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RESEARCH PAPER

Nucleic acid sensing with enzyme-DNA binding protein conjugates cascade and simple DNA nanostructures

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Abstract A versatile and universal DNA sensing platform is presented based on enzyme-DNA binding protein tags conjugates and simple DNA nanostructures. Two enzyme conjugates were thus prepared, with horseradish peroxidase linked to the dimeric single-chain bacteriophage Cro repressor protein (HRP-scCro) and glucose oxidase linked to the dimeric headpiece domain of *Escherichia coli* LacI repressor protein (GOx-dHP), and used in conjunction with a hybrid ssDNA-dsDNA detection probe. This probe served as a simple DNA nanostructure allowing first for target recognition through its target-complementary single-stranded DNA (ssDNA) part and then for signal generation after conjugate binding on the double-stranded DNA (dsDNA) containing the specific binding sites for the dHP and scCro DNA binding proteins. The DNA binding proteins chosen in this work have different sequence specificity, high affinity, and lack of cross-reactivity. The proposed sensing system was validated for the detection of model target ssDNA from high-risk human papillomavirus (HPV16) and the limits of detection of 45, 26, and 21 pM were achieved using the probes with scCro/dHP DNA binding sites ratio of 1:1, 2:1, and 1:2, respectively. The performance of the platform in terms of limit of detection was comparable to direct HRP systems using target-specific oligonucleotide-HRP conjugates. The ratio of the two enzymes can be easily manipulated by changing the number of binding sites on the detection probe, offering further optimization possibilities of the signal generation step. Moreover, since the signal is obtained in the absence of externally added hydrogen peroxide, the described platform is compatible with paper-based assays for molecular diagnostics applications. Finally, just by changing the ssDNA part of the detection probe, this versatile nucleic acid platform can be used for the detection of different ssDNA target sequences or in a multiplex detection configuration without the need to change any of the conjugates.

Keywords Nucleic acid detection · DNA binding proteins · Single-chain bacteriophage lambda Cro repressor (scCro) · Dimeric headpiece domain of *Escherichia coli* LacI repressor (dHP) · Horseradish peroxidase (HRP) · Glucose oxidase (GOx)

Introduction

The field of molecular diagnostics is one of the fastest growing sectors in clinical analysis [1], with the DNA diagnostics market expected to generate $19 billion globally by 2020 [2]. Clinical applications of molecular diagnostics are usually found in the areas of genetic diseases, cancer, infectious diseases, pharmacogenomics, as well as special applications like HLA typing and forensic science [3]. The association of specific DNA sequences with disease as well as the recent outbreaks of infectious diseases like Ebola, MERS, and Zika viruses has increased the demand for rapid, sensitive, and point-of-care testing.

Usually, the platforms used for DNA detection rely on hybridization of the target single-stranded DNA (ssDNA) sequence with complementary sequences in order to facilitate the recognition and detection with specific capture and detection probes, respectively. For the detection step, a signal

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amplification strategy is often used in order to generate a reliable signal after target identification that will subsequently allow its quantification [4]. Target-specific labeled oligonucleotides are commonly used for this purpose, resulting in elevated assay costs due to the additional labeling. In our previous work, we reported the development of a universal, target-independent signal amplification approach in DNA biosensors. A novel conjugate of horseradish peroxidase with single-chain bacteriophage lambda Cro DNA binding protein (HRP-scCro) was described and used for the detection of a model target ssDNA through the binding of the scCro DNA binding protein with its specific sequence contained in the hybrid ssDNA-dsDNA detection probe [5]. A signal enhancement effect was observed according to the number of conjugate binding sites on the detection probe leading to increased biosensor sensitivity.

In the present report, we combine the synergistic action of HRP with glucose oxidase (GOx) to provide a measurable signal with the properties of DNA binding proteins of high affinity and specificity regarding their double-stranded DNA (dsDNA) binding sequences in order to detect the target ssDNA (Fig. 1a). The system relies on target recognition by a specific capture probe while target detection is accomplished by using a hybrid ssDNA-dsDNA detection probe and the two enzymes as DNA binding protein conjugates: the previously reported HRP-scCro conjugate [5] and the conjugate described in this work of GOX with the dimeric headpiece domain of the *Escherichia coli* LacI repressor protein dHP (GOX-dHP). The detection probe contains the specific sequence required for target hybridization (ssDNA) and the two distinct sequences for the binding of the enzyme-DNA binding protein tag conjugates (dsDNA) (Fig. 1b). The bi-enzymatic HRP/GOx cascade system has been used extensively over the decades due to the numerous advantages it presents, such as rapid signal generation, high enzyme stability, possibility for immobilization without significant loss of activity, low cost, and high turnover numbers [6–10]. At the same time, the in situ generation of hydrogen peroxide from the GOx-catalyzed oxidation of glucose has promoted the wide use of this bi-enzymatic system as a convenient method to avoid the direct utilization and storage of hydrogen peroxide in point of care biosensors. The catalytic efficiency of this cascade enzyme reaction strongly depends on the diffusion of hydrogen peroxide since HRP has a much higher turnover rate than GOx [11] and this diffusion is regulated by the distance between the active sites of the two enzymes. In the system described herein, the target detection probe served as a simple DNA nanostructure for the spatial organization of the two enzyme conjugates in such a way as to allow their simultaneous binding avoiding steric hindrance effects, minimize hydrogen peroxide diffusion, and provide improved performance of the sensor for target detection. Furthermore, the signal generation step could be further optimized by altering the ratio of the two enzymes and this was easily accomplished by changing the number of the DNA binding sites on the detection probe. The preparation and characterization of the enzyme-DNA binding protein tag conjugates, along with optimization of conditions for DNA detection are discussed in detail. The proposed platform was used to detect ssDNA from the model target high-risk human papillomavirus HPV type 16.

Materials and methods

Materials

PCR reagents, restriction enzymes (NcoI and HindIII), plasmid pBADmychisA, sulfo-SMCC, HisPure Ni-NTA-agarose, SP Sepharose FF, maleimide-activated HRP, and neutravidin-coated microplate strip plates were purchased from Fisher Scientific (Barcelona, Spain). Glucose oxidase from *Aspergillus niger* and antibodies were obtained from Sigma (Madrid, Spain) and the immune-blot PVDF membrane from

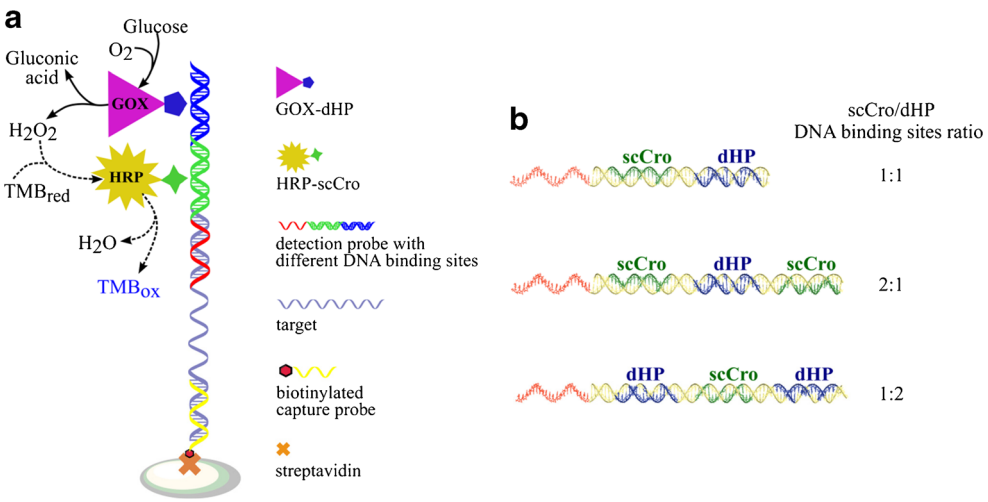


Fig. 1 **a** Detection of target ssDNA based on DNA binding proteins-enzyme conjugates. **b** Hybrid ssDNA-dsDNA detection probes with scCro/dHP DNA binding sites stoichiometry of 1:1, 2:1, and 1:2. The binding sites (highlighting the nucleotide bases that directly participate in the dsDNA-protein interactions) for scCro are shown in *green* and for dHP in *blue*

Bio-Rad (Barcelona, Spain). The DNA oligonucleotides were purchased from Biomers.net (Ulm, Germany) and Eurofins (Ebersberg, Germany) and their sequences are shown in Table S1 in the Electronic Supplementary Material (ESM). The sequences of the oligonucleotides used for HPV16 detection (ssDNA capture and detection probes, ssDNA synthetic HPV16 target) were based on previously reported sequences, which were verified for the detection of DNA extracted from cervical scrapes through PCR [12]. All other reagents were purchased from Fisher Scientific (Barcelona, Spain), Sigma-Aldrich (Madrid, Spain), and Scharlau (Barcelona, Spain).

Preparation of the enzyme-DNA binding protein tag conjugates

The HRP-scCro conjugate was prepared as described previously [5]. For the preparation of the GOx-dHP conjugate, glucose oxidase was initially reacted with a tenfold excess of sulfo-SMCC for 1 h at 25 °C in conjugation buffer (PBS, pH 7) in order to label the protein's free amines with maleimide groups. Excess crosslinker was removed using a desalting column equilibrated with conjugation buffer. Then, maleimide-activated GOx and cys-dHP (prepared as detailed in the ESM, part S1) were mixed at equimolar concentrations in conjugation buffer, and the mixture was incubated overnight at 4 °C. The GOx-dHP conjugate was purified by immobilized metal affinity chromatography (IMAC) and ion exchange chromatography (IEC). For IMAC, a HisPur Ni-NTA-agarose column was washed with equilibration buffer (10 mM potassium phosphate, 300 mM NaCl, pH 7.4) and then loaded with the conjugation mixture. The column was washed with equilibration buffer containing 10 mM imidazole and the conjugation product was eluted with the same buffer containing 500 mM imidazole. The buffer of the IMAC elution fraction was exchanged to IEC buffer (20 mM Tris-HCl, pH 7) and the sample was loaded on a DEAE Sepharose FF weak cation exchange resin-packed column equilibrated with the same buffer. The conjugate was eluted with IEC buffer containing 1 M NaCl, and the buffer was exchanged to storage buffer (100 mM potassium phosphate pH 5.1, 50% glycerol). The concentration of the conjugate was calculated using the absorbance at 452 nm of the FAD cofactor of GOx ($\varepsilon_{452\ nm}$ = 12.83 cm^{-1} mM^{-1}) [13]. The process was monitored by SDS-PAGE, native PAGE, and Western blot. For Western blot, the PVDF membrane after transfer was probed with monoclonal mouse anti-His antibody and then with polyclonal rabbit anti-mouse-HRP antibody and finally the bands were detected with 1-STEP TMB-Blot solution. For native PAGE, proteins were separated on a 7.5% polyacrylamide gel in Tris-glycine buffer (pH ~ 8.3). MALDI-TOF mass spectrometry was used to calculate the exact molecular weight of the conjugate.

Preparation and characterization of the hybrid ssDNA-dsDNA detection probes

The detection probes, containing binding sites for the enzyme-DNA binding protein tag conjugates at different stoichiometries, were prepared by the hybridization of partially complementary ssDNA oligonucleotides (pairs P88/P89, P92/P93, P94/P95) with sequences as described in the ESM Table S2. Each oligonucleotide pair were mixed in hybridization buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA) at a final concentration of 10 μM each oligo in a total of 20 μl, heated for 5 min at 95 °C and then cooled to 25 °C by 1 °C/min. Hybridization was validated by gel electrophoresis of the hybridization reactions (ESM, Fig. S2). Electrophoretic mobility shift assay (EMSA) was also used to evaluate the binding of the enzyme conjugates HRP-scCro and GOx-dHP to the prepared detection probe containing one binding site for each enzyme conjugate. The binding mixtures were prepared in a total volume of 20 μl in binding buffer (20 mM potassium phosphate pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.05% Tween-20, 5% glycerol). The detection probe (final concentration 200 nM) was incubated for 1 h at room temperature with each protein species separately (final concentration 600 nM of each maleimide-activated HRP, maleimide-activated GOx, cys-scCro, cys-dHP, HRP-scCro, and GOx-dHP) and also in mixtures of the two DNA binding proteins and their conjugates. The binding mixtures were then separated on a 5% polyacrylamide/TAE gel for 60 min at 80 V in TAE buffer and finally visualized by GelRed DNA gel staining.

Colorimetric detection of DNA with ELONA

Detection of the model target HPV16 ssDNA was achieved with a colorimetric enzyme linked oligonucleotide assay (ELONA) using neutravidin-coated microplate wells. The buffer used for DNA immobilization and the hybridization steps was ELONA buffer (20 mM potassium phosphate pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5% BSA, 0.05% Tween-20), whereas for washing, the same buffer was used without BSA. The immobilization of the biotinylated capture probe (50 μl) was performed for 15 min at room temperature and the wells were washed three times with 300 μl of wash buffer before the addition of target ssDNA (50 μl). Capture probe-target hybridization was allowed to proceed for 1 h at 37 °C followed by three washes with 300 μl of wash buffer and the addition of 50 μl of pre-hybridized ssDNA-dsDNA detection probes (with one or two binding sites for each DNA binding protein, prepared as described in the previous section). Hybridization was carried out for 1 h at 37 °C and after three washes with 300 μl of wash buffer, the equimolar mixture of the two conjugates HRP-scCro and GOx-dHP was added and incubation proceeded for 1 h at room temperature. After a final

washing step (three times of 300 μl wash buffer), 50 μl of a mixture of 250 mM glucose and 1 mM TMB (freshly prepared in 100 mM potassium phosphate pH 5) were added to the wells and the absorbance at 650 nm was monitored for 45 min at room temperature using a Cary 100 Bio UV visible Spectrophotometer. Color development was stopped by the addition of 50 μl of 1 M H_2SO_4 and finally the absorbance at 450 nm was measured. Optimization of the ELONA assay was performed in terms of capture and detection probes concentration as well as the addition order of the enzyme-DNA binding proteins conjugates using the general ELONA assay setup described above. Optimized conditions were finally used to obtain target calibration curves.

Optimization of capture probe concentration

The capture probe concentration used to immobilize on the neutravidin-coated microplate was titrated from 10 to 50 nM. The target ssDNA was used at 1 nM, the detection probes with scCro/dHP DNA binding sites ratio of 2:1 and 1:2 at 3 nM, and the enzyme conjugates (HRP-scCro and GOx-dHP) at 5 nM each with simultaneous addition.

Optimization of detection probe concentration

Using a capture probe concentration of 20 nM and the target ssDNA at 1 nM, the concentration of the detection probes was titrated from 0.2 to 3 nM. The three detection probes with scCro/dHP DNA binding sites ratio of 1:1, 2:1, and 1:2 were used, while the enzyme conjugates were added simultaneously (5 nM each).

Binding order of the enzyme-DNA binding protein tags conjugates

The capture probe, target ssDNA, and each of the three detection probes were used at constant concentrations of 50, 1, and 3 nM, respectively. The two conjugates HRP-scCro and GOx-dHP (5 nM each) were added either simultaneously (GOx-dHP + HRP-scCro) or in two steps: the first conjugate was allowed to bind initially, then the wells were washed three washes with 300 μl wash buffer and finally the second conjugate was added (first HRP-scCro and second GOx-dHP or first GOx-dHP and second HRP-scCro).

Target ssDNA calibration curves

The biotinylated HPV16 capture probe was used at 20 nM, target ssDNA at concentrations up to 1 nM, the detection probes (with one or two binding sites for each DNA binding protein, prepared as described above) at 3 nM, and finally the two conjugates HRP-scCro and GOx-dHP (5 nM each) were finally added simultaneously. Each sample was measured in triplicate with three independent experiments, whereas the blank measurements were conducted in sextuplets. Limit of detection (LOD) for each detection probe was calculated as the average of blank measurements plus three times the standard deviation of the blank measurements.

ELONA assay specificity

The specificity of the detection platform was studied by performing the ELONA assay using optimized conditions (as described above for the target calibration curves) with 5 nM of ssDNA from the target HPV16 and control non-complementary sequences from breast cancer markers (CDH1 and HUWE1) and a celiac disease marker (HLA-DQ1*0201). All the sequences used are shown in Table S4 in the ESM.

Results and Discussion

DNA binding protein tags

The DNA sensing platform described in this work is based on the synergistic action of two enzymes, HRP and GOx, in order to link the step of target ssDNA recognition and signal generation for detection (Fig. 1a). Therefore, two DNA binding proteins with different dsDNA binding sequence specificities were needed to be conjugated to the two enzymes respectively. In a previous work, we developed a single-chain dimeric DNA binding protein of the bacteriophage lambda repressor protein (Cro) with an N-terminal cysteine for conjugation purposes [5]. It was then conjugated to HRP and the enzyme-DNA binding protein tag conjugate was used for signal amplification in the detection of model target ssDNA. Here, a second DNA binding protein was chosen, the headpiece domain of LacI protein, for conjugation to GOx based on the same criteria of size, affinity, and specificity used for the first DNA binding protein tag chosen, Cro. The two proteins have different sequence specificities, a property that will ensure their specific binding to their respective sequences, avoiding cross-reactivity issues. LacI is a homotetrameric repressor protein from *E. coli* which regulates the transcriptional activity of the lactose operon [14]. It has a promoter-operator interaction region [headpiece domain, LacI(1–58)] which binds to a specific dsDNA sequence with very high affinity and specificity (K_D = 30 pM) [15]. Using this DNA binding domain, a new DNA binding protein was engineered by linking together two LacI headpiece domains since the native protein binds DNA as a homodimer. The crystal structure of the homodimeric headpiece domain bound to an operator DNA sequence [15, 16] suggested that a GGSGGS flexible peptide linker could be used to link the two monomers because of the close proximity of the polypeptide ends to be linked. In addition, it also

indicated that a C-terminal poly-histidine tag could be added for purification purposes without impacting binding. In order to facilitate conjugation of the DNA binding protein, a single cysteine residue was added to its N-terminus taking advantage of the absence of cysteine residues in the native headpiece sequence. This single-chain homodimeric LacI headpiece domain protein with an engineered N-terminal cysteine (cys-dHP) was cloned and expressed in soluble form in *E. coli* (see ESM, part S1). The majority of protein contaminants were removed by IMAC via the C-terminal poly-histidine tag and the IMAC elution fraction was further polished with cation exchange chromatography. The purified protein cys-dHP was finally obtained at more than 90% purity with a total yield of approximately 2 mg/L of bacterial culture.

Enzyme-DNA binding protein tag conjugates

The proposed DNA detection system relies on the use of two DNA binding protein-enzyme conjugates, HRP-scCro and GOx-dHP, for signal generation. The preparation and characterization of the HRP-scCro conjugate has already been described in our previous work [5]. To prepare the GOx-dHP conjugate, the heterobifunctional crosslinker sulfo-SMCC was used in order to covalently link solvent accessible amine groups of GOx with the single thiol group of dHP. GOx is a homodimeric glycosylated protein with a molecular weight of 130–320 kDa depending on the extent of the glycosylation pattern [17]. The enzyme most commonly used in biochemical assays is isolated from *A. niger* with two subunits with a molecular weight of 80 kDa each and approximately 18% sugars. According to its amino acid sequence and crystal structure (using the structure 3QVP.pdb), there are 14 amine groups on the surface of the protein that can potentially react with sulfo-SMCC. On the other hand, the DNA binding protein cys-dHP contains only one thiol group that has been specifically engineered (in the form of a single cysteine residue) at its N-terminal to enable its conjugation with maleimide-containing molecules. The amine groups of GOx were activated with an excess of sulfo-SMCC and the conjugation with cys-dHP was performed using an equimolar mixture of the two proteins in order to achieve a low conjugation ratio. After overnight incubation at 4 °C, the efficiency of the conjugation reaction was estimated at approximately 25% by comparing the intensity of the SDS-PAGE bands corresponding to cys-dHP before and after conjugation (Fig. 2a, lanes 1 and 4) using the ImageJ program. Different conditions for the activation of GOx with sulfo-SMCC as well as the use of molar excess of either protein in the conjugation reaction did not improve the conjugation efficiency (results not shown). The GOx-dHP conjugate was purified with a two-step procedure enabling the removal of unreacted proteins. With IMAC purification, it was possible to remove unreacted maleimide-GOx that did not contain any His-tag (Fig. 2a, lane 5), whereas IEC was used for the removal of unreacted cys-dHP (Fig. 2a, lane 6) based on the charge difference between the two proteins (GOx pI ~ 4.2 according to the supplier, cys-dHP pI ~7.9 calculated from its amino acid sequence). Based on SDS-PAGE analysis, three protein bands are associated with the conjugate: one at approximately 75 kDa corresponding to the GOx monomer, and two bands at 100 and 100–130 kDa corresponding to potential conjugates of GOx monomers with one and two dHP per monomer, respectively (calculated size of cys-dHP ~ 16.5 kDa). Western blot analysis revealed two distinct protein species with incorporated His-tag (Fig. 2b, lane 4) corresponding to the two bands between 100 and 130 kDa identified with SDS-PAGE. These results suggested the synthesis of two conjugation products: one product with one dHP per GOx dimer and one product with two dHP per GOx dimer. In an effort to further characterize the conjugate, we performed native PAGE as shown in Fig. 2c GOx in its native form migrates on the gel as a single band corresponding to the homodimer (Fig. 2c, lanes 1 and 2), whereas the conjugate migrates slower as a single band with higher molecular weight (Fig. 2c, lane 3), corresponding to a single conjugation product. To accurately determine the size of the conjugation product(s) and the GOx:dHP stoichiometry within the conjugate, MALDI-TOF mass spectroscopy was performed. As shown in Fig. 2d, there is one single conjugation product with a molecular weight of approximately 168 kDa corresponding to one GOx homodimer conjugated with one dHP (see ESM, part S3). The three distinct bands of the GOx-dHP conjugate identified by SDS-PAGE with molecular weights in the range of 75–120 kDa can be assigned to GOx monomer, and to GOx monomer with one dHP conjugated at different locations on the enzyme molecule surface, resulting in two molecules with different morphologies and electrophoretic mobility within the polyacrylamide gel matrix.

DNA detection probes

For optimal target detection, the design of the detection probes was performed taking into account the two distinct roles it had to perform, target recognition and signal generation. As detailed in our previous work, the detection probes were hybrid consisting of a ssDNA and a dsDNA part: target recognition is accomplished by hybridization with the ssDNA part, whereas the dsDNA contains the specific DNA binding sites for the binding of the DNA binding protein-enzyme conjugates that will ultimately provide the signal for detection. To generate these hybrid detection probes, partially complementary oligonucleotides were hybridized as detailed in the ESM, part S2. A 5-bp spacer was used to separate the two parts and the distinct functions of the probe, hybridization with the target and binding of the enzyme conjugates. In turn, the DNA binding sites were specifically arranged on the dsDNA part of the detection probes and separated by 3-bp spacers to try to minimize steric

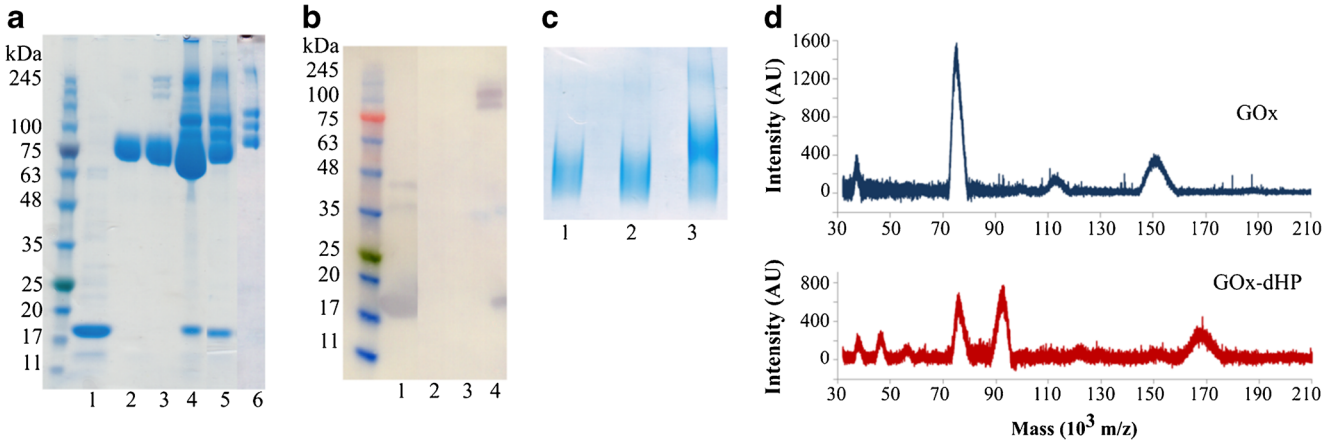


Fig. 2 Preparation of the GOx-dHP conjugate. **a** SDS-PAGE for the conjugation and purification of GOx-dHP. *1*: cys-dHP; *2*: GOx; *3*: maleimide-GOx; *4*: GOx-dHP conjugation reaction; *5*: IMAC elution fraction; *6*: IEC elution fraction. **b** Detection of conjugate by Western blot. *1*: cys-dHP; *2*: GOx; *3*: maleimide-GOx; *4*: GOx-dHP conjugation reaction. **c** Native PAGE. *1*: GOx; *2*: maleimide-GOx; *3*: GOx-dHP conjugate. **d** MALDI-TOF mass spectroscopy spectra of GOx and GOx-dHP-purified conjugate

hindrances and allow the co-binding of both HRP-scCro and GOx-dHP conjugates. Different configurations of the detection probe were designed and prepared in order to vary the stoichiometry of the two enzymes HRP and GOx and study their effect on target detection. For this reason, HRP-scCro/GOx-dHP stoichiometries of 1:1, 2:1, and 1:2 were chosen. Models of the three different detection probes were generated using the online molecular modeling tool model.it® [18], visualized with the PyMOL Molecular Graphics System (Schrödinger, LLC) and they are illustrated in Fig. 1b. As it can be seen, the constant 3-bp spacing between the binding sequences results in different relative orientations of the binding sites on the dsDNA double helix structure. This should facilitate the simultaneous binding of multiple conjugates and minimize any steric hindrance effects that might be encountered due to the size of the conjugates (61 kDa for HRP-scCro and 168 kDa for GOx-dHP).

Successful simultaneous binding of the two DNA binding protein-enzyme conjugates to the detection probe with one binding site for each conjugate was verified with an electrophoretic mobility shift assay (EMSA). The detection probe was incubated separately with different protein species and the samples were separated in a polyacrylamide gel. Free DNA and protein-bound DNA could be separated efficiently because of differences in their mobility. As it is shown in Fig. 3, the DNA binding protein tags cys-scCro and cys-dHP as well as their respective enzyme conjugates HRP-scCro and GOx-dHP were able to bind the detection probe and cause a decrease in its mobility depending on the size of the protein bound. The highest decrease was observed for the DNA detection probe bound with the two enzyme conjugates HRP-scCro and GOx-dHP simultaneously (Fig. 3, lane 9) because of the total size of proteins bound (approximately 229 kDa). Therefore, EMSA results validated the design of the detection probes and co-binding of the enzyme conjugates.

Target ssDNA detection with ELONA

In the proposed system, DNA detection is based on the use of hybrid ssDNA-dsDNA detection probes and HRP-scCro and GOx-dHP enzyme conjugates for signal generation. The combination of HRP and GOx in a cascade configuration has been reported extensively in the literature in a variety of assays and applied to commercial devices for the detection of various analytes. The system developed in this work is outlined in Fig. 1 and it is based on (a) the recognition of target ssDNA by a specific capture probe immobilized on a microtiter plate and (b) detection by the hybrid ssDNA-dsDNA probe containing binding sites for the DNA binding protein-enzyme conjugates HRP-scCro and GOx-dHP. Addition of glucose and TMB initiates the GOx-mediated oxidation of glucose and production of gluconic acid and hydrogen peroxide. Hydrogen peroxide is then scavenged by HRP that reduces

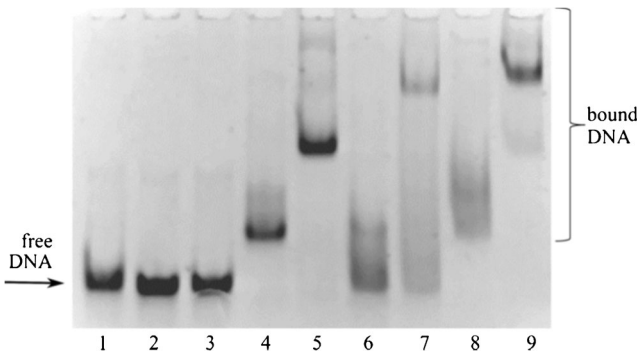
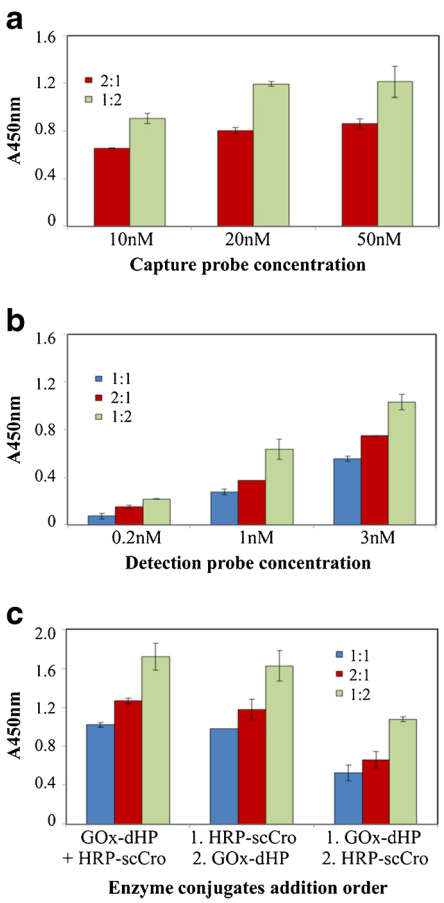


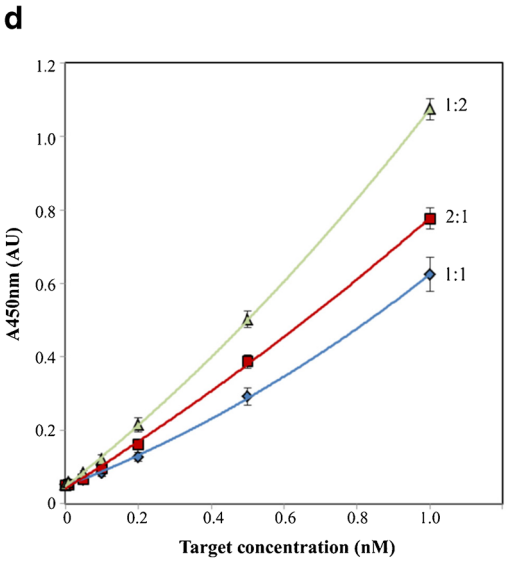
Fig. 3 EMSA assay for the binding of the DNA binding protein-enzyme conjugates to the HPV16 hybrid detection probe with scCro/dHP DNA binding sites ratio of 1:1. All *lanes 1–9* contain the DNA detection probe with different protein species. *Lane 1*: only DNA; *lane 2*: HRP; *lane 3*: GOx; *lane 4*: cys-scCro; *lane 5*: HRP-scCro; *lane 6*: cys-dHP; *lane 7*: GOx-dHP; *lane 8*: cys-scCro and cys-dHP; *lane 9*: HRP-scCro and GOx-dHP

it to water using TMB as the electron donor that eventually turns to blue after oxidation and provides the signal. For optimal assay performance, the ELONA was initially optimized in terms of capture and detection probes concentration, as well as the addition order of the DNA binding protein-enzyme conjugates. Firstly, the capture probe concentration was varied from 10 to 50 nM while maintaining constant the concentrations of target, detection probes (with one or two binding sites for each enzyme conjugate), and enzyme conjugates. As it can be seen in Fig. 4a, the use of more than 20 nM of capture probe concentration did not significantly increase the signal irrespectively of the detection probe used; therefore, a concentration of 20 nM capture probe was chosen. Next, the concentration of the detection probes was titrated from 0.2 to 3 nM. All three detection probes with a HRP-scCro/GOx-dHP stoichiometry of 1:1, 2:1, and 1:2 were used and the enzyme conjugates were added simultaneously at 5 nM each. The data in Fig. 4b show a signal increase with the increase of the detection probes concentration thus a concentration of 3 nM was chosen for further work. In addition, the effect of the order in which the enzyme conjugates are added was also explored. The GOx-dHP conjugate is bigger in size (approximately 168 kDa) compared to the HRP-scCro conjugate (61 kDa) and binding of either conjugate might be obstructed by binding of the other depending on the arrangement of the specific DNA binding sites on the detection probe. As it can be seen in Fig. 4c, the highest signal was obtained when the two enzyme conjugates were added simultaneously, whereas hindrance was observed mainly in the case where the bulkier GOx-dHP conjugate was added first and bound to the detection probe prior to the addition of the second smaller conjugate HRP-scCro.

Using the optimized conditions detailed above, target ssDNA calibration curves were obtained for each of the three detection probes as shown in Fig. 4d. All three curves were successfully fitted to a quadratic model, to relate the amount of signal (absorbance) to the target ssDNA concentration. This type of polynomial calibration curves have been reported before when these two enzymes were used for analyte detection [19–21]. The limit of detection (LOD) of the assay was also calculated as three times the standard deviation of the blank plus the average of blank measurements. The LODs achieved were all in the low picomolar range: 45, 26, and 21 pM for detection using the probes with scCro/dHP DNA binding sites ratio of 1:1, 2:1, and 1:2, respectively. Overall, higher sensitivity and lower LODs were obtained using the detection probe containing one binding site for HRP-scCro and two for GOx-dHP. Also, the performance of the described system in terms of LOD is comparable to the majority of electrochemical biosensors (LODs between 100 pM and 50 nM) that are the most common type used for DNA-based HPV detection (for an extensive review, see [22]). Regarding DNA biosensors in general, the LODs achieved vary from femtomolar to nanomolar and some examples are shown in Table S5 (see ESM). The electrochemical assays offer the advantage of sensitivity and shorter analysis times but usually require complex surface functionalization and specialized equipment that are not suitable for point-of-care applications [12, 23, 24]. On the other hand, fluorescence-based detection [25–27] is also very sensitive especially when functionalized silica nanoparticles are employed. As in the case of colorimetric systems [28, 29], they rely on biotin-streptavidin interactions or employ target-specific conjugates. For the colorimetric detection of HPV16 ssDNA using a direct HRP-labeled reporter probe for example, an LOD of 7.4 pM was achieved as reported in our previous work [5], which is comparable to the LOD of 21 pM achieved with the combination of the two universal enzyme-DNA binding protein conjugates reported here. Ultrasensitive colorimetric systems employing hybridization chain reaction have also been reported with LOD reaching low femtomolar range [30, 31], but these systems rely on complex DNA nanostructures that are target-dependent, slow to assemble, highly dependent on the specific conditions used, and also require purification before analysis. The results presented in this work highlight the efficiency, simplicity, and sensitivity of the proposed platform compared to other systems as discussed above. It offers the possibility for multiplex detection of analytes, for example in microarrays and lateral flow assays, just by changing the capture probe and the ssDNA part of the ssDNA-dsDNA hybrid detection probe for each specific target. Furthermore, since many optical DNA detection systems rely on streptavidin-biotin interactions, the system could serve as an alternative thus leading to reduced assay costs, as in the case of direct HRP systems, by eliminating the need for specific modification of each target detection oligonucleotide. The replacement of hydrogen peroxide with glucose as enzyme substrate is an additional advantage the proposed platform offers, because it avoids the direct utilization and storage of hydrogen peroxide that is susceptible to decomposition and as a consequence incompatible with paper-based assays. Point-of-care devices using paper-based lateral flow or dipstick assays have dominated the field of rapid diagnostics especially in low-resource settings because of their excellent features and versatility and the proposed bi-enzymatic DNA-based nanostructure platform is compatible with these type of assays.

The data obtained in this work also accentuates the importance of the ratio of the HRP and GOx enzymes for optimal performance, with an excess of GOx being favorable for increased assay sensitivity. Since the rate-limiting step in the cascade reaction is the production of hydrogen peroxide,





scCro/dHP DNA binding sites ratio	LOD (pM)	Equation
1:1	45	$0.200x^2 + 0.375x + 0.049$
2:1	26	$0.122x^2 + 0.615x + 0.042$
1:2	21	$0.246x^2 + 0.781x + 0.048$

Fig. 4 DNA detection with ELONA. **a** Optimization of capture probe concentration. **b** Optimization of detection probe concentration. **c** Optimization of the addition order of the enzyme-DNA binding protein conjugates. The *error bars* show the standard error of the mean (*SEM*) from triplicate samples. **d** Target calibration curves using detection probes with scCro/dHP DNA binding sites ratio of 1:1, 2:1, and 1:2. The *error bars* show the SEM from triplicate samples and three independent experiments

excess of GOx increases the amount produced which is in turn scavenged by HRP and used for signal generation. Similar observations have been reported before during the co-immobilization of the two enzymes in copper phosphate-based nanoflowers were the excess of GOx resulted in higher assay sensitivity [32]. In the sensing platform described here, the ratio of the two enzymes was easily manipulated just by changing the sequence of the hybrid ssDNA-dsDNA detection probe, thus demonstrating the flexibility of the proposed immobilization system based on the DNA binding protein tags which allowed further optimization of the signal generation step. Further improvement may be achieved by testing different spacing and orientations of the DNA binding sites, since several reports in the literature demonstrated the importance of the distance between HRP and GOx in a cascade configuration for optimal performance in order to reduce the diffusion of hydrogen peroxide in the bulk solvent, and prevent its decomposition [11, 19, 33, 34].

Finally, the specificity of the detection system, another important feature of biosensors, was evaluated by testing its response to three irrelevant sequences from different disease markers: CDH1 and HUWE1 for breast cancer and HLA-DQA1*0201 for celiac disease. All four sequences including the target were used at high concentration (5 nM) under optimized assay conditions. As it is shown in Fig. 5, the system is very specific for the target sequence from HPV16 since extremely low, background-level signal was obtained for the control sequences used.

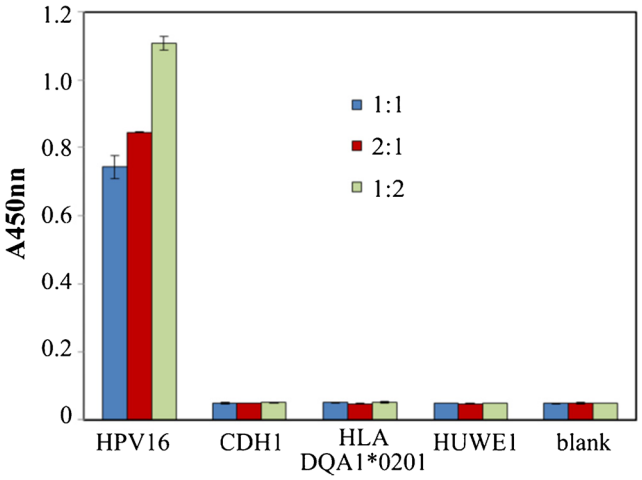


Fig. 5 Specificity of the sensing platform. Detection of different target ssDNA molecules was performed using the detection probes with scCro/dHP DNA binding sites ratio of 1:1, 2:1, and 1:2. The *error bars* show the SEM from triplicate samples

Conclusions

A DNA sensing platform was described in this work based on an HRP/GOx bi-enzymatic DNA nanostructure exploiting DNA binding protein tags. Two enzyme-DNA binding protein tag conjugates and hybrid ssDNA-dsDNA detection probes containing specific sites for the binding of the enzyme conjugates were thus designed and used for the detection of model target HPV16 ssDNA. The preparation and characterization of the GOx-dHP conjugate was detailed in this paper and the conjugate was subsequently used together with the previously reported HRP-scCro conjugate for the sensitive detection of target ssDNA. Under optimized assay conditions, increased DNA detection sensitivity and lower LOD were achieved when a detection probe containing one binding site for the HRP-scCro conjugate and two for the GOx-dHP conjugate was used compared to a detection probe providing an enzyme stoichiometry of 1:1. The performance of the biosensor in terms of LOD was comparable to direct HRP systems using target-specific oligonucleotide-HRP conjugate further highlighting the advantageous use of the proposed DNA detection platform to provide fast signal generation with lower cost. The approach described herein allows the easy control of the pattern of co-immobilization of the two enzymes by simply selecting the distribution of the specific sequences for binding of the enzyme-DNA binding protein tags conjugates, providing facile manipulation of the ratio of the two enzymes for tailored signal generation. The enzyme conjugates could also be used as universal reporters for multiplex analyte detection in conjunction with multiple target-specific capture and ssDNA-dsDNA detection probes. Finally, the versatility of the proposed system is further demonstrated by its compatibility with paper-based diagnostics for point-of-care testing since the unstable hydrogen peroxide is substituted with glucose as enzyme substrate.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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