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# Capture and Detection of DNA Hybrids on Paper via the Anchoring of Antibodies with Fusions of Carbohydrate Binding Modules and ZZ-Domains

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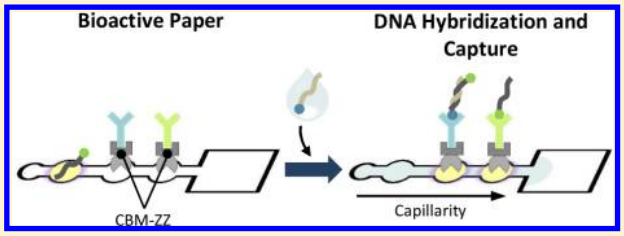
Supporting Information

**ABSTRACT:** Microfluidic paper-based analytical devices ($\mu$PADs) fabricated by wax-printing are suitable platforms for the development of simple and affordable molecular diagnostic assays for infectious diseases, especially in resource-limited settings. Paper devices can be modified for biological assays by adding appropriate reagents to the test areas. For this purpose, the use of affinity immobilization strategies can be a good solution for bioactive paper fabrication. This paper describes a methodology to capture labeled-DNA strands and hybrids on paper via the anchoring of antibodies with a fusion protein that combines a family 3 carbohydrate binding module (CBM) from *Clostridium thermocellum*, with high affinity to cellulose, and the ZZ fragment of the staphyloccocal protein A, which recognizes IgG antibodies via their Fc portion. Antibodies immobilized via CBM-ZZ were able to capture appropriately labeled (biotin, fluorescein) DNA strands and DNA hybrids. The ability of an antibody specific to biotin to discriminate complementary from noncomplementary, biotin-labeled targets was demonstrated in both spot and microchannel assays. Hybridization was detected by fluorescence emission of the fluorescein-labeled DNA probe. The efficiency of the capture of labeled-DNA by antibodies immobilized on paper via the CBM-ZZ construct was significantly higher when compared with a physical adsorption method where antibodies were simply spotted on paper without the intermediation of other molecules. The experimental proof of concept of wax-printed $\mu$PADs functionalized with CBM-ZZ for DNA detection at room temperature presented in this study constitutes an important step toward the development of easy to use and affordable molecular diagnostic tests.




Molecular tests have attractive performance characteristics (high accuracy, specificity, and sensitivity) that make them powerful diagnostic tools for infectious diseases. The field has grown immensely in the last years and molecular diagnostics of infectious diseases is now widely used in many clinical laboratories. However, these techniques usually require complex and expensive instrumentation and qualified personnel, making them inaccessible in resource-limited settings.[1,2]

Microfluidic paper-based analytical devices ($\mu$PADs) are particularly well-suited as a platform for the development of simple and cost-effective molecular diagnostic assays. $\mu$PADs combine some of the capabilities of conventional microfluidic devices with the simplicity of diagnostic test strips and may be one of the least expensive platforms available for developing assays.[3,4] Paper is a ubiquitous material composed mainly of cellulose that is inexpensive, easy to manipulate, biodegradable or easily disposable by incineration, and compatible with several biological and chemical assays. Furthermore, its porous structure facilitates lateral-flow assays which use capillary forces to move fluids without the assistance of external forces and expensive microfluidic devices.[5,6] Fabrication of $\mu$PADs is based on patterning sheets of paper with hydrophilic channels bounded by hydrophobic barriers. One of the most promising methods for patterning paper is wax printing, a technique which involves a solid wax printer and two main operations: printing patterns of wax on the surface of paper and melting the wax into the paper to form complete hydrophobic barriers across the entire thickness of paper.[7,8] The ability of these barriers to contain aqueous solutions is due solely to the fact that the hydrophilic cellulose fibers are rendered hydrophobic by the wax coating and not to the blocking of pores. Although its resolution is lower when compared to other patterning techniques, wax printing remains an excellent choice for the fabrication of $\mu$PADs due to the low cost of patterning agents, speed of fabrication, ability to produce multiple devices or

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multizones on a sheet of paper, and potential to be upgraded to large-scale manufacturing.[4,5]

Patterned paper can be modified and adapted to perform biological assays by adding appropriate biorecognition and reporting agents to the test areas. Biosensing molecules such as antibodies, enzymes, and DNA aptamers are expensive and must be used judiciously and efficiently. Attachment is not a strict requirement for incorporation of reagents into paper because dry paper itself is able to sorb aqueous solutions so that the nonvolatile components of the solutions are left in the paper structure after drying. However, impregnation without attachment may not be a robust strategy to immobilize biomolecules because subsequent exposure to aqueous solutions (washing buffers or biological samples) is likely to leach the biosensing components.[6] Furthermore, it is difficult to control the orientation of biomolecules in the paper structure, especially in the case of immunoglobulins, with recognition sites taking different positions in space after random immobilization, resulting in hindered interactions with their binding target.[9] In this context, the use of affinity immobilization strategies may provide a general solution for bioactive paper fabrication. For example, biochemical binding agents such as carbohydrate-binding modules (CBMs) that have high affinity to cellulose can be used to immobilize biomolecules on paper.[10] CBMs are noncatalytic domains involved in the targeting and binding of polysaccharide-degrading enzymes to their substrates, thus potentiating enzyme activity.[11] CBMs have been grouped into 69 defined families based on amino acid sequence similarity (see Carbohydrate Active Enzymes database at http://www.cazy.org/). The variation found in ligand specificity among these CBMs is substantial.[12] Fusion technology has been used to recombine CBMs with other proteins, producing complexes that offer the possibility of targeted immobilization of antibodies,[13,14] proteins,[15] bacteriophages,[16] and bacteria[17] onto a cellulose matrix for the purpose of developing protein purification, sensor, and microarray applications.[13−17]

In this work, the cellulose-binding properties of family-3a CBM (CBM3a) from the cellulosomal-scaffolding protein A (CipA), which is responsible for the structural organization of the cellulosomes present in *Clostridium thermocellum*,[18] were combined with the antibody-binding properties of double Z-domain from the staphylococcal protein A, which recognizes IgG antibodies via their Fc portion.[19,20] The resulting CBM-ZZ construct was then used to anchor antibiotin antibodies and subsequently capture biotin-labeled DNA strands on paper. Finally, antibodies anchored via CBM-ZZ fusions were combined with wax printed μPADs and used to capture and detect DNA hybrids. As a practical example and for experimental proof of concept, an oligonucleotide sequence taken from *esat-6*, a gene that encodes the early secreted antigenic target 6 kDa protein (ESAT-6) from *Mycobacterium tuberculosis* complex species, was used for testing in spot- and wax-printed microchannel assays. The ESAT-6 protein is a major T-cell antigen, and it is suggested that it is produced during tuberculosis infection.[21]

## EXPERIMENTAL SECTION

**Materials.** The following oligonucleotides were designed on the basis of the *esat-6* gene sequence of *M. tuberculosis* (GenBank accession number X79562) and obtained from STAB VIDA (Oeiras, Portugal): a 6-carboxyfluorescein-labeled DNA probe (6-FAM-5′-CAT TTT TGC TGG ACA CCC TG-3′), a 100% complementary DNA target labeled with biotin (biotin-5′-TTT TTT TTT TCA GGG TGT CCA GCA AAA ATG-3′), and a 100% noncomplementary DNA target labeled with biotin (biotin-5′-TTT TTT TTT TTC TAA GTC ATG TAG GCC GGA-3′). A spacer composed of ten thymines was included at the 5′ end of targets. FITC-Human IgG was obtained from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal anti-FITC (IgG2a isotype) and mouse monoclonal antibiotin (IgG2a) were purchased from Abcam (Cambridge, U.K.). Whatman no. 1 chromatography paper (180 μm thick, 25 × 25 cm sheets) was purchased from VWR and used in both spot and microchannel assays. Plasmid pET21a and *Escherichia coli* BL21 (DE3) were obtained from Novagen (Darmstad, Germany). All protein dilutions were prepared in Tris-Saline-Tween (TST) buffer (50 mM Tris buffer pH 7.6, 150 mM NaCl, 0.05% v/v Tween 20). Phosphate-buffered saline (PBS; 10 mM phosphate pH 7.2, 150 mM NaCl) with 0.05% v/v Tween 20 and PBS with 0.01% v/v Triton X-100, were used as washing buffers in the spot and in the wax-printed microchannel assays, respectively. Ultrapure water obtained with a Milli-Q purification system (Merck-Millipore, Portugal) was used in the preparation of all buffers.

**Construction, Expression, and Purification of CBM-ZZ.** The CBM-ZZ fusion protein [285 amino acids (aa)] comprising the double Z-domain from the staphylococcal protein A[19,20] and the family 3 cellulose-binding module from *C. thermocellum*[18] were constructed by inserting the coding parts of CBM3 and ZZ between the *Nde*I and *Xho*I sites of the pET21a expression vector (NZYTech, Lisbon). A 10 aa long spacer (SSGLVPRGST) was used between CBM and ZZ. Following transformation of *E. coli* BL21 (DE3), cells were grown in LB broth supplemented with 100 μg/mL ampicillin at 37 °C in an orbital shaker at 250 rpm. Expression was induced at an $OD_{550}$ = 0.5 with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested 16 h after induction by centrifugation (1900 g, 4 °C, 10 min), resuspended in a minimum volume of TST buffer, and disrupted by sonication (Branson Sonifier 250) for 6 × 30 s in ice, with interruptions of 30 s (50% duty cycle, microtip limit 5). Centrifugation (12000*g*, room temperature, 20 min) was performed to separate the supernatant containing the fusion protein from cell debris. The CBM-ZZ fusion was then purified by affinity chromatography using an IgG Sepharose 6 Fast Flow column (GE Healthcare, Uppsala, Sweden) in an ÄKTA 10 Purifier system. After equilibrating the column with 5 column volumes of TST buffer and loading 2 mL of the supernatant containing CBM-ZZ, unbound proteins were removed with 10 column volumes of TST buffer. CBM-ZZ was eluted using 5 column volumes of 0.5 M acetic acid, pH 2.8. The collected fractions containing CBM-ZZ were immediately neutralized with 3.2 M Tris buffer, pH 11. The CBM-ZZ purity was evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) in a 12% acrylamide gel stained with Coomassie Brilliant Blue, and total protein was quantified by the bicinchoninic acid method (Pierce BCA Protein Assay Kit, Thermo Scientific). The purified CBM-ZZ was stored at −20 °C until required.

**Spot Assays.** *Immobilization.* The immobilization of antibodies on paper via CBM-ZZ was evaluated by spotting approximately 40 nL of a series of CBM-ZZ solutions with different concentrations (0−8 μM) onto paper using a manual spotter MicroCaster (Schleicher & Schuell). The paper was subsequently washed three times to remove unbound biomolecules, immersed in a 0.03 μM FITC-labeled IgG


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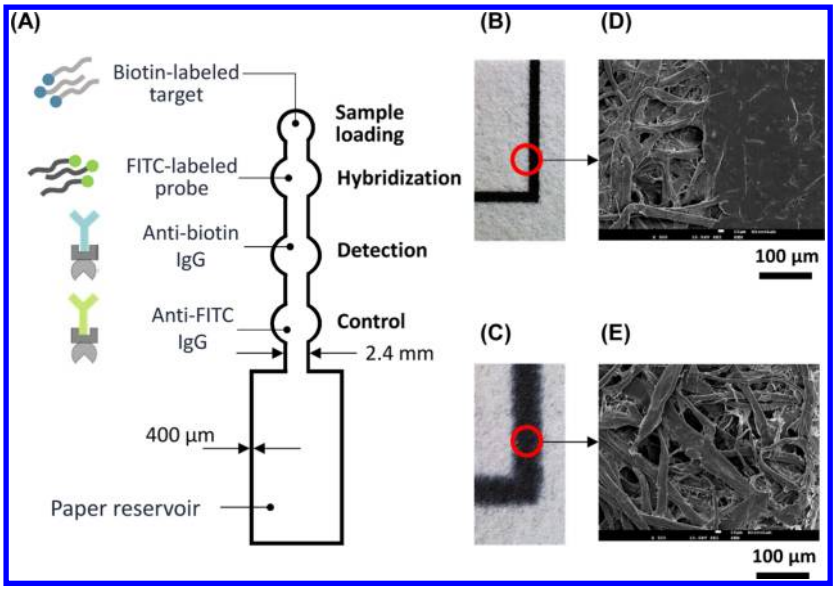


**Figure 1.** (A) Schematic illustration of the general structure of a wax-printed μPAD and the methodology used to detect biotin-labeled DNA strands. The design includes sample loading, hybridization, detection, and control zones. The μPADs are prepared by adsorbing fluorescein-labeled DNA probe to the hybridization zone and by immobilizing antibiotin and anti-FITC antibodies in the test and control zones, respectively, with CBM-ZZ fusions. Hybridization between the target and probe occurs during migration by capillarity through the hybridization zone. These hybrids are subsequently captured in the detection zone by antibiotin IgG, resulting in a fluorescent spot. Unbound fluorescein-labeled DNA probes migrate further and are trapped by anti-FITC antibodies, generating a second fluorescent spot corresponding to the migration control. Hydrophobic barriers were designed as (B) black lines on a white background and subsequently (C) melted on a hot plate. SEM images show that the tangle of fibers characteristic of cellulose is kept after the film of (D) printed wax is (E) melted.

solution, and incubated at room temperature (RT) in the dark for 2 h at 300 rpm (Heidolph Instruments Titramax 1000). The paper was then washed five times and imaged for fluorescence analysis. The immobilization of antibodies on paper by biochemical coupling with the CBM-ZZ fusion protein was compared with immobilization by plain physical adsorption using two strategies: (i) FITC-labeled IgG and (ii) anti-FITC IgG plus fluorescein-labeled DNA probes. In the first case, solutions of 5 pmol of FITC-labeled IgG or mixtures of 2 pmol of CBM-ZZ and 5 pmol of FITC-labeled IgG were preincubated at RT for 30 min, spotted on paper, and air-dried at RT in the dark. Paper was subsequently washed ten times and imaged for fluorescence analysis. In the second case, solutions of 5 pmol of anti-FITC IgG or mixtures of 2 pmol of CBM-ZZ and 5 pmol of anti-FITC IgG were preincubated at RT for 30 min and spotted on paper. Paper was air-dried at RT and washed three times. Then, 10 pmol of fluorescein-labeled DNA probe were added on top of the CBM-ZZ:anti-FITC IgG or anti-FITC IgG spots and incubated at RT for 30 min in the dark. The paper was washed again ten times and imaged for fluorescence analysis.

*Capture.* The capture of biotin-labeled DNA hybridized to fluorescein-labeled DNA probe on the paper was assessed using a CBM-ZZ:antibiotin IgG conjugate. Mixtures of 2 pmol of CBM-ZZ and 5 pmol of antibiotin IgG preincubated at RT for 30 min were spotted on paper, after which the paper was air-dried at RT and washed three times. DNA solutions (20 pmol of fluorescein-labeled probe and 20 pmol of biotin-labeled complementary target or 20 pmol of fluorescein-labeled probe and 20 pmol of biotin-labeled noncomplementary DNA target) preincubated for 30 min at RT were then added (1 μL) on top of the CBM-ZZ:antibiotin IgG spots and incubated at RT for 30 min in the dark. Paper was subsequently washed ten times and scanned for fluorescence analysis.

**Fabrication of Wax-Printed Microchannels.** Patterns of hydrophobic barriers were designed as black lines on a white background (Figure 1B) using drawing software (AutoCAD) and printed with a Xerox ColorQube 8570 wax printer set to the default parameters for enhanced resolution printing. The printed wax was then melted in a hot plate as previously described (Figure 1C).[7] Throughout this work, microchannels of 2.4 mm nominal width and 30 mm nominal length (plus a reservoir of 15 mm nominal length), defined by lines of 400 μm nominal thickness, were employed (Figure 1A).

**Hybridization by Capillary Transport in Wax-Printed Microchannels.** μPADs were prepared by pipetting either 1 μL of a solution containing 5 pmol of antibody or 1 μL mixtures of 2 pmol of CBM-ZZ and 5 pmol of antibody in the detection (antibiotin IgG) and control (anti-FITC IgG) zones. Solutions were always preincubated at RT for 30 min. Each mixture was placed on the correspondent test zone (see Figure 1A) and air-dried at RT. The paper was then rinsed three times and air-dried at RT. The hybridization zone was prepared by pipetting 30 pmol (1 μL) of fluorescein-labeled DNA probe and subsequently air-drying the paper at RT. When μPADs were completely dried, biotinylated DNA target (20 pmol diluted in a 15 μL solution of TST buffer) was applied in the sample zone with the paper "chip" hanging in the air. The channel was then washed by adding 15 μL of TST to the sample zone. Once dried, the fluorescence intensity of the test zones was measured after scanning the μPAD. The limit of detection of the method was determined by performing the hybridization protocol described above with 1, 2.5, 5, 10, and 20 pmol of the biotin-labeled complementary DNA target.

**Signal Analysis.** Following hybridization and/or capture events in both spot and microchannel assays, paper fluorescence intensity was visualized using a Typhoon Trio Variable mode Imager (GE Healthcare) at 400 V PMT with the


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488/526 nm excitation/emission filter set. The images were converted to 8-bit grayscale using ImageJ (NIH, National Institutes of Health).[22] The fluorescent spots were selected individually and the gray intensity, which is proportional to the fluorescent intensity of each spot, was measured. For every image, the total signal was calculated by adding the individual signals, which were taken as 100%. The fluorescence of each spot was then calculated as a percentage of the total fluorescence.

## RESULTS AND DISCUSSION

**Anchoring of Antibodies to Cellulose.** The ability of the CBM-ZZ fusion protein to bind antibodies to paper was evaluated by exposing paper strips spotted with increasing amounts of the fusion protein to a 0.03 $\mu$M solution of FITC-labeled IgG. Figure 2A shows that the use of increased amounts

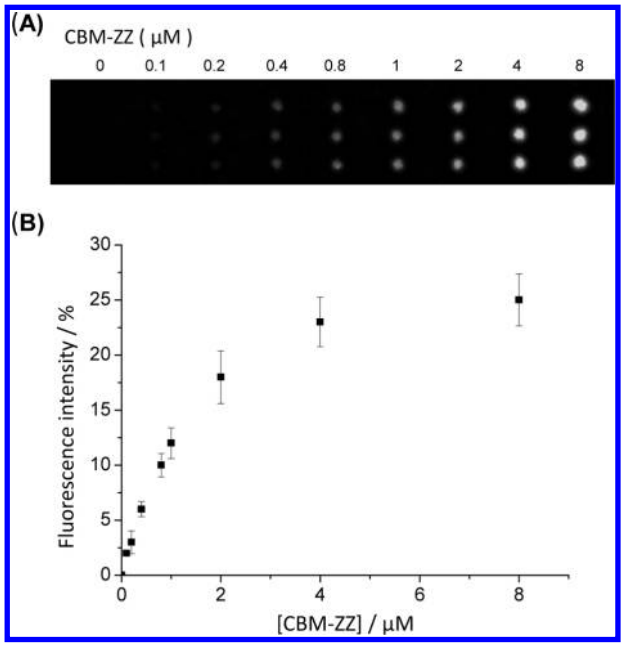


**Figure 2.** Immobilization of antibodies on paper via biochemical coupling (CBM-ZZ). Paper strips were spotted with 40 nL of CBM-ZZ solutions with concentrations ranging from 0 to 8 $\mu$M and then exposed to a common solution of 0.03 $\mu$M FITC-labeled IgG. CBM-ZZ:FITC-IgG complexes generate fluorescent spots. The graph shows the relative fluorescence intensity of spots within each row as a function of CBM-ZZ concentration. Error bars were obtained from the standard deviations of the measurements corrected with Student's parameter (95%) for three independent experiments.

of CBM-ZZ in the spots increased the amount of FITC-labeled IgG attached to paper. The fluorescence signal variation becomes smaller at higher concentrations of the fusion protein, indicating a saturation of the paper structure with CBM-ZZ molecules and, hence, a limitation in IgG binding (Figure 2B). These results clearly indicate that biochemical coupling via CBM-ZZ is a practicable strategy to immobilize antibodies on cellulose surfaces.

Additional experiments were performed to compare the immobilization of antibodies on paper via biochemical coupling with CBM-ZZ to a physical adsorption strategy. In the first case, CBM-ZZ fusions (2 pmol/spot) and antibodies (5 pmol/spot) were preincubated before spotting, whereas in the second case antibodies (5 pmol/spot) were spotted directly on paper, without the intermediation of CBM-ZZ. The CBM-ZZ:IgG molar ratio of 1:2.5 used was determined on the basis of previous tests (data not shown). Experiments were designed to assess the efficiency of the immobilization of an FITC-labeled antibody on paper (Figure 3A) and the ability of immobilized anti-FITC antibodies to capture a FITC-labeled DNA strand (Figure 3B). In either case, the fluorescence intensity is considerably higher in the spots where antibodies were immobilized via CBM-ZZ, an observation that is corroborated by the quantitative assessment of the relative fluorescence emitted by each spot. There is a significant enhancement of antibody immobilization (~2 fold, Figure 3A) and labeled-DNA capture (~3 fold, Figure 3B) when CBM-ZZ is used. Since the physical adhesion of antibodies to the paper surface is established through relatively weak interactions (e.g., van der Waals forces),[6] part of the molecules were probably removed upon washing. As a result, the density of physically adsorbed FITC-labeled antibodies in the spots is lower (Figure 3A). Furthermore, and although filter paper may still have the capacity to adsorb a large quantity of antibody, the random orientation of anti-FITC IgG on the surface could lead to a reduced recognition and capture of the target as a result of steric hindrance of binding sites (Figure 3B).[6,10] However, the fact that equivalent relative fluorescent signals from FITC-labeled IgG and fluorescein-labeled DNA were obtained suggests that the main factor causing a lower signal for the physical adsorption strategy is the removal of the adsorbed antibodies during the washing steps. On the basis of these results, we can conclude that CBM-ZZ-mediated immobilization is a valuable strategy, since it minimizes the loss of antibodies during processing steps, increasing the intensity and spatial confinement of signals.

**Detection of Hybridization.** The ability of antibodies immobilized on paper via CBM-ZZ fusions to capture hybridized DNA was investigated next using antibiotin IgG. Test solutions containing a fluorescein-labeled DNA probe and biotin-labeled, complementary DNA target or a fluorescein-labeled DNA probe and biotin-labeled, noncomplementary DNA targets were dispensed (20 pmol/spot) over CBM-ZZ:antibody (2 pmol:5−10 pmol) spots, in order to evaluate the ability of the immobilized antibiotin IgG to detect DNA−DNA hybrids. Figure 4 shows the relative fluorescence emitted by the spots as a function of the DNA mixture applied over them. Higher fluorescence intensity was detected in the spots where the mixture containing fluorescein-labeled DNA and the complementary biotin-labeled DNA was applied. This accumulation of fluorescence over the CBM-ZZ:antibiotin antibody spots indicates the capture of fluorescein-DNA:biotin-DNA hybrids via the biotin-labeled DNA strand. On the other hand, when mixtures of fluorescein-DNA probe and noncomplementary biotin-DNA were tested, no fluorescence was observed over the spots. While in this case the biotin-labeled DNA probe was in all likelihood captured by the CBM-ZZ:antibiotin antibody spot, the inability of the fluorescein-DNA probe to hybridize with the noncomplementary target resulted in no fluorescence. This lack of fluorescence signal also indicates that nonspecific attachment of DNA to the bioactive paper matrix is not significant. This is consistent with previous observations, which have shown that physical adsorption of DNA on cellulose is reversible.[23] Taken together, these results indicate that fluorescence accumulation over the spots resulted from the capture of biotin-labeled DNA hybridized with the fluorescent probe [i.e., antibodies immobilized through CBM-ZZ specifi-


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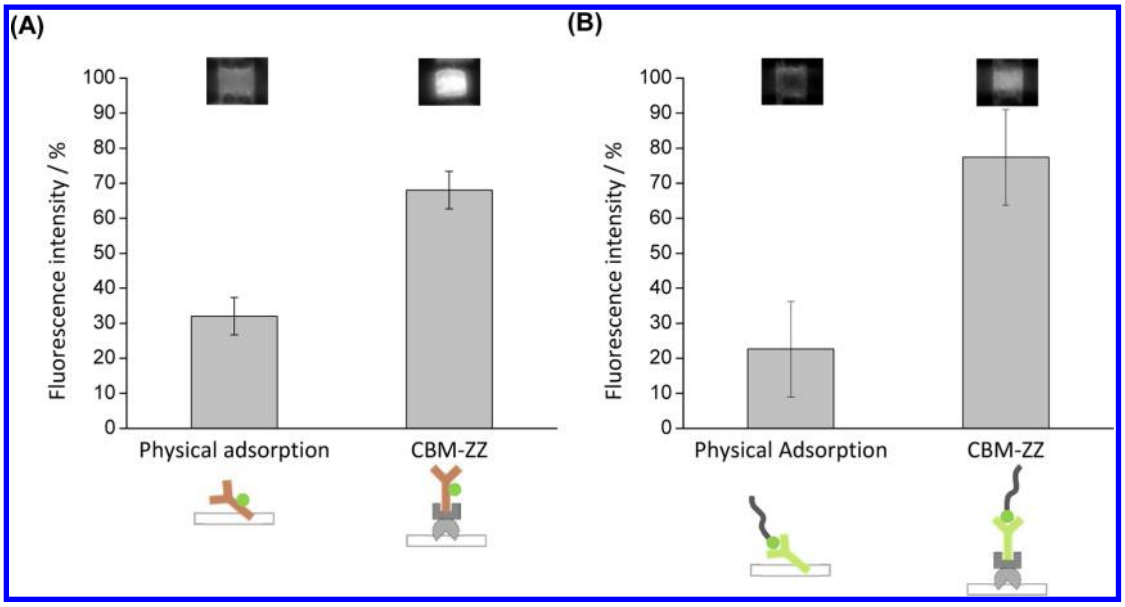


**Figure 3.** Immobilization of antibodies on paper via biochemical coupling and physical adsorption. In the first case, CBM-ZZ fusions (2 pmol/spot) and antibodies (5 pmol/spot) were preincubated before spotting, whereas in the second case antibodies (5 pmol/spot) were spotted directly on paper. The graphs show the relative fluorescence intensity of spots measured after washing, according to the immobilization method used to attach antibodies to paper. (A) Immobilization of FITC-labeled IgG and (B) capture of fluorescein-labeled oligonucleotides (10 pmol/spot) by anti-FITC IgG. In each panel, the sum of the fluorescence signals in experiments with and without CBMs was taken as 100% and the relative fluorescence of each spot was then calculated as a percentage of that total. Error bars were obtained from the standard deviations of the measurements corrected with Student's parameter (95%) for three independent experiments.

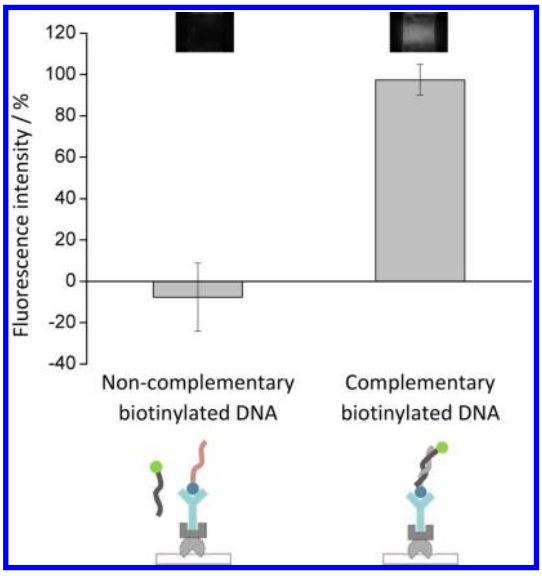


**Figure 4.** Capture of hybrids of fluorescein-labeled DNA (probe) and biotin-labeled complementary DNA targets by antibiotin IgG immobilized on paper via CBM-ZZ. The graph shows the relative fluorescence intensity of spots obtained after washing when using either noncomplementary (left) or complementary (right) DNA targets. Errors were obtained from the standard deviations of the measurements corrected with Student's parameter (95%) for three independent experiments.

cally captured biotin-labeled DNA, and a fluorescence signal appeared when the captured biotin-labeled DNA was hybridized with the fluorescein-labeled DNA (probe)].

**Hybridization of Labeled DNA in Wax-Printed Microfluidic Channels.** The feasibility of developing wax printed μPADs for molecular diagnostics based on the immobilization of antibodies via CBM-ZZ fusions was studied next. In preliminary experiments, washing conditions were optimized by testing the effect of several washing buffers containing different concentrations of nonionic and nondenaturing detergents (Tween 20 or Triton X-100) on the integrity of wax-printed hydrophobic walls. These detergents are commonly used in biochemical assays in order to remove unbound compounds and prevent nonspecific antibody binding.[24] In general, aqueous solutions wicked through wax-printed barriers after exposure to Tween 20 (0.005%−0.05%), while Triton X-100 was found to be compatible with the wax barriers when used in concentrations lower than 0.01% (Figure 5). The distinct impact of the two detergents on the ability of the wax barrier to retain aqueous solutions can be explained in terms of their hydrophilic−lipophilic balance (HLB) value. The HLB of Tween 20 (16.7) indicates that this detergent has solubilizing properties that promote the dissolution of the wax, causing the destruction of the hydrophobic barrier. On the contrary, Triton

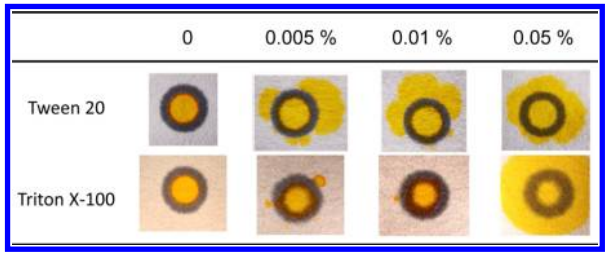


**Figure 5.** Effect of Tween 20 and Triton X-100 on the ability of wax barriers printed on paper to confine aqueous solutions. Paper strips with molten wax circular barriers (400 μm nominal width) were washed three times with PBS supplemented with increasing concentrations of Tween 20 or Triton X-100 and air-dried. The integrity of the barriers was assessed by applying aqueous solution of the yellow synthetic food dye E102 (Vahiné, Provence, France) inside the circles.


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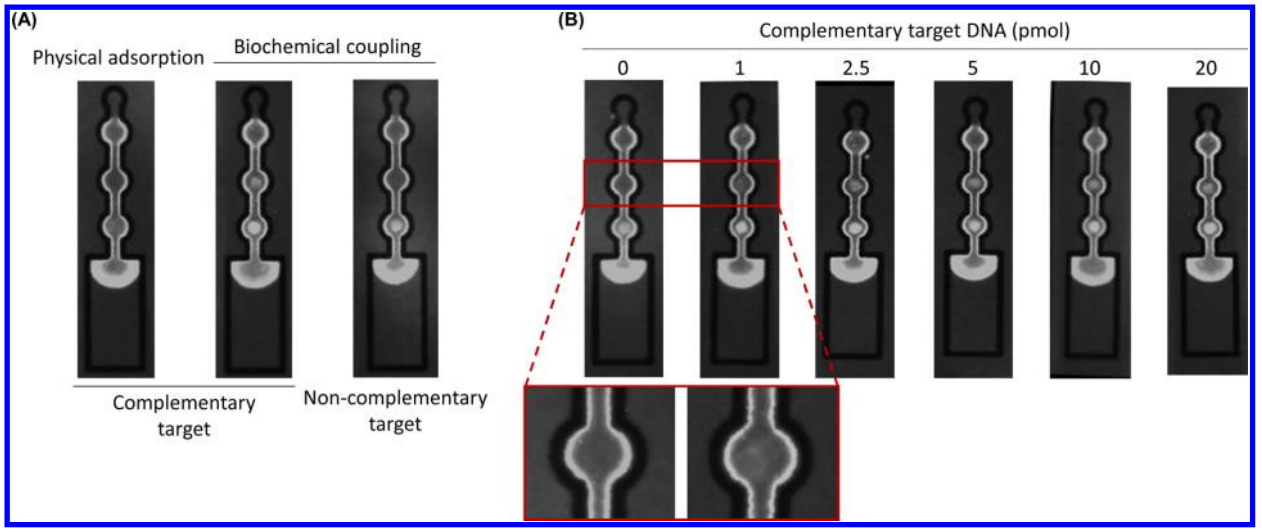


**Figure 6.** Hybridization and detection of labeled DNA in wax-printed microfluidic channels. μPADs were functionalized with CBM-ZZ:antibody (2 pmol:5 pmol) complexes and loaded with fluorescein-labeled DNA probes, according to the scheme shown in Figure 1A. (A) Samples containing 20 pmol of complementary (positive control, μPAD 2) and noncomplementary (negative control, μPAD 3) biotin-labeled DNA targets were run on the devices. A comparative test was also performed using a μPAD with physically adsorbed antibodies (μPAD 1). (B) The limit of detection of the method was assessed using samples containing 1, 2.5, 5, 10, and 20 pmol of complementary DNA target labeled with biotin.

X-100 has lower solubilizing properties (HLB = 13.5), especially at low percentages,[25,26] and therefore does not compromise the ability of the hydrophobic wax barriers to contain aqueous solutions.

The use of a μPAD-based molecular diagnostics strategy relies on the inherent low affinity of polynucleotides toward cellulose, which allows DNA targets and probes to migrate along paper channels by capilarity.[6,23] In order to assess the effectiveness of using these wax-printed microfluidic devices to promote DNA hybridization during capillary transport, experiments were performed using complementary (positive control) and noncomplementary (negative control) DNA targets, as in the previous spot assays. The μPAD design included sample loading, hybridization, detection, and control zones as schematized in Figure 1A and relied on fluorescence emission to detect hybridization. The wax-printed μPADs were prepared by adsorbing fluorescein-labeled DNA probe to the hybridization zone and by immobilizing antibiotin and anti-FITC antibodies in the test and control zones, respectively. The antibodies were anchored on paper either via biochemical coupling with the CBM-ZZ fusion protein or via physical adsorption (Figure 6A). In a preliminary study, binding buffers with or without blocking agents (1% w/v bovine serum albumin or 100 μg/mL salmon sperm DNA) were tested for reducing nonspecific antibody binding. It was found that TST buffer itself provides adequate conditions (pH and ionic strength) to guarantee specific antibody binding (data not shown).

The results demonstrate that complementary biotin-labeled DNA in samples loaded on the μPAD hybridized with the fluorescein-labeled DNA-probe adsorbed on paper, and that these hybrids were captured by CBM-ZZ:antibiotin IgG and CBM-ZZ:anti-FITC IgG complexes (Figure 6A, μPAD-2). As expected, negative controls showed only one fluorescent spot in the control zone, correspondent to the capture of fluorescein-labeled DNA by CBM-ZZ:anti-FITC IgG (Figure 6A, μPAD-3). These results confirm the analytical specificity of the CBM-ZZ:antibody capture strategy used in the assay. The intrinsic wicking ability of the paper matrix facilitated rapid sample elution through the hybridization and test zones, leading to significant, detectable hybridization in the time required for the sample liquid to travel the length of the microchannel (approximately 5 min). Once more, the biochemical attachment of the antibodies was proven to be advantageous over physical adsorption. Antibodies directly adsorbed on paper were essentially unable to capture DNA hybrids (Figure 6A, μPAD-1). In this case, the orientation of antibodies probably played a role as important as its density, since the target molecules were not directly applied over the antibody spots, as in the previous immobilization assays in spots (Figure 3B). Therefore, the rapid sample elution very likely decreased the interaction time between the antibody and its target, reducing the ability of randomly oriented antibodies to capture DNA hybrids and leading to a major signal reduction.

Aside from analytical specificity, the lowest detection limit is a fundamental feature of diagnostic tests. To investigate this parameter, serial dilutions of complementary, biotinylated DNA stands were loaded in μPADs functionalized with CBM-ZZ:antibiotin IgG and CBM-ZZ:anti-FITC IgG complexes, as previously described. Figure 6B shows representative images of results obtained for hybrid detection. Under these conditions, fluorescent signals could be visible for DNA amounts as low as 1 pmol in a 15 μL sample volume, whereas 20 pmol of noncomplementary target DNA was not detectable (Figure S-1, Supporting Information). A weak correlation between fluorescence signals and the amount of target DNA was also found, indicating that the strategy is currently limited from a quantitative point of view. Nevertheless, the data demonstrates that wax-printed μPADs activated with CBM-based fusion proteins can be used for the rapid detection of the presence of specific DNA sequences in low volume samples.

Alternative strategies for detecting DNA hybrids on paper already exist which rely on the direct covalent coupling of probe DNA to activated paper,[27] rather than on the intermediation of antibodies as proposed here. However, the compatibility of such chemical coupling strategies with wax-printing might be problematic, since organic compounds (e.g., DMSO, DMF, ethanolamine,[27]) are often used, which would compromise wax


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barriers if activation and coupling are performed after microchannel printing. On the other hand, the high temperatures required to melt wax barriers (150 °C) would most likely affect immobilized DNA probes if wax printing is performed after paper activation. On the contrary, our strategy makes it very easy to customize and also multiplex assays, since preprinted multichannel μPADs can be prepared within minutes by depositing CBM-ZZ:antibodies in the control and detection zones and fluorescein-labeled DNA probes in the corresponding hybridization zones. This flexibility and speed are advantageous when compared with the cumbersome steps typically associated with chemical activation.

## CONCLUSION

A methodology was developed to capture labeled-DNA strands and hybrids on paper via the anchoring of antibodies with a fusion protein that combines a CBM with high affinity to cellulose and a ZZ fragment which recognizes IgG antibodies. The antibodies remained active after immobilization, and the superiority of this immobilization strategy over the commonly used physical adsorption strategy was demonstrated, especially when adapted to wax-printed microchannels. Antibodies anchored to paper via CBM-ZZ were able to capture labeled-DNA strands and hybridized DNA using a fluorescein-labeled-DNA strand as the probe. The ability of an antibiotin antibody to discriminate complementary from noncomplementary biotin-labeled targets was demonstrated in both spot and microchannel assays.

The experimental proof of concept of wax-printed μPADs functionalized with CBM-ZZ for DNA detection at room temperature presented in this study constitutes an important step toward the development of affordable and easy-to-use molecular diagnostic tests for a first level screening of infectious diseases in low tech settings. Further validation of the proposed μPADs will be of extreme importance using DNA extracted from clinical isolates and amplified by simple techniques such as loop-mediated isothermal amplification (LAMP), an isothermal nucleic acid amplification method that does not require any special equipment for thermocycling reactions.[28] A full protocol from sample-to-detection can be envisaged which would encompass DNA extraction from clinical isolates, amplification by LAMP, strand-separation of resulting dsDNA, and loading of sample in preprepared μPAD. Different amplicon samples should be tested in this context to assess for specificity. Additionally, efforts should be made to replace fluorescence by a visual readout, for example, by resorting to colorimetric paramagnetic microparticles[29] or gold nanoparticles.[30] The detection of multiple targets could also be easily implemented on the basis of the CBM-based immobilization protocol described, for example, by designing μPADs with a single sample loading zone leading to multiple channels (one per target), each featuring their own hybridization, detection, and control zones.

Future developments of the device will thus target: (i) a decrease in the limits of detection, (ii) implementation of multiplexing, and (iii) the generation of a color signal readout that is immediately visualized with the naked eye. This will contribute to enhance the versatility of the diagnostic assay and to reduce the dependency on additional expensive instrumentation to a minimum.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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