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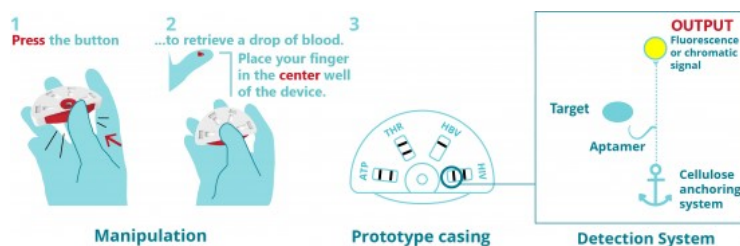
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Gotta Detect'Em All

A multi-STI sensor based on aptamers



Gotta Detect 'Em All: a multi-STI sensor based on aptamers

Authors

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Aptamers characterization and modifications: Mathieu Borel, Paul Zanoni; supervision: Yoann Louis.

Streptavidin cloning, production and characterization: Mathieu Borel, Mathilde Gonthier, Julien Orlans, Gianina Ungurean, Paul Zanoni; supervision: Viviane Chansavang, Corinne Dorel.

Biomarkers cloning and characterization: Mathieu Borel, Mathilde Gonthier, Julien Orlans, Paul Zanoni; supervision: Viviane Chansavang, Corinne Dorel.

Revelation systems (fluorescein and latex beads): Mathieu Borel, Mathilde Gonthier, Julien Orlans, Gianina Ungurean, Paul Zanoni; supervision: Yoann Louis, Corinne Dorel.

Modeling: Louis Becquey, Julien Orlans; supervision: Pedro DaSilva.

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Abstract

STIs constitute a major public health issue today. Treatments are often started too late resulting in health problems such as sterility. If prevention is probably the most effective action one can take to prevent the spread of STIs, early detection can help limit their deleterious effects. We propose in this work a new approach based on aptamers bound to paper to detect HIV and Hepatitis B biomarkers from blood sample. The detection device is based on two target generators (BBa_K1934060 and BBa_K1934061: protein subunits p51 and p66 of the HIV-1 Reverse Transcriptase, and HBsAg: biomarker of Hepatitis B) and one anchor allowing the fixation of the aptamer to the paper (see graphical abstract). Biomarker/aptamer binding is visualized by two methods. The first method is based on fluorescence. As a proof of concept, we used a paired ATP/aptamer and were able to successfully detect ATP up to $10 \mu\text{mol.L}^{-1}$. However, the signal is not detectable with naked eyes or with a cell phone equipped with a blue and green filters. Therefore a lateral flow assay with nano-sized latex black beads was tested. Our work shows that a protein biomarker such as thrombin can be complexed with latex beads coated with aptamers in liquid. The ultimate step, migration of the latex beads inside paper, needs further optimization. To easily handle several STI-tests on a single paper strip, an innovative bio-sourced PLA casing was designed and 3D printed to offer an additional intuitive user-interface.

Financial Disclosure

This work was sponsored by bioMérieux and Fondation INSA. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of this manuscript.

Competing Interests

The authors have declared that no competing interests exist.

Ethics Statement

N/A

Data Availability

All data are fully available without restriction.

Introduction

According to the World Health Organization (WHO) key numbers, more than one million Sexually Transmitted Infections (STIs) are acquired every day worldwide [1, 2]. Moreover, the majority of STIs don't present any symptom that can be easily recognized. Indeed, symptoms of common STIs tend to be non-specific and they typically have a variety of different potential causal agents that may require different treatments. Thus, STIs are a major public health issue and, in order to set up an efficient treatment, rapid diagnosis is essential.

Currently, the STIs are mostly diagnosed thanks to laboratories tests from fluid samples (blood, urine, genital sore, etc) [3]. Diagnostic tests can be divided into at least three different types [1]. First, the most relevant approach to STIs diagnosis is the direct screening of microorganisms themselves (by microscopy, antigen or nucleic acid detection, etc). Secondly, for many important STIs such as HIV and syphilis, screening of antibodies is the favoured diagnostic principle. Thirdly, the last diagnostic tests class detects microbial metabolites, such as molecules altering the pH of specific body fluids. However, these tests commonly allow a relatively late diagnosis, resulting in delayed treatments. If prevention is probably the most effective action one can take to prevent the spread of STIs, early detection can help limit their deleterious effects. As a result, pharmaceutical industries try to develop new detection systems to increase their efficiency and to diagnose people earlier.

Comments

jpgoupi: This paragraph might be strengthened by some additional citations, particularly to back up your statements about the three different types of diagnostic tests.

ylouis: Thanks for your remark, it will be added.

MackenzieRT: 'Nowadays' is a bit informal, I would suggest switching it for 'currently' and starting the next sentence with "Diagnostic tests..."

jordans: Thanks for your help

The aim of the INSA Lyon 2016 iGEM team project was to elaborate a rapid diagnostic test for multiple diseases and infections. To this end, a paper-based diagnostic test using a drop of blood was developed. This test can be easily handled and used at home without medical supervision, the results should be rapid to obtain and easy to be interpreted. In this study, a diagnosis system for HIV and Hepatitis B is presented. This detection device is based on a new technology: aptamers, which are short single stranded nucleic acids. They basically act as antibodies and present numerous advantages. Compared to antibodies, the process of selection and production of aptamers is considered as quicker, easier, and cheaper [4]. Moreover, they have the advantage to be easily chemically modifiable, so that their affinity for the target can be indefinitely improved. In this work, specific aptamers were designed to target with high specificity the HIV reverse transcriptase [5] and the Hepatitis B surface Antigen (HBsAg) [6]. Two different systems were developed to convert the Aptamer/STI biomarker complexation into a visual output signal: one using fluorescence [7] and the second one using latex beads [8]. The proof of concept of this detection device was made on ATP [9, 10] and thrombin [11-13].

Comments

MackenzieRT: I love this idea of making clinical testing cheaper and faster. However, with current antibody based detection strips, there is a window period where you cannot detect the antigen because it is too low in concentration. I am unclear on how aptamers would solve that problem. Also I would love to see some specific references to current lateral flow assays

jordans: Aptamers are smaller than antibody so they can bind antigen in hidden pocket or detect smaller epitopes, so we can target other antigens. About the window of detection, for the HIV we chose HIV RT as target instead of p24 because it appears during the primo-infection but the threshold of detection of our aptamer is not enough for now.

Materials and Methods

Bacterial strain, vector and cultural condition

All plasmids used in the following manipulations were transformed into *E. coli* NM522. This strain was grown in Luria-Bertani (LB) medium, in SOC (Super Optimal Broth with added glucose) medium or on agar Petri dishes, with the appropriate antibiotics (ampicillin [Amp] = 15 $\mu\text{g}\cdot\mu\text{L}^{-1}$, kanamycin [Kan] = 15 $\mu\text{g}\cdot\mu\text{L}^{-1}$ or chloramphenicol [Cm] = 15 $\mu\text{g}\cdot\mu\text{L}^{-1}$). Used vectors (pSB1C3, pSB1A3, pSB1K3), parts and kit of competent cells were provided by the iGEM Foundation. Restriction enzymes *EcoRI*, *XbaI*, *SpeI* and *PstI* were provided by the company New England Biolabs (NEB, USA). DNA concentrations were determined using the Nanodrop (ND-1000 Spectrophotometer UV-VIS, No. 5762 series LABTECH).

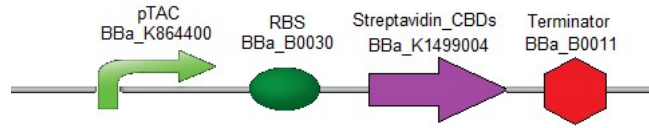
Gene construction

All parts were designed using SerialCloner software. The three parts of interest were built by assembling different sequences end-to-end: BBa_G00000 (http://parts.igem.org/Part:BBa_G00000) prefix/pTAC promotor BBa_K864400 (http://parts.igem.org/Part:BBa_K864400) [14], RBS BBa_B0030 (http://parts.igem.org/Part:BBa_B0030)/coding sequence of interest/double terminator BBa_B0011 (http://parts.igem.org/Part:BBa_B0011)/suffix BBa_G00001 (http://parts.igem.org/Part:BBa_G00001). Endogenous *EcoRI*, *XbaI*, *SpeI* and *PstI* sites (exclusive of iGEM prefix and suffix) were removed by silent mutation. Moreover, the

required spacing between characteristic sequences (for example, between transcription regulated sequences and the TATAA box) were verified. Finally, codons usage was optimized by using the associated application on the Integrated DNA Technologies (IDT, USA) website. The different sequences designed with this method were ordered from IDT as gBlocks Gene Fragment.

Streptavidin with Cellulose Binding Domains (CBDs) part.

As an anchor for the diagnostic device, the streptavidin [15] protein combined with one or two cellulose binding domain(s) [16] was chosen in order to be easily fixed onto a cellulose paper. The first streptavidin chimeric design contains two Cellulose Binding Domains (CBDs) (**Fig. 1**).

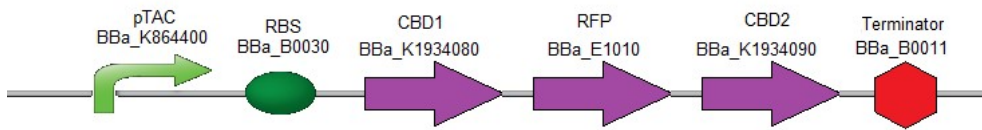


(http://parts.igem.org/Part:BBa_K1934020)

Fig. 1. BioBrick design of Streptavidin with Cellulose Binding Domains. This composite part contains the coding sequence of the streptavidin protein combined with two CBDs, which corresponds to the Stanford-Brown 2014 iGEM team part (BBa_K1499004), under the control of a pTAC promoter, a strong RBS and a bidirectional terminator.

RFP with Cellulose Binding Domains (CBDs) part.

To verify the efficiency of the CBDs for binding to the cellulose, the following composite part was created (**Fig. 2**). It contains the coding sequences for the two different CBDs (used above) which were fused respectively upstream and downstream of the coding sequence for RFP [17].

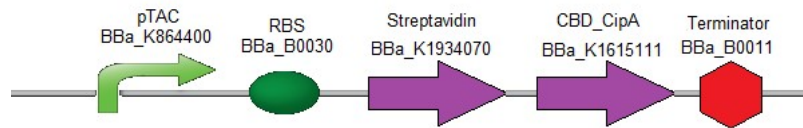


(http://parts.igem.org/Part:BBa_K1934000)

Fig. 2. BioBrick design of RFP with Cellulose Binding Domains. This composite part contains the coding sequence of the RFP protein (BBa_E1010) combined with two CBDs, which corresponds to the Stanford-Brown 2014 iGEM team part (BBa_K1499004), under the control of a pTAC promoter, a strong RBS and a bidirectional terminator.

Streptavidin with CBD-CipA (Cellulosomal scaffolding protein A).

To test another type of CBDs, the following composite part was designed (**Fig. 3**). It contains the coding sequence of streptavidin followed by the coding sequence of CBD-CipA (Cellulosomal scaffolding protein A) protein. As CBD-CipA [18] shows a better interaction with cellulose, this CBD was preferred to classical CBDs.



(http://parts.igem.org/Part:BBa_K1934030)

Fig. 3. BioBrick design of Streptavidin with CBD-CipA (Cellulosomal scaffolding protein A). This composite part contains the coding sequence of the Streptavidin protein (BBa_K1499004) combined with the CBD-CipA (BBa_K1615111), under the control of a pTAC promoter, a strong RBS and a bidirectional terminator.

Protein purification with CBDs

The Microcrystalline Cellulose (Avicel, Sigma Aldrich) was washed five times in water, and then equilibrated in washing buffer (ammonium sulfate 1 mol.L⁻¹). The culture was pelleted and resuspended in 1 mL of lysis buffer (50 mmol.L⁻¹ Tris, pH = 8, 300 mmol.L⁻¹ NaCl, 10% glycerol). The mix was sonicated on ice, five times for 30 seconds at moderate power. The lysate was centrifuged at 14,000 g for 10 min. The cellulose was packed in small (10 x 10 mm) chromatography columns or syringes barrels. The lysate supernatant was gently poured on the column. Once the liquid started to flow out, regular measurement of the OD₂₈₀ of the different fractions were performed. Buffer was poured until the OD₂₈₀ stabilized around zero. The elution was then performed with water. After a short time the OD₂₈₀ rose, fractions with high OD₂₈₀ were collected.

Aptamers & quencher

All the aptamers used in these experiments were provided by IDT (USA).

The original sequences of the aptamers used for the Electrophoretic Mobility Shift Assay (EMSA) are :

- Aptamer ATP: 5'- CACTGACCTGGGGGAGTATTGCGGAGGAAGGTTTT -3' [7]
- Aptamer Thrombin 15nt: 5'- GGTTGGTGTGGTTGG -3' [11]
- Aptamer Thrombin 29nt: 5'- AGTCCGTGGTAGGGCAGGTTGGGTGACT -3' [12]
- Aptamer Thrombin 31nt: 5'- GTGACGTAGGTTGGTGTGGTTGGGGCGTCAC -3' [13]
- Aptamer HIV Reverse Transcriptase (HIV RT): 5'- TAATACCCCCCTTCGGTGCTTTGCACCGAAGGGGGG -3' [5]
- Aptamer1 HBsAg: 5'- GGAATTCGAGCTCGGTACCCACAGCGAACAGCGCGGCAGATAATAGTGCTTACTACGACCTGCAGGCATGCAAGCTTGG -3' [6]
- Aptamer2 HBsAg: 5'- GGAATTCGAGCTCGGTACCCACATGGCATGAAGTATTATTACCCAATCCATACACAAGCTGCAGGCATGCAAGCTTGG -3' [6]
- Aptamer3 HBsAg: 5'- GGAATTCGAGCTCGGTACCGGCACAAGCATATGGACTCCTCTGAACCTACGATGTAGTACCTGCAGGCATGCAAGCTTGG -3' [6]

For the fixation on paper, a Biotin-TEG function was added on the 3-terminus of each aptamer (3BioTEG) to allow a complex to form between the aptamer and the streptavidin. For the fixation of FITC, an amino modifier C6 function was needed at the 5-terminus (5AmMC6). This function was used on the ATP aptamer. To switch off the light, a quencher was created, it had a reverse complementary sequence to the aptamer 5'-CCCAGGTCAGTG-3' [7] with a Dabcyl function at the 3-terminus (3Dab). For the fixation of latex beads, the same 5AmMC6 was used on the 15nt and 29nt thrombin aptamers. Their reverse complement for the control band of the test were:

- Reverse complement aptamer thrombin 15nt: 5'-CCAACCACCAACC-3'

- Reverse complement aptamer thrombin 29nt: 5'-AGTCACCCCAACCTGCCCTACCACGGACT-3'

Electrophoretic Mobility Shift Assay (EMSA)

The aptamer was diluted in folding buffer (SSC 1X) to a final concentration of 200 nmol.L⁻¹. It was denatured at 99 °C, then folded at 42 °C for 20 min and cooled slowly. Serial dilutions of target protein in the binding buffers (see list below) were prepared. 16 µL of proteins were mixed with 4 µL of the folded aptamer. After 20 min at room temperature loading dye was added and 15 µL of the mixture were loaded in each well of a native PAGE gel (12.5% acrylamide, TBE 1X final, 1 mmol.L⁻¹ MgCl₂, 10 mmol.L⁻¹ KCl). The gel migration was performed at 4 °C at low voltage (100 V maximum). The gel was stained with CYBR Green. Buffer list (pH = 7.4):

- Aptamer Thrombin 15 nt: TGKM, Tris 25 mmol.L⁻¹, Gly 192 mmol.L⁻¹, KCl 0.4 mol.L⁻¹, MgCl₂ 1 mmol.L⁻¹.
- Aptamer Thrombin 29 nt and 31 nt: TGK, Tris 25 mmol.L⁻¹, Gly 192 mmol.L⁻¹, KCl 0.4 mol.L⁻¹
- Aptamer HbsAg: 20 mmol.L⁻¹ HEPES, 120 mmol.L⁻¹ NaCl, 5 mmol.L⁻¹ KCl, 1 mmol.L⁻¹ CaCl₂, 1 mmol.L⁻¹ MgCl₂.
- Aptamer HIV-RT: 50 mmol.L⁻¹ Tris, 50 mmol.L⁻¹ KCl, 10 mmol.L⁻¹ MgCl₂, 1 mmol.L⁻¹ DTT.

Aptamers Modification

FITC on aptamer [19]

1 mmol.L⁻¹ of FITC solution in pure DMSO was prepared. In a microcentrifuge tube: 20 nmol of DNA aptamer modified at the 5 terminus with an amino modifier C6 and 50 µL of 1 mmol.L⁻¹ FITC solution were mixed, and the volume was adjusted to 1 mL with boron (H₃BO₃ 0.1 mmol.L⁻¹, pH = 9) buffer. The mix was incubated overnight at 4 °C.

Fluorescent aptamer purification

The DNA solutions were mixed with 10% v/v LiCl and 250% v/v ethanol (100%) or isopropanol 100% v/v (according to the size of the tube used). The mix was incubated for 10 min prior to centrifugation at 13,000 g for at least 40 min at 4 °C. The supernatant was removed thoroughly, and 1 mL of ethanol 70% was gently added. After 10 min, the tubes were centrifuged at 13,000 g 10 min, then the supernatant was removed and the pellets air dried.

Quencher on reverse complement aptamer

The purified aptamer and its quencher were mixed in equal amounts in TE buffer, heat shocked at 99 °C for 30 seconds, then hybridized at 42 °C for 30 min.

Latex bead on aptamer [20]

The latex beads (Thermo Fisher, black dyed beads, carboxy modified, 400 nm in diameter) were dissolved in PBS to get a 1 mL, 2% w/v solution. After centrifugation at 15,000 g for 10 min, the beads were resuspended in sulfonic acid buffer (1 mol.L⁻¹, pH 6) by sonication. Very quickly 20 mg of EDC (1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide) was added and the mix was incubated for 15 min. Then a new centrifugation and resuspension in PBS buffer (10 mL) was performed. To 2.5 mL of this solution, 200 µL of a 6 µM DNA solution with a free amine group were added. After a two hour incubation, the mixture was centrifuged and the pellet was washed twice with PBS + Tween 0.01% w/v and BSA 0.1% w/v. Beads can be stored a few weeks in this buffer at 4 °C.

Fluorescence detection

In a liquid phase

To test if the signal of the fluorescent aptamer could be specifically turned off in absence of the ATP target, the aptamer linked to fluorescein, and the quencher fragment were hybridized in SSC 1X at 40 °C for 20 min. 50 µL of a 10 nmol.L⁻¹ aptamer/quencher solution was prepared this way. A serial dilution of ATP in PBS pH 7.1 was prepared (ranging from 10 mmol.L⁻¹ to 1 µmol.L⁻¹). 50 µL of each ATP dilutions were poured in each well, in triplicates. Controls were: plain PBS and a 10 mmol.L⁻¹ GTP solution. The hybrid DNA was diluted in 1 mL of PBS, and quickly added (50 µL) to each well with ATP or control solutions. Fluorescence was measured (490 nm excitation, 530 nm emission) with a plate reader within 5 min to prevent bleaching.

On a paper substrate

The FITC labelled DNA was prepared as described, it was resuspended in 100 µL of TE buffer, 25% v/v loading dye was added, and the mix was distributed in nine wells of a native 15% acrylamide PAGE. 330 ng of DNA was used per well. A Southern Blot experiment was then carried out following standard procedure [21]. During the hybridization phase, 5 µg of the quencher DNA fragment was used. Once the membrane was hybridized and washed, 10 µL of ATP solution (in different concentrations) were poured very carefully on every lane. The solutions ranged from 100 mmol.L⁻¹ to 1 µmol.L⁻¹. After five minutes, the membrane was washed twice with PBS, and the fluorescence imaged with a ChemiDoc system (BioRad).

Latex beads experiments

To test the beads, 96-well plates covered with streptavidin were used. 100 µL of a PBS solution containing 1 ng of DNA were poured in each well. DNAs to be tested include the reverse complements of thrombin aptamers, and the two tested aptamers, they are biotinylated. After 1 h at 30 °C the wells were washed three times with binding buffer (PBS buffer + 0,01 % Tween + 0.1 % BSA + 1 mmol.L⁻¹ MgCl₂). In each well, 20 µL of the latex beads solution were poured (the beads were coated with the complementary aptamer of the corresponding ones in the wells), and 70 or 80 µL of binding buffer. In the test wells, 10 µL of thrombin were added (several concentrations are tested). After 30 min on a rotating plate at room temperature, the wells were washed with PBS, very carefully. A measure of the OD₆₀₀ of each well was done.

Results

Designing an anchoring system on paper

To avoid the diffusion of the aptamer sensor on paper, a protein complex is needed to anchor the aptamer on the paper surface. Chimeric proteins containing C-terminus cellulose binding domain (CBD) with a cellulosome scaffolding protein A, called CipA, were engineered. To select the best CBD candidate, ability to bind cellulose of the streptavidin-CBD (BBa_K1934020 (http://parts.igem.org/Part:BBa_K1934020), INSA Lyon 2016 iGEM team) and of the streptavidin-CiPA (BBa_K1934030 (http://parts.igem.org/Part:BBa_K1934030), INSA Lyon 2016 iGEM team) was compared (**Fig. 4**).

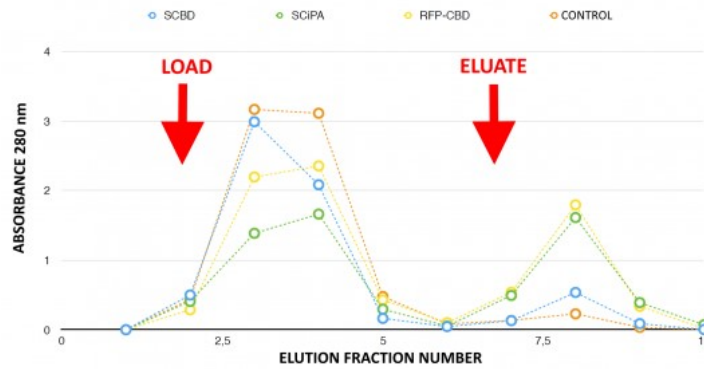


Fig. 4. Elution profiles of different Cellulose Binding Domains. CBDs confer ability to bind cellulose: demonstration on chromatography affinity column. Crude cell lysates were loaded on a cellulose column for affinity purification. Unbound proteins were injected in the column in the same proportion and were washed with water (1st peak) before elution of purified streptavidin-CBDs proteins (2nd peak). The protein purification process was followed by measuring OD₂₈₀ of the collected fractions. To visually follow the process, a RFP-CBD generator was designed (BBa_1934000). The control is a NM522 crude lysate. Fraction 8 corresponding to the elution peak contained the majority of the purified proteins. This fraction was collected for further testing. The peak corresponding to Streptavidin-CipA (green marks) in fraction 8 is higher than the Streptavidin-CBD peak (blue marks). Chimeric proteins stick to the cellulose until elution, meaning that more Streptavidin-CipA has been retained in the column during the washing run. So, Streptavidin-CipA has better adsorption properties with cellulose than Stanford's existing part. The RFP-CBD acts as a positive control with visual follow-up thanks to the red color given by the fixation of the protein to the column. No retention could be observed in the control experiment in absence of Cellulose-Binding-Domains.

Functionalization of the aptamers

In order to control the interaction of the aptamers with other pieces of the detection system, functionalization was achieved by grafting additional components to aptamers. The ATP-aptamer was decorated with fluorescein thanks to crosslinking in presence of FITC. To temporarily switch off the fluorescence, a non fully-affine reverse-complement quencher molecule (DABCYL) was linked. DABCYL quenches the fluorescence by Fluorescent Resonance Energetic Transfert. When present, ATP displays higher affinity for the aptamer and is therefore able to displace the quencher, resulting in a fluorescent signal.

Such a labelling allows further detection of the aptamer/target complex. The aptamer used here is known as ATP aptamer [7]. The mix was analyzed on a PAGE gel.

Figure 5 shows the overlays of ethidium bromide staining of the PAGE gel (red) and fluorescence imaging (purple). From the two UV visible bands after ethidium bromide staining, only the heaviest band was fluorescent. This result indicates that the fluorescein was successfully crosslinked to the aptamers. A significant amount of untagged aptamers was still observed, showing that the reaction is not complete.

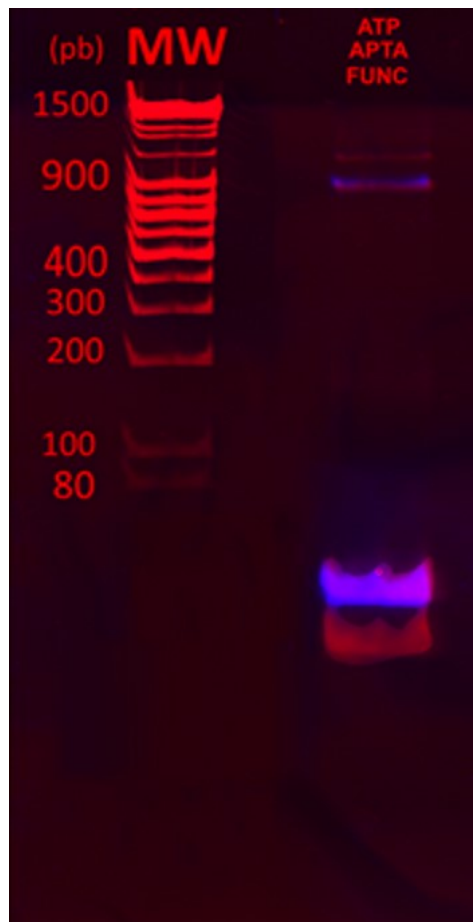


Fig. 5. Overlay of a PAGE gel stained with ethidium bromide and fluorescence imaging. Observation of ATP Aptamer crosslinked with fluorescein on PAGE stained with ethidium bromide.

Binding functionalized aptamers to protein complex

The fixation of functionalized aptamers onto a support is enabled by a Biotin/Streptavidin link. The biotin non-covalent link between a fluorescent DNA oligo (FITC on the 5' end and biotin on the 3' end) and 3 different anchoring complexes was compared (Fig. 6).

Cellulose and Streptavidin binding efficiency measurement

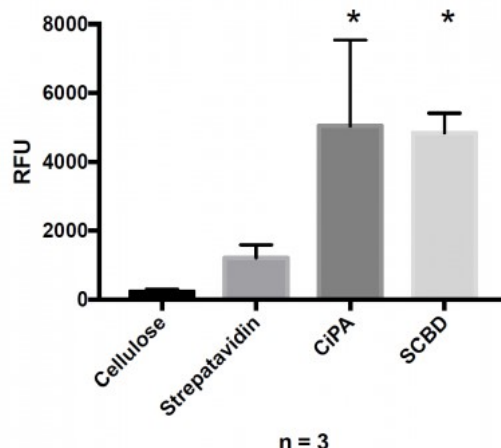


Fig. 6. Comparison of the relative binding affinity between biotinylated fluorescent DNA oligos and three different anchoring streptavidin-based complexes. FITC was used to label biotinylated oligos, and therefore serve as a reporter system. Fluorescence was measured after incubation of the oligomer in different conditions, with microcrystalline cellulose. From left to right: cellulose, single streptavidin, Streptavidin-CipA (BBa_K1934030) and Streptavidin-CBDs (BBa_K1934020).

Fluorescence of the reporter system is proportional to the amount of bound protein. Binding cellulose with the engineered proteins was statistically different than raw streptavidin (Student's test with p-value < 0.05).

Comments

jpgoupil: You may want to consider making a note of the statistically significant p-values on your graph above (maybe use an asterisk?)

jorlans: Yes, it can be helpful for the understanding of this graph

The cellulose alone showed no fluorescence, thus confirming that aptamers cannot be directly linked to paper. A protein anchoring system is required. The streptavidin experiment shows a spontaneous ability of streptavidin to link a small fraction of cellulose. Both engineered streptavidin-CBDs allowed the formation of complexes with the fluorescent reporter system and cellulose that could be detected by measuring the green fluorescence. There was no significant difference in terms of binding performance between the two engineered streptavidin designs. Nevertheless, the binding capacity of both protein was significantly higher than streptavidin alone demonstrating the efficiency of the system.

Comments

jpgoupil: Labeling the bars (1,2,3 etc. or A, B, C, etc.) on the graph above would be helpful if you want to later refer to "bar 1" or "bar 2"

ylouis: Agree ... :) it will be modified in the next version !

Electrophoretic Mobility Shift Assays

To both demonstrate the ability of the aptamers to fix their target, and measure their affinity constants, Electrophoretic Migration Shift Assays (EMSA) were carried out. A proof of concept was realized with a well described aptamer and its target protein: the human thrombin. This experiment allows to measure the affinity of an aptamer with its target. The aptamer/protein complex formed in presence of increasing amount of proteins was separated and quantified on native PAGE. **Figure 7(a)** shows the EMSA results for three different aptamers with affinity for the thrombin. This figure was obtained via integration of intensities of the lanes on several EMSAs (data not shown), with Bio-Rad ImageLab software. The curves were modeled to fit the points (thanks to a 4 parameters model and the nls fonction of R). This allowed to calculate the Kds, which are concentration values corresponding to the inflexion points. The most sensitive thrombin aptamer, aptamer of 31 nt, detected thrombin down to 350 nmol.L⁻¹. Based on these results, EMSAs were performed with two STI-biomarkers/aptamers couples (HIV-1 Reverse-Transcriptase and HBsAg), see **Figures 7 (b), (c)** and the best sensitivity associated with each biomarker is reported in the **Figure 7 (d)**.

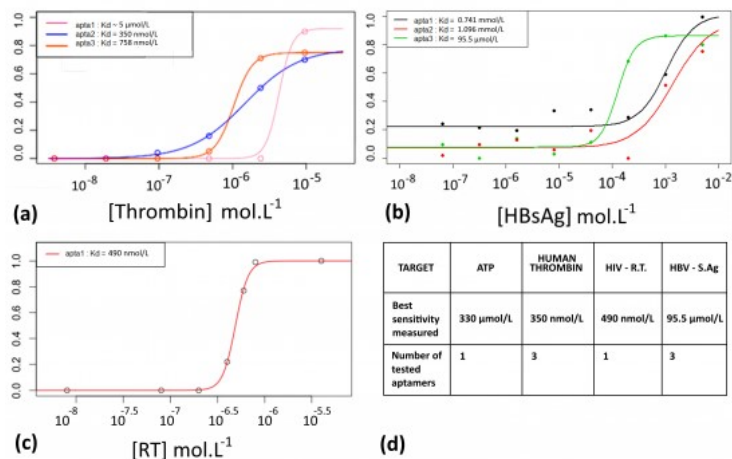


Fig. 7. Sensitivity of different aptamers catching different targets, determined by EMSA. (a) EMSA results of three different aptamers for the human thrombin. (b) EMSA result of three different aptamers for Hepatitis B virus surface antigen (HBsAg). (c) EMSA result of an aptamer for HIV-1 Reverse Transcriptase. (d) Table summary of the EMSA results for all tested targets.

Proof of concept of the detection with FITC

Fluorescent labelled ATP aptamers (with FITC) were loaded and separated on a PAGE gel. A constant amount was loaded in each well. **Figure 8** shows that the fluorescent response to ATP concentration is semi-quantitative and follows a sigmoid curve (in liquid samples).

Then, DNA was blotted on nitrocellulose using capillary transfer, with quencher oligos to block fluorescence (a reverse complement fragment of 6 nucleotides of the aptamer labelled with a DABCYL molecule at its 3' end). This quencher oligo was hybridized *in situ* by incubating the membrane in hybridization buffer, similar to the buffer for Southern Blots [21]. After washing the quencher excess, increasing amounts of ATP gradient were applied on each lane of the nitrocellulose membrane. The blotting of the complete system on a nitrocellulose sheet with a range of ATP concentrations is shown in **Figure 9**. The ATP aptamer successfully detects ATP down to $10 \mu\text{mol.L}^{-1}$.

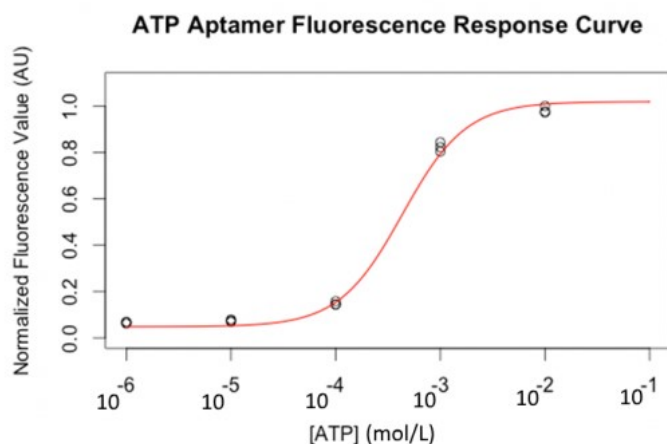


Fig. 8. ATP-Aptamer fluorescence response curve in liquid assays. Each concentration was measured in three repetitions.

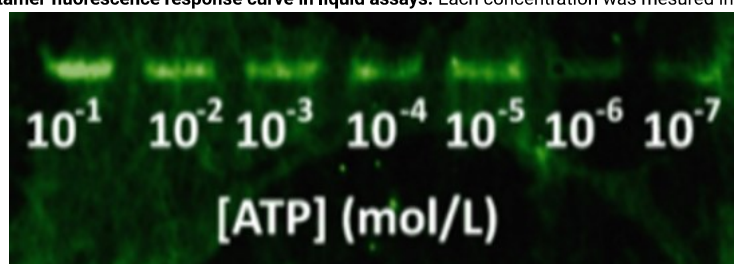


Fig. 9. Blot of the fluorescent ATP-aptamers on nitrocellulose.

Proof of concept with a second detection system that uses latex beads

In this second version of revelation system, the target molecule is taken in sandwich between two aptamers that link two different apitopes. The first aptamer is fixed on black dyed nano-sized latex beads. The second one is fixed on the support and catches the target when the assay flow comes by capillarity (**Fig. 10**). With this strategy, the user does not need any fluorescence measurement to detect aptamers: as the latex beads are black, they are visible with naked-eye.

This detection system was firstly implemented on streptavidin-coated plates. The beads fixation to the streptavidin-coated well bottom results in darkening the well. This phenomenon was quantified by OD_{600} measurement. On **Figure 11**, in absence of DNA, beads did not stick (bar 1 in black). Beads with complementary strands aptamers allowed a good fixation, and constituted the positive control (bar 2 in red). The full detection system in absence of biomarker (thrombin), the negative control, did not allow fixation of the beads (bar 3 in grey). Decreasing amount of thrombin allowed to determine the sensibility of the test, *i.e.* 100 nmol.L^{-1} in these conditions (yellow bars).

Positive test with biomarker

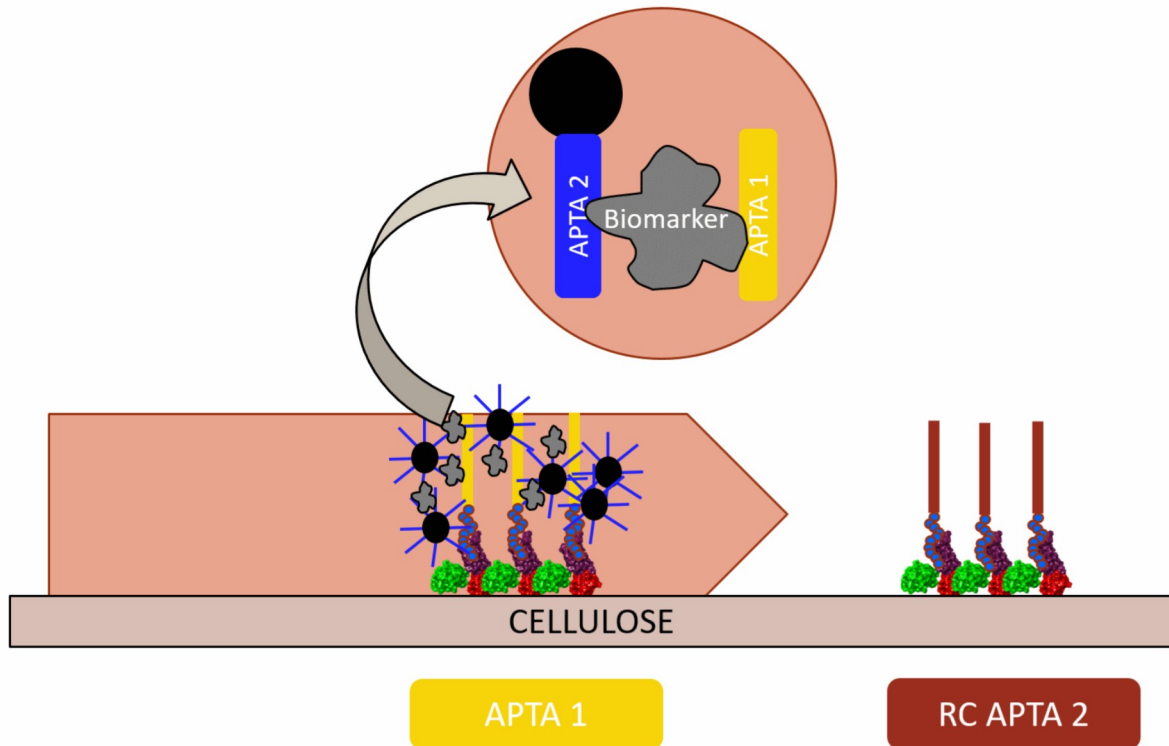


Fig. 10. Animation of the second detection system with latex beads.

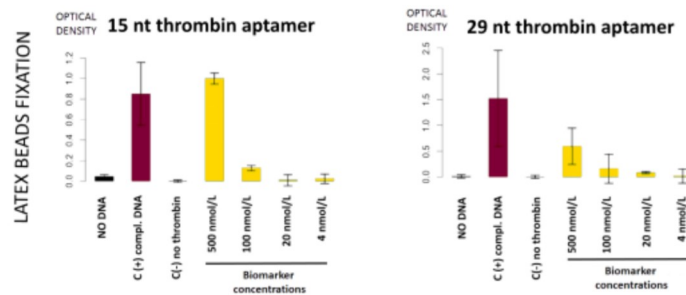


Fig. 11. DO_{600} measurement of different samples after incubation, two experiments for two different aptamers. Left panel: 15 nt thrombin aptamer was fixed on the beads. Right panel 29 nt thrombin aptamer was fixed on the beads.

Discussion

Developing a lateral flow test requires four modules: i) A biomarker referred to as target; due to experimental or safety issues, a well described molecule was used in place of the STI-biomarker to elaborate the proof of concept. ii) A paired aptamer to catch the biomarker. iii) An anchor to fix the aptamer-biomarker complex to the support. iv) A visual output signal. Our approach consists in setting and characterizing each module before assembling the whole system together.

We were able to assemble a functional system using ATP as target and a functionalized aptamer (module ii) doubled tagged with biotin (module iii) and fluorescein (module iv) (Fig. 5). The chimeric streptavidin and the aptamer put together do not lose their individual properties, showing the pertinence of our modular approach. Our results showed that the system is able to successfully detect ATP with a better affinity constant in liquid ($330 \mu\text{mol.L}^{-1}$) than on nitrocellulose support ($10 \mu\text{mol.L}^{-1}$). The sensibility is actually lowered by a 30 factor on cellulose, probably due to the diffusion of the target molecules on the paper. These results could be improved by finding a compromise between the affinity of the ATP and the reverse complement to aptamer in order to ensure a specific recognition with a low detection threshold. Moreover, paper substrate was shown to emit auto-fluorescence (Fig. 9 and data shown). This fluorescent noise competes with the emitted signal and lower the detection capacities of the system. Screening of cellulosic substrate with low fluorescence could also improve the system sensitivity.

To develop a more sensitive system and a proof of concept with a proteic biomarker more representative of the chosen STI biomarkers (HIV-RT and HbsAg), a system using Thrombin as a target (module i) and latex beads (module iv) was successfully assembled and successfully assayed in liquid in streptavidin coated plates (Fig. 11). HIV-RT, HbsAg and thrombin have in common to be recognized by 2 different and specific aptamers. This property allows these 3 biomarkers to form sandwiches with aptamers carrying different functions and is the basis of our detection system. Preliminary results obtained with HIV-RT and HbsAg indicate that module i) and ii) are functional. The complementary modules iii) and iv) have now to be added and the whole system assayed. Compared to fluorescent output, the revelation system involving black latex beads ensures an optimal reading condition for blood samples [22] and is not affected by the paper self-fluorescence.

Regarding Thrombin, we have shown that the K_d can go down to 7.2 nmol.L^{-1} [13]. The procedure can be improved by enhancing the affinity of the aptamer, *i.e.* by introducing mutation in the DNA sequence or using a SELEX method, or by optimizing the ionic concentration of the folding buffer [4]. Improvement of the antibody-antigen complex K_d could reach 2.4 nmol.L^{-1} [23]. Such an increase of the detection threshold could allow an earlier detection of the biomarker. This issue is crucial to cure and prevent the sexually transmitted infections development.

The Gotta Detect' Em All test was released with the conscience of the issues related to the false positive and false negative risk. Even if the principle of the test is quite complicated, it is dedicated to limit the undesired interaction, which is crucial to increase the reliability of the detecting system.

The main issue is that our detection procedure requires two different aptamers, and the sensitivity of the method will be limited by the highest K_d (less affinity) between the two aptamers. The need of two aptamers can be seen as a limitation of the procedure. Indeed only one aptamer is often described for one biomarker.

Comments

VeroniqueKiermer: This is a neat concept. The dual aptamer system may indeed help with specificity to avoid false positives. You may also want to discuss how you would approach the accessibility of the antigen. Would you be able to detect HIV RT in an unprocessed sample of blood? It would presumably require some preparation of the sample. Would this be possible in a 'point of diagnostic' situation?

jorlans: Thank you for your remark. For now, we aren't able to detect the HIV RT in a blood sample because of the threshold of the detection. But with some aptamer's modification we hope to improve the sensitivity. The final goal is a self-test so a diagnosis without any sample preparation.

Conclusion

Aptamers are a flexible tool, with a promising future. Utilizing this technology in a device aimed to be broadcasted vastly is nevertheless complex. The interface between aptamers interactions with their targets and the end user is difficult to set up. Here we have shown that two techniques could be implemented to solve this problem. Fluorescence detection is working, but with a poor yield and the latex beads lateral flow system is promising. These both systems would need further improvements to be implemented in detection devices commercially available. This technology is nevertheless promising, offering good results at a low price.

Acknowledgments

First we would like to thanks all the participants of this project who help us during the summer 2016 : **Mickaël Hubert, Thomas Sabate and Marianne Chouteau.**

We also want to thank the following people for their kind help with our project. Their support was precious to us and they gave us a lot of good advice! So a huge thanks to:

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Special thanks to our sponsors: **bioMerieux and Fondation INSA.**

References

Comments

jgoupil: Impressive list of references! I do believe that PLOS One uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the "Vancouver" style. These references would need to be adapted to Vancouver style if you want to be published. EndNote is a program that could help you do this.

ylouis: Thanks again for this remark, we will do the modifications according to ICMJE rules with or without Endnote !! (For us to do so : https://www.nlm.nih.gov/bsd/uniform_requirements.html)

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Comments

PLOSFeedback: Hi @jorlans (/user/jorlans) Thanks for your submission to the PLOS iGEM Project. Our editorial team have reviewed your article and have the following feedback: This study shows proof of principle of a technology that could be used in the development of an STI sensor. It is still preliminary. If the authors submitted this to PLOS ONE as a 'methods paper', it may not meet the criteria (validity and utility) if the paper claimed to have developed an STI sensor. If on the other hand the data were presented as describing a new developing technology with possible uses in various areas then it would be more suitable for consideration. Great title as well! If you'd like more feedback or advice on submitting it as a Methods paper to PLOS ONE don't hesitate in contacting collections@plos.org

ylouis: Hi, Thanks for your advice, we will make some corrections in that direction for the submission to PLOS ONE. Have a nice reviewing day !! :)

VeroniqueKiermer: Kudos for tackling an important problem! Systems that can be used at point of diagnostic will be very important. It is still preliminary but encouraging and creative.

ylouis: Thanks for your remarks and encouragement, we will do our best to improve the manuscript by answering your remarks before publication :)