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Do aptamers always bind? The need for a multi-faceted analytical approach when demonstrating binding affinity between aptamer and low molecular weight compounds

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ABSTRACT: In this manuscript, we compared different analytical methodologies to validate or disprove the binding capabilities of aptamer sequences. This was prompted by the lack of a universally accepted and robust quality control protocol for the characterization of aptamer performances coupled with the observation of independent yet inconsistent data sets in the literature. As an example, we chose three aptamers with a reported affinity in the nM range for ampicillin, a β-lactam antibiotic, used as biorecognition elements in several detection strategies described in the literature. Application of a well-known colorimetric assay based on aggregation of gold nanoparticles (AuNPs) yielded conflicting results with respect to the original report. Therefore, ampicillin binding was evaluated in solution using Isothermal Titration Calorimetry (ITC), native nano-Electrospray Ionization Mass Spectrometry (native nESI-MS) and ^{1}H-Nuclear Magnetic Resonance spectroscopy (^{1}H-NMR). By coupling the thermodynamic data obtained with ITC with the structural information on the binding event given by native nESI-MS and ^{1}H-NMR we could verify that none of the ampicillin aptamers show any specific binding with their intended target. The effect of AuNPs on the binding event was studied by both ITC and ^{1}H-NMR, again without providing positive evidence of ampicillin binding. To validate the performance of our analytical approach we investigated two well-characterized aptamers for cocaine /quinine (MN4), chosen for its nM range affinity, and L-argininamide (1OLD) to show the versatility of our approach. The results clearly indicate the need for a multi-faceted analytical approach, to unequivocally establish the actual detection potential and performance of aptamers aimed at small organic molecules.

Introduction

Aptamers are short single 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 Ligands by Exponential Enrichment). From the beginning they were considered a leap forward in many analytical and biomedical applications. Indeed, aptamers offer considerable advantages over traditional molecular biorecognition elements such as antibodies or enzymes, including stability over a wider range of temperatures and pHs, ease of synthesis and modification, lower production 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 the literature for therapeutic and drug-delivery studies,[5,6] as well as for analytical purposes.[7,8] Additionally, a wide range of SELEX approaches (such as Capture-SELEX, Cell-SELEX and Capillary SELEX[9–15]) are described in the literature since the '90s. While aptamers therefore appear promising tools for analytical chemists and biologists alike, and their potential for commercialization is broadly recognized, many challenges remain to be faced before this can be achieved. A variety of factors have been put forward to explain why aptamers have not yet penetrated the market[16]: one of the main reasons can be identified as the so called “thrombin problem”. Indeed, rather than developing assays for more clinically relevant targets, hundreds of investigators

continue to focus their attention on perfecting thrombin-binding aptamers or designing clever detection strategies for this target. The same can be said to a lesser extent for cocaine-binding aptamers in the field of small organic molecule analysis.

We strongly believe that the lack of a universally accepted and reliable quality control protocol for the characterization of aptamer performances is one of the main obstacles towards successful valorization and should therefore be tackled first.

Typically, new aptamers are characterized with affinity binding assays like equilibrium dialysis, ultrafiltration, affinity chromatography with magnetic beads or fluorescence based tests.[17] While these assays are relatively cheap, easy to perform and do not require particular equipment, these practical advantages are offset by the fact that affinity constants measured using two or more of these assays can vary considerably, up to several orders of magnitude. This casts considerable doubt on the reliability of aptamer performances reported from these assays, and two or more should at least be combined and assessed for their similarity.[17]

Instrumental analytical techniques, such as Surface Plasmon Resonance (SPR)[18] or Capillary Electrophoresis (CE)[19], are typically selected for affinity characterization.[20] For example, SPR measurements are highly accurate, providing both quantitative and reproducible results. However, the need to immobilize the aptamer (or the target) on the sensor chips can influence the affinity or even the binding mechanism.[21,22] Indeed, the addition of linkers, spacers or labels to the aptamer sequences for immobilization and sensing purposes, may perturb the recognition event to an extent that is currently unknown since a systematic comparison has not yet been presented. Lastly, CE is well suited to characterize aptamer interaction with larger molecules (such as proteins and enzymes) but is not applicable to small molecule targets. Small molecules (<1000 amu), however, represent the biggest class of environmental contaminants, and their determination is of the utmost importance in many different analytical fields. Selecting aptamers against small molecules is challenging and often leads to a poor yield of the SELEX protocol.[23] Only very few aptamers for small molecules were extensively characterized and their affinity validated. The few exceptions such as cocaine-binding aptamers[24] have become the gold standard. In general, novel aptamers are only characterized by the group that select them in the first place and are used uncritically afterwards in other applications by different groups. Caution towards (the affinity of) the aptamers in each new application (with different experimental settings) has considerably diminished over time.

In this article, we focus on ampicillin aptamers. 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) using FluMag-SELEX in which the ampicillin was covalently immobilized on magnetic beads.[15] Furthermore, they used them in a colorimetric detection strategy (Figure 1) based on gold nanoparticles (AuNPs) aggregation to detect the antibiotic in the low nanomolar range, both in aqueous solution and in milk samples. A fluorescence-colorimetry assay was used to calculate the K_d values. This was the very first example of an aptamer against a β-lactam antibiotic described in the literature. More specifically, the authors reported that the selected aptamers are capable of recognizing the side chain of ampicillin (1-phenylethylamine), assuring a high selectivity against structurally related compounds. In the last eight years these aptamers have been used by other research groups around the world, in different analytical approaches and sensor configurations. The latter mostly include electrochemical transduction, with very good results 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 no further studies on their binding mechanism have appeared.

Therefore, we undertook a systematic study of the ampicillin aptamer binding interactions, using different state-of-the-art analytical techniques which do not require immobilization: Isothermal Titration Calorimetry (ITC), native nano-Electrospray Ionization Mass Spectrometry (native nESI-MS) and ^{1}H-Nuclear Magnetic Resonance (^{1}H-NMR). These techniques were previously used for aptamer characterization,[28–31] but rarely combined although they provide complementary results. A single ITC experiment delivers information about the dissociation constant (K_d), thermodynamics, and stoichiometry of the interaction.[30,32,33] Native nESI-MS provides information about the stoichiometry (provided that the affinity is in the low μM to nM range), and allows identification and characterization of individual species. Moreover, multiple species, which are e.g. co-existing in equilibrium, can be detected separately rather than as an average or a selected, prominent state.[31,34,35] Solution state ^{1}H-NMR reveals the behavior of compounds on a molecular level and allows delineating the location of the intermolecular interaction surface involved in the aptamer-target interactions.[36–39] By combining ITC, native nESI-MS and ^{1}H-NMR one can obtain a complete overview of the binding affinity, selectivity and mechanism between aptamers and small molecule targets without immobilizing them to a substrate.[31,38–40]

In this way it is possible to validate or disprove the binding affinity and mechanism of aptamer sequences for small organic molecules, beyond reasonable doubt. Therewith, we hope to prevent a proliferation of publications which suggest aptamer sequences and applications without a proper validation of the aptamer-target affinity.

Materials and Methods

Aptamers and reagents

Ampicillin aptamers, the MN4 (quinine/cocaine binding) aptamer, the 1OLD (L-argininamide binding) aptamer and random ssDNA sequence (N36) were all purchased from Eurogentec (Belgium). In Table 1 the specifications of the five different sequences can be found. Ampicillin sodium salt, nafcillin sodium salt, quinine hydrochloride dihydrate, L-argininamide dihydrochloride and ammonium acetate solution (7.5 M) were obtained from Sigma Aldrich. Cephalexin monohydrate and chloramphenicol were 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 parameters (ITC, native nESI-MS and ^{1}H-NMR) are reported in the Supporting Information.

Table 1. Acronym, sequence, length and previously reported K_d of the ampicillin aptamers (AMP4, AMP17 and AMP18), MN4 (quinine binding) aptamer and random ssDNA sequence (N36) used in the study; *K_d value of binding with quinine.

Acronym	Sequence	Length (bp)	Target	K_d (nM)
AMP4	5'-CAC-GGC-ATG-GTG-GGC-GTC-GTG-3'	21	Ampicillin	9.4 [27]
AMP17	5' GCG-GGC-GGT-TGT-ATA-GCG-G-3'	19	Ampicillin	13.4 [27]
AMP18	5'-TTA-GTT-GGG-GTT-CAG-TTG-G-3'	19	Ampicillin	9.8 [27]
MN4	5'-GGC-GAC-AAG-GAA-AAT-CCT-TCA-ACG-AAG-TGG-GTC-GCC-3'	36	Quinine	100* [41]
1OLD	5'-GAT-CGA-AAC-GTA-GCG-CCT-TCG-ATC-3'	24	L-argininamide	$165 \cdot 10^3$ [42]
N36	5'-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-NNN-3'	36	-	-

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 this type of assay, ssDNA is first adsorbed on the gold surface to protect the nanoparticles against aggregation in the presence of an electrolyte salt.[43,44] An explicative scheme of the assay is depicted in Figure1. Colorimetric assays based on citrate capped AuNPs are widely used as an analytical tool to investigate aptamer-target interactions.[45,46] It supposedly provides a fast approach to test aptamer performances and binding capabilities. The assay used by Song *et al.* was replicated to allow for a direct comparison with the original results. Along with ampicillin, cephalexin was tested since it has the same side-chain as ampicillin, for which the aptamer should be selective, according to the conclusion of the original article. The protocol (see Materials and Methods in S.I.) was applied to ampicillin, cephalexin and two other antibiotics for which no affinity is expected, i.e. nafcillin and chloramphenicol. In addition, a random ssDNA sequence (N36) was tested in similar conditions.

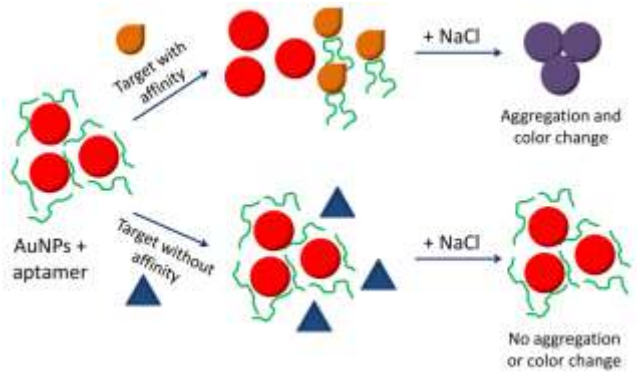

Figure 1. Schematic representation of the colorimetric AuNPs assay.

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. As both dispersed and aggregated nanoparticles are present in solution at the outset, and binding of the aptamer to its target induces aptamer release, more aggregation of AuNPs will occur upon subsequent addition of salt, thus the absorbance will increase at 620 nm (aggregated AuNPs) in intensity while the one at 520 nm (dispersed AuNPs) should decrease. This change is typically monitored by a decrease in the A_{520}/A_{620} ratio (Figure S.1) which is considered more reliable and sensitive than the change in individual absorbance alone.[46] The original paper reports an arbitrarily chosen ratio of 2 as a threshold between positive (binding, ratio <2) and negative (non-binding, ratio >2) results. In the article of Song *et al.*, only the assay with the aptamer and the specific target ampicillin showed an A_{520}/A_{620} ratio below 2, with AMP17 demonstrating the best performances. Therefore, we first repeated the measurements with AMP17 in our study (Figure 2). The histogram shows that the ratio for all the considered combinations (including nafcillin and chloramphenicol for which no binding is expected) is lower than 2, so all have to be considered as positive results, according to the original protocol. It was observed that for ampicillin and cephalexin the A_{520}/A_{620} ratio is lower than the ratio obtained with nafcillin and chloramphenicol. This observation is consistent with the report of Song *et al.*, as the aptamer should specifically recognize the side chain of ampicillin and cephalexin. For this reason, a more efficient binding can be expected for those two targets. However, the standard deviations (three repeated measurements) are relatively large, the differences in A_{520}/A_{620} between targets remain small and below the threshold value of 2 in all cases. Our results are therefore not entirely consistent with the ones reported before, and prompted us to investigate the interaction via

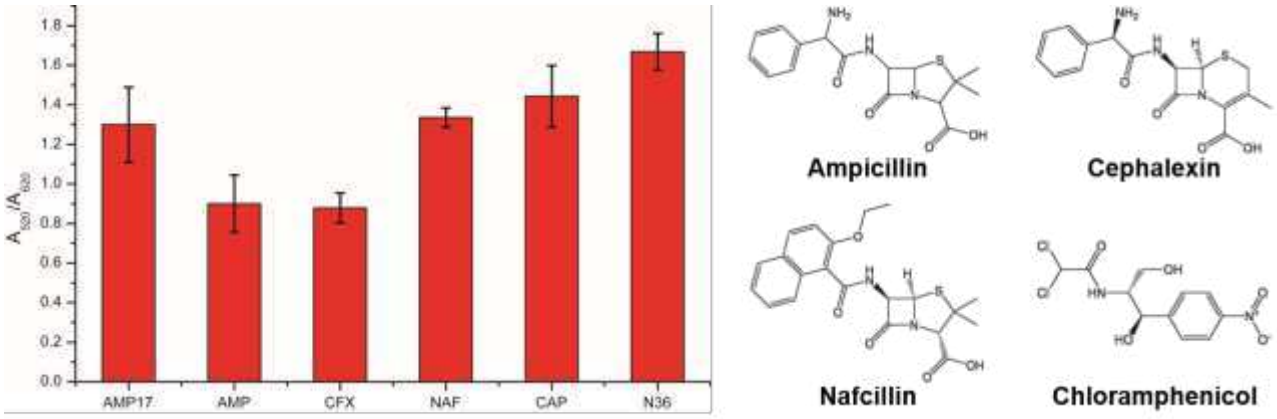

Figure 2. Absorbance ratio (A_{520}/A_{620}) for the AuNPs solutions with 100 nM of AMP17 in the absence of an antibiotic (AMP17) and with 100 nM of various antibiotics in the presence of 100 mM NaCl: ampicillin (AMP), cephalexin (CFX), nafcillin (NAF) and chloramphenicol (CAP); Negative control with a random 36 bp ssDNA (N36) in the presence of 100 nM ampicillin and 100 mM NaCl. Inset: structures of ampicillin, cephalexin, nafcillin and chloramphenicol.

other techniques to establish whether the results of AuNPs assay is linked (or not) to the binding event.

Aptamer binding in solution

The AuNP assay can be considered a label- and immobilization-free assay since the aptamer only interacts with the AuNPs via electrostatic interactions. Therefore, other techniques which do not involve labelling and immobilization were selected to characterize the behavior of the aptamer in the same conditions. ITC measurements were carried out whilst varying different parameters such as buffer composition, pH and target to aptamer molar ratio. First of all, the titration was performed in the conditions reported for the original AuNPs assay, i.e. 10 mM phosphate buffer pH 8 with the AMP17 aptamer in a concentration of 5 µM titrated with 50 µM of ampicillin. Given the reported K_d value (13.4 nM), the thermogram should show a clear exothermic binding trend. However, no heat exchange that could be linked to specific binding was observed, even when changing the aptamer (AMP4 and AMP18). Trying to understand if the buffer composition could influence the affinity of the aptamers for ampicillin, we varied several parameters, for example the buffer ionic strength by adding NaCl and KCl or changing the buffer composition (Tris instead of phosphate). Also the possible effect of the pH on the interaction between ampicillin and the aptamer was taken into consideration. Since ampicillin is a zwitterion, with pKa of 3.2 and 7.4[47] several buffers with pHs above and below the pKa of ampicillin were tested (see Table S.2 for a complete overview of all tested parameter combinations). However, none of the considered combinations allowed to observe a binding response. Figure 3 shows two sets of thermograms comparing different aptamers (Figure 3A) and different buffers (Figure 3B). The only visible heat exchange in the thermograms is linked to the injection heat.

As the heat exchanged as a result of binding may be smaller than expected and therefore not easily detected by our ITC protocols, native nESI-MS experiments were performed to further investigate the aptamer-ampicillin interaction. Figure 3C shows the mass spectrum 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. Sodium ions are a rather common contamination in MS due to impurities of the chemicals and solvents used, but they can also originate from the borosilicate needles used for nESI. For a 1:1 binding stoichiometry, the aptamer-ampicillin complex should occur at m/z = 1573.5 and m/z = 2097.4 for the 4+ and 3+ charge state respectively. However, no high-intensity peaks are visible at these m/z values (dotted lines in Figure 3C). Assuming specific binding of ampicillin to the aptamer taking place according to the previously reported K_d of 13.4 nM,[27] these peaks should be present with high intensity. A small, broad peak at the theoretical value for the 4+ complex is most likely due to non-specific binding or very weak interactions. Thus, similar to the performed ITC experiments, the nESI-MS results do not support the occurrence of a specific aptamer-target interaction under the conditions used. However one could assume that the absence of the complex might be due to the fact that only aptamers which show a K_d in the low µM to nM range can be observed using native nESI-MS, which means that binding can still occur in the high µM to mM range.[35]

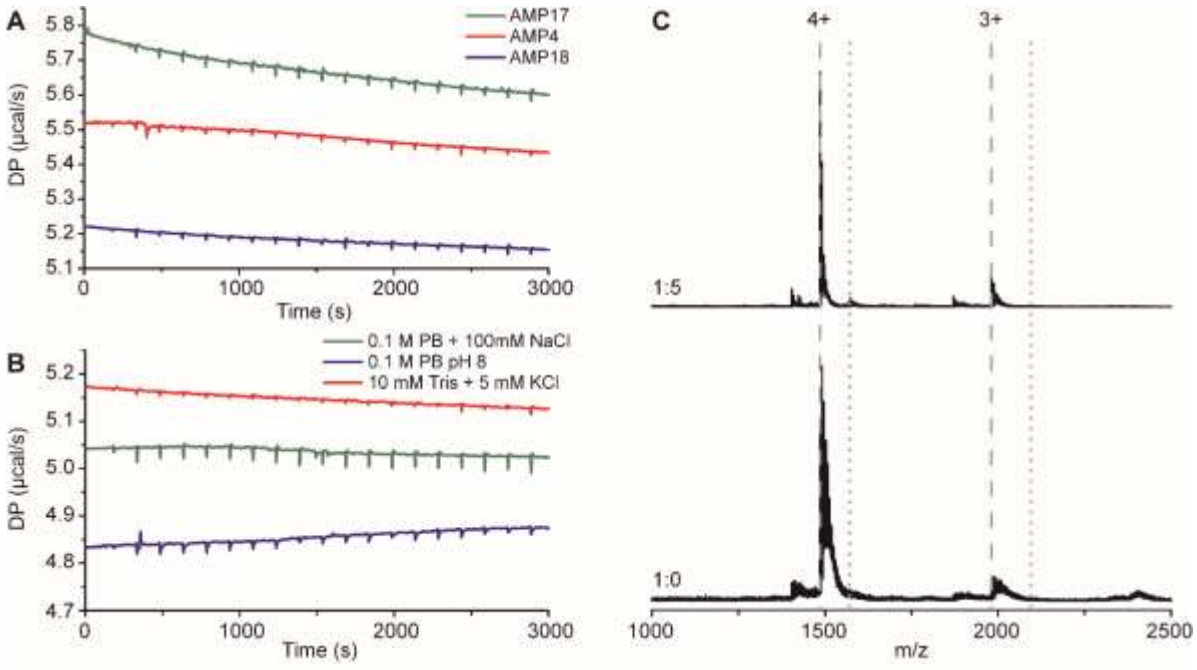

Figure 3. Different thermograms for the (absent) interaction of ampicillin aptamers with their target (ampicillin); A) 20 µM of AMP17 (blue), AMP4 (green), AMP18 (red) in 0.1 M PB pH 8 at 25 °C, titrated with 280 µM of ampicillin; B) 20 µM AMP17 at 25 °C in 0.1 M PB pH 8 with 100 mM NaCl (green), 0.1 M PB pH 8 (blue), 0.1 M Tris buffer pH 7.4 with 5 mM KCl (red), titrated with 280 µM of ampicillin; C) Native nESI-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 (dashed lines) and the 1:1 stoichiometry of the complex (dotted lines) are indicated for the 4^+ and 3^+ charge state.

To investigate the possibility of a lower affinity complexation we turned to ^{1}H-NMR spectroscopy, as this allows to also monitor specific intermolecular interactions with K_d values well into the mM range. Typically, the presence of a specific interaction may be inferred by monitoring the changes in the ^{1}H-NMR fingerprint as the target is titrated into a solution of the aptamer.[48,49] A low affinity interaction will typically manifest itself through the presence of a set of resonances for each species with concentration dependent chemical shifts due to fast exchange conditions on the NMR timescale.[49,50] In favorable cases, monitoring these enables K_d determination. Alternatively, chemical exchange may be slow on the NMR time scale, leading to a separate set of resonances for the free and complexed species in solution and immediately indicate the presence of complex formation. The titration of a 0.4 mM solution of the AMP17 aptamer with ampicillin up to a ten-fold excess of the latter is shown in Figure 4A. If the K_d value reported before for AMP17[27] is in the nM range, this should lead to full complexation and clear perturbations of the ^{1}H-NMR spectra. While the full assignment of all resonances in the spectra is not required for monitoring purposes, the resonances of ampicillin could be completely assigned (Figure S.2A), while only partial assignment of the aptamer sequence was obtained (Figure S.2B). As can be seen from Figure 4A, each spectrum recorded when titrating ampicillin to the AMP17 solution leads to a single set of resonances for each species, with constant chemical shifts that are in all cases completely identical to those of the individual species in the pure solutions, suggesting lack of interaction. In the literature, several examples exist of aptamer-NMR studies where changes that occur in the imino region of the aptamer, including the appearance of additional imino signals, are used as a sensitive indicator of binding and associated change in tertiary structure upon binding.[51–53] Here again, no change can be seen and line-widths also appear unaffected, all indicative for a lack of mutual interaction (Figure S.3). Finally, the same conclusion follows from monitoring the molecular translational diffusion coefficients of the aptamer and the ampicillin target using pulsed-field-gradient (PFG) NMR spectroscopy. Assuming rapid exchange on the diffusion time-scale[49] and depending on whether ampicillin is mostly complexed or free in solution as the ampicillin to aptamer ratio increases, the self-diffusion coefficient will increase, reflecting the changing balance between bound and free state. Within error however, the self-diffusion coefficients remain constant during the titration, and similar to that of the pure solutions (Figure 4B). To conclude, all NMR data indicates the absence of complexation in the mM concentration range and therefore, any eventual specific binding event must lie above the mM K_d range. This is the final independent indication that the aptamer does not bind the target. Together with the lack of any interaction from ITC and native nESI-MS we must conclude that there are no grounds to believe that complexation occurs within the low nM to high mM range.

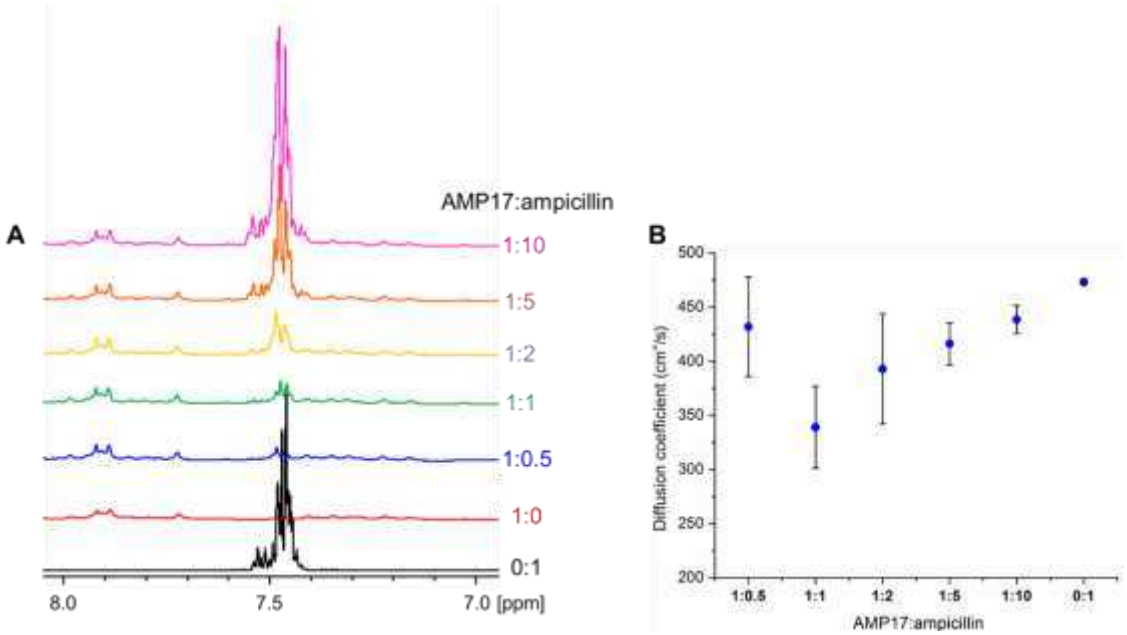

Figure 4. Impact of the titration of ampicillin in the presence of the AMP17 aptamer monitored by ^{1}H-NMR spectroscopy. A) Stacked plot of the 1D ^{1}H-NMR spectra of (from bottom to top) pure ampicillin (black), AMP17 (red) and mixtures of AMP17:ampicillin with ratios varying from 1:0.5 to 1:10. A concentration of 0.4 mM AMP17 was used throughout. B) Diffusion coefficient for ampicillin measured with PFG-NMR spectroscopy for the various ratios reported in (A). The lack of significant variations in either ^{1}H-NMR or diffusion data indicate the absence of complexation under the concentrations used (see text).

Validation of the multi-faceted analytical approach

All the results presented thus far evidence the lack of any binding between AMP17 and its target ampicillin. To validate our approach with a positive control, the same set of experiments was performed with two other aptamers. First of all, the MN4 cocaine-binding aptamer which is one of the most studied and well-characterized for analytical applications and it also binds strongly with quinine,[40,54] as evidenced by a lower K_d value (c.a. 100 nM compared to 5.5 µM for cocaine), bringing it close to the value reported for the ampicillin binding aptamers (nM range). Therefore, the quinine/MN4 system provides a suitable positive control for the combined analytical approach. The native nESI-MS experiments performed in a 1:5 MN4:quinine ratio clearly demonstrate the binding of the MN4 aptamer to quinine. In Figure 5A, the MN4 is detected at charge states 6+ (m/z = 1855.7), 5+ (m/z = 2226.7) and 4+ (m/z = 2783.1) with some non-specifically bound sodium. 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. Moreover the thermogram for the ITC titration of MN4 aptamer with quinine in 0.1 M Tris buffer pH 7.4 with 5 mM KCl (Figure 5B) shows clear evidence of an exothermic binding process (Figure 5C) from which it is possible to calculate a K_d of 171 ± 45 nM (n = 3). This value is close to the one previously reported in the literature (~100 ± 40 nM) using again ITC.[40] ^{1}H-NMR for the cocaine-binding aptamer was already extensively reported before and the tertiary structure of the aptamer and binding mechanism were already validated.[53,55] These experiments indicate that our analytical approach works well to observe aptamer-target binding in the nM range.

In order to check whether our approach can be extended to affinity studies in the µM range (to avoid missing a possible binding of ampicillin with the given aptamers in the µM-range), the 1OLD L-argininamide aptamer was chosen as second positive control. The ITC data (Figure 6A and B) clearly show binding of L-argininamide to the 1OLD aptamer. A K_d of 176 ± 15 µM was determined which is in good agreement with the one reported in the literature (K_d = 165 µM)[42]. The titration of L-argininamide into a 1OLD solution followed by 1D ^{1}H NMR shows the appearance of new signals in the imino region, as visualized in Figure 6C. From the assignment it becomes apparent that the interaction between the aptamer and L-argininamide zips up the hairpin, as base pairs 6●19 and 7●18 show up. In addition, the loop becomes structured around the target.[56] The native nESI-MS experiments using a 1:5 1OLD:L-argininamide ratio show only a small amount of (likely non-specific) complex formation between the aptamer and target (Figure S.5), which in accordance with the fact that complexes with a K_d in the µM range cannot be observed using this technique. Using both the MN4 and the 1OLD aptamer as a positive control, it is clearly demonstrated that the multi-faceted approach allows to determine aptamer-target interactions, both in the nanomolar and micromolar range.

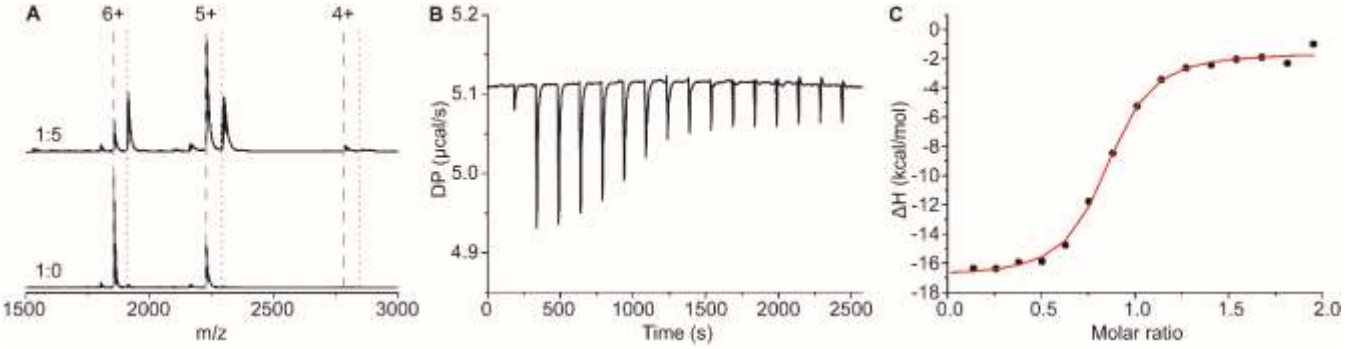

Figure 5. A) Native nESI-MS of the MN4 aptamer with and without quinine incubated at a 1:5 aptamer:quinine ratio in 150 mM AmAc (pH 6.8). Theoretical peaks of the apo form (dashed lines) and complex (dotted lines) are indicated for the 6+, 5+ and 4+ charge state, B) Thermogram for the ITC titration of 5 µM of MN4 aptamer with 50 µM of quinine in 0.1 M Tris buffer pH 7.4 with 5 mM KCl, C) Binding curve of the ITC titration for MN4 and quinine, the red line represent the fitting with the 'one set of binding sites' model.

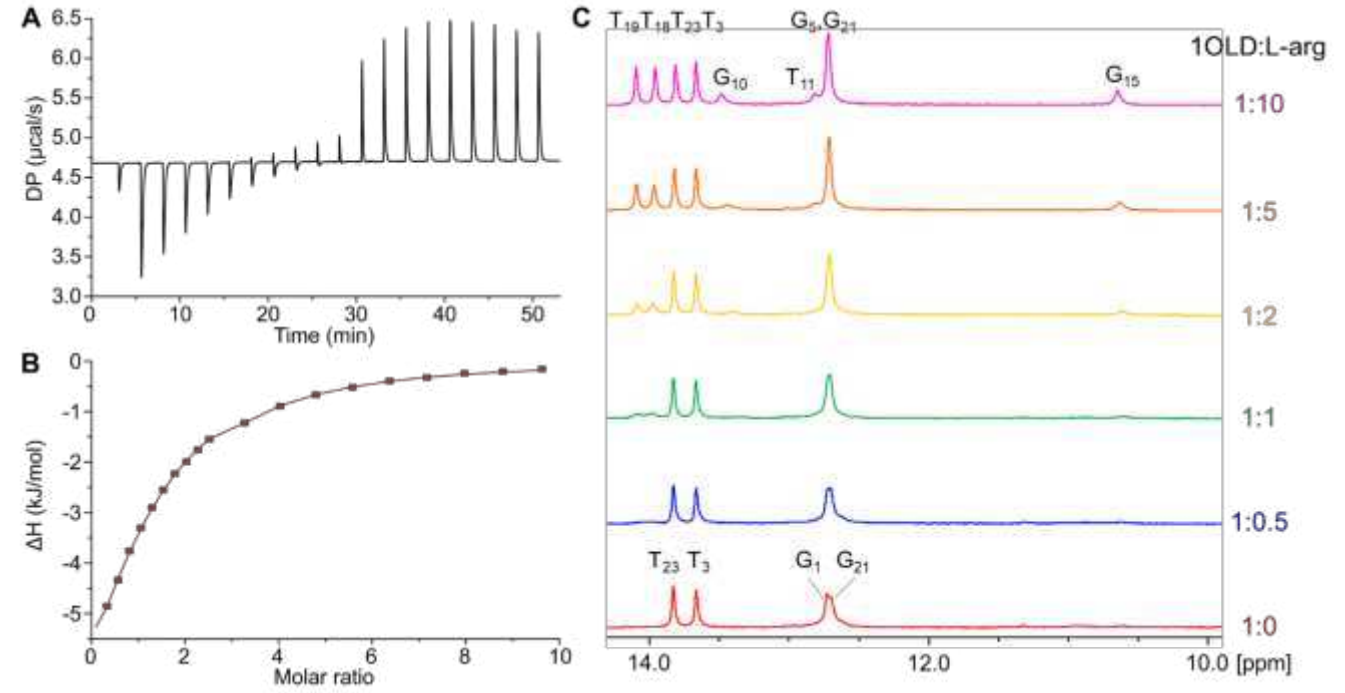

Figure 6. A) ITC thermogram and B) ITC binding curve of the 1OLD L-argininamide binding aptamer. C) Stacked plot of the imino region of six 1D ^{1}H-NMR spectra of (from bottom to top) 1OLD (red) and mixtures of 1OLD:L-argininamide (L-arg) with ratios varying from 1:0.5 to 1:10. A concentration of 0.4 mM 1OLD was used throughout.

Effect of AuNPs on ampicillin-binding aptamers

To explain the discrepancy between our findings and the results reported by Song *et al.*, it is possible to consider a beneficial effect of the AuNPs on the binding event between AMP17 and the target. Indeed, both assays used in the original paper were performed in the presence of AuNPs. However, this hypothesis was considered as many different interactions are known to take place between DNA and AuNPs.[43] Moreover, McKeague *et al.* observed that K_d values obtained using the AuNP assay were significantly improved compared to the previously reported values, indicating the presence of AuNPs may enhance the binding capabilities of an aptamer.[17]

To test this hypothesis, ITC titrations were performed with AuNPs and aptamers using the same AuNPs concentration (4 nM) as reported by Song *et al.*[57,58] Two titrations were performed to assess all possible heat exchange contributions: the first one with AuNPs, AMP17 and the ampicillin target, the second one with AuNPs and ampicillin alone.

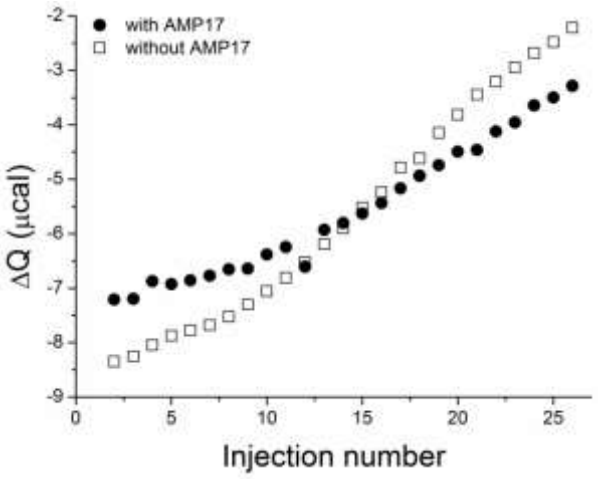

Figure 7. ΔQ for each injection for the titration with AMP 17 aptamer (full dots) and without AMP17 aptamer (empty squares) in the ITC cell.

Figure 7 illustrates the heat generated (ΔQ) at each injection for the titration of the AMP17/AuNPs solution with ampicillin (full dots) compared to the titration with only ampicillin and AuNPs (empty squares). The graph shows that the detected heat exchange mainly stems from the interaction of the antibiotic with the AuNPs, while AMP17 does not contribute to the generated heat. Observing the raw titration data (Figure S.6A and B) a clear heat exchange can be observed, with intense peaks at the beginning that tend towards saturation at the end of the titration, as expected for an exothermic binding event. This indicates that ampicillin has a strong affinity for the AuNPs. Native nESI-MS experiments could not be performed due to the presence of the AuNPs which cannot be transferred to the gas-phase. Therefore the absence of a specific interaction between AMP17 and its target ampicillin, in the presence of AuNPs, was verified again using ^{1}H-NMR. The spectra of the aptamer with and without nanoparticles (Figure S.7A) show no relevant differences apart from a slight broadening of the peaks; also the titration with ampicillin (Figure S.7B) does not indicate binding between AMP17 aptamer and the target in presence of AuNPs. As observed before when considering the diffusion coefficient measurements for the antibiotic in the absence of AuNPs (see Figure 4B), also when titrating ampicillin into AMP17 in the presence of AuNPs, the diffusion coefficient values remain constant within the experimental error associated with 6 repeated measurements for ampicillin (Figure S.7C). All these data reaffirm the absence of binding between the aptamer and ampicillin even in the presence of AuNPs in solution and this cannot explain the discrepancy between our findings and the previously reported results.

Conclusions

In line with the increasing awareness within the aptamer community, we clearly illustrated the need to address and validate the aptamer-target interaction using a multi-faceted analytical approach before "applying" aptamer sequences in other studies. More specifically, researchers should avoid relying exclusively on fast and easy-to-perform assays as analytical approaches to validate binding affinity since the risk to misinterpret the real performance of a specific aptamer is particularly high. For the majority of reported data collected in this way, the exact experimental conditions play a critical role and are often not entirely clear from the description of the work, thus difficult to replicate or adapt to a different analytical application. Therefore, extrapolating the performances of a given aptamer sequence to different experimental conditions may lead to conflicting or poorly reproducible results. The fact that no one technique is generally applicable to characterize all aptamers and the need for multiple characterization strategies was also identified by McKeague *et al.* who compared multiple techniques and found that the sensitivity of each technique affects the apparent K_d of a given aptamer.[17] Here, we offer a validation strategy to verify the performance and improve the reliability of aptamers for analytical applications. As for the aptamers used in the present study the question is still open; without completely ruling out the possibility that they do indeed bind ampicillin, it is obvious that their binding mechanism is poorly understood or superficially evaluated. This need for critical evaluation of aptamer performances was firmly identified and discussed on the occasion of the Aptamers 2019 meeting in Oxford, UK. Guidelines for standardization in aptamer selection, characterization and application are slowly finding their way into the interdisciplinary oriented aptamer community. With the present study we aim to contribute to these endeavors and provide the research community with the tools to adopt a robust analytical multi-faceted approach to validate aptamer-target interactions prior to applying them.

ASSOCIATED CONTENT

Supporting Information. Experimental details and additional data, including Figures S.1-S.6 and Tables S.1-S.2.

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