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The characterization and validation of 17β-estradiol binding aptamers

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ABSTRACT

The rapid and sensitive detection of small molecules is garnering increasing importance, and aptamers show great promise in replacing expensive, elaborate detection platforms exploiting chromatographic separation or antibody-based assays. The characterization of aptamer interaction with small molecule targets is not facile, and there is a mature need for a rapid, high-throughput technique for the analysis of aptamer-small molecule kinetics and affinity. In this work we present methodologies for the evaluation of aptamer-small molecule interactions, using the aptamers reported against the steroid 17β-estradiol as a model system. Microscale thermophoresis, apta-PCR affinity assay and surface plasmon resonance were explored to evaluate the reported aptamers' binding properties in terms of affinity and specificity, and were demonstrated to be successfully applied to the analysis of aptamer-small molecule interactions.

1. Introduction

There is an ever-increasing demand for the rapid and sensitive detection of small molecules including toxins, carcinogens, drugs, nutrients, pigments, antibiotics and heavy metals [1]. Endocrine-disrupting compounds (EDCs) are a class of emerging environmental contaminants that have a negative impact on growth, metabolism and reproduction of organisms [2–5], and include 17β-estradiol, a hormone important for the regulation of menstrual female reproductive cycles and for the development and maintenance of female reproductive tissues. High amounts of 17β-estradiol can cause adverse effects on the reproduction [6] and may also result in breast cancer [7]. 17β-Estradiol can pass as waste to sewage water with consequential negative effects on the environment [8]. There is thus, a mature need to establish simple, rapid and sensitive methods for the detection of 17β-estradiol in environmental and clinical samples.

Chromatographic-spectroscopic methods have been routinely employed for the detection of small molecules such as 17β-estradiol, including high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC/MS), achieving picomolar detection limits [9,10]. However, these techniques are time-consuming, requiring elaborate sample pre-treatment, expensive equipment and trained personnel. Alternative methods include ELISA (enzyme-linked immunosorbent assay), using antibodies as biorecognition elements and thus having an inherent disadvantage of requiring animals for their production, as well as suffering from cross-reactivity with closely related structures [11–13].

Aptamers, nucleic acid ligands, have emerged as versatile molecular recognition agents due to their ability to bind molecular targets with high specificity and affinity, offering a very attractive alternative to antibodies. They are selected through an *in vitro* selection process called systematic evolution of ligands by exponential enrichment (SELEX) [14–16]. The functional diversity of aptamers enables their use in a wide range of applications including analytical detection [17,18], diagnostics [19], therapeutics [20], drug delivery vehicles [21], intracellular imaging [22,23], scaffolding [24], gene regulation [25] and cell-based engineering [26]. Compared to antibodies, aptamers offer several advantages including long-term stability and low cost production. Aptamers

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can be easily modified with diverse types of reporter molecules or dyes with minimal consequence to their binding properties, thus enabling their integration into different biosensing and therapeutic platforms [27,28]. Furthermore, aptamers can be selected against toxic target or under non-physiological conditions as well as against molecules that do not elicit an immune response. The specific conditions of each selection can be adjusted to the final aptamer application changing the properties of the aptamers on demand.

Aptamer-based platforms offer a promising alternative to conventional methods. However, current methods including equilibrium filtration [29,30], in-line probing [31] and isocratic elution [32] as well as methods determining binding kinetics such as single-molecule fluorescence imaging [33] and electrophoretic mobility shift assay [34], are often not compatible with many small molecule targets [35]. Analytical methods including surface plasmon resonance (SPR), isothermal calorimetry (ITC) and capillary electrophoresis (CE) could also be employed, but the low sensitivity due to the low molecular mass of the target molecules remains a key limitation. A new method called backscattering interferometry (BSI) has been reported, involving the use of a microfluidic chip, a helium-neon laser, and a charge coupled device (CCD) array [36]. Binding of the aptamer to the target leads to a refractive index change allowing the accurate determination of dissociation constant (K_D) values. Recently, new approaches for detection of small molecule-aptamer interactions have been reported. The DNase I digestion assay is a label-free method typically used for locating specific binding sites of proteins on DNA [37], which has been applied to small molecule-binding aptamers, but due to the small changes in band intensity, a large error is associated with the calculation of K_D [38]. Additionally, a SYBR Green I assay and a gold nanoparticle protection assay have also been reported. They are displacement-based methods performed in solution and have been reported to be easy and rapid for the determination of aptamer affinity constants [39]. Aptamer binding affinities and specificities can vary when target or aptamer is used in solution or if one of the molecules is immobilized on a specific matrix. Moreover, the sensitivity of each technique can affect the calculation of affinity constants and can lead to discrepancies between different methods. Due to different limitations of each assay, more than one method should be used to effectively determine aptamer binding constants [39].

To date, four different research groups have reported aptamers against 17β-estradiol. The first aptamer was described by Kim et al. in 2007 [40] and its K_D was determined to be 130 nM. This aptamer has been used in a number of different sensing platforms for fluorescent [41], colorimetric [42], optical [43] or electrochemical detection [2,40,44,45]. All these platforms were successfully employed for the detection of femtomolar concentrations of 17β-estradiol, also exhibiting good binding affinities in natural waters. The second aptamer reported to bind 17β-estradiol was selected by Alsager et al. in 2014 with a reported K_D of 50 nM [46]. It was used to design aptamer-functionalized nanoparticles with transduction of aptamer receptors binding to small molecules by dynamic light scattering (DLS) and resistive pulse senses (TRPS) as well as in a label-free electrochemical aptasensor, also achieving femtomolar detection limits [47]. In 2014, Vanschoenbeek et al. [48] reported the selection of aptamers targeting different functional groups of 17β-estradiol, and in 2015 Akki et al. [49] reported aptamers that were able to selectively distinguish between the structurally similar estrogenic compounds 17β-estradiol and 17α-ethynylestradiol in natural waters.

There is an increasing number of publications detailing the use of reported aptamers but the characterization and validation of reported aptamers is not always verified thus limiting their widespread use in research and applications [39]. Many researchers are calling for more strict validation of published aptamers by characterizing them using multiple techniques and demonstrating their robustness in specific applications as in the case of antibodies [50]. The characterization and validation of aptamers selected against small molecules is even more complicated due to the limited number of adequate techniques that can be used.

The aim of this work is to compare all previously published 17β-estradiol aptamers in terms of affinity and specificity using three different techniques: MicroScale Thermophoresis (MST), Surface Plasmon Resonance (SPR) and Apta-PCR affinity assay (APAA). MST is based on the movement of molecules through temperature gradients and this movement depends on the size, charge, and hydration shell of the molecule. Upon the interaction between the molecule and the aptamer, at least one of these parameters is changed and alters its movement. This technique offers free choice of buffers and is especially suited to small molecule-aptamer binding interactions [51]. SPR is a real time and label-free optical detection platform, frequently applied for the determination of dissociation constants for larger molecules but can be challenging to adapt to small molecules. Finally, APPA is a simple method based on the PCR amplification of aptamer that is bound to the target molecule which in turn is immobilized on the surface of the matrix (positive control) compared to the matrix itself (negative control) and offers rapid information regarding the affinity and specificity of studied aptamers. With these three independent techniques we provide a screening to verify the robustness of the reported 17β-estradiol aptamers.

2. Materials and methods

2.1. Materials

Phosphate-buffered saline (PBS; 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4), phosphate-buffered saline with Tween-20 (PBST), Tris-buffered saline (TBS; 50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 8), 1-ethyl-3-(-3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), 17β-estradiol, 17β-estradiol-6-one 6-(*O*-carboxymethyl)oxime (estradiol-CMO), 17β-estradiol 6-(*O*-carboxymethyl)oxime:BSA (estradiol-BSA), progesterone, progesterone 3-(*O*-carboxymethyl)oxime (progesterone-CMO), testosterone, testosterone 3-(*O*-carboxymethyl)oxime (testosterone-CMO), testosterone 3-(*O*-carboxymethyl)oxime:BSA (testosterone-BSA) and androstenedione were purchased from Sigma Aldrich (Spain). Specific monoclonal antibodies against 17β-estradiol (E3550-03A), progesterone (P9006-04B) and testosterone (T2950-24) were from US Biological (provided by VWR International Eurolab S.L., Spain), Dynabeads M-270 amine magnetic beads from Life Technologies (Spain) and all other reagents were obtained from Scharlau Chemie S.A. (Spain) and Bio-Rad (Spain). 17β-Estradiol aptamers, unmodified and labelled with 5′-Cy5-C6, reported by Kim et al. [40], Alsager et al. [46], Akki et al. [49] and Vanschoenbeek et al. [48] were synthesized by Biomers.net (Germany) and their sequences are shown in Table S1 in the Supplementary material.

2.2. Preparation of progesterone-BSA conjugate

Progesterone-CMO was dissolved in DMSO and activated using EDC/NHS at a molar ratio of progesterone-CMO:EDC:NHS of 1:1.25:1.25. The reaction was carried out for 2 h with mild shaking at room temperature, the activated progesterone was then added slowly to a solution of BSA (12.5 mg/ml in 100 mM $NaHCO_3$ pH 7) and the mixture was incubated further for 1.5 h. The buffer was exchanged with PBS and the progesterone-BSA conjugate was