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Novel nandrolone aptamer for rapid colorimetric detection of anabolic steroids

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ABSTRACT

The illicit use of anabolic androgenic steroids (AAS) as performance-enhancing drugs remains a global issue threatening not only the credibility of competitive sports but also public health due to the well-documented adverse effects they elicit. AAS abuse is not restricted only to professional sports, but also extends to recreational athletes and adolescents as well as in livestock production as growth-promoting agents. Testosterone and nandrolone are among the AAS most frequently exploited. Gas chromatography-mass spectrometry is the reference method for AAS detection, but it is strictly laboratory-based and cannot be performed on-site. The great potential of aptamers in bioanalytical applications and specifically for the development of simple analytical tools suitable for on-site analysis has been extensively documented. In this report, we describe the selection and identification of aptamers binding nandrolone, exhibiting affinity dissociation constants in the low nanomolar range. A label-free colorimetric assay based on gold nanoparticles was developed using one of these novel aptamers for the detection of nandrolone and/or its metabolites. The assay could be deployed for the rapid, on-site, facile and cost-effective screening of samples and provide qualitative visual results with a red to purple/blue color change being indicative of a positive result.

1. Introduction

Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone, the main male sex hormone, with clinical and illicit uses. Their anabolic effects are related to their ability to increase lean body mass, muscle size and strength, and to improve protein and bone metabolism [1], with their androgenic properties resulting in masculinization. Clinically, AAS, and especially testosterone, have traditionally been prescribed to treat male hypogonadism [2]. The potential benefits of their anabolic properties to certain patient populations have also encouraged the therapeutic use of AAS for several conditions including growth impairment, infertility and depression as well as for the treatment of cachexia related to chronic diseases such as HIV, burns, renal failure, pulmonary disorders, muscular dystrophies, breast cancer and anemia [3–6]. However, the illicit use of AAS as performance-enhancement drugs, a practice commonly known as doping, has been known for decades [1]. Doping has not only been reported among competing athletes but also by amateurs, recreational athletes and adolescents with the main objective to increase muscle mass and improve bodily appearance [4,7,8]. However, a plethora of adverse effects have been associated with AAS use/abuse including hypertension, hepatic damage, reproductive disorders as well as neuropsychiatric and behavioral disorders [1,4,8]. The use of AAS is prohibited in professional sports and the World Anti-Doping Agency (WADA) annually publishes a list with prohibited substances, in- and out-of-competition, in an effort to contain the abuse [9].

Nandrolone is among these substances whose use in sports as well as in horse racing is completely prohibited [10]. Nandrolone

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(19-nortestosterone) is a synthetic testosterone analogue and one of the most frequently abused AAS together with testosterone, stanozolol and methandienone [7,8]. Its anabolic properties are more potent than those of testosterone, since it exhibits an anabolic:androgenic ratio of 10 compared to 1 for testosterone [1,11]. Besides its potential therapeutic uses as an AAS, nandrolone has also been used as a growth promoting agent in livestock intended for human consumption [12,13], which is banned in the EU [14]. Several studies report the presence of nandrolone in dietary supplements as a cross-contaminant and consumption of such supplements could lead to accidental doping [15–17]. It is therefore clear that monitoring the presence of nandrolone in human and animal biological fluids, meat products and nutritional supplements is essential to protect public health and discourage doping practices in sports.

According to WADA regulations, gas chromatography-mass spectrometry (GC-MS) and gas chromatography-isotope ratio mass spectrometry (GC-IRMS) are the official methods for the detection of endogenous (such as testosterone) [18] and exogenous AAS (like nandrolone) [19], respectively, and these techniques have been widely reported for the analysis of a range of sample types suspected of containing AAS [16,20–23]. Alternative techniques such as ultra-high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS) are garnering increasing interest [24,25], and the use of high-performance thin-layer chromatography (HPTLC)-densitometry [26] and nuclear magnetic resonance (NMR) has also been reported [27]. Even though these techniques are highly accurate, they are expensive and laboratory-based, inherently requiring significant infrastructure, specific equipment and trained personnel. Immunoassays have also been developed as simpler, lower cost and more user-friendly alternatives to the above-mentioned techniques [10,28–32]. Due to the small size of the steroids, they require the preparation of haptens for animal immunization and antibody production. ELISAs developed using these antibodies are typically performed in a competitive format, and can be very sensitive with limits of detection (LOD) in the (sub)nanomolar range. For example, an LOD of 4 pg/mL (~15 pM) was reported when a linker-optimized biotin derivative of nandrolone was used in conjunction with avidin as an immobilized competitor [32]. ELISA kits are also commercially available for various AAS, with LODs in the low picomolar range. The high sensitivity and specificity demonstrated by immunoassays emphasize the potential of biorecognition molecules for the detection of AAS and their compatibility with anti-doping drug testing.

Aptamers are biorecognition molecules considered as chemical alternatives to antibodies. They are single stranded DNA or RNA molecules, often possessing specific three-dimensional structures able to bind their cognate targets with high affinity and specificity. Starting from highly diverse oligonucleotide libraries, specific sequences binding to target molecules are identified using an in vitro repetitive process termed SELEX, Systematic Evolution of Ligands by Exponential Enrichment [33,34]. Advantages of aptamers such as facile and reproducible chemical synthesis, straightforward modification, reversible denaturation, small size and stability have expanded their application to bioanalytical applications for the detection of a plethora of targets [35], including small molecules [36]. In fact, there are a few reports in the literature detailing the selection of aptamers binding steroids, such as estradiol [37–39], progesterone [39,40], cortisol [41] and testosterone [39,42], but to date, there is no report of aptamers selected specifically against nandrolone. There is only one study in which a previously reported estradiol aptamer was split in two fragments and was re-purposed for the detection of nandrolone in a sandwich fluorescence resonance energy transfer (FRET) assay [43].

In this work, we describe the first selection designed for the identification of nandrolone aptamers. Using Next Generation Sequencing, aptamer candidates were identified and their binding affinity for nandrolone was verified using different types of assays. Finally, a homogenous colorimetric assay was developed using gold nanoparticles (AuNPs) with a red-to-blue color change to indicate the presence of nandrolone and/or its metabolites, as a proof-of-concept of an assay suitable for rapid screening.

2. Experimental

2.1. Materials

Nandrolone (NAND), 19-norandrosterone (19-NA), 19-noretiocholanolone (19-NE), trenbolone (TREN), 17β-estradiol (ESTR), 17β-estradiol-6-one 6-(O-carboxymethyloxime) (ESTR-CMO), progesterone (PROG), progesterone-3-(O-carboxymethyl)oxime (PROG-CMO), testosterone (TEST), testosterone-3-(O-carboxymethyl)oxime (TEST-CMO), 11-amino-1-undecanethiol hydrochloride (MUAM), O-(carboxymethyl)hydroxylamine hemihydrochloride and rabbit anti-mouse-HRP conjugate were purchased from Merck (Spain). Nortestosterone Sepharose 6B (10–14 μmol/mL, NAND-resin) was obtained from Polysciences (Germany). Epoxy-activated sepharose 6B, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), sulfo-NHS-acetate, maleimide-activated microtiter plates, DreamTaq DNA polymerase and lambda exonuclease were from Fisher Scientific (Spain). The DNA purification kits (Oligo Clean & Concentrator kit and DNA Clean & Concentrator kit) were from Zymo Research (supplied by Ecogen, Spain). Monoclonal antibodies to ESTR (clone 9F9), PROG (clone 9F44) and TEST (clone 5E801) were provided from US Biological Life Sciences (acquired through VWR, Spain). Streptavidin-polyHRP80 was purchased from SDT-Reagents (supplied by Bionova, Spain) and TMB Super Sensitive One Component HRP Microwell Substrate from Surmodics (USA). The ssDNA library (5′-TAG GGA AGA GAA GGA CAT ATG AT-N40-TTG ACT AGT ACA TGA CCA CTT GA-3′, 86 nt) was obtained from TriLink Biotechnologies (USA), whereas all other oligonucleotides were synthesized by Biomers.net (Germany). All other reagents were obtained from Fisher Scientific (Spain), Scharlau (Spain) and Merck (Spain). MilliQ-grade water was used for all experiments.

2.2. In vitro selection

Commercially available nandrolone sepharose 6B resin (NAND-resin) was used for the positive selection. Epoxy-activated sepharose 6B was used to prepare control-resin for the negative selection and counter-selection resins with each of the four counter selection steroids (PROG, ESTR, TEST, TREN) as detailed in the Supplementary Information. For the first round, 300 pmol of the ssDNA library containing a 40 nucleotide-long random region was dissolved in 100 μL of selection buffer (10 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$), heated for 5 min at 95 °C and cooled slowly to room temperature. The ssDNA library was then transferred to a microspin column containing 20 μL of the NAND-resin to perform the first round of selection. For the second and third rounds, the ssDNA pool was first incubated with 20 μL of control-resin before incubation with the NAND-resin. Rounds 4–7 were performed with sequential incubations with the control-resin, PROG-resin, ESTR-resin, TEST-resin, TREN-resin and finally the NAND-resin. All selection rounds were performed using 30 min incubation steps at room temperature under rotation. For the counter selection steps, 20 μL of each resin was used for round 4 whereas 10 μL were used for rounds 5–7. At the end of each selection round, unbound sequences from each resin were removed by centrifugation for 30 s at 10,000 rpm followed by washing four times with 400 μL of water and four times with 400 μL of selection buffer. The resins were resuspended in 50 μL of water and stored for further experiments, whereas ssDNA bound on the NAND-resin was used for the preparation of ssDNA for succeeding rounds. This was achieved by the amplification of resin-bound sequences using library-specific forward and phosphorylated reverse primers and a combination of asymmetric PCR with lambda exonuclease digestion. The evolution of the selection was monitored during selection as described previously [42] to ensure enrichment in sequences binding

the target NAND-resin. Following PCR amplification of resin-bound sequences and agarose gel electrophoresis, the intensity of the bands was estimated with the ImageJ software using the gel analysis option.

2.3. Next Generation Sequencing (NGS) and identification of aptamer candidates

The ssDNA pool from the last selection round (round 7) was amplified and sequenced using Ion Torrent Next Generation Sequencing (Centre for Omic Sciences, Eurecat Technology Centre, Reus, Spain). The Galaxy web server was used for the analysis of the raw data. The length of the sequences was constrained to library-length (80–95 nt) and the filtered sequences were collapsed in order to identify unique sequences. The 100 most abundant sequences were aligned for the identification of sequence families using the Clustal Omega multiple sequence alignment tool (https://www.ebi.ac.uk/Tools/msa/clustalo/). Sequence motifs within these sequences were also identified using the MEME tool (https://meme-suite.org/meme/tools/meme). The UNAFold webserver was finally used to predict potential secondary structures of the selected aptamer candidates adjusting the conditions to the ones used during selection (100 mM NaCl, 2 mM $MgCl_2$, 25 °C). The RNAComposer 3D modeling server (http://rnacomposer.cs.put.poznan.pl/) was used to build three-dimensional models of aptamers.

2.4. Apta-PCR affinity assay (APAA)

The binding properties of the aptamer candidates were first evaluated by APAA. To this end, nandrolone was immobilized on magnetic beads (NAND-beads) as described in the Supplementary Information. NAND-beads (1.5 μL of 30 mg/mL) were incubated with 50 μL of the desired concentration of each aptamer candidate (10 nM–15.6 pM performing serial two-fold dilutions in selection buffer) for 30 min at room temperature under rotation. The beads were thoroughly washed with selection buffer and finally resuspended in 10 μL of water. The beads with bound aptamer were used for PCR amplification and after gel electrophoresis, the intensities of the bands for each aptamer concentration were estimated using the ImageJ program and the gel analysis option as described previously [42]. Triplicate measurements were performed for all samples. The relative band intensities were plotted against aptamer concentration using the GraphPad software and the "One site - specific binding with Hill slope" model was used to construct the binding curves and calculate the affinity dissociation constants (K_D).

2.5. Bead-Enzyme Linked Aptamer Assay (bead-ELAA)

NAND-beads (1.5 μL of 30 mg/mL) were incubated with 50 μL of each biotinylated aptamer candidate (100 nM–6.4 pM performing serial five-fold dilutions in selection buffer) for 15 min at room temperature under rotation. For the motif sequence, 400 nM–1.6 nM were used by performing serial two-fold dilutions. The beads were washed with PBS containing 0.05% v/v Tween-20 (PBST), resuspended in 50 μL of 0.05 μg/mL streptavidin-polyHRP in PBST and incubated for 15 min. Finally, the beads were thoroughly washed with PBST and resuspended in 50 μL of TMB substrate solution. Color development was terminated by the addition of 50 μL of 1 M H_2SO_4 and absorbance was recorded at 450 nm. The K_D values were calculated as described above in the "APAA" section, by plotting the absorbance at 450 nm against aptamer concentration. Triplicate samples were analyzed for each concentration.

2.6. Enzyme Linked Aptamer Assay (ELAA)

For this assay, nandrolone was immobilized on microtiter plates as detailed in the Supplementary Information employing maleimide activated microplates and MUAM crosslinker. Solutions with different concentrations of the biotinylated aptamers (50 μL of 20 nM down to 10 pM, two-fold serial dilutions in selection buffer) were added to the wells and incubated for 15 min at room temperature under mild agitation and then washed with PBST. Subsequently, 50 μL of 0.05 μg/mL streptavidin-polyHRP in PBST were added and incubated for a further 15 min. The wells were finally washed with PBST and 50 μL of TMB solution were added, followed by the addition of 50 μL of 1 M H_2SO_4 after 5 min to stop color development. Absorbance was read at 450 nm and the K_D values were determined as described above.

2.7. Gold nanoparticles (AuNPs)-aptamer assay for NAND detection

AuNPs (diameter of ~15 nm) were synthesized using the sodium citrate reduction method as previously described [44]. AuNPs (50 μL of OD 2) were incubated with the Nand3 aptamer (15 μL) for 3 h at 22 °C under rotation. Ten microlitres of solutions containing different concentrations of NAND (with 10% of ethanol to maintain steroid solubility) were then added and the mixtures were incubated for a further 15 min. Finally, 25 μL of NaCl were added and after 2 min, the spectra of the samples were acquired in the range of 400–800 nm. The concentrations of the Nand3 aptamer and NaCl in the mixtures were optimized, using the aptamer at 100–200 nM and NaCl from 25–100 mM in the presence of various concentrations of NAND (0 - 200 μM). Optimized conditions were finally employed to determine the sensitivity of the assay by constructing a calibration curve for NAND concentrations in the range of 0.4–200 μM. The absorbance ratio of aggregated (670 nm) to dispersed (525 nm) AuNPs was plotted against the logarithm of NAND concentration using the GraphPad Prism software and a four-parameter sigmoidal model was used to fit the data. The limit of detection (LOD), defined as the bottom of the fitted curve plus three times its standard deviation (bottom + $3xSD_{bottom}$), was finally interpolated from the calibration curve. The specificity of the assay was evaluated using a non-specific aptamer to replace the Nand3 aptamer in the AuNP assay. Also, the responses of the assay to endogenous steroids present in biological fluids (TEST, PROG, ESTR) were evaluated at physiological levels, and the nandrolone urinary metabolites 19-NA and 19-NE were assayed at a range of concentrations (0–125 μM).

3. Results and discussion

3.1. Selection process

The selection of nandrolone aptamers was based on the use of nandrolone-sepharose affinity resin (NAND-resin) and a highly diverse ssDNA library with a 40 nucleotide-long random region. The selection was completed in seven rounds and a summary of the conditions used can be seen in Fig. 1A. Commercially available NAND-resin was employed for the positive selections, whereas control sepharose resin was used for the negative selections as well as for the preparation of counter selection resins with each of the four counter-SELEX molecules, progesterone (PROG), estradiol (ESTR), testosterone (TEST) and trenbolone (TREN). The structures of the target nandrolone and the other steroids used in the selection and the immobilization of these steroids on sepharose resin can be seen in the Supplementary Information (Figs. S1 and S2). For the first round, the ssDNA library was incubated with the NAND-resin (Fig. S1B) and bound sequences were amplified and used for the preparation of ssDNA for the next round. For the second round, the ssDNA pool prepared from the first round was first incubated with the control-resin to remove any sequences binding non-specifically to the matrix (sepharose resin). Unbound ssDNA was recovered and incubated with the NAND-resin for the positive selection. The amount of target-bound sequences during the second round decreased compared to the first one as a consequence of the negative selection, which effectively removed part of the ssDNA pool interacting non-specifically with the control-resin (Fig. 1B). After completion of the third round, which was performed in the same way as the second one, the ssDNA pool appeared to be enriched in NAND-resin-binding sequences with insignificant binding to the control-resin. Counter selection molecules were thus

A

Selection round	Order of selection steps	Control resin	NAND resin	Counter molecules resin
R1	NAND-resin	-	20 μL	-
R2 – R3	Control-resin → NAND-resin	20 μL	20 μL	-
R4	Control-resin → PROG-resin → ESTR-resin → TEST-resin → TREN-resin → NAND-resin	20 μL	20 μL	20 μL
R5 – R7		20 μL	20 μL	10 μL

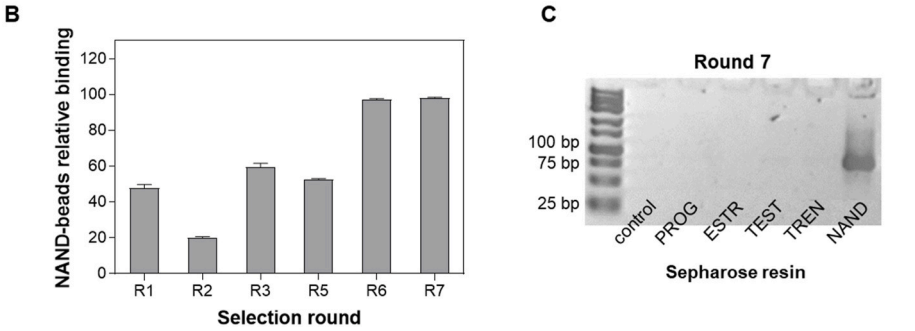

Fig. 1. Selection strategy and evolution of the process. (A) Conditions used for SELEX. (B) Evolution of the selection. (C) Specificity of the last selection round.

A

Measure	Value
Total Sequences	117,225
Sequence length	25 – 309
%GC	46
Sequences 80-95 nt	63,924 (54.5 %)
Unique sequences 80-95 nt	32,903 (51.5 %)

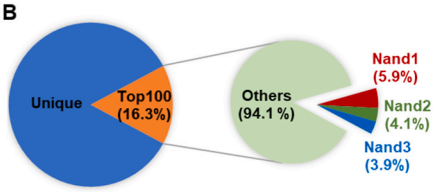

Fig. 2. NGS analysis of round 7 from the nandrolone selection. (A) General statistics. (B) Composition of the top 100 most abundant unique sequences.

introduced in the following selection round 3. After a negative selection step, sequential incubations with the PROG-resin, ESTR-resin, TEST-resin and TREN-resin were performed for counter-SELEX followed by the positive selection with the NAND-resin. This procedure was followed for rounds 4–7, with the only difference being the use of less resin per counter selection molecule in rounds 5–7 as compared to round 4 (Fig. 1A). By the final round 7, PCR amplification of bound sequences to each resin type followed by agarose gel electrophoresis showed the specific binding of the enriched ssDNA pool to the NAND-resin and no binding to the other steroid-resins (Fig. 1C).

3.2. NGS and identification of aptamer candidates

The last selection round was analyzed using Ion Torrent Next Generation Sequencing to identify aptamer candidates. The raw data was imported in the Galaxy webserver and the length of sequences was constrained to the library length (80–95 nt) to remove artefacts resulting from PCR amplification and sequencing. Unique sequences were then identified after collapsing the filtered dataset. Out of the 117225 total reads, 54.5% were sequences with library length, and approximately half of those (51.5%) were unique (Fig. 2A). The ranking and copy number of the 100 most abundant unique sequences, corresponding to 16.3% of the total unique sequences (Fig. 2B), can be found in Table S1. The next step was to perform multiple sequence alignment of these 100 sequences using Clustal Omega as well as to analyze them with the MEME tool for sequence motif discovery. No sequence families were identified (Fig. S3). Interestingly, a short sequence motif of 11 nt was identified in 52 of the 100 most abundant unique sequences (Figs. S3 and S4). It was also found in 50% of the 500 most over-represented sequences (data not shown). The first three most abundant sequences were termed Nand1, Nand2 and Nand3 and they constituted 5.9, 4.1 and 3.9%, respectively of the top 100 most over-represented sequences dataset (Fig. 2B). These sequences together with the sequence motif were finally chosen for further characterization and their sequences can be found in Table S2. The postulated secondary structures of the aptamer candidates predicted using the UNAFold webserver revealed extensive stem-loop substructures typically found in aptamer sequences (Fig. S5).

3.3. Affinity of the aptamers

Once the aptamer candidates were identified, different assays were performed to evaluate their binding properties. APAA, bead-ELAA and ELAA were employed, based on the use of magnetic beads and maleimide-activated microtiter plates to immobilize nandrolone. We have previously reported the use of these three assays for the characterization of aptamers binding to small molecules such as steroids [39,42,45] and biogenic amines [46]. For APAA and bead-ELAA, a carboxyl-derivative of nandrolone (NAND-CMO) was directly conjugated to functionalized magnetic beads. The beads were modified with amine groups through a short hydrophilic linker and cross-linking was achieved using classic carbodiimide chemistry via EDC/NHS. For the maleimide-activated microtiter plates, an 11 carbon-long crosslinker (MUAM) was used as a spacer to facilitate the immobilization of NAND-CMO to perform the ELAA. Unmodified aptamers were used for APAA, whereas modified ones (with a biotin added to the 5' end of the aptamers) were required for bead-ELAA and ELAA. The binding curves of the aptamers obtained from each of the assays and the affinity dissociation constants (K_Ds) are shown in Fig. 3 and Table S3. All three assays verified the high binding affinity of the three full length aptamers

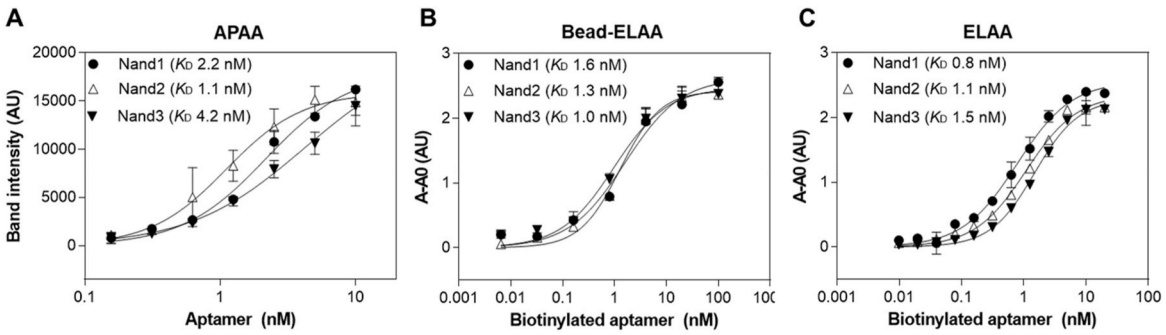

Fig. 3. Evaluation of the affinity of the aptamer candidates for nandrolone. Binding curves were obtained by (A) APAA, (B) bead-ELAA and (C) ELAA.

(Nand1, Nand2 and Nand3) with K_Ds in the low (sub)nanomolar range. The use of different surfaces for nandrolone immobilization, the length of the spacer used to spatially separate nandrolone from the surface, or the modification of the aptamers did not appear to affect their binding properties.

The motif, which is present in the sequences of the Nand2 and Nand3 aptamer candidates but not in Nand1, was also analyzed. A DNA-based spacer (T15) was introduced at the 5′-end of this 11-mer motif sequence to provide more structural flexibility and prevent any potential interference of the biotin added to facilitated detection on nandrolone-motif complex formation. A K_D of 27 nM was calculated for the motif by bead-ELAA (data not shown). As mentioned earlier, this sequence was found in 52% of the top 100 and in half of the top 500 sequences from the last selection round, indicating that the selection process resulted in the enrichment of this sequence as a nandrolone binding motif. Considering the small size of the steroids, a binding pocket formed in a three-dimensional structure of the motif predicted using the RNAComposer webserver could potentially accommodate steroid binding (Fig. S6).

3.4. AuNP-aptamer assay for nandrolone detection

A label-free colorimetric assay employing the Nand3 aptamer and gold nanoparticles (AuNPs) was finally designed for the detection of nandrolone as schematically depicted in Fig. 4. The negatively charged ssDNA (unmodified) aptamer is adsorbed on the surface of AuNPs, resulting in the stabilization of the particles and prevention of aggregation following the addition of NaCl salt and the increase of the ionic strength of the suspension. The AuNPs are thus maintained well-dispersed and exhibit their characteristic red color. When the target molecule is added to the suspension, the aptamer is displaced from the AuNP surface, either due to a higher affinity of the aptamer for its cognate target as compared to adsorption, or alternatively due to a conformational change of the aptamer upon target binding. In either case, the presence of the target provokes its desorption from the particles and displacement to the solution phase, forming a complex with the target. In this case, when salt is added, the AuNPs aggregate since their surface is no longer protected by the aptamer and the color of the suspension changes to purple/blue. This assay has been widely exploited for small molecule detection because of the several advantages it provides; it is facile and rapid, provides a clear visual result with a red-to-purple/blue color change to indicate target presence, does not require any labels to generate signal, the aptamer is used unmodified and it is a homogenous assay performed in a single tube that does not require any separation/washing steps [44,47,48]. There are already a few studies in the literature using this assay for steroid detection, including cortisol [41], estradiol [49] and progesterone [50], as detailed in Table S3.

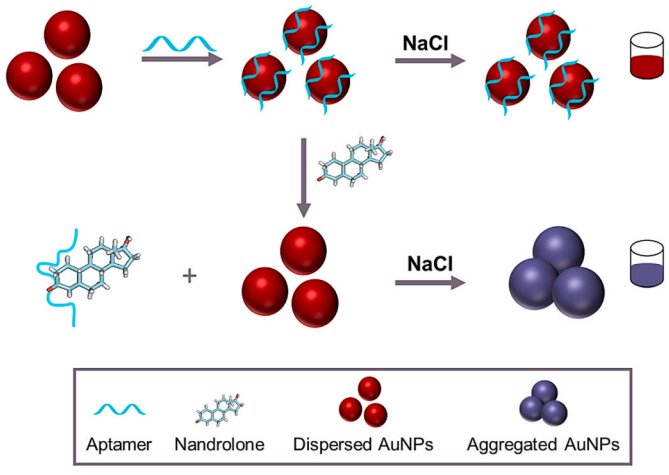

Fig. 4. Principle of the gold nanoparticle-aptamer assay for nandrolone detection. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Herein we sought to apply the selected aptamers to the detection of nandrolone using this AuNP-based assay. For optimum efficiency of the assay, a conformational change is required to enable complete aptamer displacement to the solution for target binding. Preliminary evaluation of the three nandrolone aptamers with the assay showed that the signal difference between dispersed and aggregated nanoparticles in the absence and presence of nandrolone was higher when the Nand3 aptamer was used (data not shown), it was thus chosen for further assay development. The concentrations of the Nand3 aptamer and NaCl were optimized to enhance assay performance. A range of nandrolone solutions were assayed (final 0–200 μM) for aptamer concentrations of 100, 150 or 200 nM, while NaCl was added to the mixtures at final concentrations of 25, 50, 75 or 100 mM (Fig. S7). The combination of 200 nM of aptamer with 50 mM of NaCl was chosen as the most efficient one, allowing for visual discrimination of nandrolone in the range of 2–200 μM (Fig. S7A) while resulting in a wide span of signal (absorbance ratio of aggregated to dispersed AuNPs) of 0.8 (Fig. S7B). Finally, a range of nandrolone concentrations (0.4–200 μM) were analyzed with the assay under the optimized conditions. Representative images of samples containing increasing concentrations of nandrolone showing the red-to-purple/blue color change can be found in Fig. S8. The spectra acquired for these samples are shown in Fig. 5A and the calibration curve constructed using the absorbance ratios of aggregated (A670nm) to dispersed AuNPs (525 nm) in Fig. 5B. The LOD of the assay was calculated at 2 μM nandrolone. The relatively low sensitivity of the assay might be attributed to the length of the aptamer (86 nt) limiting its efficient displacement to the solution phase. Truncation of the aptamer to remove non-essential bases could potentially improve assay performance, a strategy previously demonstrated for a bisphenol A aptamer [51]. In that work, more than 250-fold improvement of the LOD was achieved with an equivalent AuNP-based assay when the aptamer length was reduced from 63 nt to 38 nt. Additionally, removing certain parts of the sequence potentially forming secondary structures with low free

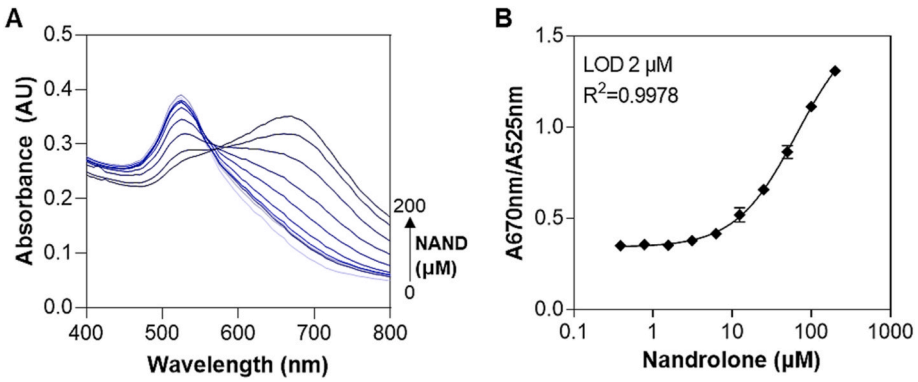

Fig. 5. AuNP-aptamer assay for nandrolone detection. (A) Representative spectra of samples containing different concentrations of nandrolone. (B) Calibration curve for nandrolone quantification.

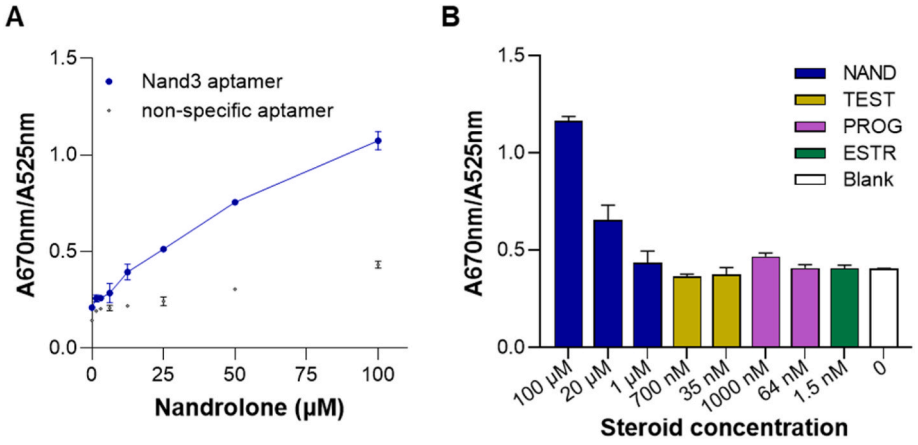

Fig. 6. Specificity of the AuNP-aptamer assay. (A) Use of Nand3 aptamer or of a non-specific aptamer for nandrolone detection. (B) Responses of the assay to a range of nandrolone concentrations (1–100 μM NAND) and different endogenous steroids at physiological levels (TEST: testosterone, PROG: progesterone; ESTR: estradiol).

energy could also improve the assay sensitivity as shown previously for an estradiol aptamer [52]. The motif sequence was evaluated for the assay as a shorter variant, but preliminary results were not encouraging (data not shown). It is known that the absorption of ssDNA on the surface of citrate-stabilized gold nanoparticles is both length- and sequence-dependent [53]. Therefore, a more systematic truncation study is required to optimize the length of the Nand3 aptamer and potentially improve the assay sensitivity (see Fig. 6).

Whilst the sensitivity of commercially available ELISA kits, with LODs in the sub-nanomolar range, is superior to the AuNP-aptamer assay reported it exhibits several advantages: (i) it is a homogenous assay not requiring any separation or washing steps (mix-and-measure assay); (ii) it only requires the use of AuNPs and the aptamer, as opposed to the different conjugates involved in the development of ELISAs; (iii) it can be performed under 20 min with room temperature incubation as opposed to 2–3 h and 37 °C required for the ELISAs; (iv) no significant laboratory infrastructure is required and (v) the results can be evaluated by naked eye inspection for rapid qualitative screening of suspicious samples. Moreover, ELISAs are strictly laboratory-based assays and require multiple incubation and washing steps.

As mentioned earlier, there is only one study in the literature demonstrating the use of an aptamer for nandrolone detection [43]. Using a split estradiol aptamer modified with a fluorophore and a quencher in each fragment, a FRET assay was developed with an LOD of 5 μM. The sensitivity of the assay developed in this work is in the same low micromolar range as the one previously described and can be used for on-site monitoring of nandrolone levels in suspicious samples. Work is ongoing to evaluate alternative truncated structures to improve the detection limit of this assay, but preliminary results have not proved successful.

The specificity of the assay was also evaluated. Initially, a non-specific aptamer was used to replace the nandrolone-specific Nand3 aptamer in the AuNP-aptamer assay. Gold nanoparticles were incubated in parallel with the Nand3 or the non-specific aptamer. Different concentrations of nandrolone were then added to the suspensions and after the addition of NaCl, the spectra were recorded. As can be seen in Fig. 6A, the responses of the assay when the non-specific aptamer was used were negligible compared to when the nandrolone-specific aptamer was employed, thus confirming the specificity of the aptamer and of the assay.

A second specificity test was designed to evaluate the responses of the assay to endogenous sex steroids found in biological fluids. These steroids share extremely high structural similarity with nandrolone (Fig. S1) and their presence could potentially interfere with the assay. These were the androgen testosterone (TEST), the progestogen progesterone (PROG) and the estrogen estradiol (ESTR). The physiological levels of these steroids in blood vary from less than 0.1 nM for estradiol to 100 nM for testosterone (Table S4) [54]. Herein, the AuNPs-aptamer assay was performed at average concentrations as defined by the National Institutes of Health (Table S5), which were 35 nM for testosterone, 64 nM for progesterone and 1.5 nM for estradiol. Testosterone was tested at a high concentration considered suspicious for doping by WADA (200 ng/mL, ~700 nM) [55]. Progesterone was assayed at high concentration associated with pregnancy (1 μM). Nandrolone was analyzed at 1, 20 and 100 μM to cover the analytical range of the assay. As shown in Fig. 6B, background-level responses were observed for all

the other steroids tested, while the presence of nandrolone was easily differentiated by visual inspection (Fig. S9), demonstrating the specificity of the assay.

For anti-doping control purposes, the presence of nandrolone in suspicious samples is determined via its main urinary metabolites 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE) [56] since the parent steroid can be readily metabolized [54,57] and it might not be present in the sample at the time of analysis. To evade detection of abuse though, masking agents such as finasteride are sometimes used to suppress the metabolism of nandrolone and effectively decrease the concentrations of its urinary metabolites [58]. In these cases, direct detection of nandrolone would be more appropriate. In this work, we sought to evaluate whether these two metabolites could also be detected with the AuNPs-aptamer assay along with nandrolone. As shown in Fig. 7, 19-NE could be detected with the same sensitivity as nandrolone, whilst lower signal responses were observed for 19-NA. This could be attributable to lower binding affinity of the Nand3 aptamer for 19-NA compared to nandrolone and 19-NE arising from the structural differences between the three steroids (Fig. S1D). Whilst this result is a little surprising, the detection of one of the metabolites is sufficient for use in screening, with the assay being able to detect nandrolone as well as its metabolites.

4. Conclusions

Doping refers to the illicit use of prohibited substances with the objective of gaining competitive advantage especially in professional sports. Anabolic androgenic steroids (AAS) are one category of these substances including testosterone and one of its synthetic derivatives called nandrolone. AAS abuse poses serious health concerns because of the numerous adverse effects they can cause, and international organizations are dedicated to improving global monitoring and prevention strategies to manage the problem. Even though highly sensitive gas chromatographic-mass spectroscopic methods have been established for the specific detection of these substances, they are limited to laboratory use and they cannot be deployed on-site for fast screening of suspicious samples. The numerous advantages of aptamers as alternative biorecognition elements successfully applied for the detection of a plethora of small molecules make them particularly attractive for the development of an assay for the screening for AAS which can be deployed for on-site analysis. In this work, we report the first selection performed specifically for the identification of nandrolone aptamers. Three aptamer candidates and a highly enriched 11-mer sequence motif were identified after sepharose-based SELEX and Next Generation Sequencing of the final enriched pool. The high binding affinity of the candidates to nandrolone was demonstrated with different types of assays, exhibiting affinity dissociation constants in the low (sub)nanomolar range. Finally, a facile and rapid colorimetric assay was developed as a proof-of-concept employing one of the full-length aptamers and gold nanoparticles, achieving a detection limit of 2 μM of nandrolone. The assay was highly specific for nandrolone, and no-cross-reactivity was observed with the endogenous steroids testosterone, progesterone or estradiol at naturally occurring levels or at high levels associated with doping or pregnancy. Moreover, negligible signals were observed when a non-specific aptamer was used to detect nandrolone, further emphasizing the specificity of the selected aptamer. Finally, the possibility to use the assay for the analysis of the nandrolone urinary metabolites was demonstrated, albeit with significantly lower sensitivity for 19-norandrosterone compared to nandrolone and 19-noretiocholanolone. Overall, this aptamer-based homogenous assay can serve as a simple on-site screening tool with the red-to-purple/blue color change to indicate the potential presence of nandrolone and/or its metabolites in suspicious samples. Further analysis by GC-MS/GS-IRMS can then be employed to confirm the specific steroid and its concentration in the sample.

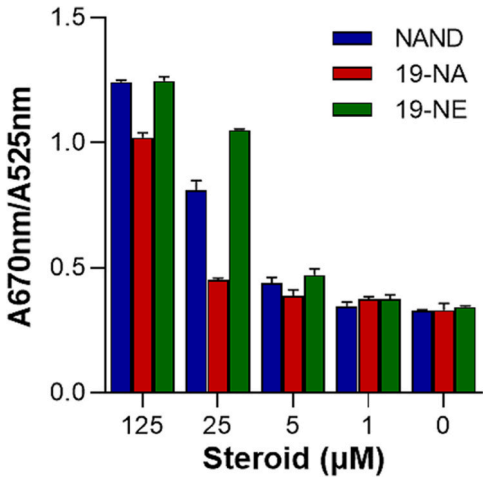

Fig. 7. Application of the AuNPs-aptamer assay for the detection of the nandrolone (NAND) and its urinary metabolites 19-norandrosterone (19-NA) and 19-noretiocholanolone (19-NE).

Compliance with ethical standards

The authors declare that they have no competing interests.

CRediT authorship contribution statement

Xhensila Shkembi: Conceptualization, Methodology. **Mary Luz Botero:** Investigation. **Vasso Skouridou:** Supervision, Writing – original draft, preparation. **Miriam Jauset-Rubio:** Investigation. **Marketa Svobodova:** Supervision, Investigation. **Pablo Ballester:** Conceptualization, Methodology. **Abdulaziz S. Bashammakh:** Conceptualization, Data curation, Project administration. **Mohammad S. El-Shahawi:** Conceptualization. **Abdulrahman O. Alyoubi:** Conceptualization. **Ciara K. O'Sullivan:** Writing – review & editing, Funding acquisition, Project administration.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2022.114937.

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