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The affinity between aptamers and low molecular weight compounds: towards a cross-referenced analytical approach

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ABSTRACT: Since their inception, DNA aptamers were regarded as the turning point for biochemical sensing in real samples; however up to now their promises are far from being fulfilled. Especially aptamers for small molecules pose a challenge for both selection and characterization. The lack of a universally accepted and robust quality control protocol for the characterization of aptamer performances coupled with the observation of inconsistent data sets in literature, prompted us to address the issue comparing different analytical methodologies to validate (or disprove) the binding capabilities of aptamer sequences. We chose three aptamers for ampicillin, a β-Lactam antibiotic; used several detection strategies described in literature. The colorimetric gold nanoparticles (AuNPs) assay used in the original paper describing the aptamer sequences was repeated with conflicting results. The three sequences were then tested with three different instrumental techniques to assess their Kd and binding mechanism in homogeneous solutions. Coupling the thermodynamic data obtained with Isothermal Titration Calorimetry (ITC) with the structural information on the binding event given by Native Electro Spray Ionization Mass spectrometry (Native ESI-MS) and 1H-NMR it was possible to verify that the three sequences do not show any specific binding with the target ampicillin. To verify the influence of the AuNPs on the binding event, the experiments were repeated in presence of AuNPs both with ITC and 1H-NMR, again without any results. By offering a cross-referenced and robust analitycal approach to aptamer characterization we aim at elucidating the potentialities of aptamer for small organic molecules, especially when ultrasensitive analytical application are involved

Introduction

Aptamers are short strands of DNA or RNA that recognize with high affinity a given target against which they are selected. Aptamers were first obtained in the 90s [1–3] following a procedure called SELEX (Systematic Evolution of Ligand by Exponential Enrichment). From the beginning they were considered a leap forward in many analytical and biomedical possible applications. Indeed, they offer considerable advantages over traditional molecular biorecognition elements such as antibodies or enzymes, like stability over a wider range of temperatures and pHs, ease of synthesis and modification, lower cost and longer shelf-life [4]. They can be selected against almost every possible analytical target, such as proteins, carbohydrates, enzymes, cells, bacteria and small organic molecules. RNA and DNA aptamers have been reported in literature for therapeutic and drug-delivery studies [5,6], as well as for analytical purposes [7,8]. As much as this scenario seems promising for analytical chemists and biologists, there are still many challenges to face before its recognized potential for commercialization is proven. First of all the selection procedure itself; while many improvements have been made since the 90s, and many different configurations and approaches, such as Capture-SELEX, Cell-SELEX, Capillary Electrophoresis-SELEX, are possible [9–15],

it still remains a randomized process. The initial probability for the target to bind specifically to a strong receptor, contained in a given library, is 10^{-9} [16–18]. Another important point is the lack of a universally accepted and reliable quality control protocol for the characterization of aptamer performance. Typically, new aptamers are characterized with affinity binding assays like equilibrium dialysis, ultrafiltration, affinity chromatography with magnetic beads or fluorescence based test [19]. All the above mentioned assays, while reliable and based on solid theoretical grounds, are prone to misinterpretation and errors in the application of the protocol are common. The affinity constants obtained with these assays are often very different and only by comparing more than one assay it is possible to obtain consistent results [19]. In spite of this, these assays are quite commonly used to characterize aptamer binding affinity especially because they are relatively cheap, easy to perform and do not require expensive equipment. Analytical techniques like Surface Plasmon Resonance (SPR) [20] or Capillary Electrophoresis (CE) [21] are to be preferred for affinity characterization. Indeed, SPR measurements are much more accurate and can give quantitative and reproducible results. However, the need to immobilize the aptamer (or the target) on the sensor chips can possibly influence the binding mechanism. The required addition of linkers, spacers or labels to the aptamer sequences may hamper the recognition event and no systematic study on the effect of these linkers and labels have been performed. Lastly, CE is well suited to characterize aptamer interaction with larger molecules (proteins, enzyme) but is not applicable to small molecule targets. Small molecules represent the biggest class of environmental contaminants, and their determination is of the utmost importance in many different analytical fields.

Finding a solution to those issues is thus crucial for the analytical application of aptamers, and especially for aptamers against small molecules (<1000 amu) such as drugs or antibiotics. Selecting aptamers against small molecules is challenging and requires particular care to avoid unspecific binding or very poor yield [22]. Even if nowadays they seem the most interesting analytical targets. Only a small percentage of the published aptamers are targeted towards these analytes (c.a. 25% in the last 25 years [23]) and they present on average a lower affinity towards their targets, compared to other target groups (e.g. proteins or cells) [19,22]. Within this small group, only very few aptamers for small molecules were extensively characterized and validated, like cocaine binding aptamers [24]. These became the golden standard to test new analytical approaches and to undertake mechanistic and theoretical studies. However many other aptamers were only used and tested by the group that select them in the first place, and even if used in other experiments, usually the results are not consistent with each other [19]. Given the observation of inconsistent data sets in literature, our focus is the development and comparison of different analytical methodologies to validate (or disprove) the affinity of aptamers sequences found in literature. By offering an overview of robust analytical methodologies we aim at preventing a proliferation of publications which suggest aptamer sequences without a proper validation.

Focusing on aptamers for small molecules with significant impact for society when reaching the stage of real sample application, we here chose ampicillin aptasensing. Ampicillin is a β-lactam antibiotic that belongs to the family of penicillins and is one of the most frequently used antibiotics for both human and veterinary medicine. Residues in the environment and the food chain may cause allergic reactions in hypertensive individuals, interfere with fermentation processes, but most importantly, increase antimicrobial resistance (AMR) [25]. As surveillance is one of the main suggested interventions to tackle AMR [26], the development of reliable sensors for antibiotics is a research topic of significant impact. In 2012, Song *et al.* [27] selected three different aptamer sequences for ampicillin (AMP4, AMP17 and AMP18) and used them in a dual colorimetric detection strategy based on AuNPs aggregation to detect the antibiotic in the low nanomolar range in aqueous solution and also in milk samples. This was the very first example of an aptamer against a β-lactam antibiotic described in literature. Moreover, the authors reported that the aptamer is capable of recognizing the side chain of ampicillin (1-phenylethylamine) specifically, assuring a high selectivity against structurally related compounds. In the last seven years these aptamers have been used by other groups around the world, with a variety of analytical approaches and sensor configurations mostly based on electrochemical transduction, with very good result both in terms of figures of merit and real sample analysis (for a complete overview see Table. S1). The affinity constant, the selectivity and the specificity reported in the original paper were always taken for granted and there are no systematic studies about the binding mechanism of ampicillin aptamers and their targets. These aptamers were selected to undertake a systematic study of aptamer-target binding interactions, coupling different state-of-the-art analytical techniques to devise a general approach for the characterization of aptamers for small organic molecules. Combining Isothermal Titration Calorimetry (ITC), Native Electrospray Ionization Mass Spectrometry (Native ESI-MS) and ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR) it is possible to obtain a complete overview of the binding affinity and mechanism in homogeneous phase. These techniques were applied to the characterization of the binding event between the ampicillin aptamers and their target, ampicillin. For comparison, the same set of experiments was performed with another aptamer sequence for small organic molecules, MN4. This cocaine binding aptamer is one of the most studied and well-characterized for analytical applications and it also binds strongly with quinine [28,29], with a much lower K_d value (≈100 nM), closer to the one of the AMP aptamers. Therefore we decide to use quinine as the target of the aptamer MN4 in this study. The information of the molecular structure and the binding mechanism confirm the specificity and selectivity of the response of MN4 towards its targets (cocaine and

quinine), thus assuring that the signal recorded with different analytical techniques is really linked to the binding event and not generated by other phenomena. All the above mentioned techniques were already used for aptamer characterization [30–33] but rarely combined and cross-referenced.

Materials and Methods

Aptamers and reagents

Ampicillin aptamers, MN_4 aptamer and a random primer were all purchased from Eurogentec (Belgium). In Table 1 the details of the four different sequences can be found. Ampicillin sodium salt, cephalexin, nafcillin sodium salt, quinine hydrochloride di-hydrate and ammonium acetate solution (7.5 M) were obtained from Sigma Aldrich. Chloramphenicol was obtained from TCI (Europe). All other chemicals were reagent grade and used without further purification. MilliQ water was obtained with a Millipore Milli-Q Academic system. Details on the AuNPs assay protocol and the instrumental analysis (ITC, Native ESI-MS and ^{1}H-NMR) are reported in Supplementary information.

Results and Discussion

Colorimetric AuNPs assay

To test the specificity of the aptamers, the colorimetric AuNPs assay employed by Song et al. was repeated. In a first step ,the adsorption of ssDNA on the gold surface protects the nanoparticles against aggregation in the presence of salt [34,35]. Upon adding a target with affinity for the aptamer, the aptamer folds to allow the binding event and leaves the nanoparticles surface. As a result, the nanoparticles are no longer stabilized in solution and aggregation or even precipitation occurs upon establishing a specific salt concentration [36]. The resulting colour change serves as proxy to establish the original binding event. An explicative scheme of the assay is depicted in Fig.1. Colorimetric assays based on AuNPs are widely used as analytical tool to investigate the aptamer-target interaction [36,37]. The principle of the assay is based on the aggregation and subsequent change in colour of gold nanoparticles in solution upon addition of salt, due to the screening of the negative charge on the nanoparticles surface by the positive ion of the salt. The electrostatic repulsion between the nanoparticles is diminished and the plasmon resonance peak in the UV-Vis spectra shifts to higher wavelengths, resulting in a visible colour change. This effect is also influenced by the size and concentrations of the nanoparticles [38].

This method provides a fast approach to test aptamer performances and binding capabilities. Along with ampicillin, also cephalexin was tested since it has the same side chain as ampicillin, for which the aptamer should be selective, following the conclusion of the original article. The protocol (see Materials and Methods) was applied to ampicillin, cephalexin and two other antibiotics, nafcillin and chloramphenicol. Also, a random primer sequence (RP36) was tested in the same conditions with ampicillin.

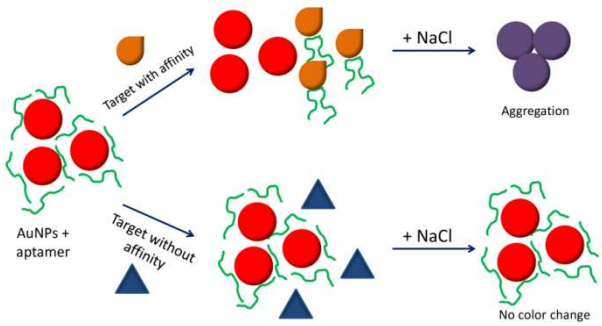

Fig. 1 Schematic representation of the colorimetric AuNPs assay

The results for the colorimetric test are reported in terms of the ratio between the absorbance of the UV-Vis band at 520 and 620 nm respectively. The band at 520 nm corresponds to the amount of dispersed particles while the one at 620 nm corresponds to that of aggregated particles. The first band should decrease and the second increase upon addition of salt and subsequent aggregation when binding induced release of the aptamer occurred from the AuNPs surface (see Figure S.1). This 520/620 ratio is considered more reliable and sensitive than the peak intensity alone [37]. The original paper reports an arbitrary ratio of 2 as a threshold between positive and negative results. The result for AMP17, the aptamer indicated as the best one by Song et al., is reported in Fig. 2. The histogram shows that the ratio for the aptamer alone (in the absence of antibiotics) remains higher than 1 even after addition of salt; the same can be said for the non-specific antibiotics, nafcillin and chloramphenicol and the random primer RP36. For ampicillin and cephalexin the 520/620 ratio is lower than 1 and the colour of the corresponding solution turns purple. This behaviour is consistent with the report of Song et al, as the aptamer specifically recognise the side chain of ampicillin and cephalexin. However, the standard deviations (3 repeated measurements) are rather large and the differences between positive and negative results remain small. Moreover, while the original paper reports an arbitrary ratio of 2 as a threshold between positive and negative results, in our case only the pure AuNPs showed a ratio higher than 2. As such all the performed measurements should be considered positive results, following the original conclusions of Song et al. The fact that these results are not consistent with the one reported before, prompted us to investigate this interaction with other techniques, to prove whether the effect seen with the AuNPs assay is really linked to the binding event between the aptamer and its target.

Table 1. Details of the ampicillin aptamers (AMP4, AMP17 and AMP18), MN4 aptamer and random primer (RP36) used in the study; *Kd value of binding with quinine.

Acronym	Sequence	Length (bp)	Kd
AMP4	5'-CAC-GGC-ATG-GTG-GGC-GTC-GTG-3'	21	9.4 nM
AMP17	5' GCG-GGC-GGT-TGT-ATA-GCG-G-3'	19	13.4 nM
AMP18	5'-TTA-GTT-GGG-GTT-CAG-TTG-G-3'	19	9.8 nM
MN4	5'-GGC-GAC-AAG-GAA-AAT-CCT-TCA-ACG-AAG-TGG-GTC-GCC-3'	36	100nM*
RP36	5'-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-3'	36	-

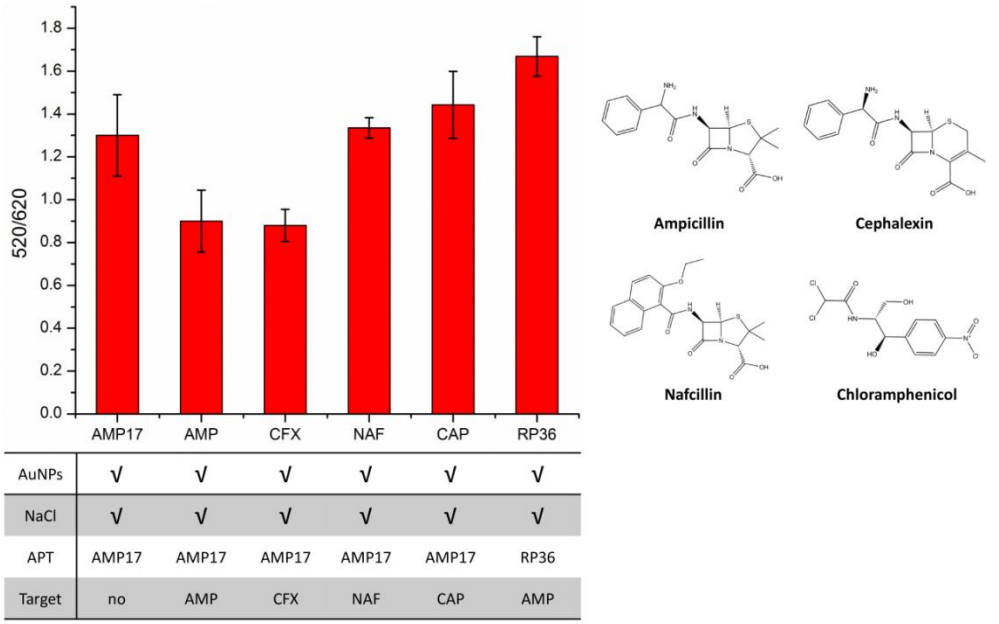

Fig.2 Absorbance ratio (520/620) for the AuNPs solutions with 100 nM of AMP17 in the absence of an antibiotic (AMP17) and with 100 nM of various antibiotics in the presence of 100 mM NaCl: ampicillin (AMP), cephalexin (CFX), nafcillin (NAF) and chloramphenicol (CAP); Negative control with random primer (36 bp) and 100 nM ampicillin (RP36) after addition of 100mM NaCl. The black line indicates the threshold value that distinguish between positive and negative results. Inset: structures of ampicillin, cephalexin, nafcillin and chloramphenicol.

Aptamer binding in homogeneous solutions

Since this assay can be considered a homogeneous assay, in which all the species involved are in solution or suspension, other techniques for homogeneous solutions were addressed to characterize the behaviour of the aptamer in the same conditions. The ITC measurements were carried out whilst varying different analytical and instrumental parameters (see Table 2); however, none of the considered combinations allow to observe clear binding. The only visible heat exchange in the thermograms is the one linked to the injection heats; in Fig. 3 three sets of thermograms are reported comparing different aptamers (Fig. 3A), different buffers (Fig. 3B) and different temperatures for the analysis (Fig. 3C).

Since ITC measurements are only informative when there is a certain amount of heat exchange upon binding, usually related to the folding and conformation change of the aptamer, to verify if the binding event was not detectable in these conditions, native ESI-MS experiments were also performed to check the occurrence of the aptamer-ampicillin interaction. The selected aptamer was AMP17 which is the most commonly used in other papers (Table S1). Fig. 3D shows mass spectra of the aptamer before and after addition of the ligand at a 1:5 aptamer:ampicillin ratio. The aptamer is detected at charge states 4+ (m/z = 1486.2) and 3+ (m/z = 1981.3) with some non-specifically bound sodium ions. These cations are a common contamination in mass spectrometry due to impurities of the chemicals and solvents used, but they can also originate from the plastic or glass vials, or from the borosilicate needles used for nano-ESI. The aptamer-ampicillin complex should occur at m/z = 1573.5 and m/z = 2097.4 for 1:1 binding and the 4+ and 3+ charge state respectively. However, there are no high-intensity peaks observed at these m/z-values (green dashed lines in Fig. 3D). In case of specific binding of ampicillin to the aptamer, with a previously reported K_d of 13.4 nM, these peaks should be present with high-intensity. In contrary, one can see a small, broad peak at the theoretical value for the 4+ complex, which is probably due to unspecific binding or very weak interactions.

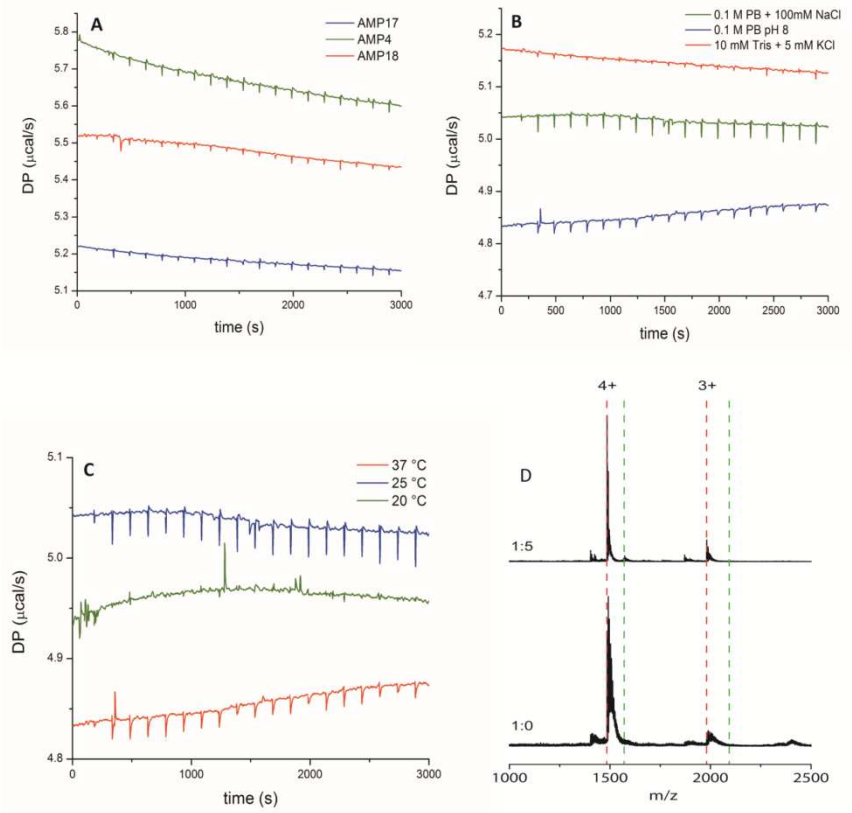

Fig.3 Comparisons of different thermograms for the interaction of AMP and ampicillin aptamers; A) AMP17 (blue), AMP4 (green), AMP18 (red) in 0.1 M PB pH 8 at 25 °C; B) AMP 17 at 25 °C in 0.1 M PB pH 8 with 100 mM NaCl (green), 0.1 M PB pH 8 (blue), 0.1 M Tris buffer pH 7.4 with 5 mM KCl (red); C) AMP 17 in 0.1 M PB pH 8 with cell temperature of 37 (red), 25 (blue) or 20 (green) °C. D) MS of the ampicillin-binding aptamer AMP17 without ampicillin and with ampicillin incubated at a 1:5 aptamer:ampicillin ratio in 150 mM ammonium acetate buffer pH 6.8. Theoretical m/z values of the apo form (red) and 1:1 complex (green) are indicated for the 4^+ and 3^+ charge state.

To gain further insight in the possible binding between AMP17 and ampicillin, ^{1}H-NMR titrations were performed. Even if the full assignment of the spectra is not required, as we are mainly interested in relative changes during the titration, it was tried to interpret the reference spectra for both AMP17 and ampicillin. The small molecule ampicillin could be completely characterized by the assignment of all signals present (Fig. S.2), but full-assignment of the aptamer sequence was not possible. As can be seen from Fig. 4A, addition of the ligand to the solution of AMP17 causes new signals to appear in the spectrum. However, at all titration points, the resonances of ampicillin and AMP17 in the mixture occur at the same position as seen in their corresponding pure solutions. It can be noted that only the relative intensity of the ampicillin to AMP17 signals changes. This indicates that, within the concentration regime of the titration, no detectable binding occurs. Indeed, depending on whether the association-dissociation kinetics of the complex is fast, or slow, in respect to the NMR frequency timescale [39] one could expect two observations. Either additional resonances should appear, corresponding to complex formation, while those of AMP and AMP17 disappear accordingly, or a single set of exchange averaged signals should be visible for AMP in respect to AMP17 with chemical shifts sensitive to the population of free and complexed states. As neither occurs, Fig. 4A is a first indication that the aptamer does not show binding affinity below the mM range.

In literature, several examples exist of aptamer-NMR studies where the imino region of the aptamer is used to prove binding/change in tertiary structure [40–42]. Figure 4B shows the imino region of the titration spectra (spectra recorded at 5 °C). It is evident that the signals do not change upon addition of the ampicillin, second indication that the aptamer does not bind ampicillin under the investigated conditions.

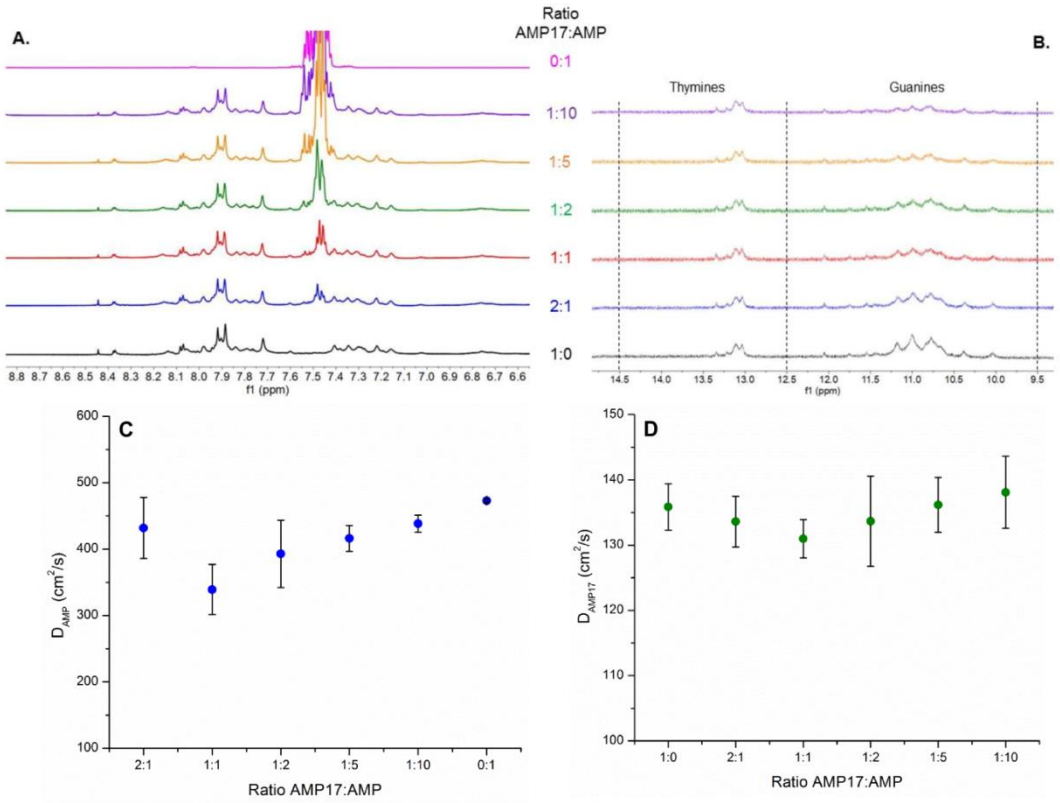

Fig. 4 Overlay of the 1D ^{1}H spectra, with at the bottom pure AMP17 (black) and on top pure AMP (pink). Ratios are indicated in the figure. A) Aromatic region. B) Imino region of the AMP17 (0.4 mM AMP17, H_2O/D_2O 90:10, pH 7, 700 MHz). Diffusion coefficients at different AMP17:AMP ratios as determined by DOSY spectroscopy. C) Diffusion coefficient of AMP17. D) Diffusion coefficient of AMP (0.4 mM AMP17, pH 7).

The same applies to the molecular diffusion coefficients as evidenced from PFG-NMR spectroscopy. Depending on whether ampicillin is free in solution or bound to the aptamer, its self-diffusion coefficient will be either higher or lower. In case of fast exchange on the diffusion time scale, a single exchange averaged diffusion coefficient is observed, that is sensitive to concentration when the ratio of bound and free compound is varied during the titration. In the case of slow exchange, two diffusion coefficients can be extracted, characteristic for free and bound ampicillin. In all cases, the diffusion coefficient remains constant within error, and similar in value to that of an ampicillin solution in the absence of aptamer(Fig. 4 C and D) This is the third indication that the aptamer does not bind the target.

Characterization of MN4 aptamer binding with quinine

All the results presented thus far evidence the lack of any binding between AMP17 and its target. In contrast the methodology employed was proven to be very accurate for the determination of binding constant and mechanism of another aptamer, MN4. ITC titrations and native ESI-MS experiments of the aptamer MN4 were performed to prove that the technique provides reliable results for a well-known aptamer. In the native ESI-MS experiments, the aptamer was measured before and after addition of quinine in a 1:5 and 1:10 aptamer:quinine ratio. In Fig. 5A, the MN4 aptamer is detected at charge states 6+ (m/z = 1855.7), 5+ (m/z = 2226.7) and 4+ (m/z = 2783.1) with some sodium non-specifically bound. After addition of the ligand, new peaks that correspond to the 6+ (m/z = 1909.8), 5+ (m/z = 2291.6) and 4+ (m/z = 2864.2) charge state of the complex are present. Upon the addition of quinine, we also observe a shift towards lower charge states. This can be explained by the loss of a charged quinine from the higher charged (n) complex which results in the apo form of the n-1 charge state. In Fig. 5B is reported the thermogram for the binding of 5 µM of MN4 aptamer titrated with 50 µM of quinine in 0.1 M Tris buffer pH 7.4 with 5mM KCl: from the binding curve (Fig. 5C) it was possible to calculate the K_d which is 171 ± 45 nM, a value close to the one reported in literature (~100 ± 40 nM) [43].

Effect of AuNPs on ampicillin binding aptamers

To explain the results reported by Song et al., one could claim a beneficial effect of the AuNPs on the binding event for AMP17, even if never reported before.

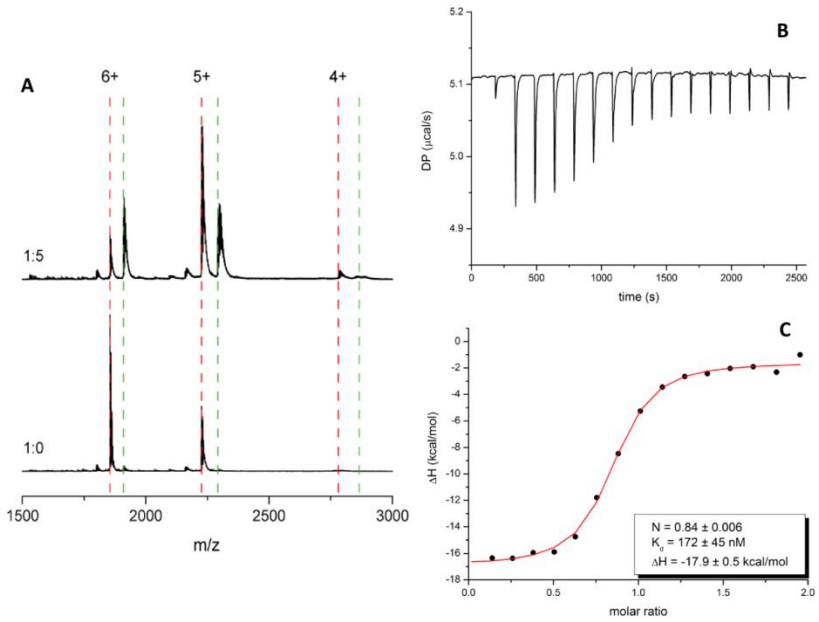

Fig. 5 A) MS of the cocaine-binding aptamer MN4 without and with quinine incubated at a 1:5 aptamer:quinine ratio in 150 mM AmAc (pH 6.8). Theoretical peaks of the apo form (red) and complex (green) are indicated for the 6+, 5+ and 4+ charge state, B) Thermogram for the titration of 5 µM of MN4 aptamer titrated with 50 µM of quinine in 0.1 M Tris buffer pH 7.4 with 5mM KCl, C) Binding curve obtained by averaging three repeated titration for MN4 and quinine, inset: thermodynamic parameters: Kd, N (number of binding sites), ΔH (enthalpy)

For this reason, ITC titrations were repeated with the AuNPs in the cell along with the aptamer [44,45]. Since many different interactions are known to take place between DNA and AuNPs [34] the idea that the aptamer is able to bind its target only in the presence of the gold surface was taken into consideration. The titration was performed using the same concentration of gold nanoparticles (4 nM) of the colorimetric assay reported by Song et al. The nanoparticles were dialyzed overnight against MilliQ water, because the titrations performed without this step gave too much background experimental noise.

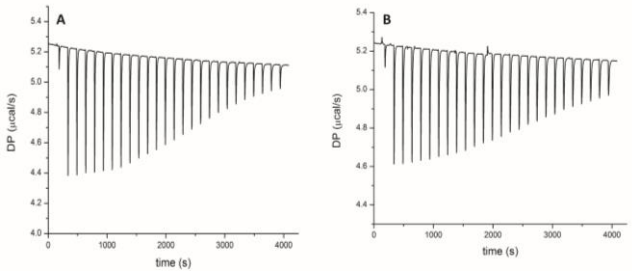

Fig. 6 Thermograms of the titrations with AuNPs; A) 1 µM AMP17 with 4 nM AuNPs, titrated with 1 mM AMP in MilliQ water, B) control titration with 4 nM AuNPs titrated with 1 mM AMP in MilliQ water.

In Fig. 6A the complete titration is reported with 1 µM AMP17 with 4 nM AuNPs, titrated with 1 mM AMP in MilliQ water. In this case some clear heat exchange can be observed, with intense peaks at the beginning that tend towards saturation at the end of the titration. Also, a control titration was performed, removing the aptamer from the cell (Fig. 6B). By comparing the control titration with the original one it is evidenced that the most part of the detected heat exchange stems from the interaction of the antibiotic with the AuNPs (Fig. S3). The absence of a specific interaction between AMP17 and its target ampicillin, in the presence of AuNPs, was verified again using NMR. The spectra of the aptamer with and without nanoparticles (Fig. S.4A) shows no relevant differences apart from a slight broadening of the peaks; also the titration with ampicillin (Fig. S.4B) does not evidence binding between the AMP17 and the target. As observed before when considering the diffusion coefficient measurements for aptamer and antibiotic in the absence of AuNPs (see Fig. 4C and D), these values remain constant within the experimental error associated with 3 repeated measurements for both AMP17 (Fig. S.4C) and AMP (Fig. S.4D). All these data are evidences of the absence of binding even in the presence of AuNPs in solution.

Non-specific binding for the "naked" aptamer

Another control titration was performed to exclude any other heat contribution in the titration with the AuNPs (see Fig. 6A). In contrast to what was observed before (see Fig. 3) the binding event seems to take place and the thermogram presents the typical shape for an exothermic process (Fig. 7A). However, the titration was performed in MilliQ water and the same titration performed in the binding buffer (10 mM PB pH8) reported for the colorimetric assay by Song et al., does not show any signs of binding (Fig. 7B). The binding curve fitting parameters of the control titration without AuNPs (inset Fig. 7A) moreover reported a calculated number of bindings sites (N) higher than 10, while this value was close to 1 for the cocaine aptamer MN4 (inset in Fig. 5C). All this evidence hints at non-specific interaction between the "naked" aptamer sequence and the antibiotic. The effect of ions in

solution on the structure and conformational changes of DNA is well known [46,47]; the behaviour of the aptamer AMP17 in solution with very low ionic strength and no added counter ions (MilliQ water) could be easy explained. The ssDNA sequence is not shielded by the counter ions and thus cannot fold properly to allow the specific interaction between the aptamer and the target. The apparent binding event is probably due to electrostatic interactions or other weak non-specific bonds.

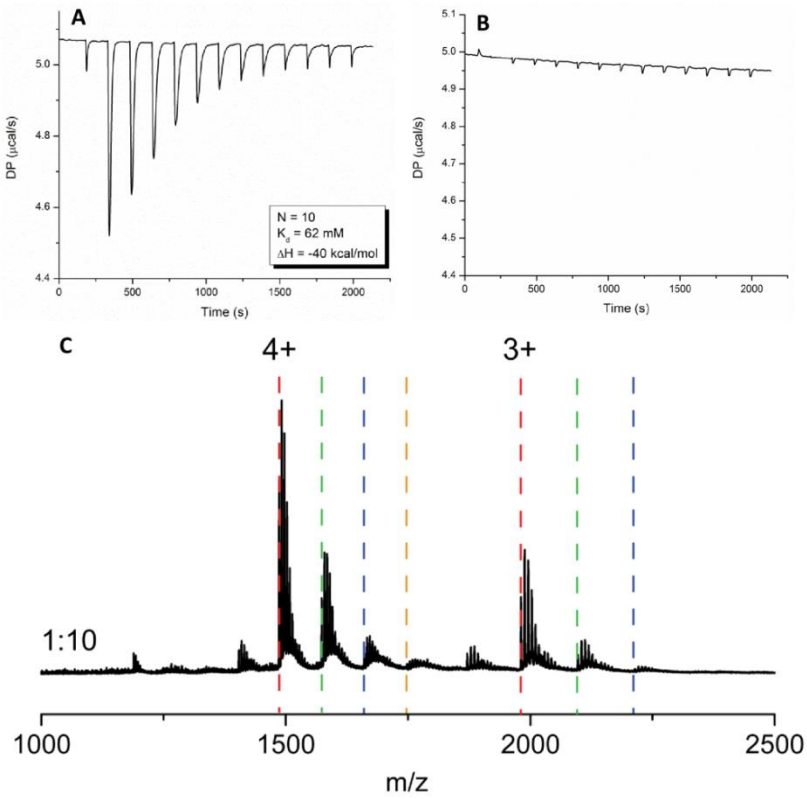

Fig. 7 Thermograms of the titrations without AuNPs in MilliQ water; A) 1 µM AMP17 titrated with 1 mM AMP in MilliQ water, B) 1 µM AMP17 titrated with 1 mM AMP in 10 mM PB pH 8. C) MS of the ampicillin-binding aptamer AMP17 with ampicillin incubated at a 1:10 aptamer:ampicillin ratio in MilliQ water. Theoretical peaks of the apo form (red) and complex in a 1:1 (green), 1:2 (blue) and 1:3 (orange) stoichiometry are indicated for the 4+ and 3+ charge state

To provide further evidence that the binding of ampicillin to AMP17 in MilliQ water is unspecific, native ESI-MS experiments were performed. Fig. 7C shows the mass spectrum of the aptamer after addition of ampicillin in a 1:10 aptamer:ampicillin ratio. The aptamer is detected at charge states 4+ (m/z = 1486.2) and 3+ (m/z = 1981.3) with some nonspecific sodium ions bound as contaminants. Furthermore, peaks corresponding to the complex in a 1:1 stoichiometry are observed at m/z = 1573.5 and m/z = 2097.4 for the 4+ and 3+ charge state respectively. There are however also peaks present that correspond to the 1:2 (m/z = 1660.4 and m/z = 2213.6 for the 4+ and 3+ respectively) and 1:3 stoichiometry (m/z = 1747.5 for the 4+ charge state) of the complex which indicates that there is binding of up to 3 ampicillin molecules to the aptamer. Again this is in contrast to what observed before with the MS analysis in buffer solution (see Fig. 3) and confirms the results of the ITC experiment on the non-specific nature of the binding in MilliQ water.

Conclusions

The experimental results presented here show, first of all, that the interaction between an aptamer and its small molecule target can and must be addressed with a coupled and cross-referenced analytical approach rather than the assays commonly used for aptasensor development. A shared strategy and validation protocols are needed to verify the performance and improve the reliability of aptamers for analytical applications (i.e. ligand binding). Indeed, very often the reported binding constants are linked to the specific assay with which they were obtained and cannot be safely used as a reference for different experimental conditions. The coupled and cross-referenced approach proposed should be enforced as a standard for validating or disproving the data obtained with the assays. As for the aptamers used in the present study the question is still open; without ruling out completely the possibility of their binding to ampicillin, it is obvious that their binding mechanism is poorly understood or superficially

evaluated. With the present study we would like to raise awareness and prompt the research community to consider more carefully the implication of the use of aptamers for analytical applications.

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