Paper-based Bioassays for Dengue Diagnostics Using Gold Nanoparticle Colorimetric Probes

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Abstract The availability of rapid and portable tests for a field-diagnostics of Dengue, an acute febrile disease caused by the mosquito-borne dengue virus that affects almost 40% of the world population, would be a valuable asset for the developing countries most hit by the disease. Such point of care (POC) tests should work with small sample volumes (100 nl to 1 mL) with low concentrations of analytes (femtomolar to millimolar) and provide a result in seconds to hours. One option to create a POC test is to combine microfluidic paper-based devices with gold nanoparticles (AuNPs) in order to obtain a colorimetric result that is visible with naked eye. In this work, paper-based tests were developed to detect the hybridization between DNA probes and a dengue RNA target (DENV2) that rely on the capture of hybrids by CBM64-ZZ:anti-biotin antibody conjugates anchored on paper and AuNP detection. This system uses two DNA probes, one coated onto AuNPs and another labeled with biotin, to detect the label-free dengue RNA target. The presence of the target in a sample is signaled by the presence of a red spot.

Dengue is an acute febrile disease caused by the mosquito-borne dengue virus (DENV). This virus is a member of the *Flavivirus* genus of the *Flaviviridae* family and includes four antigenically related serotypes (DENV 1-4), all of which cause the disease. Flaviviruses are enveloped viruses with a single stranded 11kb, positive-sense ribonucleic acid (RNA) genome. It encodes a single long open reading frame (ORF), flanked by highly structured 5' and 3' untranslated regions (UTRs). The dengue virus genome has been completely sequenced and data shows that there is 60 to 70% of homology between the four serotypes.[1-3] The virus is transmitted to humans through *aedes aegypti* or *aedes albopictus* vectors, commonly known as the dengue mosquitos. These mosquitos cover most of the tropical and subtropical areas of the world. About 2500 million people, or 40% of the world's population, live in areas where there is a risk of dengue transmission. Dengue infection is a systemic and dynamic disease. It has a broad clinical spectrum that includes both severe and non-severe clinical manifestations. After the incubation period, the illness arises brusquely and is followed by three phases – febrile, critical and recovery. This disease is complex in its manifestations and the key for recovery is early recognition and understanding of the clinical problems during the different phases of the disease. There is an urgent need for simple, user-friendly, inexpensive and instant diagnosis tests. Such point of care (POC) tests require small amounts of sample (100 nL to 1 mL) with low concentrations of analytes (femtomolar to milimolar), little or no sample preparation and provide a result or answer in seconds to hours. Also, the costs involved are lower when compared with those of conventional laboratory analysis and the involved instruments are normally smaller and less specialized, so they cost less. In 2007 the group of Prof. George Whitesides at Harvard University described the principles and key features behind microfluidic paper-based analytical devices (μPADs). These systems, which combine the potentials of conventional microfluidic devices with simple detection methodologies, have been since applied for several purposes[4]. μPADs contain patterned microfluidic hydrophilic channels – capillary channels - on paper that enable aqueous solutions to flow on delimited areas of paper. In order for these μPADs to be useful they have to be functionality bioactive. Bioactive paper is any low-cost and easy-to-use paper product laced with biologically active chemicals that provides a quick way to biorecognition. In this recognition system the target analyte reacts with the bioreceptor, in this case nucleic acids, leading to a measurable effect. The anchoring of these receptors onto paper has bee accomplished by bioaffinity attachment

[5,6], using carbohydrate binding modules (CBM), which have high affinity to cellulose.

In order for µPAD diagnostics to be completely functional one last step is required, which is the detection. Gold nanoparticles (AuNPs) are one of the most promising tools for colorimetric reporting since they have unique opto-physical properties, which enable sensitive detection of biomarkers and are receptive to modification for use in different assay formats. AuNPs are small (normally 10-50 nm in diameter) and spherical, they have an intense red color and their extinction coefficients are significantly higher than those of common organic dyes and proportional to their size. The intensive red color allows the use of AuNPs as colorimetric labels and their qualitative interpretation does not require a reader. Because of their relatively small size AuNPs can be distributed in high density onto the test regions, leading to a more intense colorimetric response. Also, high color intensity helps the discrimination of weak positive signals. [7-9].

Experimental Section

Gold nanoparticles synthesis Gold nanoparticles, with around 13 nm, were synthesized by the citrate reduction method[10]: a boiling solution of chloroauric acid ($HAuCl_4$, 1 mM) was mixed with a trisodium citrate solution (TST, 38.8 mM; 0.279 g for 25 mL of Mili-Q water). This mixture is heated under reflux for 30 minutes, leading to a change in color from pale yellow to deep red.

AuNP functionalization A thiol-DNA probe (sh_5' – cct gtt ctc gga gag – 3') was purchased from STAB-Vida and attached to AuNPs as follows. Probes were dehydrated prior to functionalization in a rotary evaporator (Thermo Scientific – Savant DNA 120 Speed Vac Concentrator) for about 1 hour (h). Probes were then resuspended in 50 µL of dithiothreitol (DTT, 1 M) and incubated at room temperature for 1 h followed by the addition of 450 µL Mili-Q water and incubation for 1 h at room temperature. Next, extraction was performed 3 times with ethyl acetate, using a ratio of 1 volume of DNA solution to 2 volumes of ethyl acetate and a 5-minute centrifugation at 21,380g and room temperature for phase separation. The DNA-containing sample was purified in a NAP-5 column (GE HealthCare®) according to the manufacturer's instructions. The final concentration of DNA was measured by reading the absorbance at 260 nm in a Nanodrop spectrophotometer (NanoVue Plus, General Electrics) and applying the Lambert-Beer equation. The functionalization of AuNPs with DNA probes (at a theoretical molar ratio of 1:200 AuNP:DNA) was made by salt-aging. The nanoparticles were mixed with the DNA probe at a molar ratio of 1:200 with addition of an AGE I solution (10 mM phosphate buffer (PB), 2% SDS, pH 8) in a ratio of 1:200 (v/v) (AGE I:AuNP-DNA (v/v). After 10 seconds in an ultrasound bath the solution was incubated in the dark for 30 minutes. Next, AGE II solutions (10 mM PB, 0.01% SDS, 1.5 M NaCl, pH 8) with 50, 100, 200 and 300 mM NaCl were added sequentially. Between each NaCl solution concentration, solutions are kept for 10 s in the ultrasound bath followed by a 30-minute incubation in the dark. After the last addition, the solution is incubated overnight. In the following day, AuNPs were washed four times, two with phosphate buffer (10 mM, pH 8) and two with phosphate buffer saline (PB 10 mM, NaCl 0.1 M, pH 8). Each wash step is intercalated with centrifugation for 40 minutes at 21,380g. By measuring the absorbance at 526 nm in a Nanodrop spectrophotometer and using the Lambert-Beer equation, assuming an extinction coefficient of $2.33 \times 10^8\ M^{-1}\ cm^{-1}$ for the plasmon resonance band[11], a final concentration of 15 nM is obtained.

DNA:RNA hybdridization The goal of this work was to analyze the ability of the bioactive paper areas described above to yield a positive, red signal, when challenged with a dengue virus RNA target sequence that is pre-hybridized with a probe DNA sequence functionalized onto AuNPs. The first element in the system, which is used to capture dengue RNA targets, is the recombinant fusion protein CBM64-ZZ (25.2 kDa). This protein results from the fusion of an N-terminal double Z-domain of protein A from *Staphylococcus aureus,* and a C-terminal Carbohydrate Binding Module from *Spirochaeta thermophila,* CBM64. The CBM part anchors the fusion to paper, while the double Z-domain provides a way to capture an anti-biotin monoclonal

antibody through its Fc portion. Mouse monoclonal anti-biotin (IgG2a) was obtained from Abcam (Cambridge, UK). The Fc portion of this antibody has high affinity to the ZZ portion of the CBM, whereas the variable regions are able to bind to biotin that will be added to the nucleic acid strands. The other part of the system is composed of three nucleic acid sequences: 1) a capture DNA strand modified with biotin at the 5' extremity (5'_ Biotin – ttt ttg aag tcg agg – 3', STABvida) 2) a reporter DNA probe that is immobilized in gold nanoparticles, and finally 3) the target viral RNA (from serotype 2 of the DENV genome, GenBank JX470186) (5'- ctc tcc gag aac agg cct cga ctt caa-3') that is complementary to the other two strands (Figure 1). The functionality of this system is assessed by observing the appearance of a red color due to the accumulation of the AuNPs on the surface of the test region.

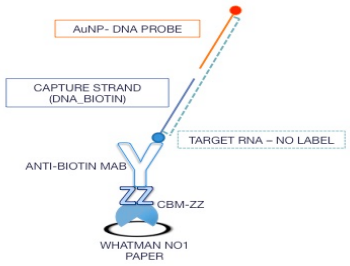

Figure 1 Detection of dengue virus based on nucleic acid hybridization and colorimetric reporting with gold nanoparticles. From the bottom: chromatographic paper, CBM64-ZZ fusion protein, anti-biotin antibody, biotin-labeled DNA capture strand, target RNA and AuNPs functionalized with detection probe.

Spot assays Hybridization assays were performed in circular reaction areas (hereafter called "spots") delineated on Whatman N. 1 chromatographic paper by printing 4 mm circumferential hydrophobic barriers with a wax printer. The assays comprise two major steps: 1) the biochemical immobilization of the CBM64-ZZ:anti-biotin IgG conjugate on paper and 2) the detection of the hybridization between DNA probes and a dengue RNA target. The CBM64-ZZ:anti-biotin antibody conjugate was prepared in a tube by incubating fixed amounts of CBM64-ZZ and antibody at RT for 30 minutes in TST buffer (50 mM Tris buffer, pH 7.6; 150 mM NaCl; 0.05 % Tween 20). A volume of 2 µL was then dispensed on the circular reaction areas. A proportion of 5 pmol CBM64-ZZ to 5 pmol anti-biotin antibody was used. In the second step, fixed amounts of the biotin-labeled complementary (CS) (10 pmol) or non-complementary strand (NCS) (10 pmol) of DNA), and fixed amounts (0.01 to 10 pmol) of the label-free RNA target (exclusive of the), were mixed with 45 fmol of AuNPs modified with the DNA probe in an Eppendorf tube and incubated for 20 minutes at room temperature in TST buffer. 3.1 µL of this mixture were then dispensed on the spots and allowed to dry at 60°C. To assure that there was no unspecific signal due to the presence of AuNPs, a test with only the CBM64-ZZ and AuNP-DNA was always used as control.

Microfluidic channels: lateral flow assay To demonstrate the versatility of the detection system, a lateral flow test was performed in a µPAD with a microchannel of 2.4 mm nominal width and 28 mm nominal length and a reservoir of 13 mm nominal length as seen in figure 2. The spots are labeled with T and C for test and control region, respectively.

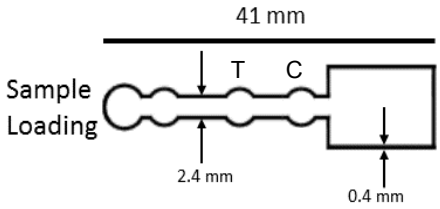

Figure 2 – Design of the µPAD used for detection of hybridization. The regions labelled with T and C correspond to the Test and Control spots respectively. This identification will be used throughout the work. The thickness of the printed wax lines is 0.4mm. After melting and diffusion of the wax, the width of the wax barriers increased to 1mm.

Two µL of a mixture of anti-biotin monoclonal antibody (5 pmol), CBM64-ZZ (5 pmol) and TST buffer were applied to the T and C regions of the micro channel (0.5 µL at a time with air drying at RT between applications). A mixture of 5 µL of AuNP-DNA (45 fmol), biotin-labeled probe DNA (10 pmol), RNA target strand (10 pmol), 1.5 µL of TST buffer and 4 µL of running buffer (PBS 1%, BSA 0.5%, Tween 0.01%, saccharose 2.5%) was applied in the sample-loading region of the micro channel. In order to flow the test solutions across the micro channel until the T

and C regions, 3 x 10 µL of the same running buffer was added to the loading region. The paper device was allowed to dry at room temperature.

Cellulose particles To further demonstrate the flexibility of the system, tests were implemented using cellulose microparticles. To do so, 5 mg of cellulose particles (Sigmacell cellulose, type 20, ~20 µm) were weighted and suspended in 100 µL of buffer. Then, 2 µL of the CBM64-ZZ:anti-biotin complex (5 pmol: 5 pmol, previously incubated at room temperature for 30 minutes) was added to the particle suspension and incubated for 30 minutes. To test the hybridization, 10 pmol of a fully complementary strand (CS) or of a non-complementary DNA strand, both modified with a biotin, and 10 pmol of target RNA were mixed and incubated for 20 minutes at RT with 90 fmol of AuNPs-DNA in TST buffer. Finally, a total volume of 5 µL of the hybridization mixtures was added to the CBM64-ZZ:anti-biotin antibody particle suspension, allowed to settle at room temperature and then centrifuged for 10 minutes at 21, 380 x g. To assure that there was no unspecific signal due to the presence of AuNPs, a test with only the CBM64-ZZ:anti-biotin antibody complex and AuNPs was used as control. Images were captured with an Olympus E-PM1 camera.

Spot Analysis The results of all paper-based assays were recorded using a HP Scanjet 4400c scanner. Images were then processed with the public domain, image processing software ImageJ (National Institutes of Health) by converting to 8-bit grey scale, inverting colors and then measuring the mean grey intensity of the test region.

Scanning Electron Microscopy A micro and nano scale observation of the paper regions generating positive and negative signals was performed by scanning electron microscopy (SEM) using a FEG-SEM JEOL JSM7001F equipment. This equipment includes an Energy Dispersive Spectroscopy (EDS) detector (Oxford Instruments) that allows the identification of particular chemical elements and their relative proportions in the sample.[12] Preceding the analysis, the paper spots were coated with a gold layer using a Polaron E5100 coating system (Quorum Technologies). One sample contained the anti-biotin antibody:CBM64-ZZ complex, the non-complementary, biotin-labeled DNA probe (ESAT-6), the RNA target (DENV2) and AuNPs functionalized with DNA detection probe. The second sample contained the CBM64-ZZ:anti-biotin antibody complex, a complementary, biotin-labeled DNA probe, the RNA target and AuNPs functionalized with probe DNA.

Results and Discussion

Capture of gold nanoprobe by paper-immobilized CBM64-ZZ:anti biotin mAB complex To evaluate the viability of thr detection system a paper-based assay was performed and three conditions were tested. The three conditions contain the CBM64-ZZ:Ab complex (5:5 pmol) and the gold nanoprobe (45 fmol), and vary on the capture strands: in the control no target and capture strand is present, and the other two contain the label free RNA target (10 pmol) but the NCS condition includes a non-complementary capture strand, while the CS conditions involves a complementary capture strand, both modified with a biotin (10 pmol). This test was performed in 4 mm paper spots (Figure 3).

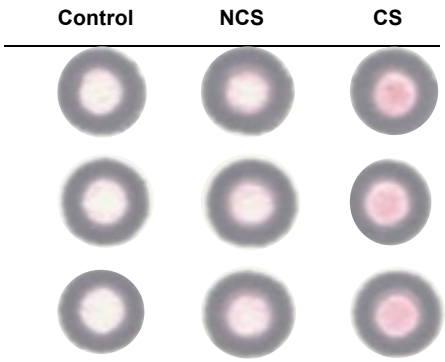

Figure 3 - Detection of DNA:RNA hybridization on paper-based 4 mm spots modified with CBM64-ZZ:anti-biotin antibody complex. Test samples were incubated with Au-nanoprobes and added to the spots. Three conditions were tested: control (no DNA or RNA), non-complementary capture probe (NCS) and complementary capture probe (CS). For proof-of-concept the assay was performed in triplicates

The success of the test was confirmed since the control and NCS spots show a white test area, while the CS spot presents a wide spread red signal that covers the whole test area (Figure 3).

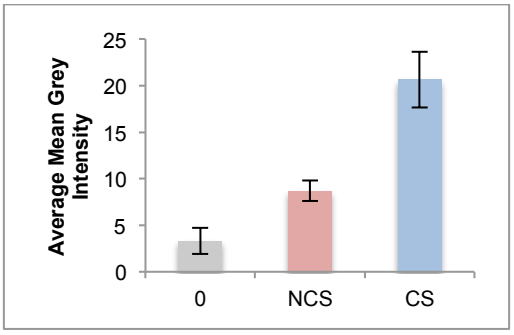

Figure 4 - Average mean grey intensites of the DNA:RNA hybridization spots shown in Figure 3. Grey bar: control spot; red bar: non-complementary strand + target RNA; blue bar: complementary strand target RNA. Tests were made in triplicate and the error bars represent the standard deviation.

The average mean grey intensities of the test areas (Figure 4) are in agreement with the visual observations of the paper test spots. As expected the intensities of the control (3.3 ± 1.4 a.u.) and NCS spots (8.7 ± 1.1 a.u.) are much lower than the one of the CS spot (21 ± 3.0 a.u.) that shows a very intense red signal. This proves that the system work and that 4 mm spots are suitable for performing these tests.

Determination of minimal target concentration for detection To determine the minimal target (RNA_DENV2) concentration necessary to obtain a signal, an assay with target concentrations ranging from 0 to 10 pmol was implemented (Figure 5). A control condition was included to assure that the presence of AuNPs alone do not generate a signal and a test with a non complementary capture strand was used to confirm the specificity of the system. Here the target was at 10 pmol. In the case of the complementary strand, 10, 1, 0.1 and 0.01 pmol of target were tested. Figure 5 shows that the minimal amount of target required to obtain a positive red signal is 1 pmol. A decrease in the average mean grey intensity of the signals was observed as the target amount decreased (Figure 6) showing that the best signal is obtained for 10 pmol. Even though there's no evident red signal in paper spot below 1 pmol, this chart suggests that some intensity is present.

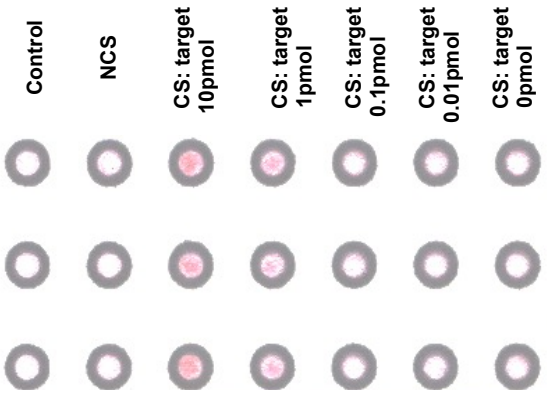

Figure 5 – Determination of the minimal target (RNA DENV2) concentration necessary to obtain a signal an assay with target concentrations ranging from 0 to 10 pmol. CBM64-ZZ:anti-biotin antibody was applied in every spot (5 pmol:5 pmol) and control and non complementary (NCS) conditions were included. The test was made in triplicate.

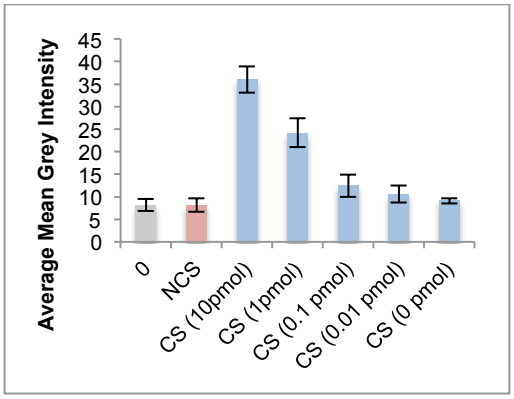

Figure 6 - Average mean grey intensites of the tests to determine the minimal target concentration needed to obtain a positive signal. Target concentrations ranged from 0 to 10 pmol (Figure 5). CBM64-ZZ:anti-biotin antibody was applied in every spot (5 pmol:5 pmol) and control and non complementary (NCS) conditions were included. Grey bar: control spot; red bar: non-complementary strand +RNA target; blue bars: complementary strand + RNA target at different concentrations. The test was made in triplicate and the error bars represent the standard deviation.

Scanning Electron Microscopy (SEM)

To understand in more detail the behavior of the AuNPs on paper and in different conditions a Scanning Electron Microscopy analysis was performed. SEM is a type of microscopy that uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that arise from electron-sample interaction give information about the sample including texture, chemical composition and crystalline structure. This technique presents a magnification ranging from 20x to 30,000x and a spatial resolution of 50 to 100 nm.[12] Samples containing the CBM64-ZZ:Ab complex, the gold nanoprobe, RNA target and complementary or non-complementary strand were analyzed. () Figures 7 A, C and E correspond to sample with the non-complementary strand and Figures 7 B and D correspond to the sample with the complementary strand. Figures 7 B-E were obtained with the most common detection mode, secondary electron imaging (SEI), in which the secondary electrons are emitted from very close to the specimen surface. Figure 7 A is a back scattered image in compositional mode, using a back-scattered electron detector (BSE). This detector will point out the difference between the present atomic species based on their mean atomic number (Z). Consequently a brighter BSE intensity relates with a larger average Z in the sample while darker areas correlate with lower average Z. [13]

In the images corresponding to the assay with the non-complementary strand an agglomeration of AuNPs in the cellulose fibers is visible (Figure 7 A, C and E), while in assays with the complementary strand we can see a more uniform distribution of the nanoparticles throughout the paper surface (Figure 7 B and D). This difference is due to the fact that the CBM64-ZZ has high affinity for cellulose and it will orientate the anti-biotin antibody in such a way that the capture strand, and consequently the complementary strand coated onto the gold nanoparticles, will be forced to stay attached to the paper's surface. In the case of the non-complementary strand the basis complex is still attached to the paper but the DNA strand coated onto the nanoparticles will not be able to pair with the capture strand, meaning the nanoparticles will move freely through the paper during evaporation, ultimately forming particle aggregates (white blur in Figure 7 A). In figures 7 B and D it is

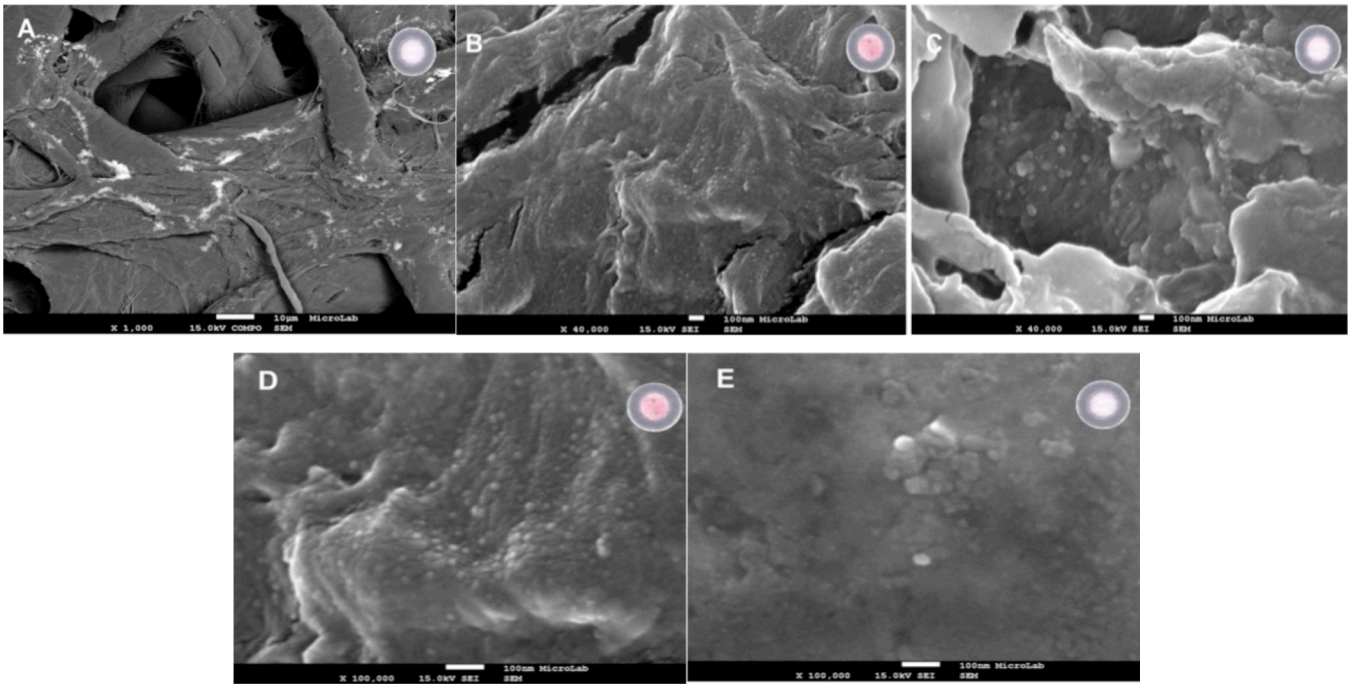

Figure 7 – SEM images of spots containing CBM64-ZZ+anti-biotin and a non-complementary (A,C and E) or a complementary DNA strand (B and D). Image A was obtained with a 1,000x magnification and with a COMPO detector (10µm scale). Images B and C were obtained with a 40,000x magnification with SEI detector (100nm scale). Finally images D and E were acquired with a magnification of 100,000x with the SEI detector (100nm scale).

almost possible to identify and observe an individual gold nanoparticle, unfortunately the particles are small and the capacity of the equipment does not go beyond this. However there is a clear difference between these last images and figures 7 C and E, where is impossible to identify a round shape gold nanoparticle due to its aggregation state.

Lateral flow assays The molecular detection system was also implemented using a lateral flow test in a μPAD. CBM64-ZZ:anti-biotin antibody complex and CBM64-ZZ fusion alone were dispensed over the test region (T) and control (C) regions of the channel.

Figure 8 – Capture of RNA target stand by a complementary strand in the presence of CBM64-ZZ:anti-biotin antibody complex in paper microfluidic channels. The reaction mixture migrates across the channel with the aid of a running buffer (PBS 1%, BSA 0.5%, Tween 0.01%, Saccharose 2.5%).

Next, a test sample containing biotin-labeled capture DNA, RNA target and AuNP nanoprobes was added and allowed to flow through the channel into the test zone with the aid of a running buffer. A red signal with a half-moon shape was detected, confirming that the assays can also be implemented in a paper microchannel format (Figure 8).

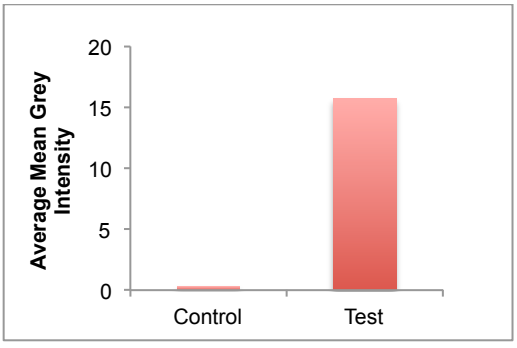

Figure 9 - Average mean grey intensites of the DNA:RNA hybridization ability test B in a paper microfluidic channels that are represented in Figure 8. The bars correspond to the control and test conditions.

The average mean grey intensities of the test and control regions of the microchannel (Figure 9) confirm the visual observations made before. Another fact that stands out is the much lower intensity values of the control region compared to the control regions of the previous paper spot assays. This difference may result from the fact that the gold nanoparticles that are in excess in the solution are washed away and are not confined in some boundaries as in the spots. This suggests that microfluidic channels could be a good alternative to paper spots because the relationship between what is seen in the paper and what is measured in Image J is much closer and reliable.

Cellulose microparticles To further demonstrate the versatility of the molecular detection system developed, tests were made using Sigmacell cellulose microparticles (~20 μm). The first step of the assay comprised the modification of the microparticles with the CBM64-ZZ:anti biotin antibody complexes. After settling, the microparticles present a white uniform color with a milk-like aspect (Figure 10).

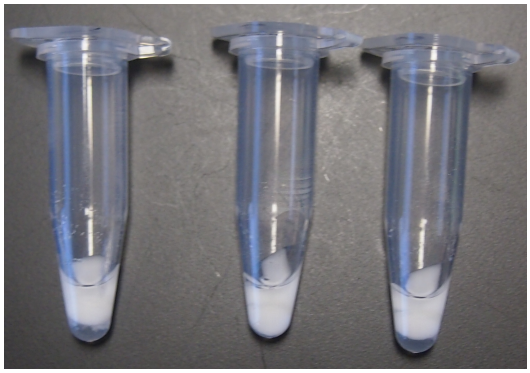

Figure 10 – Cellulose particle suspension (5mg/ μL) mixed with the CBM64-ZZ:anti-biotin conjugate (5:5 pmol), in TST buffer. Images were captured by an Olympus E-PM1 camera after a 30 minutes incubation at room temperature.

The second step of the assay involved the addition of the capture probe, RNA target and gold nanoprobe. A control was performed where only the gold nanoprobe was added. A second test was made that involved the addition of samples containing a non-complementary, biotin-labeled strand, the free RNA target and the gold nanoprobe. The third test comprised a complementary, biotin-labeled strand, the free RNA target and of the gold nanoprobe. After centrifugation, the cellulose microparticles in the tubes corresponding to the control and NCS situations remained white, whereas the gold nanoparticles accumulated in the form of a

small pellet in the bottom of the tube (Figure 11).

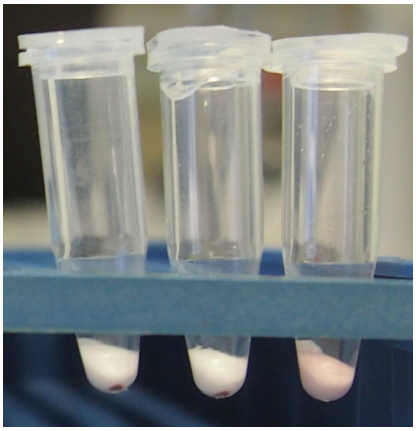

Figure 11 - Detection of DNA:RNA hybridization, on a cellulose particle suspension (5mg/ µL) with CBM64-ZZ+anti-biotin antibody complex (5:5 pmol). Tests samples were incubated with Au-nanoprobes (30nM, 90 fmol) and added to the spots. Three conditions were tested: control, non-complementary DNA (NCS) (10pmol) and complementary RNA (CS) (10 pmol). Images were captured by an Olympus E-PM1 camera.

Since no hybridization occurred in either situation, the gold nanoprobe were not captured by the immobilized CBM64-ZZ:anti-biotin antibody conjugate and remained dispersed in the particle suspension until they were forced to settle upon centrifugation. On the other hand, when hybridization occurs (CS) the gold nanoprobes are captured by the immobilized CBM64-ZZ:anti-biotin antibody conjugate, rendering the microparticles pink/red (). In this, case the absence of a red pellet in the bottom of the tube confirms that the majority of the Au-nanoprobes were captured. These assays indicate that cellulose microparticles may be a good alternative format to paper spots. The result is visible with naked eye just like in paper spots. However in terms of preparation, the cellulose particles may be more time consuming.

Conclusion

Gold nanoparticles with an average diameter of 12 nm were successfully synthesized using the citrate sodium reduction method. The salt-aging method was further used to functionalize gold nanoparticles with nucleic acids, in this case DNA.

A detection system was successfully designed to capture target RNA (label free). Spot assays were performed on paper to test the ability of the system to detect RNA using AuNP-DNA probes. In the assays, a CBM64-ZZ fusion was immobilized on paper and combined with an anti-biotin monoclonal antibody to capture hybrids of the target RNA and Au-nanoprobes. Tests were performed with both complementary and a non-complementary strands. A positive signal was obtained in the case of the complementary strand and no signal with the non-complementary strand. While the system was functional, it is important to highlight the fact that tests were only made with synthetic oligonucleotides and that these tests with biological samples from individuals affected with dengue virus. The real number of viral particles in blood will be the challenge when working with biological samples and a PCR may be required.

The molecular detection system was also successfully implemented using a lateral flow, microchannel format and cellulose microparticles. Even though good results were obtained using the microchannels the design of this paper support may not be the most tempting for assays involving multiple conditions. Good results were also obtained with cellulose particles, however this type of device support may be more time consuming than paper-based assays.

In terms of future work, a key challenge will be to test the ability of the paper-based devices to capture real viral RNA. If the system is insufficiently sensible, amplification techniques must be coupled to this paper-based device. Recent studies show the incorporation of amplification techniques, like LAMP in paper-based devices showing, once again, the flexibility of these devices and their very promising future.[14,15]

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