incubations of the three components (i.e. capture probe, aptamer and target). Briefly, the first protocol (P1) consists of the following steps: *i)* incubation of the capture probe in the well while the aptamer is incubated separately with the testosterone in an Eppendorf; *ii)* addition and incubation of the mixture in the well. The second protocol (P2) includes: *i)* hybridization of the capture probe with the aptamer in an Eppendorf; *ii)* incubation of this mixture in the well; *iii)* incubation with testosterone. The third protocol (P3) follows: *i)* the capture probe, aptamer and testosterone are incubated in an Eppendorf; *ii)* the complex mixture is added and incubated in the well. Finally, all protocols were followed by an incubation with the antibody anti-digoxigenin (DIG) before the reading of the ECL signal.

3. Results and Discussion

Yang and co-workers developed a fluorescence-based aptasensor, without electrochemical initiation and in solution-phase, for testosterone, which comprises two DNA strands: *i)* a TESS.1 labelled aptamer with fluorescein together with *ii)* a partially complementary oligonucleotide sequence carrying a quencher.[5] Upon addition of the target, a high-affinity binding of TESS.1 to testosterone occurs, which leads to dissociation of the double helix between TESS.1 and its complementary strand and results in a fluorescent signal [5]. Our ECL assay is inspired by the same principle of competitive binding, but with different modifications on the TESS.1 aptamer, *i.e.* our aptamer does not have a quencher neither fluorescein attached. In this case, the TESS.1 carries the DIG label, to which the $Ru(bpy)_3^{2+}$-labelled anti-DIG will bind. Hence, the aptamer, which is not immobilized, will be washed away in presence of the target. Therefore, the signal of the label is only observed when the aptamer is not bound to testosterone, and decreases upon adding more target (**Figure 1**). Besides, our TESS.1 complementary ssDNA, named capture probe in this manuscript, is not in solution but is instead immobilized on the surface of the working electrode (in the 96-well plate to allow assay based detections). Following the same principle as Yang *et al.*, the capture probe only hybridizes with the aptamer when it is not bound to the target (**Figure 1i**). In this work, the ECL detection technology uses an anti-DIG antibody as co-reactant [13] which has high affinity and specificity for the DIG label. Concurrently, the antibody carries multiple $Ru(bpy)_3^{2+}$ labels which emit light upon electrochemical stimulation whereas signal amplification is provided by tripropylamine (TPrA) present in the reading buffer. Considering that the aptamer does not bind to the capture probe upon binding of testosterone, the ECL signal decreases upon increasing concentration of the target (**Figure 1ii and 1iii**).

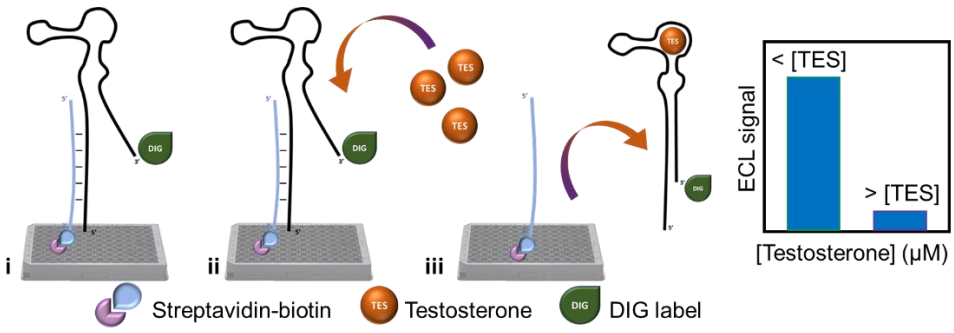

Figure 1. Schematic representation showing: **i.** the hybridization between the capture probe (blue) with the aptamer (black); **ii.** the subsequent addition of testosterone, **iii.** how the aptamer leaves the capture probe after binding with the target; and the ECL signal produced depending on the lower and higher concentrations of testosterone.

3.1. Characterization of the TESS.1 Aptamer

As it was previously mentioned, the proposed principle assumes that the aptamer cannot form a duplex with the capture probe upon binding to testosterone. This hypothesis was confirmed using native nano-electrospray ionization mass spectrometry (nESI-MS) in which an aptamer-capture complex was disrupted upon addition of testosterone and an aptamer-testosterone complex was formed (**Figure 2**). In absence of testosterone, a clear signal for the aptamer-capture complex is observed (**Figure 2 – bottom**). Simultaneously, there is some free aptamer and free capture detected. Upon addition of testosterone, the aptamer-capture interaction was disrupted and an aptamer-testosterone complex was formed (**Figure 2 – top**).

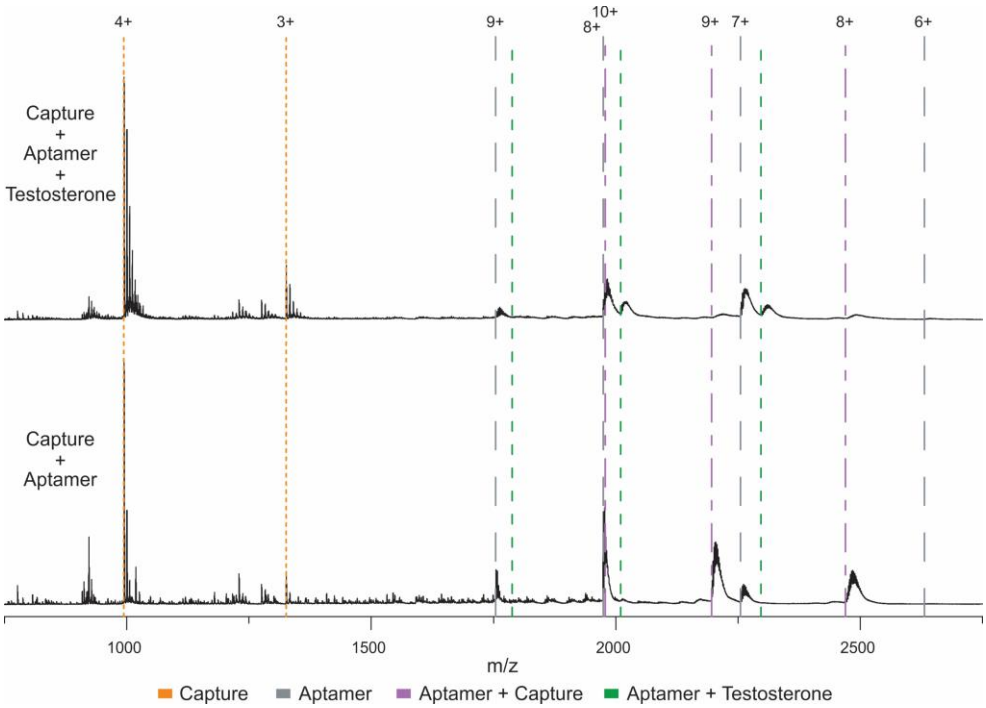

Figure 2. Native nESI-MS spectrum of the aptamer and capture in a 1:1 ratio (**bottom**) and the same mixture with testosterone added in a 1:10 ratio (**top**). The dotted orange lines, dashed grey lines, dotted green lines and dashed-dotted purple lines represent the theoretical m/z-values of the capture probe, aptamer, aptamer-testosterone complex and aptamer-capture probe complex, respectively.

In order to validate the binding between the TESS.1 aptamer and testosterone, ITC and native MS experiments were carried out (plots not shown). The thermogram for the ITC titration of TESS.1 with testosterone showed a clear exothermic binding process. From these results it is possible to calculate a K_d of 240 ± 29 nM, which is slightly higher than the previously reported value of ~80 nM by Yang *et al.* [5]. To investigate whether the stems of TESS.1 facilitate the binding of testosterone, native MS experiments were performed with the full length TESS.1 aptamer and a shorter variant, called TESS.1short, which is only eight bp shorter. These results suggest that even though the TESS.1short aptamer still contains the binding region, the stems of TESS.1 are a crucial structural part for the folding of the aptamer into its binding-competent state. Hence, the presence of the stems clearly enhances the affinity of the aptamer towards testosterone. Since stem 1 (closer to 5′ end) and stem 2 (closer to 3′) of TESS.1 hybridize upon binding of the target, stem 1 is no longer available to interact with the capture probe, as shown in **Figure 2.**

3.2. Optimization of the Incubation Protocol for ECL Detection

Different incubation protocols were tested in order to obtain the optimal analytical performance of the ECL assay and to study the interactions among the capture probe, the

aptamer TESS.1 and the target (testosterone). An equimolar interaction between aptamer and capture probe was expected but higher and lower concentrations were tested in order to verify and optimize the entire protocol. Considering the obtained results (data not shown), the concentration of 200 nM for the TESS.1 aptamer (and capture probe) and the protocol 1 were chosen as the optimal conditions.

3.3. ECL detection of testosterone

TESS.1 aptamer. Experiments with multiple concentrations of testosterone were performed to gain better insight in the binding of testosterone to its aptamer. **Figure 3** (bottom curve, black squares) shows data of the TESS.1 aptamer in the presence of testosterone in a concentration range from 0 to 25 μM, showing a linear range from 0.39 to 1.56 μM and a limit of detection of 0.29 μM. After the incubation of the aptamer with the different concentrations of testosterone, only the free aptamer will hybridize to the capture probe. The ECL signal already decreases with 14% upon addition of the lowest concentration of testosterone used (0.39 μM). This decrease is due to the lower availability of the free TESS.1 aptamer to hybridize with the capture probe in presence of the target. The ECL response decreases until 3.13 μM to 79% of the initial value indicating an increasing amount of testosterone is binding to its aptamer in this range. From 3.13 μM onwards the signal is almost constant which suggests saturation of the aptamer. From the ECL experiments, the K_d was estimated ca. 0.97 μM, which is slightly higher than the K_d determined by ITC, although it remains in the same range. It is important to considerer that the presence of the capture probe (not present during ITC measurements) can likely influence within the increment of the value.

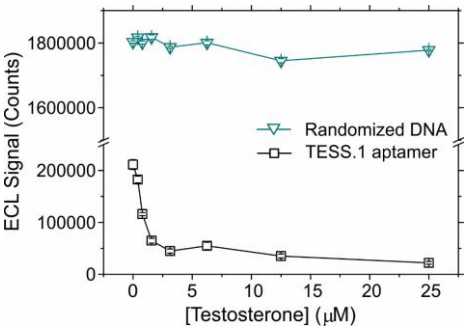

Figure 3. ECL data of testosterone (from 0 to 25 μM with a total of 8 concentrations, two fold dilutions) in combination with the TESS.1 aptamer and randomized ssDNA sequence in the optimal concentration of 200 nM and following the protocol P1.

Specificity of the ECL approach: randomized ssDNA. To investigate whether the decrease in signal can be ascribed to specific binding, the same experiments were performed using a randomized ssDNA sequence instead of TESS.1 as shown in **Figure 3** (upper calibration). Native MS experiments with the randomized ssDNA were carried out to confirm and demonstrate that no binding occurs between this sequence and the target (data not shown). In this case, the ECL signal at each concentration of testosterone is in the same range with a maximum deviation of 3% compared to the blank. Important to notice is that the signal of the randomized sequence is much more intense than the one of the TESS.1 aptamer (**Figure 3**). This is most likely due to two factors: *i)* the randomized ssDNA is attached to the capture probe through the preserved region of the sequence and, *ii)* the scrambled part was designed to avoid the complementarity showed by TESS.1 between the stem 1 and the stem 2. As a result, this unfolded structure of the randomized sequence promotes a further position of the DIG label and therefore also the anti-DIG antibody with the $Ru(bpy)_3^{2+}$ labels with respect the surface of the electrode. In consequence, the labels were closer to the light source during the generation and reading of the ECL signal, offering the highest values (~1.8×10^6 counts, **Figure 3** upper calibration). The increase of the

ECL response due to the higher distance between the ruthenium complex and the surface of the electrode has already been explained and demonstrated in the literature [14].

3.4. Selectivity of the TESS.1 Aptamer and the Developed ECL Approach

Deoxycholic acid (DCA) and hydrocortisone (HC) steroids were selected, after preliminary native MS experiments, as negative controls to replace testosterone in order to confirm the selectivity of the aptamer. Hence, the native MS results showed no interactions between DCA and the TESS.1 aptamer, and negligible binding between HC and the aptamer (data not shown).

4. Conclusions

A novel ECL assay based on aptamer recognition towards testosterone as model for small molecules was introduced. In parallel, a characterization of the aptamer-target interaction was validated using a multifaceted approach with analytical techniques such as ITC and native nESI-MS. The best analytical performance of the ECL assay was obtained following a previous incubation of the TESS.1 aptamer (concentration of 200 nM) together with the target before adding them to the 96-well plate where the capture probe was immobilized. Although aptamer-based ECL sensors emerged only over one decade ago, they have already found broad applications in both fundamental research and biomedical diagnostics applications. The present work aims to open a new avenue for the development of reliable and robust ECL biosensor assays for biochemical analysis, promoting a deeper understanding of the potential of biosensors based on aptamers for the accurate detection of numerous biomolecules relevant in biomedical applications.

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