

Immunodetection in paper-based devices using Carbohydrate Binding Modules fusions for antibody anchoring and gold nanoparticles for colorimetric reporting

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Abstract Microfluidic paper-based analytical devices (μ PADs) are a new platform that combines some of the capabilities of conventional microfluidic devices with the simplicity of diagnostic strip tests and may be one of the least expensive platforms available for developing assays. The patterning of paper is based on patterning sheets of paper with hydrophilic channels bounded by hydrophobic barriers. Wax printing was used to delineate circular reaction areas (spots) and microchannels on paper. Then anti-biotin antibodies were anchored on paper via the CBM3-ZZ fusion and its ability to recognize biotin was tested using gold nanoparticles coated with biotin (biotin-AuNPs). The capture of biotin-AuNPs by the CBM3-ZZ:antibiotin IgG complex in both the spot and microchannel configuration could be visually detected by the generation of a red color that is virtually absent in controls. These results open up the possibility of combining CBMs and gold nanoparticles with PADs for immunodetection purposes.

Microfluidic paper-based analytical devices (μ PADs) are a new platform that combines some of the capabilities of conventional microfluidic devices with the simplicity of diagnostic strip tests and may be one of the least expensive platforms available for developing assays. This strategy is based on the fact that paper-based assays have been used for a variety of simple diagnostic tests and this polymer has several advantages over other materials. Paper is a material composed mainly of cellulose, and it is ubiquitous, inexpensive, easy to manipulate, biodegradable, as well as compatible with several biological and chemical assays [1].

The patterning of paper is based on patterning sheets of paper with hydrophilic channels bounded by hydrophobic barriers. One of these methods is wax printing, which is very attractive due to its simplicity, efficiency, rapidity and its low cost. Wax is used as a hydrophobic barrier to form well-defined millimeter-sized channels on the hydrophilic paper. The fabrication process of microfluidic channels using this method involves two main operations: printing patterns of wax on the surface of paper, and melting the wax into the paper to form complete hydrophobic barriers [2], [3].

Patterned paper can be modified and adapted to perform biological assays by adding appropriate biorecognition and reporting agents to the test areas [4], [5]. One of the problems associated is the difficulty to control the orientation of biomolecules in the paper structure, like in the case of immunoglobulins, with recognition sites taking different positions in space after random immobilization, may result in hindered interactions with their binding target. 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 [6].

CBMs are non-catalytic domains present in glycoside hydrolases which target the associated catalytic modules to their substrates, thus potentiating enzyme activity [7]. Based on their amino acid similarity, CBMs have been grouped into 69 families. CBMs can be engineered into fusion with other proteins producing complexes that offer the possibility of targeted immobilization of antibodies, proteins, bacteriophages, and bacteria onto a cellulose matrix for the purpose of developing protein purification, sensor, and microarray applications [6].

One example of this type of fusion proteins is CBM3-ZZ, which is comprised of the cellulose-binding properties of family-3a CBM (CBM3a) from the cellulosomal-scaffolding protein A (CipA), combined with the antibody-binding properties of double Z-domain from the staphylococcal protein A, which recognizes IgG antibodies via their Fc portion. Ana M. Rosa et al.[6] demonstrated the ability of antibodies immobilized via CBM-ZZ being able to capture appropriately labeled DNA strands and DNA hybrids.

An important aspect of μ PADs is the detection method. One of the most attractive is the colorimetric detection, which is typical related to enzymatic or chemical color-change reactions, having the advantage that the results can be assessed by the naked eye. Gold nanoparticles (AuNP) are one of this kind of promising colorimetric reporting system that has already been used in dipsticks and lateral flow tests

coupled with antibodies. Gold nanoparticles are an attractive resource because they can be functionalized with DNA strands, proteins and antibodies and used as a colorimetric detection system[8], [9].

The objectives of this work is the development of microfluidic paper-based analytical devices (μ PAD) for immunodetection which use gold nanoparticles as a colorimetric detection method and the CBM3-ZZ fusion protein as an intermediate for antibody immobilization.

Experimental Section

Materials. Mouse monoclonal anti-FITC (IgG2a isotype) and mouse monoclonal antibiotin (IgG2a) were obtained from Abcam (Cambridge, UK). Species and antibody class were chosen based on their strong binding to protein A. InnovaCoat™ Gold Biotin-40 nm (OD 10.0) was obtained from Innova Biosciences (Cambridge, UK). Through this work the biotin labeled gold nanoparticles will be mentioned as Biotin-AuNPs.

Device fabrication. The wax printing method was used to pattern hydrophobic barriers onto Whatman No.1 chromatography paper. A Xerox ColorQube 8570 color printer was used to print wax-based inks. The print head dispenses ink (melted wax) on the surface of the paper, where it cools and solidifies instantaneously without further spreading. The ink contains hydrophobic carbamates, hydrocarbons, and dyes that melt around 120 °C. The default printer parameters were used for enhanced resolution printing. Two designs of patterned paper devices were used, 4 mm diameter circles with 0.4 mm line thickness (referred as spots from now on), and microfluidic channels of 2.4 mm nominal width and 28 mm nominal length (plus a reservoir of 13 mm nominal length), defined by lines of 0.4 mm nominal thickness. After printed, the paper devices are heated on a magnetic stirrer/heat plate with temperature sensor (MR Hei-Standard, Heidolph Instruments®, Schwabach, Germany) with temperature set at 150 °C for 2 minutes. In this step it is important to use a flat and uniformly heating surface to create a uniform three-dimensional hydrophobic barrier [3]. The paper device is ready to use after cooling at room temperature.

Production and Purification of CBM3-ZZ. The CBM3-ZZ fusion protein (~30 kDa) was cloned in *E. coli* by NZYTech, Lda.. The fusion combines an N-terminal double Z-domain of the staphylococcal protein A and the family 3 cellulose-binding module from *C. thermocellum* CipA. The genes were cloned into the NdeI-XhoI sites of a pET21a (Novagen) expression vector. The corresponding pET_ZZCBM3 plasmid was used to transform the *E. coli* DE3 strain BL21 (Novagen). For CBM3-ZZ production, 100 μ L of *E. coli* DE3 strain BL21 transformed with

pET_ZZCBM3 were cultivated in 5 mL of LB media supplemented with 100 μ g/mL ampicillin. Cells were cultured overnight at 37 °C with a shaking rate of 250 rpm. After reading the OD600 nm, the cell suspension was diluted down to an OD600 nm of 0.1 and used to inoculate 250 mL of LB media supplemented with 100 μ g/mL ampicillin. Cells were grown at 37 °C with a shaking rate of 250 rpm and expression was induced at an OD550 nm of ~0.5 with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 16 hours at 37 °C (250 rpm). Cells were harvested by centrifugation at 1900 g (Sorvall® RC-6 Plus superspeed centrifuge with SS34 rotor), 4 °C, for 10 minutes, and resuspended in a minimum volume of Tris-saline Tween 20 buffer (TST: 50 mM Tris buffer, pH 7.6, 150 mM NaCl, 0.05% Tween 20). Cells were disrupted by sonication (Branson Sonifier 250) for 6 x 30 seconds on ice with interruptions of 30 seconds (50% duty cycle, microtip limit 5). Centrifugation at 12,000 g, at room temperature, for 20 minutes was performed to separate the supernatant containing the fusion protein from cell debris

The CBM3-ZZ protein was purified by affinity chromatography using an IgG Sepharose 6 Fast Flow column (GE Healthcare) in an ÄKTA 10 Purifier system. The column was equilibrated with 5 column volumes of TST buffer and then loaded with 2 mL of the supernatant containing CBM3-ZZ, unbound proteins were washed away in a single step with 10 column volumes of TST. The bound CBM3-ZZ was eluted with 0.5 M acetic acid, pH 2.8. The pH of the collected fractions of CBM3-ZZ was immediately neutralized with 3.2 M Tris buffer, pH 10.96.

The purity of CBM3-ZZ fusion protein was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% acrylamide gel. All samples were diluted prior to denaturation in reducing conditions with 5 μ L of 1 M dithiothreitol at 100 °C for 5 minutes. Gels were stained with Coomassie Brilliant Blue. A 10-250 kDa protein ladder (Bio-Rad) was used as a molecular mass marker. Images of SDS-PAGE gels were obtained with a GS-800™ Calibrated Densitometer (Bio-Rad). The purified CBM3-ZZ concentration was determined by the BCA (Bicinchoninic Acid) Protein Assay using the Pierce® BCA Protein Assay kit (microplate procedure), according to the manufacturer's instructions. A buffer composed of 10 mM NaHEPES, pH 7.5, 2 mM imidazole, 200 mM NaCl and 1 mM CaCl₂ was used as diluent. The purified CBM3-ZZ was stored at -20 °C before use.

Spot Assays. Immobilization. In all the performed assays, 2 μ L of the complex CBM3-ZZ:antibiotin IgG (ratio1:2.5) was applied on the patterned spots in fractions of 0.5 μ L, letting it air dry at room temperature between applications.

The mixture of 2 pmol of CBM3-ZZ and 5 pmol of antibiotin IgG in TST was pre-incubated for 30 minutes at room temperature before being spotted on paper.

Capture. The capture of biotin labeled gold nanoparticles was evaluated by applying different amounts of biotin-AuNPs on spots patterned on paper (0, 0.5775, 1.155 and 2.31 fmol) that contained either the CBM3-ZZ:antibiotin IgG complex or physically adsorbed antibiotin IgG. As a control, biotin-AuNPs were applied to a plain spot. The mixture of the CBM3-ZZ:antibiotin IgG conjugate and its consequent immobilization in paper patterned spots was performed as explained previously. After the immobilization is done and the paper is dry, 2 μ L of TST containing the amounts of biotin-AuNPs specified above are applied in the quantity previously referred were added

Capture control conditions. To perform this assay, spots in three different conditions were prepared. A spot was functionalized by physical adsorption of 5 pmol of antibodies per spot, another spot was given a function by biochemical immobilization of 2 pmol of CBM3-ZZ and 5 pmol of antibodies, and a last spot was prepared with no function. In the capture test, an antibiotin IgG was used while in the negative controls an anti-FITC IgG was used. To all the spots 2.31 fmol of biotin-AuNP in 2 μ L of TST were applied.

Optimization of AuNP. The procedure used to apply sample solutions on the patterned spots on paper was studied by applying a total of 2 μ L of a solution containing 2.31 fmol of biotin-AuNPs in TST buffer to spots functionalized as described before. One spot without anything applied on its surface, one functionalized by physical adsorption of 5 pmol of antibiotin IgG, and one spot functionalized by the conjugate CBM3-ZZ:antibiotin IgG prepared as previously described. The 2 μ L solution of AuNPs was applied on each of the different spots as 4 x 0.5 μ L, 2 x 1 μ L or 1 x 2 μ L.

SEM Observation of the spots in different conditions was done by scanning electron microscopy (SEM) using a FEG-SEM JEOL JSM7001F equipment. Prior to analysis, samples were coated with an Au/Pd layer using a Polaron E5100 coating system (Quorum Technologies). The samples analyzed were the 3 different conditions evaluated before, one spot with only the Biotin-AuNPs, one spot with antibiotin IgG physical adsorbed and Biotin-AuNPs, and one spot with the CBM3-ZZ:antibiotin IgG conjugate immobilized and Biotin-AuNPs. In all the mentioned conditions, 2.31 fmol of Biotin-AuNP in 2 μ L of TST buffer was used.

Microfluidic channel assays. **Capture.** μ PADs were prepared by applying 2 μ L of a solution containing 5 pmol of antibiotin IgG or 2 μ L mixtures of 2 pmol of CBM3-ZZ and 5 pmol of

antibiotin IgG in the detection zone (spot 2/T). Solutions were always pre-incubated at room temperature for 30 min. The dispensing of the 2 μ L solutions was done in fractions of 0.5 μ L to assure that the antibiotin IgG and the CBM3-ZZ:antibiotin IgG conjugates remained mostly in the center of the detection zone, without flowing by capillarity through the channel. After drying, different quantities of Biotin-AuNPs (0.5775, 1.155, 2.31, 4.62 fmol) diluted in 15 μ L of TST were applied in the sample loading zone with the microfluidic device hanging in the air (Figure 3-5). Each μ PAD was then washed by adding 15 μ L of TST to the sample loading zone. Once dried, μ PADs were scanned using a HP Scanjet 4400c scanner. The mean grey intensity of the capture spots was then measured with the ImageJ software as previously described. The mean grey intensity takes the background signal into consideration.

Results and Discussion

One of the objectives of this work was to evaluate, through proof-of-concept experiments, whether the fusion protein CBM3-ZZ could be used in the context of antigen detection in PADs. In a first instance, several spots were fabricated on paper by wax printing, outlining 4 mm circular reaction areas by hydrophobic barriers. Different immobilization strategies were used to functionalize the fabricated spots with antibodies, a biochemical coupling with a CBM3-ZZ fusion versus a physical adsorption approach. In the first case, CBM3-ZZ fusions (2 pmol/spot) and antibiotin IgG (5 pmol/spot) were pre-incubated before spotting, while in the physical adsorption strategy antibiotin IgG (5 pmol/spot) was spotted directly on paper. The ratio of CBM3-ZZ:antibody IgG was determined in previous work of Rosa *et al.* [6]. Different quantities of biotin labeled gold nanoparticles (biotin-AuNPs) were then applied to those spots. Spots where only biotin-AuNPs were added were used as controls.

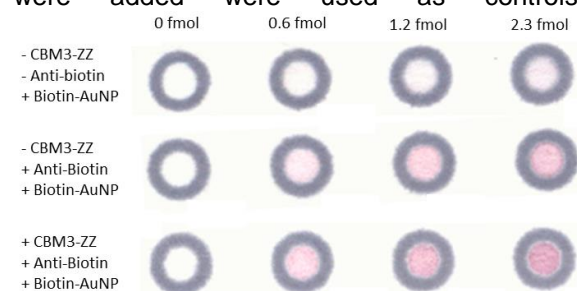


Figure 1 Capture of biotin-AuNPs by antibiotin IgG immobilized in paper spots. The first row shows control experiments where different amounts of biotin-AuNPs (0- 2.31 fmol) were applied to non-functionalized spots. In the second row, biotin-AuNPs were captured by physically adsorbed antibiotin IgG (5 pmol/spot). In the third row, the biochemical coupling of CBM3-ZZ (2 pmol/spot) and antibiotin IgG (5 pmol/spot) was evaluated. The 4 mm circular areas were defined on paper by wax-printing.

Analyzing the spots in Figure 1, it is noticeable the virtually absence of color in the spots where only the biotin-AuNPs were applied, while in the spots where either of the antibodies immobilization strategies were used, there is an increasing intensity of red color with the increase of biotin-AuNPs applied. To further analyze the results, the mean grey intensity of each of the spots was measured using ImageJ software (Figure 2).

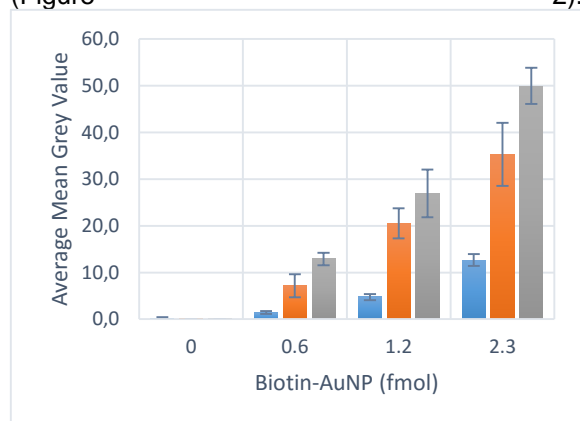


Figure 2 Capture of biotin-AuNPs by antibiotin IgG immobilized in paper spots. The figure shows a comparison of the average mean grey intensity of the spots prepared with the different immobilization strategies represented in Figure 2. Each one of the represented test conditions was done in triplicate and the error bars represent the standard deviation. **Legend:** Blue – Biotin-AuNP; Orange – Antibiotin IgG + biotin-AuNPs; Grey – CBM3-ZZ:antibiotin IgG + biotin-AuNP.

The higher color intensity of the spots where the biochemical coupling of the antibiotin IgG by CBM3-ZZ was used as an immobilization strategy is observable by analyzing figures 1 and 2. The physical adsorption of the antibody also

display a higher intensity of the color developed when compared with the simple deposition of biotin-AuNPs on paper, but inferior to the spots with CBM3-ZZ:antibiotin IgG conjugate. This weaker signal is probably due to the random orientation of antibiotin IgG on the surface of the paper, which can lead to a reduced recognition and ability to capture the biotin-AuNPs due to the steric hindrance of binding sites [4], [6], [10]. The physical adsorption of antibodies to the paper surface is established mainly by weak interactions, such as Van der Waals and electrostatic forces [4], and as such if a washing step, of the strips of paper with the spots, was done between the immobilization step and the addition of the sample, a portion of the physically adsorbed antibodies would be removed from the spots [6]. Even though the filter paper may still have a capacity to adsorb a great quantity of antibodies the signal measured would be lower, while the color intensity of the spots where biochemical immobilization was applied would be constant.

Figure 3 data concern the two bottom spots of the last column of figure 1 where the two different approaches to immobilization are shown. The plot profile analysis was done in ImageJ by drawing a line through the middle of the spot, and the surface plot was acquired by delineating a circular area covering the whole spot and using the surface plot tool. In the plot profile graphs, the maxima of the curves correspond to the wax barriers limiting the reaction area. Analyzing the spot with physically adsorbed antibodies (Figure 3A) it is possible to notice that there is an

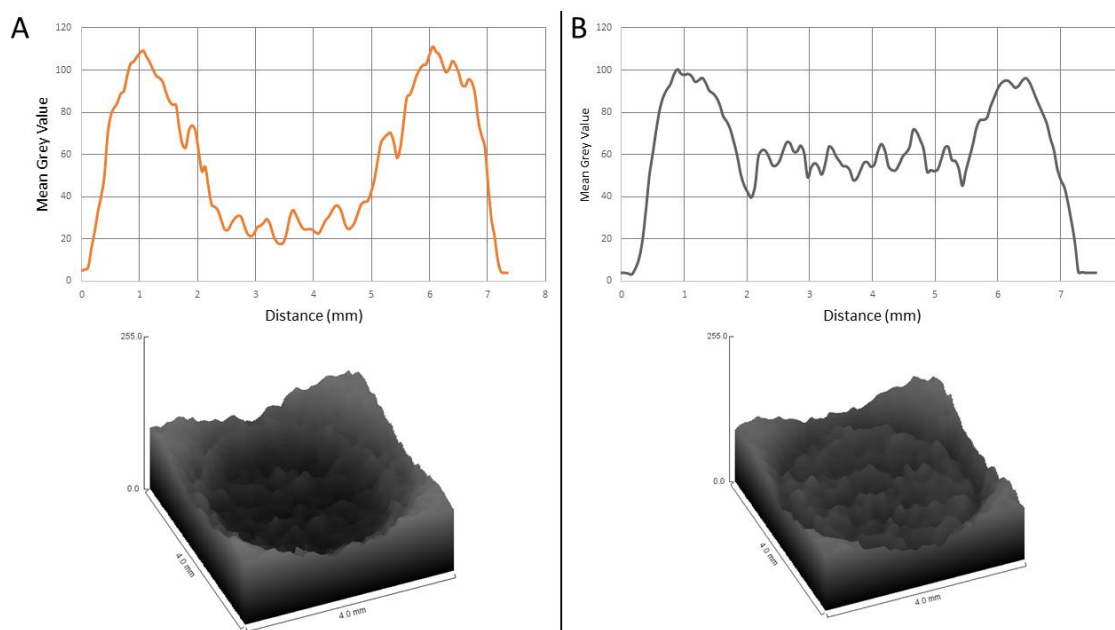


Figure 3 Plot profile (upper graph) and surface plot of spots where Biotin-AuNPs were captured by antibiotin antibodies immobilized by adsorption or biochemical coupling. A) Physical adsorption of antibiotin IgG (5 pmol/spot) and 2.31 fmol of biotin-AuNPs. B) Immobilization through biochemical coupling of CBM3-ZZ (2 pmol/spot) and antibiotin IgG (5 pmol/spot) pre-incubated and addition of a sample with 2.31 fmol of biotin-AuNP.

accumulation of color near the walls of the spot, at around 2.0 mm and 5.2 mm. This phenomenon is called the coffee ring effect, and it occurs because a drop of a liquid tends to spread by capillarity flow from the center to the edges, bringing the particles suspended in the solution to the walls of the spot [11]. After subsequent evaporation, the concentration of the particles will be higher on the edges than in the center. In the case of biochemical immobilization, this effect is not observable, being the intensity of the color evenly distributed through the spot, although not being completely homogeneous (Figure 3B). CBMs are molecules with high affinity to cellulose and spontaneously bind to it. As such when the CBM3-ZZ:antibiotin IgG conjugate is immobilized in paper and the sample with biotin-AuNPs is applied, the antibiotin IgG will readily capture the biotin-AuNPs, which will not be able to flow to the edges.

Control conditions. To investigate whether the increase in the intensity of the colors generated in the spots was due to the recognition of the biotin-AuNPs by the antibiotin IgG, a control assay was designed where an anti-FITC IgG was used instead of antibiotin IgG. To test this, the same conditions were used for the immobilization methodologies. The physical immobilization was done by applying 5 pmol of either antibiotin IgG or anti-FITC IgG per spot. The biochemical immobilization was performed by dispensing a pre-incubated mixture of 2 pmol of CBM3-ZZ and 5 pmol of either of the referred antibodies. Plain paper spots were used as a control.

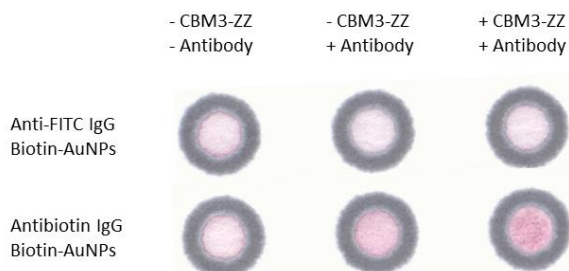


Figure 4 Capture of biotin-AuNPs by antibiotin IgG using different immobilization strategies versus a control assay using an anti-FITC IgG. Negative controls with anti-FITC IgG are shown in the top row, and assays with antibiotin IgG are shown in the bottom row. On the left column are the biotin-AuNPs applied on paper, the middle column refers to the physical adsorption assays and the last column are the biochemical coupling assays. 2.31 fmol of biotin-AuNPs were applied to each spot.

Analyzing the spots of figure 4 by naked eye, it is possible to notice a higher intensity of the color in the spot corresponding to the biochemical immobilization of the CBM3-ZZ:antibiotin IgG conjugate when compared to all the other spots, corroborating the data previously acquired. The spots where the antibody used was an anti-FITC IgG show no variation in the intensity of the color.

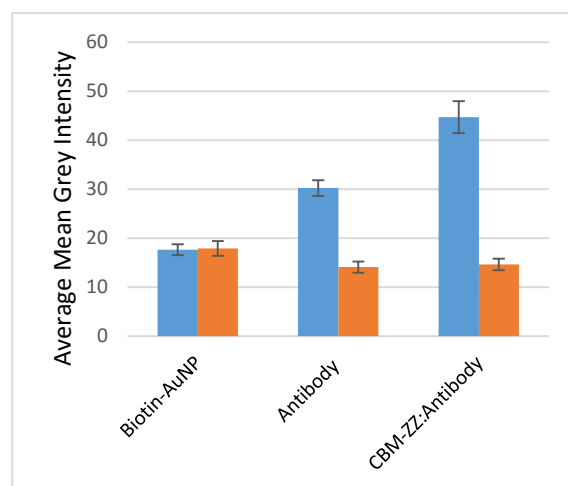


Figure 5 Capture of biotin labeled gold nanoparticles by antibiotin IgG using different immobilization strategies versus a control assay using an anti-FITC IgG. The assay was performed in triplicates and the error bars represent the standard deviation. **Legend:** Blue – antibiotin IgG; Orange – Anti-FITC IgG.

Figure 5 shows the analysis of the intensity of the color developed in the paper spots. The negative control, using anti-FITC IgG, did not show a significant variation, even though the average mean grey intensity was slightly higher in the spot with no immobilization strategy applied. Significant increases in color were detected when physically or biochemically immobilizing antibiotin IgG. The development of a more intense red color in the spots with the biochemically-coupled antibiotin IgG can be attributed to a more correct orientation of the antibody provided by the CBM3-ZZ fusion and hence to a more efficient recognition of the antigen.

Optimization. To study the way the sample addition affect the final color developed in each immobilization condition, an assay where the sample solution with AuNPs was added in fractions of the total volume was designed. In spots non-functionalized, and functionalized by either physical adsorption or biochemical coupling as previously described, a total volume of 2 μ L of solution containing 2.31 fmol of biotin-AuNPs in TST was applied in fractions of 0.5, 1 or 2 μ L. Analyzing figure 6, it is noticeable the same pattern between the different immobilization techniques in study. While the spots where the physical adsorption of antibiotin IgG was used and the spots where only the biotin-AuNP were applied display the coffee ring effect, which is noticeable by the accumulation of the gold nanoparticles near the edges of the reaction zone, the biochemical coupling of the CBM3-ZZ:antibiotin IgG results in a more uniform and concentrated signal.

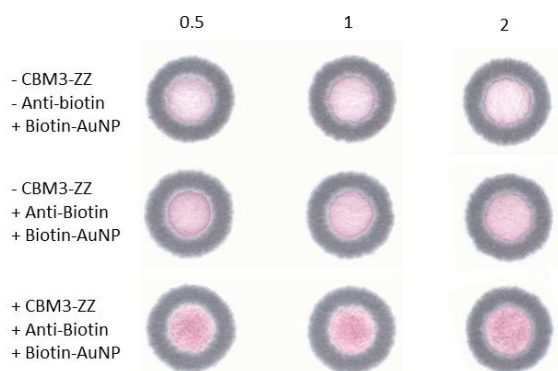


Figure 6 Evaluation of the intensity of the color obtained in the different immobilization approaches in study when the sample is applied in different volume. Each column concerns the fraction of volume (in μL) which was applied in each spot, always totaling 2 μL applied per paper spot. The top row concerns the spots with only the biotin-AuNPs applied. The middle row regards the physical immobilization of anti-biotin IgG. The bottom row concerns the biochemical coupling of the anti-biotin IgG by CBM3-ZZ.

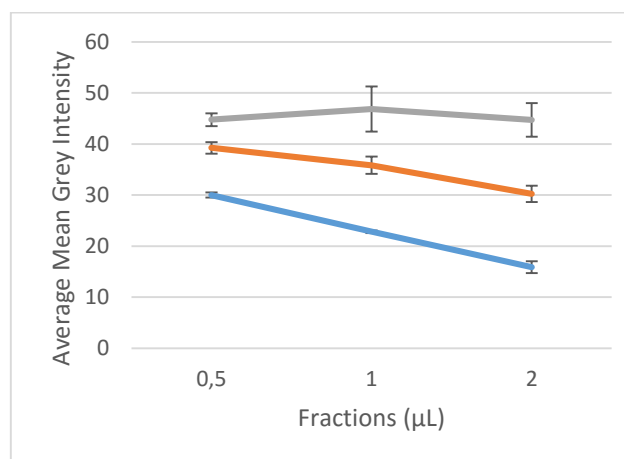


Figure 7 Evaluation of the intensity of the color obtained in the different immobilization approaches in study when the sample is applied in fractions of the total volume. The assay was performed in triplicates and the error bars represent the standard deviation. **Legend:** Blue – Biotin-AuNP; Orange – Anti-biotin IgG + biotin-AuNPs; Grey – CBM3-ZZ:anti-biotin IgG + biotin-AuNP

Figure 7 show the average mean grey intensity as a function of the volume of fractions applied as the spots in figure 6. The intensity of the color in the spots with the CBM3-ZZ:anti-biotin IgG conjugate immobilized display a color without a significant variation in its intensity, while both the physical adsorption approach and the spots with only the biotin-AuNP applied on paper show a decreasing intensity of the color with the increase of the volume applied with the sample. This phenomenon is, once more, related to the coffee ring effect [11]. As the molecules in these spots are not strongly bound to the paper, when a single addition of 2 μL is used the molecules in suspension tend to be pushed to the edges. Although the addition of the samples in four fractions of 0.5 μL is enough to wet all the reaction zone of the spot, the volume of buffer is not enough to make the molecules in suspension get dragged towards the edges of the spots, thus

being more deposited near the center of the spots and giving a higher intensity when analyzed by ImageJ.

SEM Spots containing biotin-AuNPs captured by physically or biochemically immobilized anti-biotin IgG were analyzed by SEM (Figure 8).

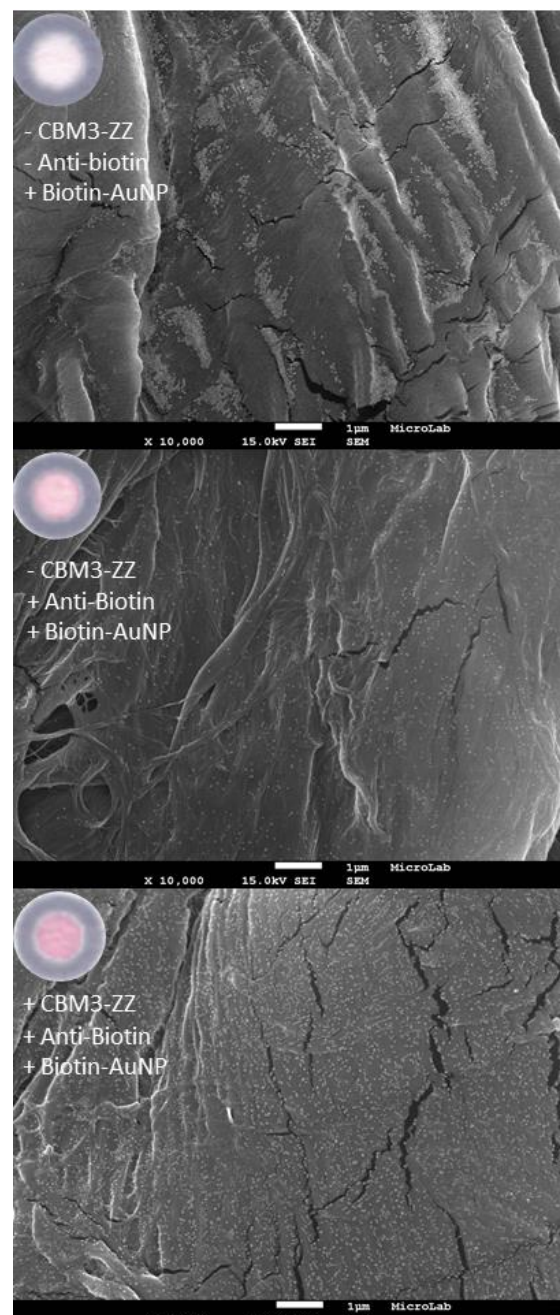


Figure 8 SEM images of spots containing biotin-AuNPs captured by physically or biochemically immobilized anti-biotin IgG. The upper left image corresponds to a control spot with only biotin-AuNPs applied on paper. The upper right image concerns a spot where biotin-AuNPs were captured by physically adsorbed anti-biotin IgG. The bottom image corresponds to a spot where biotin-AuNPs were captured by a biochemically immobilized CBM3-ZZ:anti-biotin IgG conjugate. All images were acquired at a 10,000x magnification. The scale of each photograph is defined by the horizontal white bar which corresponds to 1 μm .

The SEM image of the control spot with only the biotin-AuNPs shows that the gold nanoparticles accumulate together in the

grooves created by the cellulose fibers (Figure 8, top left). On the contrary, in the SEM image of the spot prepared by biochemical immobilization of antibiotin-IgG with the CBM3-ZZ fusion, a uniform distribution of the gold nanoparticles throughout the whole observable surface of the paper is visible (Figure 8, bottom). The image obtained from a spot where the physical immobilization of the antibiotin IgG was used, show a in between state of the other conditions (Figure 8, top right). In this condition the gold nanoparticles are not accumulated as in the first case, but they also are not so evenly distributed as in the biochemical coupling. This is due to two main reasons: the random orientation of antibiotin IgG on the surface of the paper, which can lead to a reduced recognition and ability to capture the biotinylated gold nanoparticles [4], [6], [10]; the physical adsorption of antibodies to the paper surface is established mainly by weak interactions, and as such, these can be washed to the edges of the spot [4].

The difference in the developed color between the spots with only the biotin-AuNPs and the spots where the nanoparticles were captured by the CBM3-ZZ:antibiotin IgG conjugate may be due to two factors: i) as the CBM3-ZZ has affinity for cellulose and will orientate the antibiotin IgG correctly, the biotin labeled gold nanoparticles will be forced to stay at the surface of the paper, while without any biosensor immobilization for recognition of the gold nanoparticles those will flow through the paper fibers and can become hidden between the fibers translating in a low intensity color observed; ii) the optical properties of gold nanoparticles change when there is an aggregation of the particles leading to the conduction electrons near each particle surface to become delocalized and shared amongst neighboring particles. Consequently, the surface plasmon resonance shifts to lower energies, causing the absorption and scattering peaks to shift to longer wavelengths. UV/Visible spectroscopy can be used as a simple and reliable method for monitoring the stability of nanoparticle solutions. As the particles destabilize, the original extinction peak will decrease in intensity, due to the depletion of stable nanoparticles, and often the peak will broaden or a secondary peak will form at longer wavelengths, due to the formation of aggregates [9], [12]. This phenomenon which happens in solution is probably occurring in the paper spots in this study.

μPADS Capture. The capture of biotin-AuNPs by antibodies immobilized in wax-printed microfluidic channels on paper via biochemical

coupling with CBM3-ZZ was studied versus the physical adsorption of the antibody. To perform this assay either 5 pmol of antibiotin IgG or 2 pmol of CBM3-ZZ and 5 pmol antibiotin IgG pre-incubated were dispensed in the test zone (marked T in Figure 9). After drying, a sample with different quantities of biotin-AuNP was applied in the sample loading zone at the beginning of the microfluidic channel. An additional amount of buffer was added to elute the AuNPs alongside the channels.

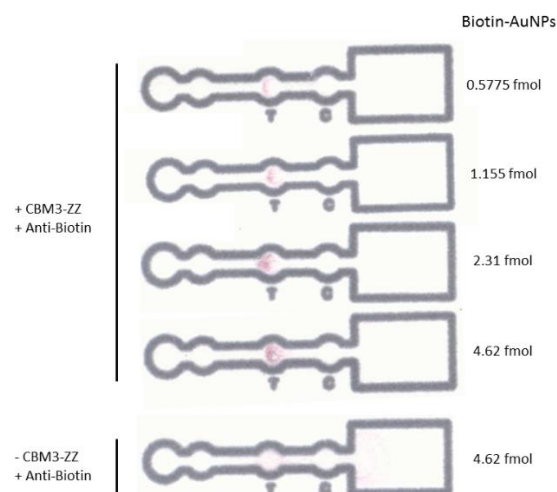


Figure 9 Capture of biotin-AuNPs by antibiotin IgG immobilized in paper microfluidic channels via biochemical coupling with CBM3-ZZ and physical adsorption. The bottom microfluidic channel was prepared using physical adsorption of the antibiotin IgG in the test zone, while the remaining were prepared through biochemical immobilization.

The intensity of the color developed in the test zone increases with the increasing quantities of loaded biotin-AuNPs. The μ PAD prepared by physical adsorption of the antibiotin IgG does not present a signal visible with the naked eye when compared with the immobilization by biochemical coupling. The preliminary results shown in Figure 10, suggest a linear increase in the intensity of the color developed with increasing quantities of biotin-AuNPs in the sample added. The μ PAD functionalized by physical adsorption of antibiotin IgG in the test zone shows a very low intensity of color developed, most probably because the interactions of the antibodies with the cellulose fiber are weak [4], [6]. The addition of a buffer like TST, which contains 0.05% of Tween 20, will drag the antibody through the channel. Tween 20 is a surfactant commonly used in biochemical assays to remove unbound compounds and prevent nonspecific antibody binding [4], [6]. Still some antibody molecules may be retained in the cellulose fibers that will be able to capture some of the biotin-AuNPs.

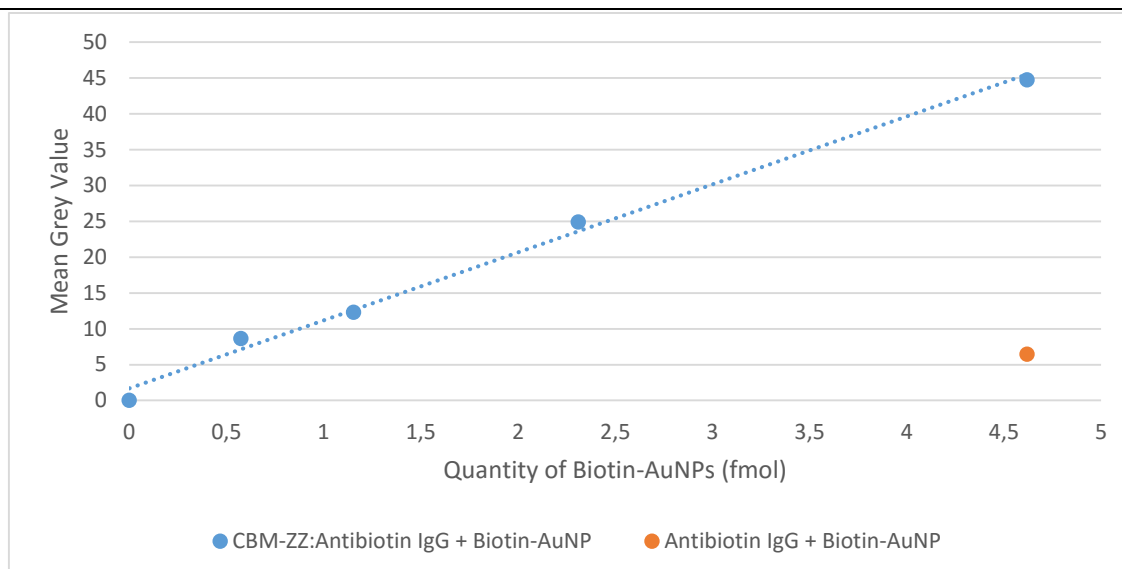


Figure 10 Capture of biotin-AuNPs by antibiotin IgG immobilized in paper microfluidic channels via biochemical coupling with CBM3-ZZ and physical adsorption.

Conclusions

The development of μ PAD has been growing at an accelerated pace, as it is a promising technology to develop easy-to-use and affordable disease diagnosis and environmental monitoring for both developed and developing countries. This work had as an objective, the gain of insight in the development of μ PAD for immunodetection using antibodies anchored to paper through carbohydrate-binding modules fusion proteins, and gold nanoparticles as a colorimetric report agent.

The first stage of this work was to evaluate the capture ability of an antibiotin IgG immobilized on paper either by physical adsorption or biochemical coupling, in 4 mm circular reaction areas delineated by hydrophobic wax barriers. The assays using the CBM3-ZZ:antibiotin IgG complex as an immobilization approach yielded a more intense red color than the physical adsorption of the antibodies when gold nanoparticles were present. As a control, an anti-FITC IgG was used and the red color was virtually absent. SEM imaging showed that when using the antibiotin IgG immobilized by biochemical coupling the gold nanoparticles coated with biotin are uniformly distributed, while in the absence of the antibody the gold nanoparticles tend to form clusters.

The ability of the gold nanoparticles to be used as a colorimetric report agent either for a qualitative as a quantitative analysis was demonstrated, by the development of a red color of different intensity according to the quantity of gold nanoparticles applied.

The second part of the work was to evaluate the capture ability of an antibiotin IgG anchored to paper by CBM3-ZZ in a microfluidic channel with 28 mm of length (and 2.4 nominal width)

plus a reservoir with 13 mm nominal length. The capture of the gold nanoparticles coated with biotin was successful, displaying an intense red color formed in the capture zone, while in a physical adsorption assay no signal is observable.

The work developed gives an insight on the development of immunoassays involving carbohydrate-binding modules as an immobilization approach and gold nanoparticles as a colorimetric report agent in microfluidic devices.

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