



Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

DNA aptamer selection in methanolic media: Adenine–aptamer as proof-of-concept

Thinhinane Chaou, Brune Vialet, Laurent Azéma*

Univ. Bordeaux, ARNA Laboratory, F-33000 Bordeaux, France
INSERM, U869, ARNA Laboratory, F-33000 Bordeaux, France

ARTICLE INFO

Article history:

Received 15 October 2015

Received in revised form 3 January 2016

Accepted 4 January 2016

Available online xxxx

Keywords:

Aptamer

Organic buffer

Modified nucleotide

Adenine

ABSTRACT

The major objective of this study is to investigate the usefulness of aptamers as *in situ* detection tool in organic solvents, which are often used for environmental extraction. But two problems related to the use of methanol-containing buffers have to be addressed. Firstly, the folding of nucleic acids can be impaired, because of weaker hydrogen bonding interactions. Secondly, the affinity of aptamers selected in aqueous buffers can be altered by the presence of methanol. Thus, in order to improve hydrophobicity of the DNA pool, nucleotide with hydrophobic modification 5-(octa1,7-diynyl)-2'-deoxyuridine (ODT) has been chosen instead of thymidine.

As a proof of concept, an adenine aptamer operating in presence 25% of methanol has been selected. We have shown that the modified nucleotide is essential for target binding in organic media, in addition to essential structural pattern as proposed through analysing truncated sequences analysis. The strategy described in this paper offers preliminary insight on the adaptability of the implementation of aptamers as key instrument for *in situ* detection. It could be broadened to identify other aptamers directed against other chemical species after alcoholic extraction or for monitoring by-product traces in drugs production.

© 2016 Elsevier Inc. All rights reserved.

1. Introduction

Development of *in situ* detection tools is required for specific real time monitoring of various chemical and biochemical species. The detection sensitivity is related to the affinity of the molecular recognition element (MRE) for the intended target and the specificity depending on its ability to discriminate between close molecular species. Moreover, the analytical tool must be adapted for operating under conditions imposed by the context of the application. Among others, high temperature, pH variation and presence of organic solvents may be mentioned. From this viewpoint, antibodies, that are traditional detection tools, are of limited interest for use under conditions far from that of biological media.

Over the last twenty years, reports accumulated demonstrating that aptamers offer a promising alternative [1,2]. These short single-stranded oligonucleotides (DNA or RNA) are identified through a combinatorial method named SELEX. They bind specifically to their targets by complementary shape interactions. Indeed, aptamers function by folding into unique three-dimensional

conformation that dictates high-affinity and specificity binding to a variety of targets (proteins [3], peptides [4], nucleic acids [5], small molecules [6]). Aptamer-based tools were designed for analytical chemistry, diagnostic or therapeutic applications over the last decades [7,8]. They are a promising alternative to monoclonal antibodies in many applications, including pollutants detection [9], molecular imaging [10,11] or guided cancer therapy [12].

Consequently, aptamers can constitute powerful detection tools if factors resulting in alteration of aptamer specific folding are overcome. It is known that aptamers have great potential of self-renaturation after heat stress or changes in pH compared to antibodies. Nevertheless, it remains difficult to use aptamer in organic media even though a couple of examples are available [13,14]. It is noteworthy that this peculiarity is of high interest, for the detection of chemical species after extraction, or for the monitoring of by-product traces in drugs production.

But it has been shown that adaptation to organic conditions of an aptamer selected in aqueous media is an issue [14]. Change in dielectric constant can modify three-dimensional structure of the aptamer and recognition pattern, through weaker hydrogen bonding and increased van der Waals interactions [15]. Therefore, aptamer selection directly in organic media is of high-interest. We decided to undertake the identification of aptamers able to bind

* Corresponding author at: Univ. Bordeaux, ARNA Laboratory, F-33000 Bordeaux, France.

E-mail address: laurent.azema@u-bordeaux.fr (L. Azéma).

their target in the presence of high methanol concentration. To this end, the use of unnatural nucleobases is an attractive option to select aptamer with new properties, especially if the corresponding nucleotide is incorporable by DNA polymerases. Modifications introduction on the 5-position of thymidine fulfils these conditions and led to aptamers with improved binding efficiency, through a decrease in off-rate with respect to unmodified corresponding nucleic acid. According to this approach, modified aptamers have been successfully raised against small molecule [16,17] or proteins [18,19]. In order to increase hydrophobicity of the oligonucleotide library, we have chosen to introduce 5-(octa1,7-diynyl)-2'-deoxyuridine (ODT) instead of thymidine. Both triphosphate (ODTTP) and phosphoramidite derivatives of this modification are available, thus allowing enzymatic and chemical synthesis [20]. We firstly demonstrated that this modification on the thymine carbon 5 was tolerated by DNA polymerase in methanol-containing buffer, as it stands in the major groove of the double helix and does not interfere with the Watson–Crick base pairing [21]. We subsequently prepared a ODT-containing DNA library for selecting aptamers to adenine in solvent containing 25% methanol. We report the successful selection procedure, as well as the characterisation of aptamers under these conditions.

2. Experimental section

2.1. Chemistry

Reagents and solvents were purchased from Aldrich or Alfa Aesar and used without further purification. All reactions involving air- or moisture-sensitive reagents or intermediates were performed under an argon atmosphere. 5-(octa1,7-diynyl)-2'-deoxyuridine triphosphate, and 6-carboxyfluorescein azide were purchased from Baseclick.

2.1.1. 8-(1,7-Octadiynyl)-adenine (compound **1**; Fig. 1)

The protocol was adapted from literature [22]. To a degassed solution under argon of 8-bromoadenine (1 g, 4.65 mmol) in 25 mL of anhydrous dimethylformamide was added copper iodide (0.1 g, 0.95 mmol). The solution was degassed once more. Pd(PPh₃)₄ (0.54 g, 0.465 mmol), 1,7-octadiyne (2.48 g, 23.5 mmol) and Et₃N (1.3 mL, 9.4 mmol) were sequentially added. The slurry was stirred at 60 °C overnight, and volatiles were evaporated. The crude product was purified by chromatography on silica gel (CH₂Cl₂/AcOEt 1/1 and MeOH gradient 0–20%) to give **1** in 55% yield.

¹H NMR (300 MHz, DMSO-*d*₆, δ): 13.3 (b, 1H, NH), 8.1 (s, 1H, Ar H), 7.3 (b, 2H, NH₂), 2.5 (2s, 3H, CH and CH₂), 2.2 (s, 2H, CH₂), 1.6 (s, 4H, CH₂).

¹³C NMR (75 MHz, DMSO-*d*₆, δ): 17.7, 18.4, 27.1, 27.5, 71.9, 72.7, 84.6, 93.3, 119.4, 132.6, 150.3, 153.7, 155.9.

MS (ESI) *m/z* 240 (M+H⁺), 262 (M+Na⁺).

2.1.2. Fluorescein-adenine conjugate

Compound **1** (17 mg, 72 μ mol) was dissolved in ^tBuOH/DMSO 1/3 (2 mL). CuBr (10 mg, 70 μ mol), tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 74 mg, 140 μ mol) and sodium ascorbate (60 mg, 0.3 μ mol) were sequentially added, followed by addition of fluorescein-N₃ (144 μ mol) [23]. The slurry was stirred overnight at 45 °C and purified through HPLC to obtain the conjugate as an orange solid. Compounds were characterized through Mass Spectrometry. Compound **3**: *m/z* 696.2 (M–H⁺).

2.1.3. Oligonucleotide syntheses

Oligonucleotides were synthesized on an ABI Expedite 8909 synthesizer using conventional β -cyanoethyl phosphoramidite

chemistry with the standard DNA protocol for 1 μ mol-scale synthesis and purification. 5-(octa1,7-diynyl)-2'-deoxyuridine cyanoethyl phosphoramidite was purchased from Baseclick. Reagents were purchased from Link and Glen Research.

2.1.4. Analysis

Flash column chromatography was carried out on silica gel (Merck, SDS 60 Å, 40–63 μ m, VWR). Analytical thin-layer chromatography (TLC) was conducted on Macherey–Nagel precoated silica gel 60F254 plates, and compounds were visualized by irradiation (254 nm) or by staining with iodine, ninhydrin or anisaldehyde stain. ¹H and ¹³C NMR spectra were recorded on a Bruker AC 300 MHz spectrometer. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ¹H resonance of the solvent. Mass spectra were recorded on Thermo LCQ advantage. HPLC was performed using a Hitachi L2455 chain equipped with a Macherey–Nagel RP-18 column (250 mm $\times$ 4.6 mm, 5 μ m for analytical HPLC and 250 mm $\times$ 10 mm, 5 μ m for semipreparative HPLC). All HPLC analyses were run at RT. A gradient of CH₃CN containing 0.1% TFA (eluent B) in water containing 0.1% TFA (eluent A) from 5% to 100% was used at a flow rate of 1 mL/min for analytical HPLC and 5 mL/min for semipreparative HPLC.

2.2. Beads preparation

2.2.1. Adenine-beads (A-beads) [24]

Magnetic carboxylic functionalised beads (Dynabeads MyOne) were purchased from Invitrogen. 10 mg of beads (1 mL) were washed three times with MES buffer (2-[N-morpholino] ethane sulfonic acid, 15 mM, pH 6). Beads were collected and incubated 3 h at 25 °C with N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC, 1.5 M in MES, 50 μ L) and O-(2-aminoethyl)-O'-(2-azidoethyl) diethylene glycol [25] (1.2 M in MES, 50 μ L). Beads were collected, washed three times with TT buffer (tris(hydroxymethyl)aminomethane 0.25 M, pH 8, 0.01% Tween-20). These azido-beads were subsequently engaged in CuAAC reaction. 5 mg of azido-beads were washed two times with 500 μ L of ^tBuOH/H₂O 1/1 and suspended in 100 μ L of ^tBuOH/H₂O 1/1. Compound **1** (1 mM in ^tBuOH, 50 μ L), CuSO₄ (0.12 M in H₂O, 50 μ L) and sodium ascorbate (0.6 M in H₂O, 50 μ L) were sequentially added and the suspension was stirred overnight at 45 °C. Completion of the reaction was monitored by HPLC (Fig. S1). Beads were collected and washed three times 30 mn with TE buffer (tris(hydroxymethyl)aminomethane 10 mM, pH 7.5, EDTA 1 mM), and three times with TT buffer. Beads were stored in TE buffer at 4 °C.

2.2.2. Counter selection (N-beads)

As for adenine beads, 5 mg of azido-beads were washed two times with 500 μ L of ^tBuOH/H₂O 1/1 and suspended in 100 μ L of ^tBuOH/H₂O 1/1. (1-Trimethylsilyl)-1, 7 octadiyne [25,26] (1 mM in ^tBuOH, 50 μ L), CuSO₄ (0.12 M in H₂O, 50 μ L) and sodium ascorbate (0.6 M in H₂O, 50 μ L) were sequentially added and the suspension was stirred overnight at 45 °C. Beads were collected and washed three times 30 mn with TE buffer and three times with TT buffer and subsequently suspended in Et₃N, 3HF (0.5 mM in THF, 100 μ L). After 2 h shaking at room temperature, beads were collected, washed three times with TE buffer and stored in TE buffer at 4 °C.

2.3. Production of alkyne-containing library

2.3.1. DNA pool

ssDNA pool containing 30 random nucleotides central core, flanked by two constant regions, required for enzymatic amplification (5'-ACTGACTGACTGACTGACTA-6C₃-GGGAGACAAGAA

TAAACGCTCAA-N₃₀-TTCGACAGGAGGCTCACAACAGGC₃) was synthesised by phosphoramidite chemistry process and purified by gel electrophoresis. 5' constant region includes 6 propylene linkers (C₃) that constitute polymerase reaction breakpoint, leading to generation of shorter complementary strands easily separated by electrophoresis on denaturing gel. This short strand constitutes the library for the selection.

2.3.2. Library production

3 nmoles of library was used in 3 ml PCR reaction. The PCR reaction mixture contains 2 μM of heavy P3 primer, 2 μM of P5 primer, 0.2 mM of each nucleotide triphosphate (dATP, dCTP, dGTP and dTTP), 0.1 U/μl of TaqDNA polymerase (New England Biolabs) in PCR buffer (67 mM Tris–HCl pH 8.8, 16.6 mM (NH₄)₂SO₄, 10 mM β-mercaptoethanol, 1.17 mM MgCl₂). Thermal cycle comprises the following steps: one cycle of 10 min at 95 °C, followed by 19 thermal cycles of 1 min 95 °C, 1 min 60 °C, 1.5 min 72 °C and one cycle 10 min 72 °C. Recovered PCR products were desalted, filtered and concentrated on 10 K Nanosep devices (Pall), then purified by 8% PAGE. The shorter complementary strand incorporating dODTP was electro-eluted in buffer containing 40 mM Tris, 20 mM glacial acetic acid, 1 mM EDTA pH 8.0 and 0.1% SDS. Recovered ssDNA library was desalted on G25 Sephadex column (GE healthcare), dried and suspended in water.

2.4. Aptamer selection protocol

2.4.1. Selection

Oligonucleotide candidates containing ODT (from 500 pmol in round 1 to 15.6 pmol in round 11) were diluted in 10 μl water and heated 5 min at 75 °C, cooled 5 min at 4 °C, and incubated 5 min at room temperature in a total volume of 100 μl of selection buffer adjusted to final concentrations of 1× PBS, 1 mM MgCl₂, 100 mM KCl (SLE buffer) and 25% methanol. Negative selection beads (N-beads) were washed three times with SLE, equilibrated for 5 min in SLE at RT, and finally suspended in 100 μl of prepared oligonucleotides for 30 min at RT. Supernatant was collected and introduced in a second counter-selection round. The supernatant was then incubated with adenine beads (A-beads) for 30 min at room temperature with a total concentration of immobilized adenine of 200 μM (round 1) down to 100 μM (round 10). Supernatant was discarded and A-beads were washed with SLE buffer/25% MeOH (from 1 × 100 μL in round 1 to 10 × 100 μL in round 11). Bound oligonucleotides were subsequently eluted from A-beads with 1 mM free adenine in 100 μL SLE buffer/25%MeOH. Recovered candidates were PCR amplified in a total volume of 1 mL, following the library production protocol (final percentage of MeOH was therefore 2.5% in PCR mixture).

2.4.2. Cloning-sequencing

Upon completion of 11 rounds of selection/amplification, DNA candidates were cloned according to TOPO TA Cloning kit protocol (Invitrogen). Positive colonies from transformed *Escherichia coli* bacteria were PCR amplified, and resulting products were sequenced (Eurofins, MWG-Operon).

2.5. Fluorescence beads assay

2.5.1. Adenine beads binding assays

Oligonucleotides were chemically synthesised with a 5' termini fluorescein. Candidates (150 nM, SLE buffer, 100 μL) were structured in the absence or presence of 25% of methanol and incubated for 30 min at RT with A-bead or N-beads (100 μM). Magnetic collection beads in interaction with ssDNA allowed measurement of supernatant fluorescence of non-bound candidates and determination of binding percentage, which was normalised to control beads.

Thus, binding percentage was $B\% = (1 - (Fa/Fc)) * 100$, where *Fa* is the fluorescence of unbound ssDNA to A-bead and *Fc* is fluorescence of unbound ssDNA on the surface of N-beads. Fluorescence measurements were performed on Berthold Twinkle LB 970 microplate fluorimeter.

2.5.2. Determination of dissociation constant of A'12

The aptamer was incubated at 150 nM for 30 min with A-beads in the presence of increasing total adenine concentration (0–100 μM), in SLE buffer. Experiment was performed in 100 μl total volume. Binding percentages are determined according to adenine concentration variations. Curves were fitted using Hill equation, as $(1 - (Fa/Fc)) * 100 = \%B = \frac{1}{1 + (\frac{K_D}{L})^n}$ where *Fa* is the fluorescence of unbound aptamer at given adenine concentration, *Fc* the fluorescence in absence of adenine, *L* the unbound adenine, *K_D* the dissociation constant and *n* the Hill factor of cooperativity.

2.6. Fluorescence anisotropy assay

2.6.1. Fluorescence polarization binding assay

Fluorescein-adenine (10 nM in SLE buffer, 25% MeOH) was incubated with increasing concentration of aptamer (0–100 μM) in the same buffer. Measurements were performed on a TECAN INFINITE M1000PRO device set with following parameters: 470 nm excitation wavelength, 525 nm emission wavelength, 5 nm excitation and emission bandwidth, settled time of 25 ms, flashes number of 10 and G-factor of 1.076 (as calibrated from free fluorescein: 20 mP for 1 nM fluorescein solution in 0.01 M NaOH). Curves were fitted using Hill equation, as $\Delta r = \frac{1}{1 + (\frac{K_D}{L})^n}$ where Δr represents the variation of anisotropy of adenine-fluorescein conjugates at a given aptamer concentration, *L* the aptamer concentration, *K_D* the dissociation constant and *n* the Hill factor of cooperativity.

2.7. Graphical TOC

The predicted secondary structure of A'12c was subsequently modelled in 3D with RNAcomposer software and rendered with Chimera software.

3. Results and discussion

3.1. Selection design

In the framework of an exobiology project, we were seeking for functional oligonucleotide aptamers towards adenine in methanol-containing solvent. In one hand, adenine is claimed to be a good marker of life trace [27]. On the other hand, extraction conditions of extra-terrestrial rocks involve methanol as a primary solvent. To this aim, we synthesized a DNA library with a 30 positions random window in which we substituted standard TTP by 5-(octa-1,7-diynyl)-2'-deoxyuridine triphosphate (ODTTP) (**2**, Fig. 1) in order to improve the solubility of oligomers in organic solvents. Whereas benzyl or naphthyl modified thymidine have been successfully introduced in the design of SOMAmers [28], we went for an alkyne-bearing nucleobase. Besides the assumed hydrophobicity increase, we were interested in a modification that could allow further labelling of the aptamer, through CuAAC ligation [29]. For the production of the DNA pool we first checked that Taq DNA polymerase (New England BioLabs) incorporates ODTTP properly (Fig. S2A and B). Non-denaturing gel electrophoresis showed that the DNA pool amplified in aqueous solution with the same enzyme (in the presence of ODTTP), yields one band that moves slower than the one amplified with standard dTTP suggesting that ODTTP is well incorporated in DNA random library (Fig. S2C). Template

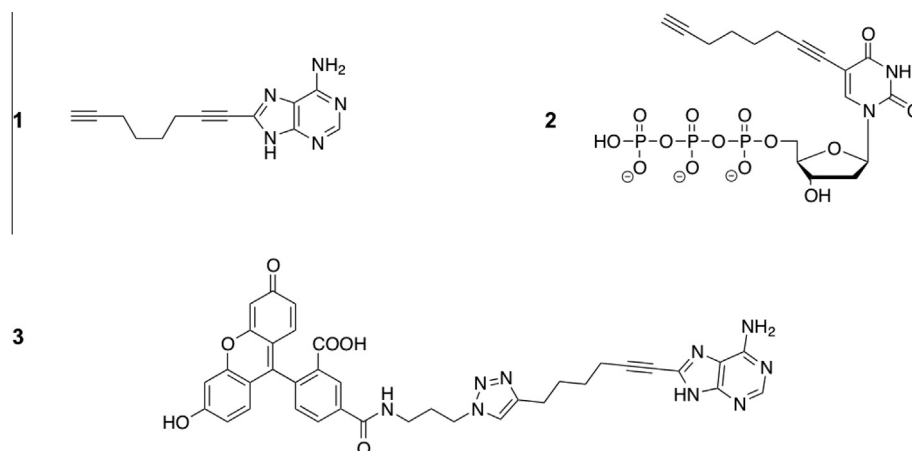


Fig. 1. Chemicals used in this study. 1/8-(1,7-octadiynyl)-adenine. 2/5-(octa1,7-diynyl)-2'-deoxyuridine triphosphate (ODTTP). 3/8-(5-carboxyfluorescein-aminopropylamido-1,2,3-triazolo)-adenine.

amplification was then evaluated in the presence of ODTTP by simulating post selection amplification conditions in the presence of methanol. Results showed that methanol is not a limiting factor for selection in the presence of up to 50%: remaining methanol (following ten fold dilution of the selection sample) had no influence on DNA polymerase activity. Incorporation of ODTTP under these conditions was not affected (Fig. S3). In general family A DNA polymerase such as Taq DNA polymerase are described as not good at successive incorporation of modified nucleotides [30]. However, other groups demonstrated that Taq DNA polymerase incorporates properly thymidine bearing C5 rigid modification [31]. Our results based on PCR amplification are in agreement with analysis at molecular level. Moreover, we observed high incorporation efficiency with the Taq DNA polymerase whereas the use of Vent DNA polymerase (family B) led to low amplification yield. We assume that the type of modification and experiment conditions are important factors for efficiency of amplification.

Targeting a small compound such as adenine requires its immobilization on a solid support, in order to selectively capture oligonucleotides displaying affinity for the target molecule. We performed its covalent immobilisation on functionalised carboxylic acid magnetic beads using *O*-(2-aminoethyl)-*O'*-(2-azidoethyl) diethylene glycol linker [25,32]. The azido function was subsequently engaged in a CuAAC reaction [33] with 8-(octa-1,7-diynyl)-adenine (**1**, Fig. 1) to generate the target-bound beads. Linkage on the C8 position of adenine was preferred, as it preserves π -stacking and hydrogen bonding capacity for interaction with aptamers. In the same manner, azido function was engaged in CuAAC reaction with octa-1, 7-diyne thus providing a control for counter-selection steps: oligonucleotides interacting with such functionalised beads were discarded.

Control beads were also used for evaluating the rate of non-specific interaction at increasing methanol concentration. Above 25% non-specific binding increased dramatically, compared to aqueous conditions. Therefore, selection buffer (SLE) containing 25% methanol was chosen to carry out 11 selection/amplification rounds (see Section 2).

3.2. Identification of adenine-binding aptamers

Analysis of 49 sequences obtained following cloning of rounds 10 and 11, revealed limited homology. Nevertheless, almost all candidates exhibited several purine-rich tracks, the mean rate of GA bases in the random window being about 61%. 58% of

sequences displayed at least 2 blocks of purines, one of them being at least 6 consecutive purines long. Secondary structure prediction showed that purine-rich stretches are located in internal loops in 41% of sequences (family 1), in apical loops in 21% of sequences (family 2). In remaining sequences purine rich stretches are mostly involved in stem pairing. 21% of sequences constitute family 3 that is characterized by the presence of short pyrimidine loops including ODT; and 17% of sequences constitute family 4 that has no similarity to those described previously.

We evaluated the binding properties of representative candidates with the adenine-functionalized beads used for the SELEX process. Four candidates belong to family 1 (A'12, A16, B8, A'3), three candidates belong to family 2 (A36, C3, B'14), two candidates have pyrimidine loops including ODT (A44 and B19) and finally one candidate has no particular features shared with the other sequences (A'47). Selected candidates were chemically synthesised with a 5' fluorescein residue. After incubation with beads, the percentage of bound candidates was determined by fluorescence intensity measurement (Fig. 2) [34].

Candidates within purine-rich loops (family 1 and 2) show the most efficient binding for adenine. The oligonucleotide A'12 that displays the strongest binding was chosen for further studies. The A'12 sequence displays five purine rich regions PuR1 **GAAACG**, PuR2 **AGAGAG**, PuR3 **AGAGGA**, PuR4 **GCGG** and Pur5 **GAGCG** (Fig. 3). They are all located in the random window, except PuR1 that is part of the 5' fixed region. We used mfold [35] to predict A'12 secondary structure, substituting for folding regular thymine nucleotides to ODT in the aptamer sequence.

Two different secondary structures, A'12-1 and A'12-2 (Fig. 3) of similar energy ($\Delta G \leq -21$ kJ mol⁻¹) were predicted. In A'12-2, PuR1 and PuR3 form a short apical purine loop (L4) and an internal purine loop (L5), respectively, whereas PuR3 and PuR4 are involved in double-stranded stems. In A'12-1 PuR1 and PuR2 are part of double-stranded regions whereas PuR3 and PuR4 form an internal purine loop (L2), similar to that identified previously in two aptamers directed towards adenosine monophosphate (AMP) selected independently from RNA or DNA libraries in aqueous buffers [36,37]. These two aptamers differ in secondary structures and binding stoichiometry but they display the same purine richness in the ligand recognition pocket. AMP in both DNA and RNA aptamers is stabilized by stacking between purine bases and forms identical G.AMP specific hydrogen bounds [38]. Moreover, similar purine rich motifs identified in the human genome have been demonstrated to be involved in ATP binding with a dissociation constant 10 fold lower than its physiological concentration [39].

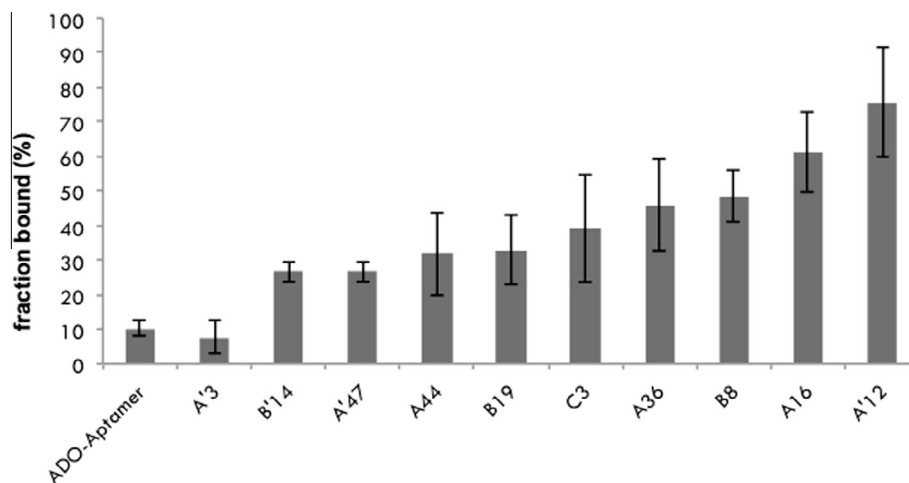


Fig. 2. Binding of different candidates to adenine-conjugated beads. 100 μ M adenine, and 150 nM candidate in SLE buffer (containing 25% methanol) were used. DNA adenosine aptamer was used as control.

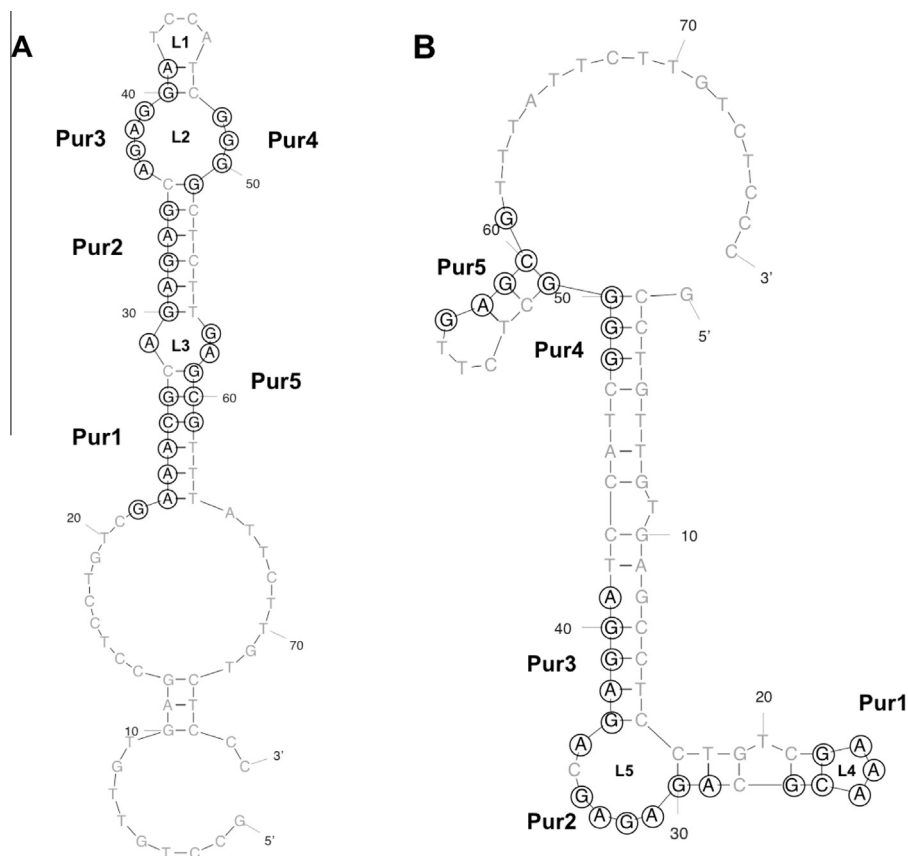


Fig. 3. Secondary structures for the A'12 aptamer, A'12-1 (panel A) and A'12-2 (panel B) as predicted by mfold. Purine-rich regions are highlighted.

Therefore, purine rich motifs can be considered as a hallmark of specific interaction between nucleic acid recognition elements and adenosine derivatives.

3.3. Contribution of ODT to interaction in presence of methanol

We have evaluated the contribution of ODT to the A'12 aptamer adenine binding. For this purpose, two 5' fluoresceine-conjugated variants were evaluated; the first one (A'12+) comprises the ODT,

and the second (A'12-) comprises thymidines that substitute ODT. In addition, DNA adenosine aptamer (ADO-aptamer) previously described, was evaluated [37]. Tests were carried out in the presence and in the absence of 25% methanol through adenine-bead capture strategy (Fig. 4A).

A'12+ binds adenine both in the presence and in the absence of methanol, with a slightly higher efficiency in methanol (Fig. 4A). We determined the dissociation constant of adenine-A'12 complex in methanol. Triplicate adenine-beads binding assays allowed us to

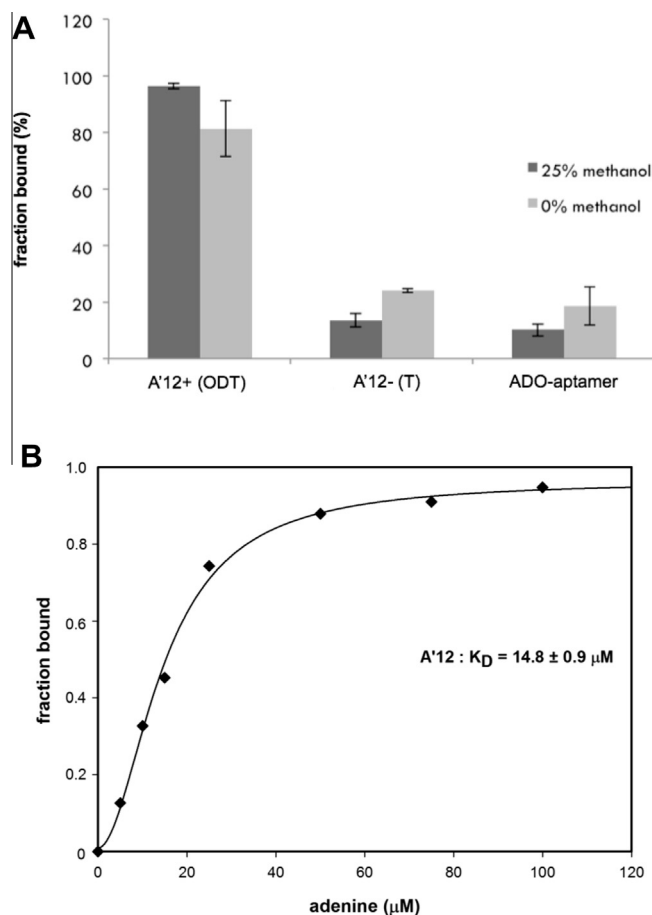


Fig. 4. Binding assay of aptamer analogues. (A) Contribution of octadecyl modification to aptamer affinity for adenine, evaluated through adenine-beads binding assay, in presence of 25% methanol (solid bars) or in aqueous buffer (grey bars). Binding percentage of fluorescent A'12 incorporating ODTTP (A'12+), of the TTP-containing aptamer (A'12-) and the adenosine aptamer (ADO-aptamer). (B) Titration curve of A'12 (150 nM), derived from adenine-beads binding assay.

evaluate an apparent K_D of $14.8 \pm 0.9 \mu\text{M}$ (Fig. 4B). In contrast, a weaker binding is observed with A'12- as well as with the adenosine DNA aptamer (selected in aqueous buffer) [37].

These results suggest that our approach enabled the selection of a high affinity aptamer efficient in organic solvents thanks to the incorporation of ODTTP. We assume that the modified residues might be involved in aptamer folding and target recognition.

3.4. Specificity assay

Aptamers directed towards nucleobases share similar interaction patterns, involving π -stacking and specific hydrogen bonding, that are required for discriminating between the different nucleic acid bases [38]. The specificity of A'12 aptamer for adenine was evaluated by competition with other nucleobases or ribonucleosides: the binding of A'12 was determined by the adenine-bead assay after incubation with a large excess (1 mM) of different nucleic acid base derivatives. Fig. 5 shows that A'12 is highly specific for adenine as adenosine, guanosine, cytosine and uracile did not displace it from the beads. It is noteworthy that we went for guanosine not guanine, because of the poor solubility of guanine in aqueous buffers and its insolubility in methanol.

The lack of interaction with adenosine in our conditions could suggest a tight recognition of adenine, especially through hydrogen bonding to N9 nitrogen, which is involved in the glycosidic bond in the case of adenosine. Regarding AMP aptamer, deletion of both the 2'- and the 5'-hydroxyles of adenosine resulted in an affinity decrease [37], whereas the phosphate has less significant interaction [40]. As discussed in previous section, this result could suggest that rational design of an adenine-binding aptamer from an existing aptamer, that binds adenosine in water, would not have been successful.

3.5. Evaluation of truncated structures

Design of functional shortened aptamers is usually based on the mfold predicted structure. Functional shorter sequences have to be designed with regards to essential recognition motifs. If we hypothesize that purine rich regions (PuR) are crucial for the interaction with adenine, they must be part of A'12 derived minimal sequences. We first synthesized two short ODT-containing sequences: A'12-a and A'12-b (Fig. 6). A'12-a includes PuR3 and PuR4 organised in loop L2 as in A'12-1 structure. A'12-b is designed to test the involvement of PuR1 and PuR2 in adenine recognition and derives from A'12-2, with loop L4 and L5. Evaluation of A'12-a revealed efficient adenine binding only in aqueous buffer (Fig. 7). On the other hand, evaluation of A'12-b revealed lower adenine affinity compared to A'12 and to A'12-a in aqueous buffer. However addition 25% of methanol increases significantly affinity for adenine compared to A'12-a.

Thus, this suggests that both L2 loop including PuR3 and PuR4 from A'12-a structure and L4 and L5 including respectively PuR1 and PuR2 from A'12b are required for efficient interaction with adenine in presence of methanol. We therefore removed the 5'

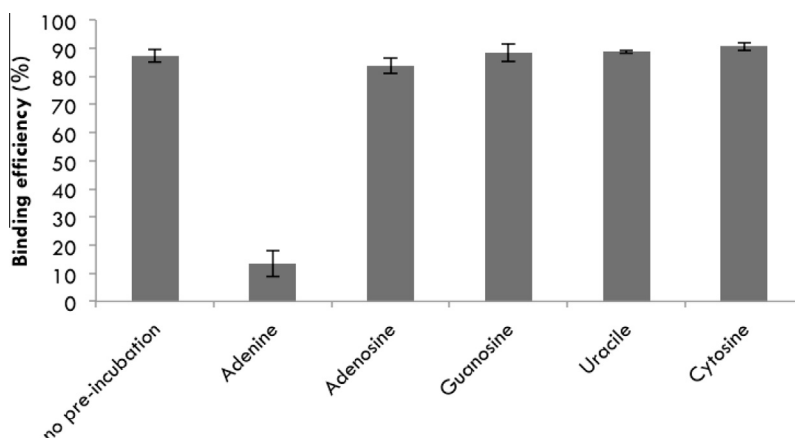


Fig. 5. Specificity of A'12 aptamer for adenine, compared to other nucleobases or nucleosides. A'12 is pre-incubated with 1 mM competitor before evaluation of adenine binding by bead capture (100 μM total adenine concentration).

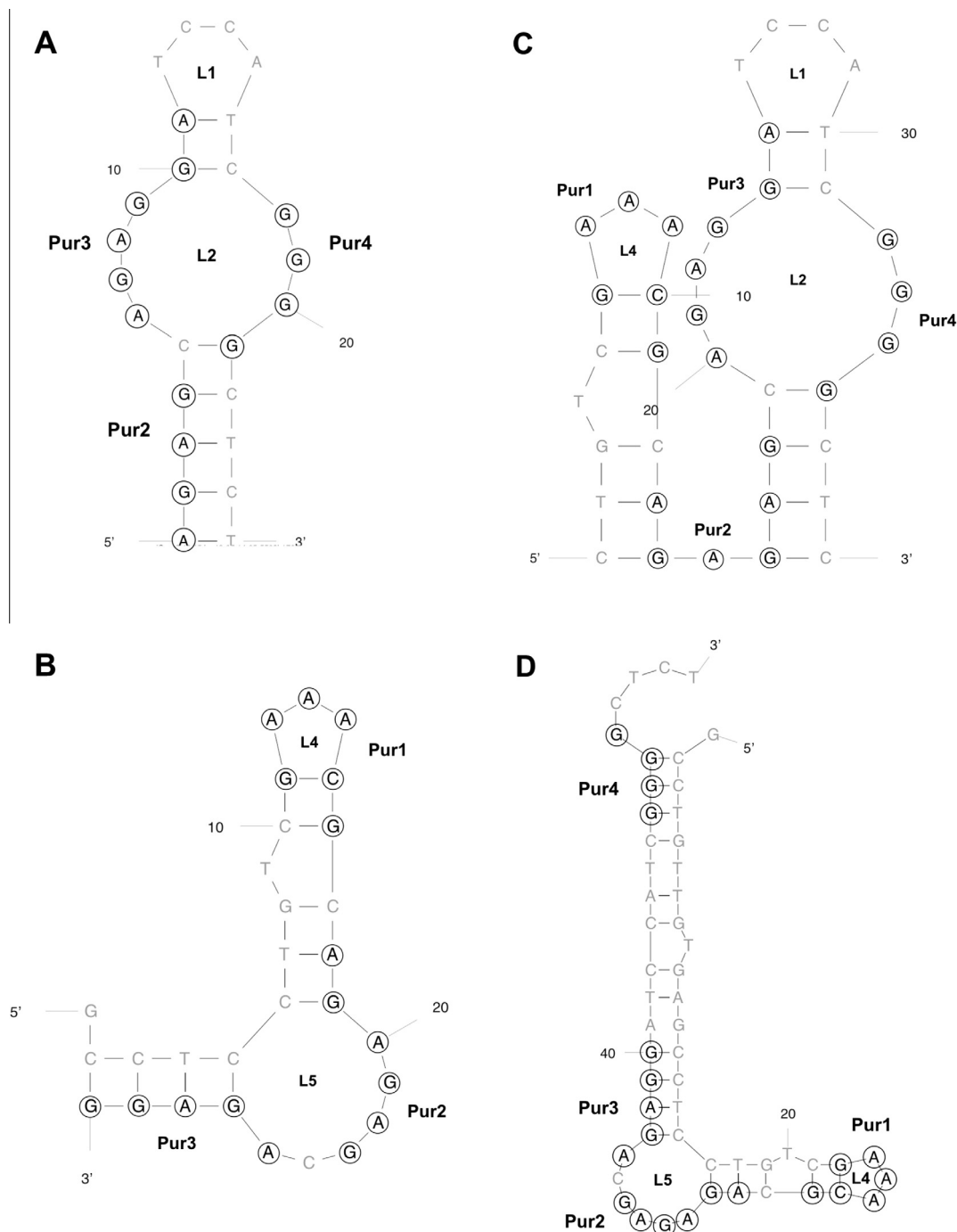


Fig. 6. Truncated derivatives of the aptamer A'12. (A) A'12a; (B) A'12b; (C) A'12c; (D) A'12d. Purine-rich are highlighted. Secondary structures as predicted by mfold program.

and 3' PuR complementary sequences of A'12 which leads to A'12-c (Fig. 6C). It is predicted to be organized into two stem loop motifs, including L1 and L2 as in A'12-a and L4 as in A'12b whereas PuR2 track is involved in stems formation. We also designed A'12-d (Fig. 6D) by lengthening the 5' part of A'12-c; this will prevent organisation in two loop motifs like A'12-c.

A'12-c binds efficiently adenine in presence of 25% of methanol: at a concentration of 100 μ M adenine, 85% of binding is observed. A'12-d binds efficiently adenine in aqueous buffer, whereas a strong decrease is observed in the presence of methanol. The folding in double loop motif, which could be considered as a merge of

structural organisations of full-length A'12 aptamer, seems to be required for adenine recognition in methanol.

Finally, we measured the equilibrium dissociation constant of the adenine-A'12c complex using fluorescence polarization compared to fluorescence beads assay. For this purpose, we synthesized an adenine-fluorescein conjugate (3, Fig. 1). The binding of the labelled adenine to the aptamer will result in a decreased mobility of the reporter, i.e. in an increased anisotropy. The titration curve obtained indicated that the fluorescein modification on the adenine C8 position did not prevent binding and consequently that this position is not involved in the interaction with

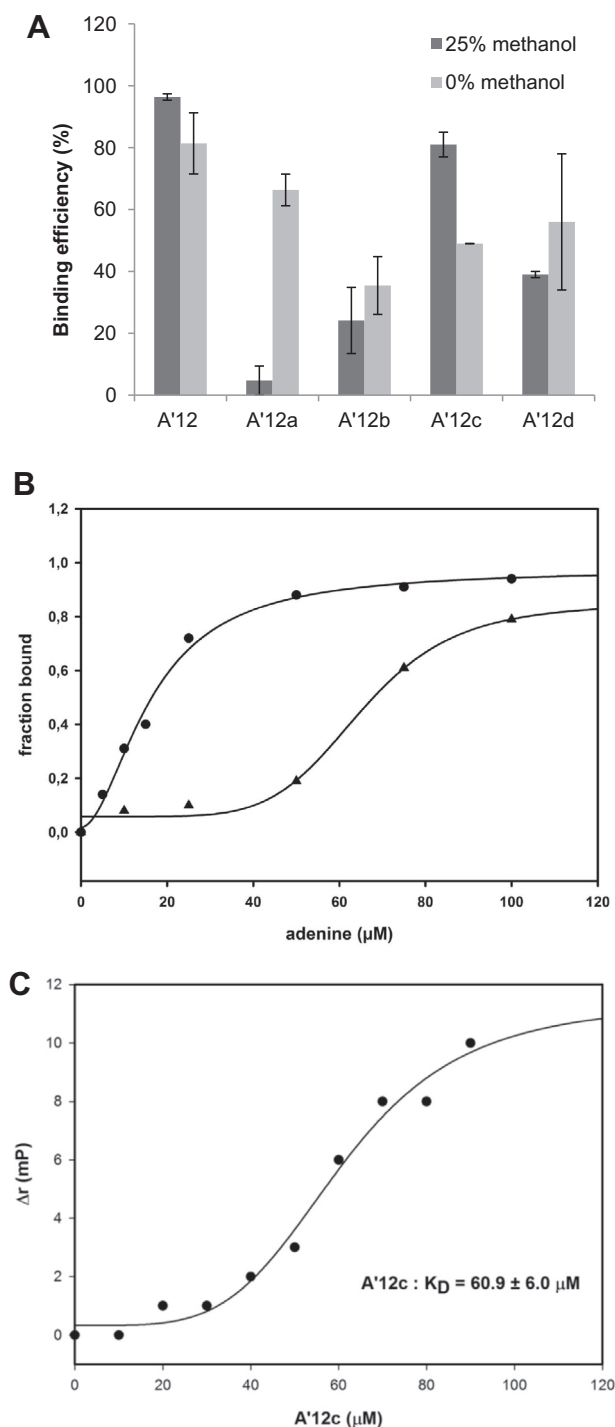


Fig. 7. (A) Evaluation of the binding efficiency of truncated versions of the aptamer A'12, using adenine-beads binding assay, in presence of 25% methanol (solid bars) or in aqueous buffer (grey bars). (B) Compared titration curves of A'12 (black squares) and A'12c (black triangles), derived from adenine-beads binding assay. Aptamers concentrations: 150 nM, in 25% methanol buffer. Apparent K_D of A'12: 15 μM ; A'12c: 65 μM . (C) Binding constant evaluation of A'12-c, through fluorescence anisotropy assay. The K_D of 60 μM is consistent with the first experiment.

the aptamer. This was anticipated as adenine was grafted onto the beads through the same position for SELEX. We deduced an apparent K_D of $60.9 \pm 6.0 \mu\text{M}$ in 25% MeOH (Fig. 7C), consistent with the value obtained using fluorescence assay with adenine-beads (65 μM , Fig. 7B).

4. Conclusion

The development of tools for the detection of small chemicals in organic conditions is of high interest, with potential applications in various fields such as pollutant detection, drug manufacturing or product traceability. Aptamers constitute a class of sensing tools, thanks to their high affinity and specificity for a wide range of target. But their use in organic conditions is hampered by solubility and weaker hydrogen bonding capability. Adaptation to organic condition of an aptamer selected in aqueous buffer is an issue. We have therefore chosen to directly select aptamer in methanol-containing solvent, with incorporation during the SELEX process of a lipophilic modified residue, ODT. We assumed that this modification might enhance oligonucleotide solubility and could participate to the stabilization of the complex with our proof-of-concept target, adenine. We obtained an adenine-binding aptamer A'12, with an apparent K_D of ca. 15 μM for adenine in the presence of 25% methanol. This aptamer is of interest for detecting adenine in organic solvents, in which aromatic compounds usually present higher solubility constant than in aqueous buffer. Interestingly, the aptamer A'12 is more efficient for binding adenine than a closely related previously described aptamer, even in aqueous buffer. Moreover, the incorporation of regular thymidines instead of ODT led to an A'12 variant with dramatically decreased affinity. These results demonstrate that our approach allowed the selection of aptamer that could not be derived from an existing aptamer obtained in aqueous buffer. Moreover, this work proposes an application of octadiyne derivatives, enhancing the chemical diversity of aptamers.

Following this proof-of concept, we will generalize our selection procedure and develop aptamers directed towards poorly water-soluble chemicals, as pollutants. Efforts for getting access to signalling aptamers could take advantage of the folding structure of A'12c. Indeed, this stem-loop aptamer could be engineered to lead to structure switching kissing complex [41]. The use of beads immobilised target limited the concentration of methanol to 25%. Whereas the implement of selection strategies where the target is free in solution will open the door in higher methanol concentration usefulness.

Acknowledgement

We acknowledge the support of the Conseil Régional d'Aquitaine (grant 20101303002) and the Centre National d'Études Spatiales (n°115451/00). Eric Dausse (structures analysis) and Jean-Jacques Toulmé are gratefully acknowledged for their help.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ymeth.2016.01.002>.

References

- [1] C. Tuerk, L. Gold, *Science* 249 (4968) (1990) 505–510.
- [2] A.D. Ellington, J.W. Szostak, *Nature* 346 (6287) (1990) 818–822.
- [3] E.W. Ng, D.T. Shima, P. Calias, E.T. Cunningham, D.R. Guyer, A.P. Adamis, *Nat. Rev. Drug. Discov.* 5 (2) (2006) 123–132.
- [4] P. Nadal, M. Svobodova, T. Mairal, C.K. O'Sullivan, *Anal. Bioanal. Chem.* 405 (29) (2013) 9343–9349.
- [5] F. Ducongé, J.J. Toulmé, *RNA* 5 (12) (1999) 1605–1614.
- [6] R.D. Jenison, S.C. Gill, A. Pardi, B. Polisky, *Science* 263 (5152) (1994) 1425–1429.
- [7] I. Palchetti, M. Mascini, *Anal. Bioanal. Chem.* 402 (10) (2012) 3103–3114.
- [8] E. Dausse, S. Da Rocha Gomes, J.-J. Toulmé, *Curr. Opin. Pharmacol.* 9 (5) (2009) 602–607.
- [9] J.A. DeGrasse, *PLoS One* 7 (3) (2012) e33410.

- [10] S. Da Rocha Gomes, J. Miguel, L. Azéma, S. Eimer, C. Ries, E. Dausse, H. Loiseau, M. Allard, J.-J. Toulmé, *Bioconjugate Chem.* 23 (11) (2012) 2192–2200.
- [11] S.D.R. Gomes, L. Azéma, M. Allard, J.-J. Toulmé, Aptamers as imaging agents, *Expert Opin. Med. Diagn.* 4 (2010) 511–518.
- [12] J. Li, H. Zheng, P.J. Bates, T. Malik, X.-F. Li, J.O. Trent, C.K. Ng, *Nucl. Med. Biol.* 41 (2014) 179–185.
- [13] W.-B. Shim, M.J. Kim, H. Mun, M.-G. Kim, *Biosens. Bioelectron.* 62 (2014) 288–294.
- [14] A.K. Behera, K.J. Schlund, A.J. Mason, K.O. Alila, M. Han, R.L. Grout, D.A. Baum, *Biopolymers* 99 (6) (2013) 382–391.
- [15] T.T. Herskovits, J.P. Harrington, *Biochemistry* 11 (25) (1972) 4800–4811.
- [16] Y. Imaizumi, Y. Kasahara, H. Fujita, S. Kitadume, H. Ozaki, T. Endoh, M. Kuwahara, N. Sugimoto, *J. Am. Chem. Soc.* 135 (25) (2013) 9412–9419.
- [17] H. Fujita, K. Nakajima, Y. Kasahara, H. Ozaki, M. Kuwahara, *Bioorg. Med. Chem. Lett.* 25 (2) (2015) 333–336.
- [18] D.R. Davies, A.D. Gelin, C. Zhang, J.C. Rohloff, J.D. Carter, D. O'Connell, S.M. Waugh, S.K. Wolk, W.S. Mayfield, A.B. Burgin, T.E. Edwards, L.J. Stewart, L. Gold, N. Janjic, T.C. Jarvis, *Proc. Natl. Acad. Sci. U.S.A.* 109 (49) (2012) 19971–19976.
- [19] A.D. Gelin, D.R. Davies, T.E. Edwards, J.C. Rohloff, J.D. Carter, C. Zhang, S. Gupta, Y. Ishikawa, M. Hirota, Y. Nakaishi, T.C. Jarvis, N. Janjic, *J. Biol. Chem.* 289 (12) (2014) 8720–8734.
- [20] J. Gierlich, K. Gutmiedl, P.M.E. Gramlich, A. Schmidt, G.A. Burley, T. Carell, *Chem. Eur. J.* 13 (34) (2007) 9486–9494.
- [21] J.D. Watson, F.H. Crick, *Nature* 171 (4356) (1953) 737–738.
- [22] P. Lang, G. Magnin, G. Mathis, A. Burger, J.-F. Biellmann, *J. Org. Chem.* 65 (23) (2000) 7825–7832.
- [23] P.M. Gramlich, S. Warncke, J. Gierlich, T. Carell, *Angew. Chem., Int. Ed.* 47 (18) (2008) 3442–3444.
- [24] R.M. Williams, C.L. Crieff, S. Gattu, L.A. Holland, L.J. Sooter, *Int. J. Mol. Sci.* 15 (8) (2014) 14332–14347.
- [25] F. Tran, A.V. Odell, G.E. Ward, N.J. Westwood, *Molecules* 18 (9) (2013) 11639–11657.
- [26] B.M. Trost, M.T. Rudd, *Org. Lett.* 5 (24) (2003) 4599–4602.
- [27] M.P. Callahan, K.E. Smith, H.J. Cleaves, J. Ruzicka, J.C. Stern, D.P. Glavin, C.H. House, J.P. Dworkin, *Proc. Natl. Acad. Sci. U.S.A.* 108 (34) (2011) 13995–13998.
- [28] J.C. Rohloff, A.D. Gelin, T.C. Jarvis, U.A. Ochsner, D.J. Schneider, L. Gold, N. Janjic, *Mol. Ther. Nucleic Acids* 3 (2014) e201.
- [29] F. Tolle, G.M. Brändle, D. Matzner, G. Mayer, *Angew. Chem., Int. Ed. Engl.* 54 (37) (2015) 10971–10974.
- [30] H.A. Held, S.A. Benner, *Nucleic Acids Res.* 30 (17) (2002) 3857–3869.
- [31] S.E. Lee, A. Sidorov, T. Gourlain, N. Mignet, S.J. Thorpe, J.A. Brazier, M.J. Dickman, D.P. Hornby, J.A. Grasby, D.M. Williams, *Nucleic Acids Res.* 29 (7) (2001) 1565–1573.
- [32] K. Shimokawa, K. Yamada, O. Ohno, Y. Oba, D. Uemura, *Bioorg. Med. Chem. Lett.* 19 (1) (2009) 92–95.
- [33] H.C. Kolb, M.G. Finn, K.B. Sharpless, *Angew. Chem., Int. Ed. Engl.* 40 (11) (2001) 2004–2021.
- [34] L. Gold, D. Ayers, J. Bertino, C. Bock, A. Bock, E.N. Brody, J. Carter, A.B. Dalby, B.E. Eaton, T. Fitzwater, D. Flather, A. Forbes, T. Foreman, C. Fowler, B. Gawande, M. Goss, M. Gunn, S. Gupta, D. Halladay, J. Heil, J. Heilig, B. Hicke, G. Husar, N. Janjic, T. Jarvis, S. Jennings, E. Katilius, T.R. Keeney, N. Kim, T.H. Koch, S. Kraemer, L. Kroiss, N. Le, D. Levine, W. Lindsey, B. Lollo, W. Mayfield, M. Mehan, R. Mehler, S.K. Nelson, M. Nelson, D. Nieuwlandt, M. Nikrad, U. Ochsner, R.M. Ostroff, M. Otis, T. Parker, S. Pietrasiewicz, D.I. Resnicow, J. Rohloff, G. Sanders, S. Sattin, D. Schneider, B. Singer, M. Stanton, A. Sterkel, A. Stewart, S. Stratford, J.D. Vaught, M. Vrkljan, J.J. Walker, M. Watrobka, S. Waugh, A. Weiss, S.K. Wilcox, A. Wolfson, S.K. Wolk, C. Zhang, D. Zichi, *PLoS One* 5 (12) (2010) e15004.
- [35] M. Zuker, *Nucleic Acids Res.* 31 (13) (2003) 3406–3415.
- [36] M. Sassanfar, J.W. Szostak, *Nature* 364 (6437) (1993) 550–553.
- [37] D.E. Huizenga, J.W. Szostak, *Biochemistry* 34 (2) (1995) 656–665.
- [38] C.H. Lin, D.J. Patel, *Chem. Biol.* 4 (11) (1997) 817–832.
- [39] M.M.K. Vu, N.E. Jameson, S.J. Masuda, D. Lin, R. Larralde-Ridaura, A. Lupták, *Chem. Biol.* 19 (10) (2012) 1247–1254.
- [40] P.L. Sazani, R. Larralde, J.W. Szostak, *J. Am. Chem. Soc.* 126 (27) (2004) 8370–8371.
- [41] G. Durand, S. Lisi, C. Ravelet, E. Dausse, E. Peyrin, J.-J. Toulmé, *Angew. Chem., Int. Ed.* 53 (27) (2014) 6942–6945.

Supporting Information

DNA APTAMER SELECTION IN METHANOLIC MEDIA: ADENINE-APTAMER AS PROOF-OF-CONCEPT

Thinhinane CHAOU^{1,2}, Brune VIALET^{1,2} and Laurent AZÉMA^{*1,2}

1. Univ. Bordeaux, ARNA laboratory, F-33000 Bordeaux, France

2. INSERM, U869, ARNA laboratory, F-33000 Bordeaux, France

1. Adenine beads functionalization

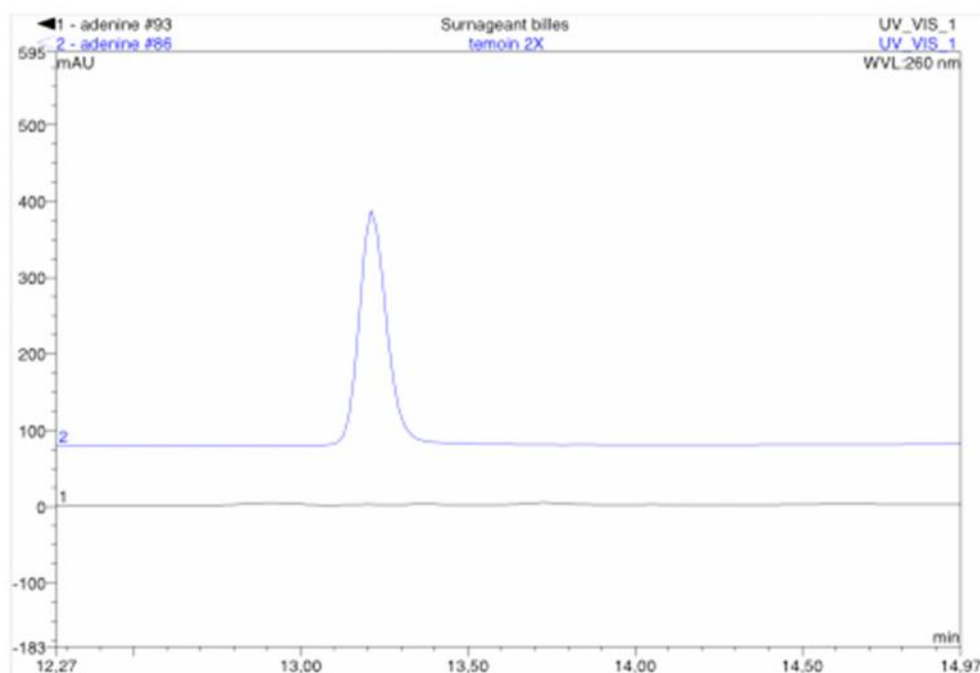


Figure S1. HPLC post-synthesis analysis of bead supernatant. Chromatogram1(gray) demonstrates absence of adenine in supernatant after click chemistry reaction, as adenine was captured by octadiynyl containing beads. Chromatogram 2 (blue) represents analysis of bead supernatant before click reaction; peak at 13 min corresponds to 8-(1,7-octadiynyl)-adenine.

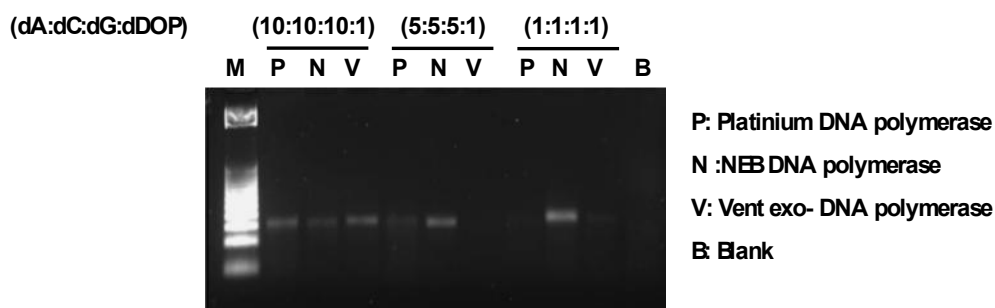
2. PCR

incorporation of ODTTP

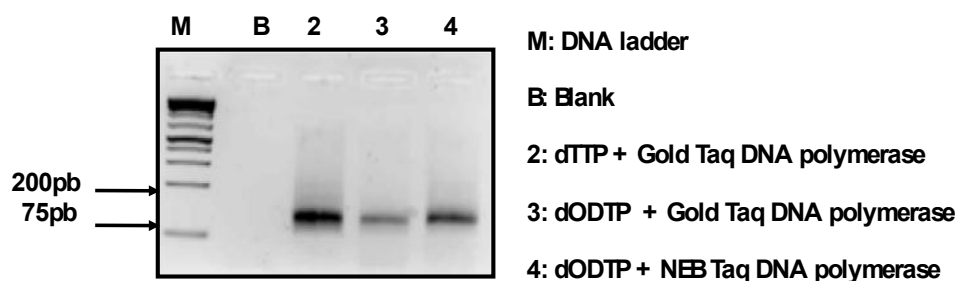
PCR conditions for C5-alkyne dUTP. The PCR reaction mixture contained 2 μM of each primer, 0.2 mM of each nucleoside triphosphate (dATP, dCTP, dGTP) except dODTP (40-200 μM), 0.1U/ μl of DNA polymerase (New England Biolabs) in PCR buffer (67 mM Tris-HCl pH 8.8, 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM β -mercaptoethanol, 1.17 mM MgCl_2). Thermal cycle comprises the following steps: one cycle of 10 min at 95°C, followed by 19 thermal cycles of 1min 95°C, 1 min 60°C, 1.5 min 72 °C and

one cycle 10 min 72°C. PCR product were loaded on a 3% agarose gel containing ethidium bromide and revealed on G-box apparatus .

A.



B.



C.

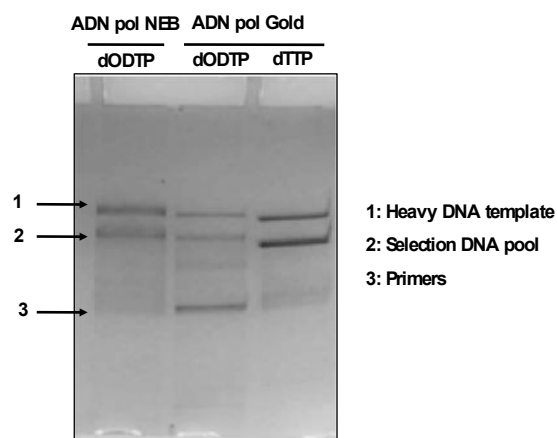


Figure S2. PCR incorporation analysis of ODTTP. **A:** Incorporation of ODTTP present in increased ratio in aqueous buffer. Test performed with various DNA polymerases. **B:** Incorporation of ODTTP compared with TTP; test performed with two Taq DNA polymerases, NEB and Gold. **C:** Observation under denaturing conditions of incorporation of ODTTP compared with TTP; test performed with two Taq DNA polymerases NEB and Gold.

3. Amplification efficiency in the presence of methanol and dDOTP

PCR conditions. The PCR reaction mixture contained 2 μ M of each primer, 0.2 mM of each nucleoside triphosphate (dATP, dCTP, dGTP and ODTTP), 0.1U/ μ l of DNA polymerase (New England Biolabs) in PCR buffer (67 mM Tris-HCl pH 8.8, 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 10 mM β -mercaptoethanol, 1.17 mM MgCl_2) containing various amount of methanol (0%, 2.5% and 5%) in order to estimate the methanol maximal concentration. Thermal cycle comprises the following steps: one cycle of 10 min at 95°C, followed by 19 thermal cycles of 1min 95°C, 1 min 60°C, 1.5 min 72 °C and one cycle 10 min 72°C. PCR product were loaded on a 3% agarose gel containing ethidium bromide and revealed on G-box apparatus .

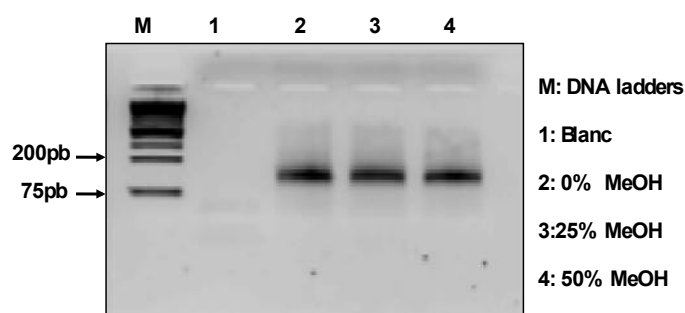


Figure S3. Incorporation of ODTTP by NEB Taq DNA polymerase in the presence of increased methanol concentrations. Indicated percentages correspond to selection percentage. Methanolic concentration during PCR was ten fold lowered, due to dilution.

4. Sequences of analysed candidates

A, T, C and G represent regular deoxyribonucleoside. O represents 5-(1,7 octadiynyl)-2'-deoxyuridine. The sequence we obtain are classified on the basis of the presence of purine riche sequence organisation for this purpose the frequencies are unemployable

A'12>

GCCTGTTGTGAGCCTCCTGTGCGAAACGCAGAGAGCAGAGGAOCCAOCGGGGCOCOOGAGCGOOOA AOCOOGOCOC
CC

A16>

GCCTGTTGTGAGCCTCCTGTGCGAAGGGAGCAGGCCAOGGCGAOGGGGGOOAGAGCGOOOA AOCOOGOCOC

B8>

GCCTGTTGTGAGCCTCCTGTGCGAACAGAGGGGGCGCAOCCGAACGCAGGCCOOGAGCGOOOA AOCOOGOCOC

A36>

GCCTGTTGTGAGCCTCCTGTGCGAAAGGAAGGACGAOAGGGCAGCCGOGGGAOGCOOGAGCGOOOA AOCOOGOCOC
CC

C3>

GCCTGTTGTGAGCCTCCTGTGCGAAAGAGGAGGCCCCGACGGGGAGAGCCGGCCCCOOGAGCGOO AOCOOGOCOC
C

B19>

GCCTGTTGTGAGCCTCCTGTGCGAACGCGGGGACCAGCAOCGCCAOGAGCOCOOGAGCGOOOA AOCOOGOCOC

A44>

GCCTGTTGTGAGCCTCCTGTGCGAAGGGCAGAGACOACAGCOCOCGAGGAGCCOCOOGAGCGOOOA AOCOOGOCOC
CC

A'47>

GCCTGTTGTGAGCCTCCTGTGCGAAGGGGAGAGAGACACCOACGAOCCCOGCOOGAGCGOOOA AOCOOGOCOC

B'14>

GCCTGTTGTGAGCCTCCTGTGCGAACACAAGGCGAGACAAGGAOAGAACAGCOCOOGAGCGOOOA AOCOOGOCOC
C

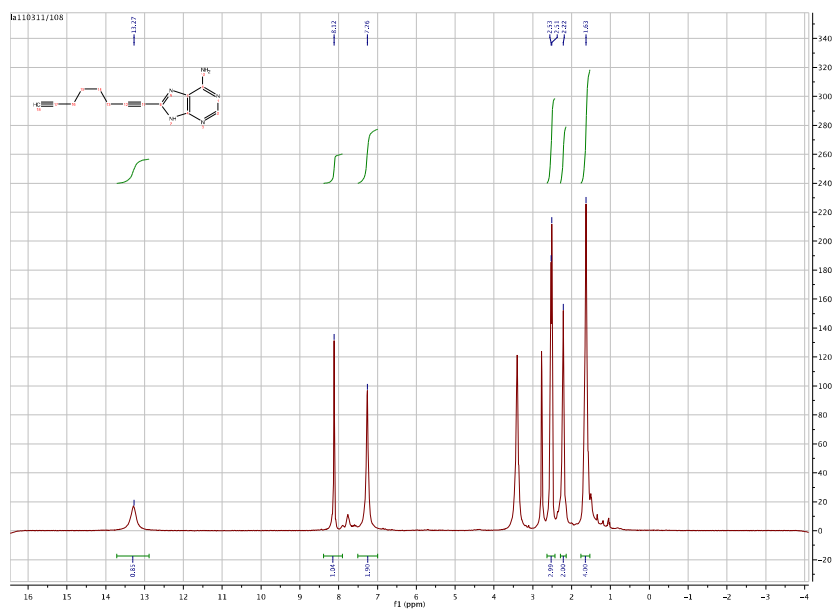
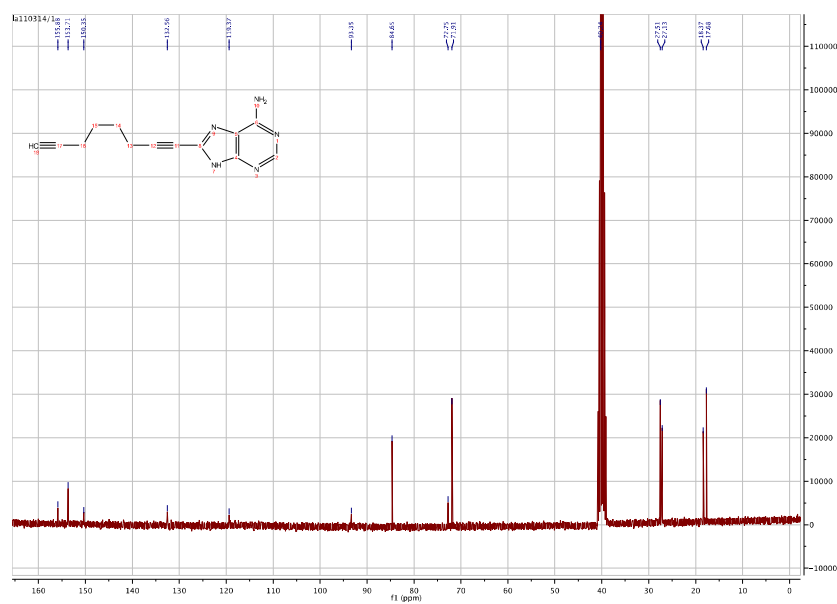
A'3>

GCCTGTTGTGAGCCTCCTGTGCGAACGAAGGAGGAOAGACCCOAGCAGCCAGGCCOOGAGCGOOO OCOOGOCOC
C

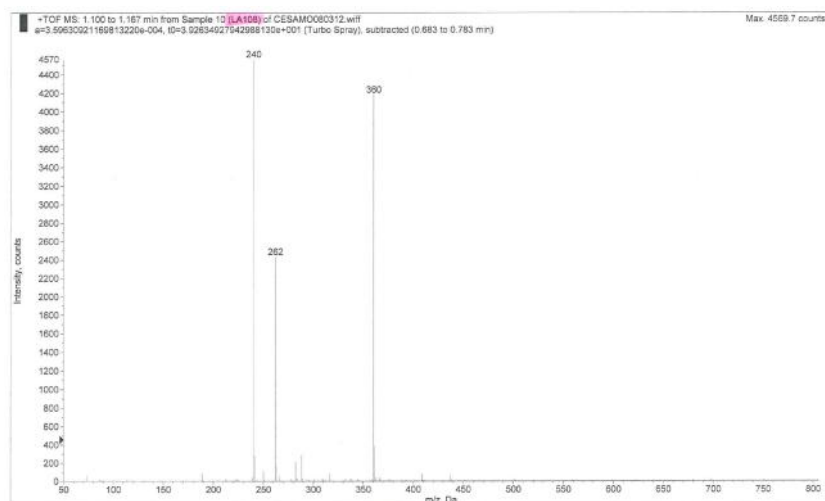
5. Spectral Data

Compound 1: 8-(1,7-octadiyne)-adenine

¹H NMR (300 MHz, DMSO-*d*₆)

 ^{13}C NMR (75 MHz, DMSO- d_6)

ESI Mass Spectrometry



Compound 3: fluoresceine-adenine conjugate

ESI-Mass spectrometry

140221-Exac-1410-LA-TC-TC01

21/02/2014 17:08:50

140221-Exac-1410-LA-TC-TC01 #103-129 RT: 2.39-2.98 AV: 27 NL: 3.38E4
T: FTMS - p ESI Full ms [100.00-2000.00]

