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Synthesis of steroid-oligonucleotide conjugates for a

DNA site-encoded SPR immunosensor

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TITLE RUNNING HEAD. SPR Steroid-oligonucleotide conjugates

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ABSTRACT: The excellent self-assembling properties of DNA and the excellent specificity of the antibodies to detect analytes of small molecular weight under competitive conditions have been combined in this study. Three oligonucleotide sequences (N_{1up} , N_{2up} , and N_{3up}) have been covalently attached to three steroidal haptens (8, hG and 13) of three Anabolic-Androgenic Steroids (AAS), stanozolol (ST), tetrahydrogestrinone (THG) and boldenone (B), respectively. The synthesis of steroid-oligonucleotide conjugates has been performed by the reaction of oligonucleotides carrying amino groups with carboxyl acid derivatives of steroidal haptens. Due to the chemical nature of the steroid derivatives two methods for coupling the haptens and the ssDNA have been studied: a *solid-phase coupling strategy* and a *solution-phase coupling strategy*. Specific antibodies against ST, THG and B have been used in this study to assess the possibility of using the self-assembling properties of the DNA to prepare biofunctional SPR gold chips based on the immobilization of haptens, by hybridization with the complementary oligonucleotide strands possessing SH groups previously immobilized. The capture of the steroid-oligonucleotide conjugates and subsequent binding of the specific antibodies can be monitored on the sensogram due to variations produced on the refractive index on top of the gold chip. The resulting steroid-oligonucleotide conjugates retain the hybridization and specific binding properties of oligonucleotides and haptens as demonstrated by thermal denaturation experiments and surface plasmon resonance (SPR).

KEYWORDS: Anabolic-Androgenic Steroids (AAS), steroid-oligonucleotide conjugate, DNA-directed immobilization (DDI), Surface Plasmon Resonance (SPR)

INTRODUCTION

The food safety, biomedical research and environmental monitoring areas require methods of screening with the ability to detect, rapidly and sensitively, trace amount of small targets (<1000 Da). As an example, Anabolic Androgenic Steroids (AAS) are completely prohibited substances by the WADA (World Antidoping Agency) (1) and the European Community (2), for their respective use to improve athletic performance and to increase meat production in the agro-alimentary field, due to the associated adverse health effects. Immunosensors based on the surface plasmon resonance (SPR) principle provide a rapid and convenient analytical alternative for monitoring (bio)chemical substances in a label-free way (3-9). Several strategies have been used to anchor the immunoreagents onto gold chips to develop SPR biosensors to detect small molecules under competitive immunochemical configurations. In indirect formats, usually a protein functionalized with the hapten is immobilized on the surface of the transducer (10-12) however; alternative methods to avoid the use of a carrier protein have been described. For example, an oligo(ethyleneglycol) (OEG) linker was used on a SPR biosensor to detect 2,4,6-trinitrotoluene (TNT) (13) and testosterone (14). In direct detection formats, the antibody can be immobilized directly through a self-assembled layer (SAM) of heterobifunctional linkers possessing a thiol group, although alternative methods to appropriately orientate the antibody molecule have also been described. Thus, it has been reported the immobilization of antibodies by affinity interaction with a layer of protein A previously immobilized on the gold substrate (15). Moreover, the use of DNA-protein conjugates has also been reported on nanoscale immunosensors microstructured biochips (16). Hence, a protein G-DNA conjugate has been employed to ensure controlled immobilization of antibodies to intended areas of a chip surface or to particles (17). The DNA-directed immobilization (DDI)

strategy provides a chemically mild process for the simultaneous binding of multiple proteins to a solid support using DNA surfaces as immobilization matrices. It consists on tagging biomolecules with oligonucleotides chains that are complementary to ssDNA previously linked on the surface. An additional advantage of this strategy is the possibility to produce regenerable sensor surfaces due to the reversible nature of the DNA hybridization reaction. This strategy has been used to immobilize antibodies covalently linked to ssDNA onto a SPR surface where the complementary strands have been previously immobilized, to detect three fertility hormones in a direct competitive format (18, 19). However, this approach has been rarely used to immobilize haptens on a SPR gold chip. Previously, we have demonstrated the possibility to use DDI to create hapten microarrays for fluorescent detection (20). This time, our objective has been to demonstrate the biofunctionality of steroid-oligonucleotide bioconjugates synthesized and characterized to use them on a SPR biosensor and to assess the possibility to develop multiplexed SPR immunosensors for small organic molecules by directly immobilizing the haptens on the gold chip through a DDI approach (12, 22-27).

EXPERIMENTAL PROCEDURES

Chemicals, reagents and buffers

General. Phosphoramidites and ancillary reagents used during oligonucleotide synthesis were from *Applied Biosystems* (*PE Biosystems Hispania S.A.*, Spain), *Link Technologies* (*Link Technologies Ltd.*, Scotland) and *Glen Research* (*Glen Research Inc.*, USA). The rest of the chemicals were purchased from *Aldrich*, *Sigma* or *Fluka* (*Sigma-Aldrich Química S.A.*, Spain). NAP-10 columns (Sephadex G-25) were purchased from Pharmacia Biotech. The preparation of the stanozolol (ST), tetrahydrogestrinone (THG) and boldenone (B)

haptens (8, hG and 13, respectively) and the production of the antiserums (As147, As170 and As138, respectively) has been described previously (28-30). *N,N'*-dicyclohexylcarbodiimide 99% (DCC) was purchased from Aldrich and the *N*-hydroxysuccinimide (NHS) from Fluka. Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) was from *Bachem* (*Bachem*, Switzerland). Long chain alkylamine Controlled Pore Glass (LCAA-CPG) was from *CPG* (*CPG Inc.*, New Jersey, USA).

Instrumentation

Oligonucleotide sequences were synthesized on an *Applied Biosystems* DNA synthesizer model 3400 (*Applied Biosystems*, USA). Mass spectra (matrix-assisted laser desorption ionization time-of-flight, MALDI-TOF) were provided by the Mass Spectrometry service of the University of Barcelona. UV-Visible spectra were recorded on a *Shimadzu* UV-2101PC spectrophotometer. HPLC (SHIMADZU-9A) with manual injector volume of 150 μ L, bomb LC-9A and detector SPD-M6A UV-VIS.

Commercial SPR chips with bare gold surface, SPRchip[™] and spotReady[™] (*GWC Technologies*, USA) substrates, were used for all the experiments. Imaging SPR measurements were collected with a *GWC Technologies* SPR Imager II instrument (*GWC Technologies*, USA). Measurements were performed at a fixed angle of incidence collecting the changes on the reflected light intensity through imaging the SPR chip with a CCD camera. The spotReady[™] chips contain an array of gold spots where positive and negative controls can be included and measured simultaneously. Individual chips were index-matched to the prism and fitted with a 20 μ l flow cell connected to a peristaltic pump from *Ismatec* (Glattbrugg, Switzerland). A total volume of 0.5 mL was needed to

completely fill the tubing and flow cell with buffer or analyte. The SPR images were collected and analyzed using V⁺⁺ software (*Digital Optics*, New Zealand).

Synthesis and purification of oligonucleotides and steroid-oligonucleotide conjugates

Three pairs of oligonucleotide complementary sequences were designed (see Table 1). Three oligonucleotides were prepared with an amino group at the 5'-end (N_{1up} , N_{2up} and N_{3up}) and the three complementary sequences contained a thiol group at 5'-end (N_{1down} , N_{2down} and N_{3down}). Oligonucleotide sequences carrying an amino and thiol group at the 5'-end were assembled on a 1 μ mol scale. The benzoyl (Bz) group was used for the protection of the amino group of C and A, and the isobutyryl (ibu) group for the protection of G. The phosphoramidite derivative of 6-aminohexanol carrying the monomethoxytrityl (MMT) group (*Glen Research* and *Link Technologies*) (31) was used for the introduction of the amino group at the 5'-end. The thiol group at the 5'-end was introduced using the phosphoramidite derivative of 6-hydroxyhexyldisulfide protected with the dimethoxytrityl (DMT) group (5' thiol modifier C6 S-S CE phosphoramidite, *Link Technologies, Inc.*, Scotland). Coupling yields were >98%. After the assembly of the sequences, the resulting solid supports (except for N_{1up} and N_{2up}) were treated with concentrated ammonia for 6 h at 50°C. Oligonucleotides carrying thiol groups were deprotected with concentrated ammonia carrying 0.1M dithiotreitol (DTT). The resulting $N_{1-3down-SH}$ oligonucleotides were stored in presence of DTT to prevent the disulfide formation. Prior to immobilization thiol-oligonucleotides were desalted with Sephadex G-25 (NAP-10 columns, *GE Healthcare*) eluted with water to remove the excess of DTT.

The corresponding $N_{1-3up-NH_2}$ oligonucleotides have been conjugated with the steroids (8, hG and 13, respectively). For the preparation of these conjugates we have used two

different approaches: 1) the active ester method in aqueous solution (*solution-phase strategy*), which consists on the activation of the acid of the steroidal haptens using DCC and NHS, and 2) the *solid-phase strategy*, using PyBOP as activating agent and diisopropylethylamine (DIPEA).

1) Synthesis of oligonucleotides carrying steroids using the solution-phase coupling protocol

The resulting solid support obtained after the assembly of the oligonucleotide sequence ($N_{3up}-NH_2$), and removal of the last MMT group, was treated with concentrated ammonia for 6 h at 50°C. The mixture was filtered and the ammonia solution concentrated to dryness. The residue was passed over a Dowex 50x4 (Na^+ form) column to exchange ammonium for sodium ions. The resulting amino-oligonucleotide was dissolved in 0.2 mL of water, and mixed with 0.1 mL of an aqueous buffer. We tried three different aqueous buffers: 1) 1 M sodium carbonate, pH 9.0; 2) 50 mM Tris pH 8.5 and 3) 0.2 M sodium borate pH 8.6. In a separate container the steroidal hapten with the carboxylic acid group (13, 10 molar excess) was dissolved in 0.1 mL of DMF and mixed with NHS (25 molar excess) and DCC (50 molar excess). The mixture was left with magnetic stirring for 3 h at room temperature, until the solution became opaque due to the precipitation of the urea. Then, we centrifuge (10.000 rpm for 15 min) to remove the precipitate and the supernatant was added to the aqueous solution of the amino-oligonucleotide. The reaction mixture was kept at 37°C with magnetic stirring overnight. The mixture was concentrated to dryness and the residue was dissolved in water and passed through a NAP-10 column. Finally, the oligonucleotide fractions were analyzed by reverse-phase HPLC-UV. HPLC solutions were solvent A: 5% ACN in 100mM triethylammonium acetate (TEAA) pH 6.5 and solvent B: 70% ACN in

100 mM triethylammonium acetate pH 6.5. Column: PRP-1 (*Hamilton*), 350 x 8 mm. Flow rate: 3 mL/min. A 20 min lineal gradient from 5-35% ACN was used. The desired oligonucleotide conjugate carrying the steroid eluted at around 11 min, while the unreacted amino-oligonucleotide eluted at around 2-5 min. The desired product was characterized by UV-spectra and mass spectrometry (MALDI-TOF), with a global yield for 13-*N₃up* of 20 (carbonate buffer) -30% (Tris, borate buffers). MALDI-TOF m/z (negative mode) 13-*N₃up*, calc 6639, found 6400 (M-239) corresponding to the fragmentation of the ester linkage of the hapten during the acquisition of MALDI spectra. Electrospray m/z (negative mode) 13-*N₃up*, calc 6639, found 6639. Increasing the molar excess of active ester from 10 to 30 times excess, allows the conversion of the amino-oligonucleotide to the desired conjugate to 45%.

2) Synthesis of oligonucleotides carrying steroids using the solid-phase coupling protocol

The oligonucleotide sequences carrying an amino group at the 5'-end were prepared as described above (*N_{1,2}up-NH₂*). The resulting solid supports were treated with 3% trichloroacetic acid/CH₂Cl₂ to eliminate the MMT group, washed with ACN and reacted with the carboxylic acid derivatives of the steroids as follows. A mixture containing 10 molar excess of the hapten (hG or 8), 20 molar excess of *N,N*-diisopropylethylamine (DIEA) and 10 molar excess of PyBOP was prepared in dry DMF (0.2 mL). The mixture was left for 10 min at room temperature and added to the support. After 30 min at room temperature, the mixture was filtered and washed with DMF and ACN. The support was dried and concentrated ammonia (1 mL) was added. The ammonia solution was left for 6 h at 50 °C. The mixture was filtered and the ammonia solution was concentrated to dryness. The fractions containing oligonucleotide were analyzed by HPLC-UV as described above.

The desired steroid-oligonucleotide conjugates eluted at around 11 min, and were obtained with global yields of 52% for 8-*N*_{1up} and 40% for hG-*N*_{2up}. Finally, the conjugates were characterized by UV and mass spectrometry (MALDI-TOF). MALDI-TOF *m/z* (negative mode) 8-*N*_{1up}, calc 6668, found 6672; hG-*N*_{2up}, calc 6619, found 6614. In this last compound we also observed a second peak at 6333 *m/z* corresponding to the hapten fragmentation at the oxime bond.

DNA site-encoded SPR immunosensor

General considerations. All the SPR experiments began with a buffer baseline (10mM PBS with 100 mM NaCl, assay buffer) and the same buffer was used to wash between each step and to prepare all the solutions of the bioreagents. All the solutions were flowed at a rate of 3 $\mu\text{L}\cdot\text{s}^{-1}$. Solutions carrying the thiolated oligonucleotides (*N*_{1,2,3 down-SH) and the steroid-oligonucleotide conjugates (8-*N*_{1up}, hG-*N*_{2up} and 13-*N*_{3up}) were prepared at 150 $\mu\text{g}\cdot\text{mL}^{-1}$ and 60 $\mu\text{g}\cdot\text{mL}^{-1}$, respectively in the assay buffer. Nonspecific adsorption of proteins was avoided by using a blocking solution of 2% of bovine serum albumine (BSA). Antisera for ST (As147), THG (As170), B (As138) and for negative controls (CTR-NEG, pre-immune serum) were diluted 250 times in the assay buffer.}

Individual SPR procedure. (see

Figure 1, A) Biofunctionalization of the SPRchipTM was accomplished by flowing a solution of the thiolated oligonucleotides down (*N*_{1,2,3down-SH}) for 15 min. A solution containing the corresponding steroid-oligonucleotide conjugate (8-*N*_{1up}/ hG-*N*_{2up}/ 13-*N*_{3up}) in the assay buffer was flowed to the cell for about 20 min, until a steady-state situation. Following the chip was washed (10 min) and the blocking solution was passed (15 min). After a new washing step, the solution of the specific antisera (As147/ As170/ As138,

1/250 dilution) was flowed for about 20 min until the signal reached the maximum, and washed again with the assay buffer. At the end of the cycle, the chips were regenerated by flowing a solution of 0.3M NaOH for about 15 min. The regeneration step provided gold chips with the *Nxdown*-SH oligonucleotides immobilized. Absence of nonspecific binding was assessed by passing a solution of a pre-immune serum.

Multiplexed SPR procedure. (see

Figure 1, B) For this experiment a spotReady™ substrate with 16 gold spots per chip, with a spot diameter of 1 mm, was used and the solutions of *Nxdown*-SH oligonucleotides ($150\ \mu\text{g}\cdot\text{mL}^{-1}$ in the assay buffer) were deposited ($0.5\ \mu\text{L}/\text{spot}$) per triplicate for 1 hour at room temperature. Then, with the chip inside the SPR set up, a solution containing a mixture of the three steroid-oligonucleotide conjugates (*8-N_{1up}*/ *hG-N_{2up}*/ *13-N_{3up}*) was flowed to the cell for about 20 min, until a steady-state situation. Following the chip was washed (10 min) and the blocking solution was passed (10 min). After a new washing step, a solution with a mixture of the three specific antisera (*As147*/ *As170*/ *As138*, called *cocktail As*), was flowed for about 25 min until the signal reached the maximum, and washed again with buffer. The existence of no cross-hybridization between the different oligonucleotides pairs and the affinity of each antibody for the different steroid-oligonucleotide conjugates were previously studied (20).

RESULTS AND DISCUSSION

Synthesis and purification of steroid-oligonucleotide conjugates

A set of three complementary pairs of ssDNA (*N₁*, *N₂* and *N₃*, *up* and *down*) were synthesized. These sequences have 50% G:C content (purines/pyrimidines) and have the same composition but different sequence. In this way it is expected that the three resulting

duplexes will have similar hybridization properties. The sequences should display high hybridization efficiency for maximum hapten density at the surface after the immobilization. The specificity of the hybridization of these sequences has been previously studied in our group in a microarray multiplexed competitive format (20) and the results indicated hardly any cross-hybridization between the three sequences.

Once a complete set of oligonucleotides sequences were chosen, the steroid-oligonucleotide conjugates were prepared. The synthesis of oligonucleotides carrying steroids such as cholesterol (32, 33), hydroxyprogesterone (34), dexamethasone (35), digoxigenin (36), cholic (37), lithocholic (38) and bile acids (39) have been described in order to obtain hydrophobic derivatives of oligonucleotides for antisense, triplex and RNA interference studies (40). Most of these compounds are synthesized by conjugation of carboxylic acid derivatives to oligonucleotide carrying amino groups although the direct incorporation to oligonucleotides using the corresponding phosphoramidites have also been described for compounds that can be prepared in large amounts (32-34). In our case the amount of carboxylic acid derivatives available was low so post-synthetic methods were selected. Thus, ST, THG and B steroid-oligonucleotides conjugates were prepared through the formation of amide bonds (see Table 1). The steroidal haptens used in these conjugations have been previously used to develop enzyme linked immunosorbent assays (ELISAs) to detect AAS in real samples (28-30).

The conjugation of boldenone hapten (13) to amino-oligonucleotide *N₃up* was performed with the unprotected amino-oligonucleotide in aqueous solution. The hapten 13 has an ester group which in basic medium could be hydrolyzed. For this reason conjugation was not possible before ammonia deprotection. Therefore, we used the solution-phase coupling to obtain the desired conjugate 13-*N₃up* (B) in 20-30% yield (Figure S2, supporting

information). The NHS-ester of 13 was prepared by reaction of the carboxylate moiety with NHS using DCC (41). Increasing the molar excess of active ester from 10 to 30 times excess, allows the conversion of the amino-oligonucleotide to the desired conjugate to 45% (Figure S2, supporting information).. The mass obtained for this conjugated was 6400 m/z while the mass expected was 6639 m/z. We attribute this difference at the fact that during MALDI-TOF analysis fragmentation can occur as a result of heating (laser ionization) in the acidic environment (the matrix) and then what we could observe is the oligonucleotide with the steroid attached hydrolyzed at the ester point. This was confirmed by using electrospray MS analysis. The softer ionization conditions provided the expected mass (13-*N_{3up}*, calc 6639, found 6639).

On the other hand, it had been also reported that solid phase synthesis may be advantageous to the standard synthesis in solution (42) since it allows forming the amide bond in organic solvent in which the steroidal haptens have better solubility. Thus, the conjugates 8-*N_{1up}* (ST) and hG-*N_{2up}* (THG) (Table 1) were obtained in higher yields (52-40%), demonstrating that the conjugation reaction is clearly advantageous using the solid-phase coupling protocol. Both conjugates were characterized with MALDI-TOF mass spectrometry (8-*N_{1up}*, calc 6668, found 6672; hG-*N_{2up}*, calc 6619, found 6614).

In order to assess the influence of the steroids in the duplex structure denaturation curves were analyzed. Table S1 (supplementary information) shows the melting temperatures of duplexes carrying the steroids stanozolol (8), tetrahydrogestrinone (hG), and boldenone (13). As expected, the presence of the steroid at the 5'-end of the duplex has very little effect in the stability of the duplex.

The recognition of the oligonucleotide-steroid conjugates by specific antibodies was analyzed by immobilization of the duplex DNA carrying steroids on glass surfaces

followed by incubation with the specific antisera and the visualization of the antigen-antibody interaction with a fluorescently labeled anti-rabbit secondary antibody as described previously [20]. The presence of the oligonucleotides chain does not affect to the recognition of antigen antibody as it can be observed in the detectability parameters (LOD and IC₅₀) in the table S2 in the supplementary material.

Evaluation by SPR of the biofunctionality of the steroid-oligonucleotide conjugates

Hybridization. The specificity of the hybridization step was assessed by SPR by flowing the solutions of the steroid-*N*₁₋₃*up* conjugates into a cell containing the *N*₁₋₃*down* functionalized chips and recording the SPR signal (see Figure 2). The DNA double helix is stabilized by hydrogen bonds between the bases of the two strands. The strength of the double-stranded formation depends on a) the number of matching bases in a sequence, b) the number and loci of mismatches and c) the base composition. The prevailing DNA architecture, the double helix, has well stacked nearly parallel bases with overlapping π -electron systems. Such π -electron systems may be good candidates for long-distance and one-dimensional (linear) charge transport (43). Yoo et al. (44, 45) demonstrated differences in conductivity due to variation in the base pair sequence. Poly(dG).Poly(dC) showed a conductivity of 40 S/cm, whereas that for Poly(dA).Poly(dT) was about 0.1 S/cm. This fact could be an explanation to the different behavior in the hybridization between the three hapten-oligonucleotide conjugates, because as it can be observed in Figure 2, *N*₁*down*/*N*₁*up*-8 pair has a lower change in the SPR signal than *N*₂*down*/*N*₂*up*-hG and *N*₃*down*/*N*₃*up*-13 pairs. In Table 1, it can be observed that the sequence of the oligonucleotide *N*₁*down* at the 5'-end has CAG whereas in the sequences *N*₂*down* and *N*₃*down*, in both cases we have CGG. Therefore, changes in the conductimetric properties

of the double helix, could affect the refractive index and influence in the SPR signal (46, 47).

Immunochemical recognition. Three specific antisera (As147, As170 and As138, 1/250 dilution) and a non-specific serum (CTR-NEG) were used to test if after the conjugation of the steroids with the oligonucleotides chains, the antibodies could still recognize their corresponding steroidal haptens. The features of these antibodies have been previously described (28-30). Results presented in the Figure 3 and Figure S1 (supporting information), show the change in the SPR signal recorded as result of three consecutive cycles in different chips for each system (ST, THG and B). The first cycle, shows the specific antibody binding to each steroid-oligonucleotide conjugate (steroid- N_{1-3} -up) hybridized on the surface of the sensor. On each graph it can also be observed the absence of nonspecific signal when the CTR-NEG serum was injected, after regenerating the surface. Similarly, subsequent injection of the specific antiserum on systems ST and THG allows recovering again the same signal. In the case of B system the signal of the third cycle is lower than the signal obtained of the first cycle, but both first and third cycle are clearly much higher than the non-specific (CTR-NEG) second cycle. The difference between the first and the third cycle for the B system (N_{3down} - N_{3-up} -13/As138) could be attributed to the low S/N ratio obtained or to a small lost of the binding response. The differences observed in the maximum of the signal and in the slope of the binding step, between the three systems N_1 , N_2 , N_3 (see Figure 3) is due to the distinct antisera affinity for the corresponding steroid-oligonucleotide conjugates. And another remarkable fact is the complete regeneration of the surface with a simple washing step with diluted NaOH solution, which offers to these chips the possibility to be reused.

Finally, as a proof of concept, the possibility to develop a multiplexed SPR immunosensor was demonstrated. In this experiment the DNA-directed immobilization of a cocktail of steroid-oligonucleotide conjugates was used to detect the three AAS simultaneously, under competitive conditions (see Figure 4). The DNA biofunctionalized chip was prepared using a spotReady™ substrate with 16 gold spots per chip in which the oligonucleotides *N1down*-SH, *N2down*-SH and *N3down*-SH were deposited per triplicate spots. Then, with the chip placed inside the SPR set up, the assay was performed as described in the experimental section by flowing a solution containing a mixture of the three steroid-oligonucleotide conjugates (8-*N1up*/ hG-*N2up*/ 13-*N3up*) (Figure 4 left), followed by the *cocktail* As (Figure 4 right). As it can be observed the change in the refractive index produced on the hybridization step and as result of the antibody binding is recorded as an SPR signal on each respective spot demonstrating the possibility of using DDI to prepare hapten-biofunctionalized SPR chips responding simultaneously to the presence of the corresponding specific antibodies.

CONCLUSIONS

Immunochemical methods show great potential for detection of small targets for a large number of applications. In spite of the powerful analytical tools that are being developed, one of the important issues is the efficient immobilization of analytes to the sensing surfaces. The present work demonstrates the potential of oligonucleotide-steroid conjugates for DNA-directed immobilization of haptens on sensor surfaces. To this end, we have demonstrated that three different steroids can be conjugated to oligonucleotides using the same steroid derivatives used for the obtention of protein-conjugates needed for

immunization. Moreover, the results show that coupling of the hapten to the oligonucleotides preserves the antibody recognition properties allowing the construction of a biosensor for the specific detection of steroids. The presence of the steroid do not affect the hybridization properties of the oligonucleotides. The immobilization of the conjugates to the gold sensing surface is performed by immobilization of the complementary strand carrying a thiol group followed by hybridization. The DNA-directed immobilization strategy presents the advantage of the possibility to re-use the same sensor chip for multiple measurements after an easy dehybridization step. Moreover, consecutive measurement cycles addressed to detect other molecules could be performed on the same DNA-chip, by just binding distinct haptens, proteins, or antibodies, to the complementary oligonucleotides strands. The strategy presented here shows a valuable approach for small organic molecule detection on biosensors. It has been demonstrated the coupling of the hapten to the oligonucleotide preserves the antibody recognition properties allowing the construction of a biosensor for the specific detection of esteroids. For these reasons, the use of the DNA-direct immobilization strategy opens the way to get a multiplexed imaging SPR immunosensor by easily immobilizing distinct haptens on different spots of the ssDNA biofunctionalized chip. This methodology opens the possibility to detect a large number of analytes simultaneously using a DNA site-encoded chip in multichannel SPR devices (12, 22) and SPR-imaging biosensors (23-27), or in a future, using also encoded nanoparticles biofunctionalized with the specific antibodies for multiplexes localized surface plasmon resonance (LSPR) sensing. Finally, the methodology described for the preparation of the steroid-oligonucleotide conjugates may be useful for the generation of potential nucleic acids drugs.

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Supporting Information Available. Examples of complete SPR sensograms obtained for each system, stanozolol (ST, 8), tetrahydrogestrinone (THG, hG) and boldenone (B, 13). HPLC analysis and mass spectra of oligonucleotide conjugate 13-N₃up. Melting temperatures of duplexes carrying steroids and analysis of the recognition of the conjugates with specific antisera. This material is available free of charge via the internet at <http://pubs.acs.org>

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Table 1: Oligonucleotides sequences and chemical structures of the steroidal haptens used in this study.

Name	Sequence
$N_{1down-SH}$	5' HS-(CH ₂) ₆ -CAGGACAATATCGTTGCGTG 3'
8- N_{1up}
$N_{2down-SH}$	5' HS-(CH ₂) ₆ -CGGAGGTACATTGCGACTTGA 3'
hG- N_{2up}
$N_{3down-SH}$	5' HS-(CH ₂) ₆ -CGGATAGAGCACTACGTTGT 3'
13- N_{3up}

Scheme 1: Synthesis of steroid-oligonucleotide conjugates where a) ammonia treatment, b) hapten coupling using DCC and NHS and c) hapten coupling using PyBOP and DIPEA.

Figure 1: Schematic representation of the experimental procedure employed to assess the steroid-oligonucleotide bioconjugate biofunctionality by SPR. ssDNA biofunctionalized chips are used to immobilize the haptens through hybridization with the corresponding steroid-oligonucleotide bioconjugates. Subsequently, biofunctionality is assessed by exposing the chips to specific antibodies. A) Evaluation of the biofunctionality of each steroid-oligonucleotide conjugate (8- N_1up , hG- N_2up and 13- N_3up) using the individual SPR procedure. B) Evaluation of the DNA site-encoded applicability of these conjugates for the development of a multiplexed SPR immunosensor.

Figure 2: SPR sensograms recorded on the hybridization step between the steroid-oligonucleotide conjugates and the oligonucleotides down previously immobilized on the chip surface.

Figure 3: SPR sensograms recorded on the antibody binding step: ST (top), THG (middle) and B (bottom). Each graph shows the response of three consecutive cycles, after regeneration of the surface at the end of each cycle: a) first cycle: specific antibody; b) second cycle: preimmune serum; c) third cycle: specific antibody.

Figure 4: Sensograms recorded in the DNA-directed SPR immunosensor; on the left, the specific hybridization of the steroid-oligonucleotide conjugates with their complementary strands previously immobilized on the gold chip; on the right, the immunochemical recognition of each specific antibody for the corresponding steroid.

Graphical abstract