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RESEARCH PAPER

DNA immobilization and detection using DNA binding proteins

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Abstract

The immobilization of sensing bioreceptors is a critical feature affecting the final performance of a biosensor. For DNA detection, the (strept)avidin-biotin affinity interaction is often used for the immobilization of biotin-labeled oligonucleotides or PCR amplicons. Herein, DNA binding proteins are proposed as alternative universal anchors for both DNA immobilization and detection, based on the strong and specific affinity interaction between certain DNA binding proteins and their respective dsDNA binding sites. These binding sites can be incorporated in the target DNA molecule during synthesis and by PCR, eliminating the need for post-synthesis chemical modification and resulting in lower costs. When scCro DNA binding protein was immobilized on microplates and nitrocellulose membrane, both ssDNA and dsDNA targets were successfully detected. The detection limits achieved were similar to those obtained with the streptavidin-biotin system. However, the scCro system resulted in higher signals while using less amount of protein. The adsorption properties of scCro were superior to streptavidin's, making scCro a viable alternative as an anchor biomolecule for the development of DNA assays and biosensors. Finally, a nucleic acid lateral flow assay based solely on two different DNA binding proteins, scCro and dHP, was developed for the detection of a PCR amplicon. Overall, the proposed system appears to be very promising and with potential use for multiplex detection using various DNA binding proteins with different sequence specificities. Further work is required to better understand the adsorption properties of these biomolecules on nitrocellulose, optimize the assays comprehensively, and achieve improved sensitivities.

Keywords scCro DNA binding protein · Streptavidin · DNA immobilization · Microplate · Nitrocellulose · Protein adsorption

Abbreviations

CNP	Carbon nanoparticles
dHP	Dimeric headpiece domain of the *Escherichia coli* LacI repressor
ELISA	Enzyme-linked immunosorbent assay
ELONA	Enzyme-linked oligonucleotide assay
EMSA	Electrophoretic mobility shift assay
LFA	Lateral flow assay
scCro	Single-chain bacteriophage Cro repressor

Introduction

The demand for sensitive, accurate, fast, and low-cost detection of analytes has led to an immense growth of the field of biosensors. Continuous (bio)technological advancements have made it possible to detect virtually any kind of target analyte using biosensors which are compatible with routine bioanalysis, point-of-care diagnostics, and home testing and are expected to replace traditional bioanalytical methods [1, 2]. The specificity of biosensors relies upon the sensing bioreceptors which are typically proteins (e.g., enzymes, antibodies, cell receptors) and nucleic acids (DNA and RNA). Consequently, the performance of biosensors can be considerably affected by the immobilization of these biomolecules on the desired surface [3].

The attachment of biorecognition elements to surfaces can be covalent or non-covalent. In the case of proteins, direct adsorption (non-covalent) is the mainstay since it is simple, straightforward, and inexpensive [4, 5]. However, the weak association of proteins with surfaces is susceptible to desorption [6]. If direct adsorption is not feasible and/or minimal chemical modification of the bioreceptors is desired to preserve their native structure and activity, specific capture molecules can be used as anchors to mediate their immobilization [7–9]. This is typically achieved with affinity interactions which result in the oriented and site-directed immobilization of the bioreceptors. Different tags are used for this purpose

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and typical examples include nickel for the immobilization of histidine-tagged proteins [10, 11], glutathione for glutathione-S-transferase (GST) fusion proteins [12], protein A/protein G for immunoglobulins via their Fc fragments [13], and lectins (e.g., concanavalin A) for the carbohydrates of glycosylated proteins [9]. Yet, the most common non-covalent affinity interaction used for biosensor development is based on biotin-binding proteins [14, 15]. The extremely low disassociation constant of the (strept)avidin-biotin interaction ($K_D \approx 10^{-14}$ M) results in virtually irreversible immobilization, which is resistant to desorption and is compatible with harsh regeneration conditions [16]. These properties, in combination with the small size of biotin, its facile and low-cost incorporation, and the resulting oriented and site-specific immobilization, have made the system very popular for the immobilization of nucleic acids and the development of DNA biosensors [17, 18]. DNA immobilization through covalent bonding is an alternative approach for DNA biosensors. Modified DNA probes are tethered to functionalized surfaces via chemisorption or covalent attachment, leading to the preparation of biosensor surfaces with typically high stability and density and DNA probes with good orientation for high hybridization signals [18, 19]. However, this approach presents several drawbacks compared to immobilization via affinity interactions which can limit their use, such as requirements for post-synthesis modification of the DNA probes and the preparation of appropriately functionalized surfaces, the dependence of immobilization efficiency on numerous parameters (e.g., pH, temperature, ionic strength, time), and especially the high non-specific interactions leading to lower detection specificity.

When anchor proteins are used, their efficient immobilization on the surface is essential to achieve the desired sensitivity, minimize steric constraints, and facilitate analyte accessibility as well as surface coating stability, and eliminate non-specific binding [13, 18]. Physical adsorption of proteins on material surfaces is a complex process resulting from hydrogen bonding, hydrophobic interactions, and/or electrostatic forces [20]. Since electrostatic interactions are believed to be the principal driving force, it is expected that the charge of both the surface and the protein, in combination with the pH used during deposition, will play a major role on the efficiency of the immobilization process [21]. Therefore, positively charged proteins are likely to adsorb more efficiently on a negatively charged surface like nitrocellulose membrane [22] at an operating pH higher than the isoelectric point (pI) of the protein and vice versa. This is exemplified by the work of Holstein et al. [23], where nitrocellulose-binding anchoring proteins recognizing an influenza surface protein were designed computationally and used for the development of rapid lateral flow assays. The best performing anchor protein was found to be a small protein with high pI. On the other hand, the adsorption of proteins on the hydrophobic polystyrene surface of microplates tends to be very strong but can result in protein denaturation at the initial protein-surface interface [24]. For this reason, polar hydrophilic groups are introduced to the surface in order to allow both hydrophobic and electrostatic interactions. These "high binding" plates are the preferred ones for the development of ELISA-type assays.

Streptavidin is widely used for biosensor development even though its immobilization properties are expected to be limited by its overall charge (pI of 5–6 [25]). Indeed, this is reflected by the commercial availability of streptavidin variants with improved adsorption properties on certain biosensor-related surfaces. For example, streptavidin-plus was manufactured for enhanced adsorption to polystyrene (e.g., Agilent/ProZyme #SA20) whereas the streptavidin-NC variant was engineered to bind more efficiently to nitrocellulose membrane (e.g., Ray Biotech #228-11471-2). The improved adsorption properties and biotin binding capacity of the streptavidin-plus variant have been demonstrated experimentally [26]. However, the considerably higher cost of these variants compared to the already high cost of native streptavidin limits their widespread application.

To address these limitations, we sought to exploit DNA binding proteins as anchors to mediate the immobilization of target DNA on surfaces typically used for biosensor development and bioanalytical applications. Certain DNA binding proteins like transcription factors have naturally evolved to bind dsDNA sequences with very high affinity and specificity [27]. In this work, we propose the use of sequence-specific DNA binding proteins in combination with their respective binding sequences as an alternative to the streptavidin-biotin interaction, widely exploited for DNA biosensor development, to mediate DNA immobilization and detection, based on their high specificity and affinity as well as their biophysical properties favoring their immobilization on specific surfaces. In addition, this would enable the use of the streptavidin-biotin interaction in other parts of the biosensor depending on the needs of the specific application. Specifically, two sequence-specific DNA binding proteins were chosen: the engineered single-chain dimeric variants of the bacteriophage Cro repressor protein (scCro) and the dimeric headpiece domain of *Escherichia coli* LacI repressor protein (dHP). The scCro has an affinity dissociation constant (K_D) for its specific dsDNA sequence of 4×10^{-12} M [28] and a calculated pI of 9.6. Similarly, the dHP also displays high affinity ($K_D = 3 \times 10^{-11}$ M) for its dsDNA binding sequence and a calculated pI of 7.9 [29]. The sequence specificities of these two proteins are different; therefore, they can be used simultaneously [30]. Both are small in size (~ 17 kDa) and positively charged; they were engineered to monomers and are readily expressed and purified using *Escherichia coli*-based bacterial expression systems [30, 31]. Their proposed use for DNA immobilization and detection is illustrated in Fig. 1. The classic streptavidin-biotin system for ssDNA

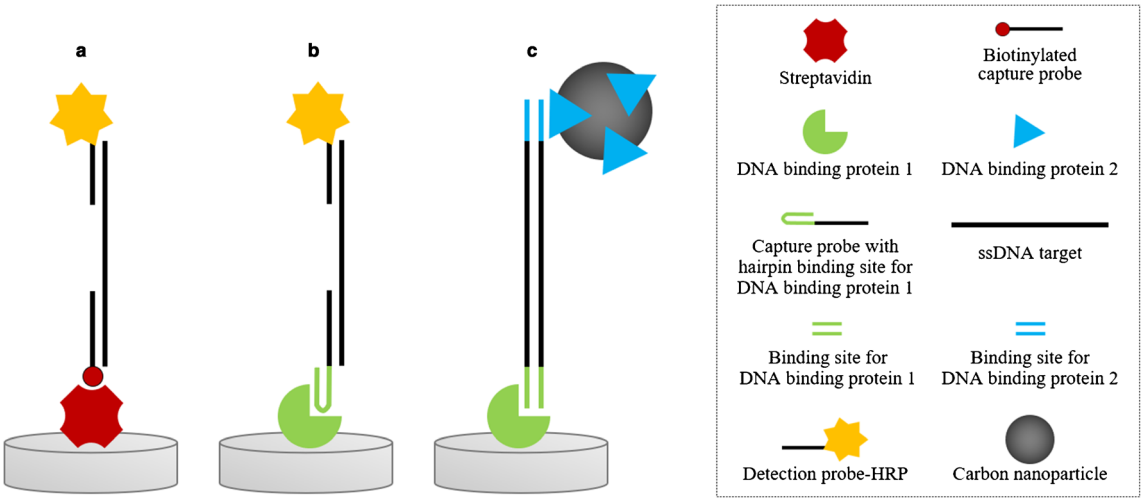

Fig. 1 DNA immobilization and detection using DNA binding proteins. ssDNA target detection using **a** the standard streptavidin-biotin system or **b** a DNA binding protein-dsDNA interaction for anchoring of the capture probe. **c** dsDNA PCR amplicon detection using two different DNA binding proteins and carbon nanoparticles

detection is shown in Fig. 1a for comparison. In the case of the detection of ssDNA targets using microplate-based (ELISA-type) and lateral flow (LFA), only one DNA binding protein is required according to the configuration shown in Fig. 1b. The target ssDNA capture probe is extended with the specific dsDNA binding site for the DNA binding protein during oligonucleotide synthesis in the form of a hairpin, whereas the DNA binding protein is immobilized on the surface by physical adsorption. This allows for the association of the capture probe with the surface through affinity interactions. For the detection of dsDNA, two DNA binding proteins with different sequence specificities are required, one for capture and one for detection as shown in Fig. 1c. The target dsDNA is generated by PCR using target-specific forward and reverse primers each extended with the DNA binding site of each of the DNA binding proteins. The performance of the proposed DNA immobilization and detection strategies was evaluated on two highly relevant surfaces for biosensor development and bioanalytical applications, microtiter plates and nitrocellulose membrane, and compared to streptavidin-based systems. Different aspects were assessed: the immobilization efficiency of each protein on the surface and their performance as anchoring elements for the detection of model ssDNA and dsDNA in microplate-based and lateral flow assays. The model DNA target used was high-risk human papillomavirus HPV type 16.

Materials and methods

Materials

The DNA oligonucleotides were purchased from Biomers.net (Ulm, Germany) and Eurofins (Ebersberg, Germany) and their sequences are shown in Table 1. Nunc MaxiSorp high protein-binding capacity polystyrene microplates and PageBlue protein staining solution were provided from Fisher Scientific (Madrid, Spain). The scCro and dHP DNA binding proteins were prepared as previously detailed [29, 30]. Their conjugation to carbon nanoparticles (CNPs) was performed according to a previous report [31]. Streptavidin from *Streptomyces avidinii* was purchased from Sigma-Aldrich (Madrid, Spain) whereas Hi-Flow Plus HFB13502 nitrocellulose membrane and absorbent pad were provided from Millipore (Germany). All other reagents were purchased from Fisher Scientific (Barcelona, Spain) and Sigma-Aldrich (Madrid, Spain).

Immobilization and detection of ssDNA in microtiter plates

The efficiency of ssDNA immobilization on microtiter plates using scCro DNA binding protein was evaluated and compared to the standard streptavidin-biotin system by enzyme-linked oligonucleotide assay (ELONA). Specifically, the wells of a microtiter plate were coated with 50 μL of 200 nM scCro or streptavidin in 50 mM carbonate buffer pH 9.6 overnight at 4 °C. The plates were washed three times with 300 μL PBST and then blocked with 5% BSA in PBST for 1 h at room temperature. Then, 50 μL of 50 nM of the corresponding capture DNA probe (HPV16 ssDNA hairpin capture probe with scCro binding site or biotinylated capture probe) in ELONA buffer (20 mM potassium phosphate pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% BSA, 0.05% Tween-20) was added and incubated for 30 min at room temperature. The wells were then washed with PBST and 50 μL of target ssDNA (2–200 pM in ELONA buffer) was added and incubated for 1 h at room temperature. The wells were washed three times and 50 μL of 10 nM HPV16 detection

Table 1 Oligonucleotides used in this work. The binding sites for the DNA binding proteins are underlined

Oligonucleotide	Sequence (5′–3′)
HPV16 ssDNA target	GCCCATTAACAGGTCTTCCAAAGTACGAATGTCTACGTGTGTGCTTTGTACGCACAACCGAA GCGTAGAGTCACACTTGCAACAAAAGGTTACAATATTGTAATGGGCTCTGTCCGGTTCTG CTTGTCCAGCTGGACCATCTATTTCATCCTCCTCCTC
Biotin-HPV16 capture probe	biotin-GAGGAGGAGGATGAAATAGATGGT
scCro-HPV16 capture probe	TATCACCGCAAGTGATATTTTTATCACTTGCGGTGATATTTTTTTTTTGAGGAGGAGGATGAAA TAGATGGT
HPV16 detection probe	TTGGAAGACCTGTTAATGGGC-HRP
scCro-HPV16 forward primer	TATCACCGCAAGTGATAGCCCATTAACAGGTCTTCCAA
dHP-HPV16 reverse primer	AATTGTGAGCGCTCACAATTCGAGGAGGAGGATGAAATAGATGGT
Control ssDNA	ATACCAGCTTATTCAATTGCATCACACACCGATACTCACCCGCCTGATTAACATTAGCCCAC CGCCCACCCCCGCTGCAGATAGTAAGTGCAATCT
Control ssDNA forward primer	ATACCAGCTTATTCAATT
Control ssDNA reverse primer	AGATTGCACTTACTATCT

probe conjugated to HRP was added and incubated for 30 min at room temperature. After the last washing step, 50 μL of TMB substrate was added to the wells and color development was stopped after 15 min by adding 50 μL of 1 M H_2SO_4. The absorbance was recorded at 450 nm. Triplicate sample measurements were performed with three independent experiments and six repetitions for the blank measurements. Data was fitted to linear curves and the limits of detection (LOD) for each immobilization approach were calculated as three times the standard deviation (SD) of the blank measurements divided by the slope ($3 \times SD_{blank}$/slope). The reproducibility of the assay was evaluated by testing triplicate samples of two different concentrations of DNA within a single experiment, whereas the repeatability was assessed from the analysis of at least duplicate samples within three independent experiments. The coefficients of variation (CV values) were calculated from the mean values of the samples (MV) and their standard deviation (SD) as %CV = 100 × SD/MV. The effect of the overall duration of the assays on the sensitivity achieved was also evaluated. When each step was performed separately, the total assay duration was 165 min. When the capture probe was pre-incubated with the ssDNA target prior to the addition to the plate and the final detection using the HRP-conjugated detection probe, the assay was performed in 105 min. Finally, when the ssDNA target was pre-incubated with both the capture probe and the detection probe before addition to the plate, the assay was performed in 65 min.

Immobilization of scCro DNA binding protein on nitrocellulose membrane

The immobilization of scCro DNA binding protein on nitrocellulose membrane was studied with regard to its resistance to desorption after washing. For this purpose, 1 μL of scCro or streptavidin was manually spotted on the nitrocellulose membrane, using solutions of equal mass (1 mg/mL) or molar concentrations (10, 25, and 50 μM) prepared in PBS (pH 7.4). After drying for 15–30 min at room temperature, the membranes were washed in two different challenging solutions: deionized H_2O and PBS with 0.1% v/v Tween-20 (PBST). The protein remaining immobilized on the membranes after washing was visualized after staining with the protein dye Coomassie Brilliant Blue. Briefly, the membrane was stained in PAGE Blue protein staining solution under shaking conditions for 10 min at room temperature and finally destained using a solution containing 40% (v/v) methanol and 10% (v/v) glacial acetic acid in water.

Immobilization and detection of ssDNA on nitrocellulose membrane

The performance of scCro DNA binding protein and streptavidin as anchoring proteins for the immobilization of a ssDNA capture probe on nitrocellulose membrane and the subsequent detection of a model ssDNA target (HPV16) was evaluated with an LFA. The capture probe to be immobilized via the scCro DNA binding protein was extended with the binding site of scCro in the form of a hairpin. The respective one for streptavidin was only modified with a 5′-biotin. The sequences of the probes can be found in Table 1. The LFA strips were prepared as follows: the protein solutions, of equal mass (1 mg/mL) or molar (60 μM) concentrations, were mixed with 50 μM of the corresponding capture probes in PBS (pH 7.4) and incubated for 30 min at room temperature. The solutions were then deposited on the nitrocellulose membrane using a micropipette tip and incubated for 1 h at room temperature. The membrane was blocked with 2% (w/v) skim milk and 0.1% (v/v) Empigen BB in 10 mM carbonate buffer pH 9.6 for 15 min and then left to air-dry. The prepared strips

were stored at 4 °C until use. For the detection of the ssDNA target, the prepared strips with immobilized ssDNA capture probe were initially flowed with 20 μL of ssDNA target (up to 160 nM in ELONA buffer). After the strips adsorbed completely the introduced solution, 20 μL of 25 nM ssDNA detection probe conjugated with HRP (in ELONA buffer) was introduced to the strips. Finally, the strips were incubated in a freshly prepared chromogenic dye solution (1 mM of each 3-amino-9-ethylcarbazole and hydrogen peroxide in 100 mM potassium phosphate pH 5) for 5 min for signal (color) generation. The strips were air-dried and ssDNA target was detected visually (naked eye).

Detection of dsDNA on nitrocellulose membrane using DNA binding proteins

An LFA was developed for the detection of dsDNA on nitrocellulose membrane employing two DNA binding proteins. A PCR amplicon containing the binding sites for scCro and dHP DNA binding proteins was used as the dsDNA target. The HPV16 ssDNA target was amplified using primers extended with the binding sites of the scCro and dHP DNA binding proteins (scCro-HPV16 forward primer and dHP-HPV16 reverse primer, see sequences in Table 1). The forward primer contained the sequence for scCro (Cro-HPV16 primer) and the reverse primer the sequence for dHP (dHP-HPV16 primer). To validate the correct incorporation of the binding sites, the amplicon was column-purified and was used in an electrophoretic mobility shift assay (EMSA). Binding mixtures were prepared in EMSA buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 mM EDTA, 5% glycerol) containing 100 nM of the HPV16 PCR amplicon and 500 nM of each protein species (dHP, scCro, or both). After a 30-min incubation at room temperature, the samples were resolved on an 8% polyacrylamide gel in TAE buffer. Finally, the gel was stained with GelRed for visualization of the DNA bands.

The LFA strips were prepared by depositing one of the DNA binding proteins on the test zone and a monoclonal anti-His tag antibody on the control zone for the specific capturing of the recombinant His-tagged DNA binding proteins. The specific conditions used for preparation of the LFA strips were optimized as detailed in the Supplementary Information (ESM). Specifically, 0.5 μL of scCro (80.8 μg/mL in PBS pH 7.4) and mouse anti-His tag antibody (680 μg/mL in PBS pH 7.4) were spotted on the test and control zones, respectively. The distance between the two zones was 5 mm. The strips were dried under vacuum for 10 min followed by blocking using 2% (w/v) skim milk and 0.1% (v/v) Tween-20 in 10 mM carbonate buffer pH 9.6 for 30 min and then left to air-dry. A cellulose absorbent pad (3 cm in height) was attached to the nitrocellulose membrane through a homemade backing pad and the strips were assembled, cut manually (35 mm height and 3–4 mm width), and stored at 4 °C until use. The DNA binding protein-carbon nanoparticle (CNP) conjugates were prepared as previously described [32].

The target calibration curve was constructed as follows: the strips were dipped in the wells of a 96-well plate containing 35 μL of the sample solution with 3.9–1000 ng of the purified PCR amplicon in running buffer (1% (w/v) skim milk in 10 mM Tris-HCl pH 7.5, 75 mM NaCl, and 0.1% Empigen BB). Once the strips absorbed the solution, 1 μL of the DNA binding protein-CNP conjugate was mixed with 34 μL of running buffer and the strips were dipped in this solution. When the sports became visible, the strips were imaged, and the intensity of the black spots was estimated with the ImageJ software. Each sample was measured in triplicate and the limit of detection (LOD) was interpolated from the fitted sigmoidal calibration curve as the bottom of the curve plus three times the standard deviation of the bottom (bottom + 3 x SD_{bottom}).

Results and discussion

Immobilization and detection of ssDNA in microtiter plates

The performance of DNA biosensors relies on the effective immobilization of DNA capture probes on the desired surface. Streptavidin is the most frequently used biomolecule mediating the immobilization of biotinylated DNA sequences on various surfaces via affinity interactions. In this work, it was hypothesized that the interaction between a DNA binding protein and its specific binding sequence could be a valid alternative to this standard streptavidin-biotin system (Fig. 1). Many parameters affect the immobilization efficiency of an anchor biomolecule on a specific surface. Among them, the porosity and hydrophobicity of the surface as well as the buffer (composition and pH) used during protein deposition can potentially determine how well the protein is physically absorbed, like, for example, on the polystyrene surface of a microtiter plate well [33]. How much of the protein remains adsorbed on the surface after repeated washing steps using detergent-containing buffers is also an important factor, considering that detergents are essential for eliminating non-specific adsorption of reagents and background signals.

Streptavidin is a homotetrameric protein with an approximate size of 53–60 kDa and isoelectric point (pI) ranging from 5 to 7.5, depending on the source of the protein (native or recombinant). The scCro DNA binding protein on the other hand is a single-chain homodimeric protein of 16 kDa and pI of 9.6 [28]. Unlike streptavidin, which can bind multiple (up to four) biotin molecules simultaneously, scCro can bind only one dsDNA sequence at a time. The possibility of using scCro DNA binding protein as a DNA anchor biomolecule instead of streptavidin was initially explored on microtiter plates in the context of an ELONA. The microplates used were

standard immunoassay plates with hydrophilic modification of the polystyrene surface to allow maximum adsorption of a variety of proteins. When streptavidin was used for immobilization, the ssDNA capture probe was modified with a biotin as shown in Fig. 1a. In the case of scCro DNA binding protein, the sequence of the ssDNA capture probe was extended with the dsDNA binding site for scCro through the formation of a hairpin (Fig. 1b). The ssDNA target was added to the plates and it was detected using a ssDNA detection probe conjugated to HRP. As shown in Fig. 2, the increase of target ssDNA concentration resulted in a linear increase of the signal (absorbance), independent on the anchor protein used for the immobilization of the capture probe. The analytical sensitivity of the scCro-based approach though was more than fivefold higher compared to the streptavidin-biotin system and this is reflected by the slopes of the linear calibration curves, 6.55 AU/nM for scCro and 1.27 AU/nM for streptavidin. This enhanced sensitivity might be attributed to more efficient adsorption of scCro on the plate surface when the carbonate buffer, one of the most popular buffers used for passive adsorption of proteins, was used during the coating of the protein on the hydrophilic polystyrene surface of the plate. Another possible explanation could be a relative resistance of scCro to desorption after several washing steps using a detergent-containing buffer. The limits of detection (LODs) of both systems were very similar, calculated in the low picomolar range (5.1 pM for streptavidin and 10.4 pM for scCro). The precision of the two systems was also compared by means of the coefficients of variation (CV) as shown in Table S1 (see ESM). Their reproducibility was similar with the CVs calculated within the narrow range of 2.1–4.5%. The repeatability of the streptavidin system was slightly better though with CVs ≤ 10% whereas the respective ones for the scCro system were around 13%. Non-specific interactions of negatively charged DNA with the positively charged scCro could be partially responsible, thus requiring washing steps with higher ionic strength buffers. The analytical performance of both systems is comparable to our previous work, where the streptavidin-biotin interaction was used for DNA capture probe immobilization in combination with a detection probe-HRP conjugate or a hybrid detection probe-scCro binding site and HRP-scCro conjugate (sensitivity of 1.6–5.6 AU/nM and LOD of 2.1–7.4 pM) [31]. In order to reduce the overall assay duration, certain incubation steps were combined. The performance of the two proteins was compared, and as shown in Table 2, scCro performed better than streptavidin as the anchoring protein in all cases in terms of sensitivity resulting in higher signals. Additionally, regardless of the protein used, the decrease of the total assay time resulted in improved LODs but the signals were lower (lower sensitivity). These results suggest that the scCro-dsDNA anchoring system may be more compatible with large complexes, providing the target ssDNA with more accessibility to the surface compared to the streptavidin-biotin system. The latter one appears to be more susceptible to steric hindrance effects considering its multiple biotin binding sites and the importance of favorable orientation for multiple biotin binding. No significant differences between the two systems are expected due to possible secondary structures of the probes and target DNA, since the only difference between them is the stable hairpin sequence part of the capture probe used for the scCro-based system. It should also be noted that the use of a directly HRP-conjugated detection probe could result in decreased hybridization efficiencies, therefore lower signal intensities. However, this should not affect the comparison between the two proteins since the same detection probe was used for both systems. Overall, the potential use of scCro DNA binding protein to mediate the immobilization and detection of ssDNA on microtiter plates as an alternative to streptavidin appears to be promising. This is especially important considering some limitations of streptavidin-biotin systems such as the possible interference from endogenous biotin in certain assays, background signals from non-specific binding to cell surface receptors, high production costs for streptavidin (both native

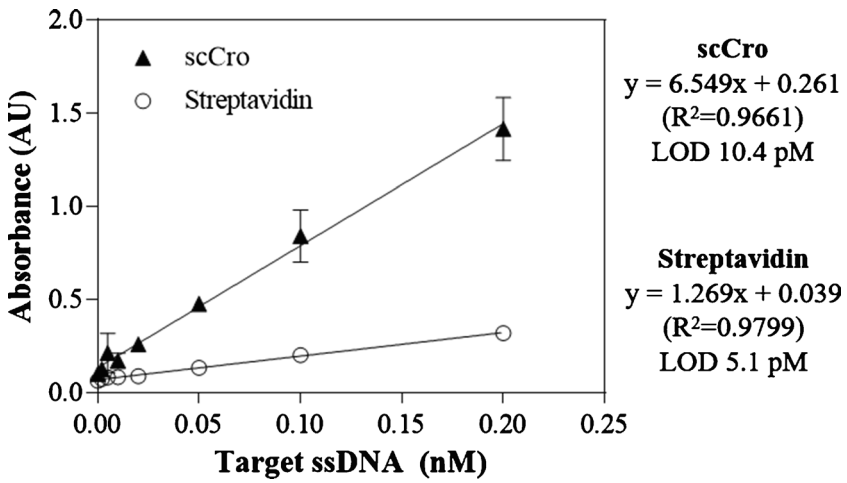

Fig. 2 Detection of target ssDNA in microtiter plates using the scCro-dsDNA or the streptavidin-biotin ssDNA anchoring system. The error bars correspond to the standard deviation ($n = 5$)

Table 2 Sensitivity and LOD of the ELONAs performed with different overall duration using streptavidin or scCro to mediate ssDNA capture probe immobilization on the microtiter plates

Assay duration	Streptavidin		scCro	
	Sensitivity (AU/nM)	LOD (pM)	Sensitivity (AU/nM)	LOD (pM)
165 min	1.27 ± 0.04	5.1	6.55 ± 0.26	10.4
105 min	0.32 ± 0.03	16.4	4.46 ± 0.21	12.4
75 min	0.61 ± 0.06	8.5	4.21 ± 0.01	2.7

and recombinant), and the requirement for chemical post-synthesis modification of the oligonucleotides leading to increased costs.

DNA binding protein adsorption on nitrocellulose membrane

The adsorption efficiency of scCro DNA binding protein on another biosensor surface, specifically nitrocellulose membrane, was evaluated next. Nitrocellulose membrane is the principal material used for manufacturing lateral flow assay tests. Thus, effective protein immobilization on nitrocellulose is highly relevant with home testing and point-of-care diagnostics. To enable hybridization-based detection as dictated by the LFA format, site-specific immobilization of the DNA probe is required. Furthermore, even though DNA adsorbs readily on nitrocellulose via ionic and non-ionic interactions [34], its retention is compromised after hybridization and washing [35]. The streptavidin-biotin system is commonly exploited for anchor-based DNA immobilization on nitrocellulose. The association of proteins with nitrocellulose membrane mainly results from hydrophobic interactions and possibly hydrogen bonds, and it is considered weak and sensitive to desorption during processing steps like washing [35]. Holstein et al. showed that streptavidin, for example, is prone to stripping off the nitrocellulose membrane after washing with detergent-containing buffers, as opposed to IgG antibodies [23]. Also, as mentioned earlier, to address the limitations of streptavidin adsorbed on nitrocellulose, an engineered variant is commercially available with improved properties, but its high cost limits its extensive use for biosensor development. Thus, it is evident that there is a need for alternative anchoring systems, more efficient than the streptavidin-based ones and cost-effective, for sensitive and efficient target detection. For this purpose, the use of scCro DNA binding protein was also tested as an anchor for the immobilization of DNA on nitrocellulose primarily intended to be used in paper-based LFAs. The adsorption of scCro and streptavidin on nitrocellulose was evaluated by spotting each protein solution using equal mass or equal molar concentrations. When solutions of equal molar concentrations are used for immobilization, more than threefold increased mass of streptavidin is being used over scCro considering the size of the proteins, 17 kDa and 60 kDa for scCro and streptavidin, respectively. After drying, the membranes were washed with water or PBST to evaluate the retention of each protein after such processing steps which are typically performed in LFAs. Finally, the membranes were stained with a Coomassie blue solution to detect the remaining adsorbed proteins. As it can be seen in Fig. 3, the adsorption of streptavidin on the nitrocellulose membrane resulted in ring-like spots. In contrast, scCro appeared to form spots of uniform density. Ring stains typically result from the evaporation of dispersed solutions on surfaces [36]. Our results suggest that the adsorption and subsequent immobilization of scCro on nitrocellulose are faster than the evaporation of the solution, leading to uniform immobilization and absence of ring formation. The data also indicates different degrees of retention of the two proteins on nitrocellulose based on the washing solutions used after adsorption, even though hand spotting of the proteins was not totally uniform. The water and PBST washing solutions were used to evaluate the retention of each protein adsorbed on the matrix,

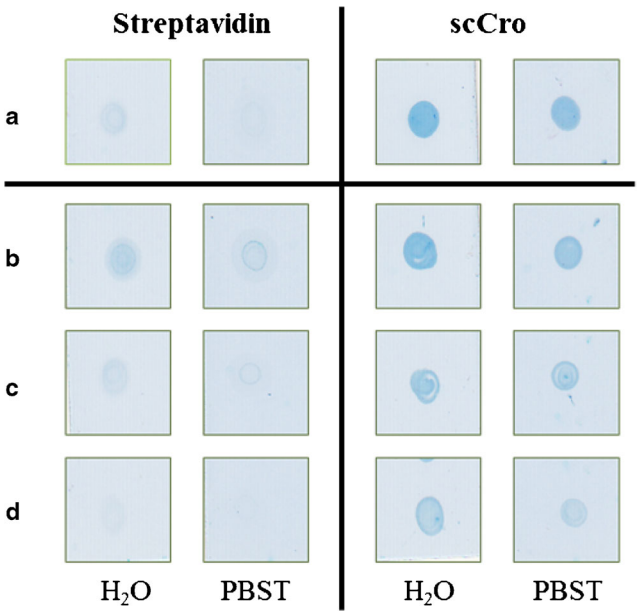

Fig. 3 Immobilization efficiency of streptavidin and scCro on nitrocellulose membrane after washing with H_2O or PBST. Each protein was spotted on the membrane at **a** an equal mass concentration of 1 mg/mL or at equal molar concentration of **b** 50 μM, **c** 25 μM, or **d** 10 μM

especially when a detergent (e.g., Tween-20) was included in the wash solution to prevent non-specific binding. After the PBST wash, desorption of streptavidin from the nitrocellulose membrane was more pronounced compared to scCro. Both the size and overall charge (in the context of pI) of the protein seem to affect its relative rate of adsorption on nitrocellulose, since scCro adsorbed faster than streptavidin. Therefore, small and positively charged proteins appear to adsorb faster on nitrocellulose and form uniform spots which are more resistant to stripping. Protein structural plasticity has also been proposed as another important factor affecting adsorption, with relaxed structures adsorbing more efficiently compared to tightly structured ones [37]. This is partly attributed to possible structural rearrangements on the surface leading to increased interactions and spreading on the surface. Streptavidin is a highly stable protein with a compact beta barrel-based folding [38]. On the other hand, the folding of scCro is based on a helix-turn-helix motif providing it with the necessary flexibility and plasticity for binding and bending DNA [39], thus potentially contributing to its superior adsorbing properties on nitrocellulose compared to streptavidin. These properties are highly desired for the development of LFAs, making scCro an ideal protein candidate to be used in such applications. Also, these results provide a plausible explanation for the more sensitive detection of ssDNA using microtiter plates and scCro which can be partially attributed to improved resistance of scCro to washing using a detergent-containing solution, as opposed to streptavidin.

Immobilization and detection of ssDNA on nitrocellulose membrane using one DNA binding protein

The suitability of a protein as an anchor biomolecule for the immobilization of DNA on nitrocellulose depends not only on how well the protein itself adsorbs on the surface but also on how well it performs in terms of DNA detection. For this reason, scCro was used in a lateral flow assay to immobilize a ssDNA capture probe and facilitate the detection of target ssDNA. For comparison, streptavidin was tested in parallel and the performance of the two proteins was evaluated. The capture and detection probes were the same ones used for the ELONAs detailed in a previous section. The test lines in the LFA strips were prepared by depositing equal volumes of solutions of equal molar (60 μM) or equal mass (1 mg/mL) concentrations of the two anchoring proteins. A concentration of 1 mg/mL is equivalent to 60 μM of scCro and 16.7 μM of streptavidin. The strips were washed with PBST and an HRP precipitating chromogenic substrate was used for signal development. The format of the assay can be seen in Fig. 1a and b for streptavidin and scCro, respectively. As it is shown in Fig. 4, the intensity of the test lines increased with increasing concentration of the target ssDNA, regardless of the protein

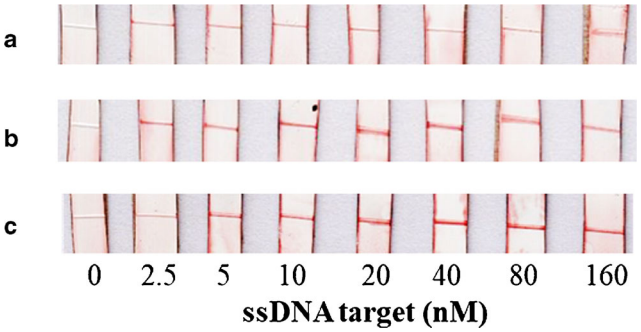

Fig. 4 Detection of ssDNA with lateral flow assays. The capture probe was immobilized via **a** streptavidin (1 mg/ml), **b** streptavidin (60 μM), or **c** scCro (1 mg/mL, 60 μM)

anchor used. In the case of streptavidin, more signal was generated when the protein was used at 60 μM, corresponding to 3.2 mg/mL (Fig. 4b), more than three times the mass concentration of scCro used (Fig. 4c). Higher signal was obtained when using scCro as indicated by the darker lines compared to the ones obtained with streptavidin for the same target concentration. However, a better visual LOD of 2.5 nM ssDNA was obtained with 60 μM of streptavidin as opposed to 5 nM in the case of scCro, but the tetravalency of streptavidin must be taken into account. Overall, even though the experimental setup was not optimized, the scCro-based DNA immobilization system appeared to be a valid alternative to the streptavidin one. It is also cost-effective in terms of protein production costs, considering that more than three times of protein mass is required for the streptavidin-based approach (3.2 mg/mL streptavidin) compared to the scCro one (1 mg/mL) to obtain similar target detection efficiency in LFAs.

DNA immobilization and detection on nitrocellulose membrane using two DNA binding proteins

In the previous sections, the use of scCro DNA binding protein to mediate the immobilization and detection of ssDNA on microtiter plates and nitrocellulose membrane was demonstrated. The next step was to explore the compatibility of DNA binding proteins with the detection of PCR amplicons on nitrocellulose. Detection of dsDNA instead of ssDNA is far more important considering the extensive applications of PCR in medicine, genetic research, forensic science, and environmental microbiology. To achieve this, two different DNA binding proteins are required as shown in Fig. 1c. For this purpose, scCro was used in combination with a second DNA binding protein, dHP. This is the single-chain dimeric form of the headpiece domain of the *Escherichia coli* LacI repressor protein. In our earlier works we demonstrated the use of the two proteins as enzyme conjugates for the detection of ssDNA [30] and protein targets [40]. The biotin-binding protein neutravidin was used in both cases for mediating the immobilization of ssDNA capture probes on the surfaces.

Also, we designed lateral flow assays exploiting scCro-CNP conjugates for the detection of a PCR amplicon captured on the test line by an antibody [32]. In this work, we developed a lateral flow assay using the two DNA binding proteins for both capture and detection of a PCR amplicon, thus eliminating the use of either antibodies or biotin-binding proteins for the construction of the test line. The primers used for the generation of the PCR amplicon were extended with the binding sequences for scCro and dHP (Table 1). The resulting target amplicon was therefore flanked between the two dsDNA binding sequences for each of the DNA binding proteins. An electrophoretic mobility shift assay (EMSA) was used to demonstrate the correct incorporation of these sequences to the PCR amplicon as shown in Fig. 5. The HPV16 PCR amplicon (lane 1) was incubated with each DNA binding protein individually or simultaneously. The changes in the electrophoretic mobility of the amplicon demonstrate that the two proteins were able to bind the PCR amplicon individually (dHP and scCro in lanes 2 and 3, respectively) or simultaneously (lane 4), thus verifying the presence of their respective binding sequences.

The next step was to design and optimize the lateral flow assay. One of the DNA binding proteins was deposited on the test zone for capturing the PCR amplicon whereas the second one was used as a CNP conjugate to facilitate detection. The control zone was constructed with an anti-His tag antibody recognizing the respective tag added to the N-terminal of the recombinant proteins during cloning. An initial optimization of the LFA was performed as detailed in the ESM. The conditions leading to higher signal-to-noise ratio (ratio of the intensity of the test zone in the presence and absence of target) or saturated signals were considered optimum and were chosen for the final assays. Specifically, these were the combination of scCro immobilized on the membrane and dHP used as a CNP conjugate for detection (Fig. S2a, ESM), 75 mM of salt (NaCl) in the running buffer (Fig. S2b, ESM), and 40.4 ng of scCro deposited on the test zone (Fig. S2c, ESM). With regard to the detergent used in the running buffer, Tween-20 is the typical choice in ELISA-type assays to eliminate non-specific binding. On the other hand, the use of Empigen BB has been reported before in nucleic acid LFAs employing gold nanoparticles for detection [41]. In this CNP-based LFA, the performance of Empigen BB was superior to that of Tween-20 (Fig. S2d, ESM) and it was chosen for the final assays.

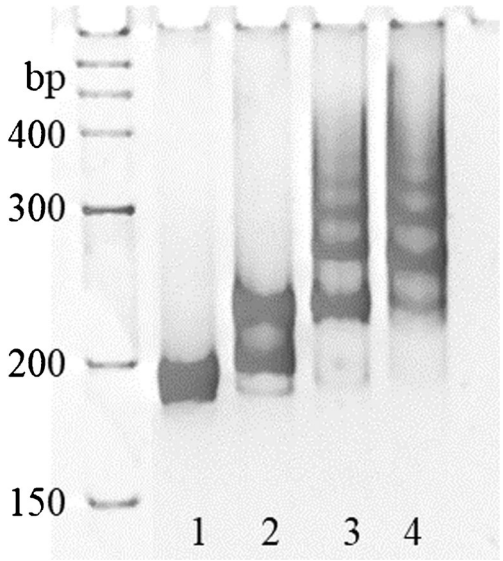

Fig. 5 Validation of the incorporation of the binding sites for the DNA binding proteins in the PCR amplicon by EMSA. The HPV16 PCR amplicon (lane 1) containing the specific binding sites for dHP and scCro was incubated with dHP (lane 2), scCro (lane 3), or both proteins (lane 4)

Finally, the optimized conditions were used for the construction of a calibration curve for the target PCR amplicon. As mentioned above, the test zone was constructed with 40.4 ng of scCro and 340 ng of an anti-His tag antibody were deposited on the control zone. Different amounts of the PCR amplicon (3.9–1000 ng) were flown on the strips, and then, the dHP-CNP conjugate was added for detection. The results shown in Fig. 6 demonstrate the compatibility of the proposed LFA configuration based on the two DNA binding proteins with PCR amplicon detection. The intensities of the spots at the test zones (Fig. 6a) were plotted against the PCR amplicon amounts as shown in Fig. 6b. The data was fitted to a sigmoidal model and the LOD was calculated at 33.5 ng of PCR amplicon. This LOD is within the same low nanogram range of LFAs previously reported by our group where an antibody was used to capture the labeled PCR amplicon with incorporated DNA binding site and detection was achieved using a DNA binding protein-CNP conjugate with or without HRP enzyme signal amplification (LODs of 7.8–10.4 ng of PCR amplicon) [32]. Finally, the specificity of the strips was demonstrated by the absence of signal on the test zones in the presence of an irrelevant PCR amplicon not containing the binding sites for the DNA binding proteins (Fig. 6c). Even though the sensitivity of the LFA obtained is by no means optimal, the work described herein confirms our initial hypothesis that two DNA binding proteins could be used for the detection of dsDNA with nucleic acid lateral flow assays without significant compromises in terms of sensitivity compared to other systems. This approach can lead to lower costs since the primers can be extended with the required binding sites during synthesis instead of being chemically modified post-synthesis.

Conclusions

In this work, we sought to exploit DNA binding proteins to facilitate the immobilization of DNA on surfaces typically used for biosensor development and bioanalytical applications via affinity interactions and serve as an alternative to the

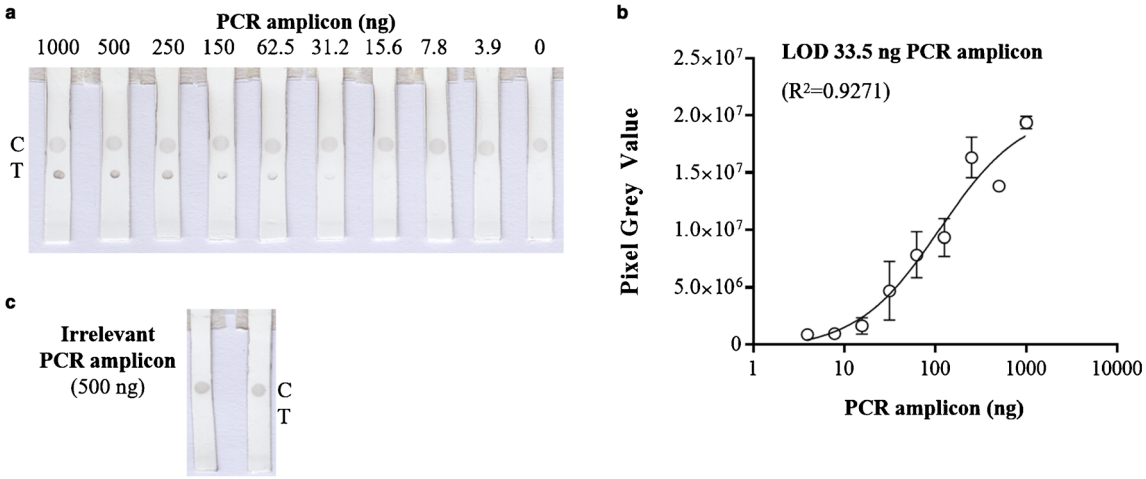

Fig. 6 Lateral flow assay for the detection of dsDNA using DNA binding proteins. **a** Representative strips. **b** Calibration curve for target PCR amplicon detection ($n = 3$). **c** Specificity of the assay using an irrelevant PCR amplicon. T, test zone (scCro DNA binding protein); C, control zone (anti-His tag antibody)

classic (strept)avidin-biotin system. Initially, scCro DNA binding protein was evaluated as an anchor protein for the immobilization of ssDNA on microplates. The analytical sensitivity of the ELONA based on scCro was more than fivefold higher compared to the streptavidin-biotin system performed in parallel for comparison, whereas similar limits of detection were achieved. The reproducibility of the two systems was also comparable, although the repeatability of the streptavidin-based one was slightly better. Next, the immobilization of the two proteins on nitrocellulose membrane was compared and scCro appeared to adsorb faster than streptavidin resulting in spots of uniform density. scCro also resisted to desorption after washing with a detergent-containing buffer as opposed to streptavidin. These properties, which are ideal for any bioanalytical application, could be partially attributed to the small size, high positive charge, structural plasticity, and high affinity of scCro for its specific dsDNA binding sequence. Regarding the detection of ssDNA on nitrocellulose with a lateral flow assay, the performance of the two proteins was similar even though more streptavidin was required than scCro to obtain similar signals. Finally, a PCR amplicon was detected with a nitrocellulose-based lateral flow assay using two DNA binding proteins with different sequence specificities for capture and signal generation. The main advantages of the proposed system compared to the streptavidin-based one were mainly the resistance to desorption from microplates and nitrocellulose in the presence of detergents, the fast and efficient adsorption on nitrocellulose, and lower assay development costs. These costs are related to the labeling step which is performed during synthesis by means of sequence extension instead of being chemically modified afterwards. Also, less DNA binding protein is required compared to streptavidin for obtaining similar signals. Finally, multiplex detection can be achieved using various DNA binding proteins with different sequence specificities like scCro and dHP. An extensive study is required though to gain a deeper understanding on the factors contributing to the attractive adsorption properties of scCro, especially on nitrocellulose considering the ever-expanding market of point-of-care diagnostics and home tests. Overall, the utility and great potential of DNA binding proteins for DNA immobilization and detection were demonstrated on both surfaces studied. Comprehensive optimization of assay conditions will allow the approach to reach its full potential and be established as a viable alternative to the classic streptavidin-biotin system.

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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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