Do Aptamers Always Bind? The Need for a Multifaceted Analytical Approach When Demonstrating Binding Affinity between Aptamer and Low Molecular Weight Compounds

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Table S.1. Summary of analytical parameters for biosensors based on AMP aptamers, (ACV = alternate current voltammetry, SWV = square wave voltammetry, ND = not determined)

Analytical approach	Aptamer	K_d	K_d obtained with	Linear range	LOD	Selectivity	Real samples	Ref.
Dual colorimetric detection with AuNPs	AMP4 AMP17 AMP18	AMP4 = 9.4 nM AMP17 = 13.4 nM AMP18 = 9.8 nM	Fluorescence titration	0.1 to 140 nM	0.1 nM	Amoxicillin Benzyl penicillin Other non β-lactam antibiotics	Milk	[1]
Microfluidic impedimetric biosensor	AMP17	ND	ND	10 pM to 1 µM	10pM	Non-specific ssDNA sequence	Milk	[2,3]
Electrochemical aptasensor based on quadratic recycling amplification	AMP17	ND	ND	5 pM to 10 nM	1.09 pM	Amoxicillin Benzyl penicillin Penicillin	Milk	[4]
Electrochemical aptasensor based on dual recycling amplification	AMP17	ND	ND	0.2 to 40 nM	4 pM	Amoxicillin Benzyl penicillin Penicillin Lincomycin	Milk	[5]
Signaling probe displacement electrochemical aptasensor	AMP17	ND	ND	100 pM to 1 mM	10 pM	Kanamycin A Tetracycline Streptomycin Sulfadimethoxine	no	[6]
Fluorescent AuNPs aptasensors with nicking enzyme	AMP17	ND	ND	0.1 to 100 nM	0.07 nM	Amoxicillin Benzylpencillin Sulfadimethoxine Chloramphenicol	River water	[7]
Probe displacement electrochemical aptasensor	AMP18	ND	ND	0.2 to 15000 µM	30 nM	Levofloxacin Amoxicillin Trimethoprim Sulfamethoxazole Nitrofurantoin	Urine Tap water Milk Saliva	[8]
Reusable signal-off electrochemical aptasensor	AMP18	ND	ND	5 to 5000 µM (ACV) 100 to 5000 µM (SWV)	1 µM (ACV) 30 µM (SWV)	Levofloxacin Amoxicillin Trimethoprim Sulfamethoxazole Nitrofurantoin	Milk Saliva Calf serum	[9]
Electrochemical-SPR aptasensor	AMP4	ND	ND	2.5 to 1000 µM	1 µM	Penicillin G Penicillin V Oxacillin Cephalexin	River water	[10]
Metal-organic framework impedimetric biosensor	AMP18	ND	ND	0.001 to 2000 pg/ml	$0.2 \cdot 10^{-3}$ pg/ml	Doxorubicin Trombamycin Streptomycin Kanamycin Penicillin Cefazolin	Serum River water Milk	[11]
Electrochemical aptasensor with endonuclease digestion amplification	AMP17	ND	ND	0.1-100 nM	32 pM	Chloramphenicol Florfenicol Thiamphenicol Tobramycin Streptomycin Tetracycline Kanamycin Oxytetracycline Amoxicillin Penicillin Lincomycin Melamine	Milk Tap water	[12]

Metal-organic framework AuNPs (MOF) colorimetric sensing platform	AMP17	ND	ND	50-100 nM	13 nM	Amoxicillin Chloramphenicol Sulfadimidine Kanamycin Oxytetracycline Tetracycline	no	[13]
Metal-organic framework impedimetric aptasensor	AMP18	ND	ND	0.01 pg/m–2 ng/ml	6 fg/ml	Tetracycline Kanamycin Tobramycin , Na^+, K^+, Streptomycin Oxytetracycline	Diluted human serum	[14]
Ladder-shaped DNA based electrochemical aptasensor	AMP17	ND	ND	7 pM−100 nM	1 pM	Amoxicillin Levofloxacin Chloramphenicol Kanamycin Tetracycline	Milk	[15]
Dual AuNPs colorimetric assay	AMP17	ND	ND	1-600 nM 1-400 nM	0.1 nM 0.5 nM	Amoxicillin Penicillin Lincomycin Benzylpenicillin	Milk	[16]
Exonuclease III-powered DNA walking machine electrochemical aptasensor	AMP17	ND	ND	1 pM – 10 nM	0.7 pM	Kanamycin Chloramphenicol Oxytetracycline Terramycin Carbenicillin	Milk	[17]
DNase I-assisted cyclic enzymatic signal amplification graphene oxide aptasensor	AMP17	ND	ND	10 ng/mL – 500 ng/mL	2.4 ng/ml	Chloramphenicol Penicillin Carbenicillin Amoxicillin	Milk	[18]

Materials and Methods

AuNPs synthesis

Gold nanoparticles were prepared according to Storhoff *et al.* [19]. All glassware was cleaned in aqua regia (3 parts HCl, 1 part HNO_3), rinsed with MilliQ, and then oven dried prior to use. An aqueous solution of 1 mM $HAuCl_4$ was brought to boiling under stirring, and then 10 mL of a 38.8 mM trisodium citrate ($Na_3C_6H_5O_7$) solution was added quickly, which resulted in a color change from pale yellow to deep red. After the color change, the solution was left to boil for additional 15 min and then allowed to cool down to room temperature. The resulting NPs were filtrated under vacuum to remove aggregates and impurities. The size and concentration of the nanoparticles was estimated according to Haiss et al. [20]. AuNPs with a diameter of 13 nm and a concentration of 20.4 nM were obtained.

AuNPs colorimetric assay

The colorimetric AuNPs assay was performed according to Song *et al.* [1]. A solution of AuNPs (≈4 nM) was incubated with 100 nM of the selected ampicillin aptamers in 10 mM phosphate buffer (PB) pH 8 for 1 h with tilting and rotation. A 100 nM solution of the chosen target in 10 mM PB pH 8 was then added to the vial and incubated for 1 h. Subsequently, 100 mM of NaCl was added to the solution to promote the eventual aggregation of the nanoparticles and the color change (from red to purple) linked to the binding event. UV-Vis spectra were recorded after each step. The results were reported in terms of the ratio between the absorbance at 520 and 620 nm (A_{520}/A_{620}). UV-Vis spectra were acquired between 400 and 800 nm with a Cary100 Conc spectrophotometer (Agilent Technologies) operated by Cary Win UV 4.20 software and with a NanoPhotometer N60 (Implen) operated by NanoPhotometer NPOS software.

ITC protocol

Isothermal titration calorimetry experiments were performed on a MicroCal PEAQ-ITC instrument (Malvern Panalytical) operated by MicroCal PEAQ-ITC control software. Data analysis was performed with the MicroCal PEAQ-ITC Analysis software. Since many different parameters have been tested a summary is reported in Table S.2 for ease of reference. For the control experiments only one set of parameters was used. For the MN4 aptamer: 50 µM quinine was titrated in 5 µM of aptamer. The assay buffer was a 0.1 M Tris buffer with 5 mM KCl at pH 7.4. A total of 16 injection was performed with a volume of 2.5 µL (initial delay 180 s, spacing 150 s and reference power 5 µcal/s). For the 1OLD aptamer: 7.5 mM L-argininamide was titrated in 150 µM of aptamer at 25 °C. The assay buffer was a 10 mM phosphate buffer containing 100 mM NaCl at pH 7.5. A total of 20 injections was performed with the volume of the first 11 injections set at 1 µL and of the last 9 set at 3 µL with a spacing of 150 s and reference power of 5 µcal/s. The reference cell was filled with degassed ultrapure water. The aptamer solutions were always put in the sample cell, after a two minutes pre-equilibration time with assay buffer. The target solution was administered in the injection syringe. The instrument temperature was set to the run temperature before loading and kept constant during the complete run. In order to determine the dilution heats, control titrations were performed consisting of injection of the ligand into the sample cell filled only with buffer. Thermograms for the binding of quinine to MN4 and L-argininamide to 1OLD were analyzed using the 'one set of sites' binding model, by including the corresponding control titration.

ITC with AuNPs

Prior to the ITC experiments, AuNPs were dialyzed overnight against MilliQ water with a dialysis cellulose membrane (Dialysis tubing cellulose membrane, avg. flat width 10 mm, Sigma Aldrich). ITC measurements were performed in MilliQ at 25 °C, with 26 injections of 1.5 µL of 1 mM ampicillin solution, with 1 µM of AMP17 in the cell (reference power 5 µcal/s, 180 s initial delay and 150 s spacing). Different titrations were performed without aptamer and without nanoparticles, to assess the heat exchange contribution of all interactions.

Table S.2. Instrumental and analytical parameters of the ITC experiments.

Total number of injections:	13-19-25	Stirring speed:	750 rpm
Cell temperature:	20-25-30-37-40 °C	Injection volume:	1-2-3 µL
Reference power:	2-5-10 µcal/s	Spacing:	150 s
Initial delay:	180 s		
Aptamers	AMP4 AMP17 AMP18	Molar ratios	1/2 1/5 1/10
Targets	Ampicillin Cephalexin	Concentration range	Aptamer: 1-25 µM Target: 50 µM – 1 mM
Buffers	0.1 M Phosphate buffer, 100 mM NaCl 10 mM Phosphate buffer, 100 mM NaCl 0.1 M Tris buffer, 5 mM KCl 10 mM Tris Buffer, 5 mM KCl	Annealing[1]	Yes/No
pHs	2-5-6-7-7.4-7.6-8	Sonication[2]	Yes/No

[1]Annealing [21] = 3 min at 90 °C, followed by 1 min at 4°C, then 1 min at RT before injection; [2]Sonication = all solutions were sonicated in an ultrasonic bath for 10 min to remove air bubbles.

Native nESI-MS protocol

Prior to native MS analysis, the aptamers were buffer exchanged into 150 mM aqueous ammonium acetate (pH 6.8) using Micro-Biospin P-6 columns (Bio-rad Laboratories, USA), in order to desalt the samples and to provide a volatile electrospray buffer of appropriate ionic strength. Solutions of 5 to 10 µM aptamer with a five-fold excess of ampicillin, quinine or L-argininamide were prepared for binding to the AMP17, MN4 or 1OLD aptamer respectively, and 3-5 µL of each sample was injected into the mass spectrometer using in-house prepared, gold-coated borosilicate nano-electrospray ionization (nESI) emitters. The experiments were performed on a Synapt G2 HDMS (Waters, Manchester, UK) in positive ionization mode using careful tuning to maintain fragile noncovalent interactions during ionization and inside the instrument. Experiments were performed in sensitivity and mobility mode. The spray capillary voltage ranged between 1.3-1.5 kV and the sampling cone voltage was 25-50 V. The trap and transfer collision energy were set at 5 V and 0 V, respectively and the trap DC bias was fixed to 35 V. The IMS wave velocity was set to 800 m/s and the IMS wave height to 35 V. Gas pressures were 2.57 mbar and $2.16 \cdot 10^{-3}$ mbar for the backing and source gas, respectively. All data was analyzed using MassLynx 4.1 (Waters).

^{1}H NMR protocol [22]

Nuclear magnetic resonance spectra were recorded at 25 or 5 °C on a Bruker Avance II spectrometer operating at a ^{1}H frequency of 700.13 MHz, under Topspin 3.1pl7 and using a 5 mm triple channel Prodigy N_2-cryocooled probe with a Z-gradient of 6.56 G/mm. Standard pulse sequences from the Bruker library were used throughout. Samples for reference spectra consisted of 550 µL of 1 mM solutions of AMP17 and ampicillin or 1OLD and L-Argininamide in D_2O, buffered at pH 7 using aliquots of acid or base (DCl or NaOD). All 1D ^{1}H spectra were recorded using a spectral width of 20 ppm and consisted of 128 to 256 scans of 64K TD points each preceded by a 2.0 s relaxation delay. Processing consisted of one order zero-filling prior to multiplication and Fourier transformation. Chemical shifts are referenced against internal DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid). An additional experiment involved ^{1}H-{^{13}C} HMBC for assignment of the ampicillin and L-argininamide resonances. Titrations were executed by adding aliquots from a solution containing 8 mM ampicillin or L-argininamide and 0.4 mM AMP17 or 1OLD, to a 0.4 mM solution of AMP17 or 1OLD, thus keeping the aptamer concentration constant throughout the titration. Final aptamer:target ratios varied from 1:0 to 1:10. In addition, for the AMP17 aptamer PFG-NMR was performed to detect aptamer-ligand interactions by monitoring the diffusion coefficient of the ampicillin resonances.

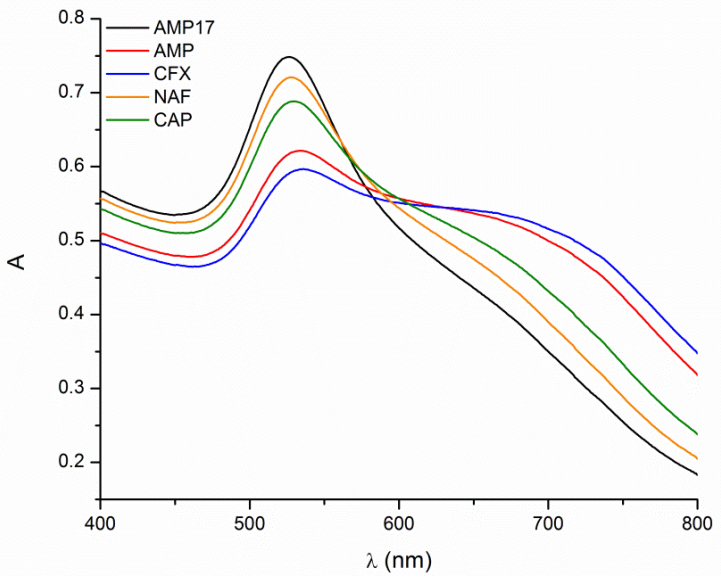
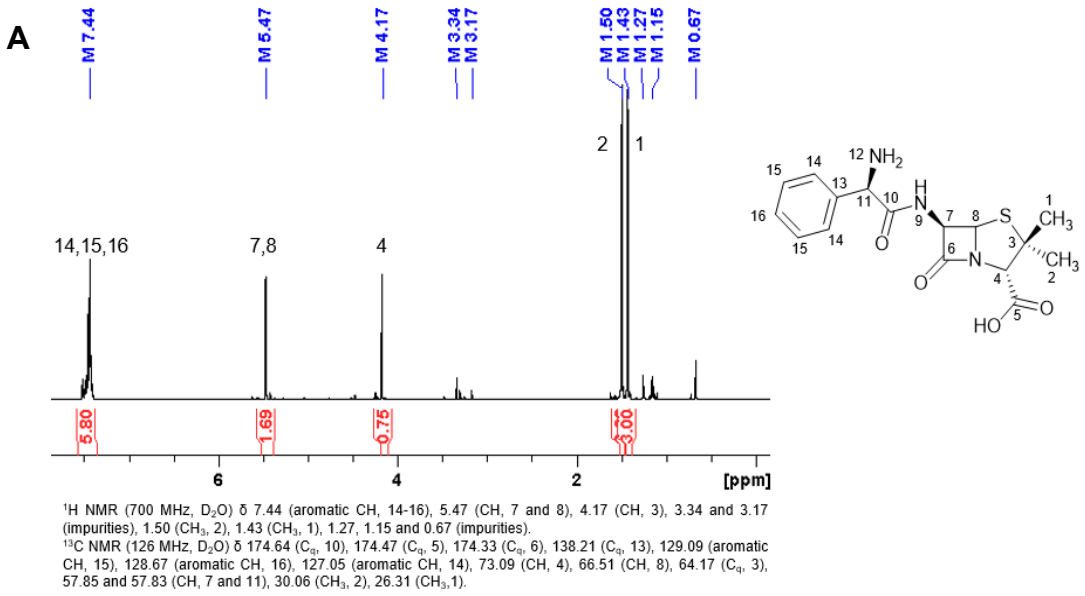


Figure S.1. UV Vis spectra for the AuNPs solutions with AMP17 in the absence of an antibiotic (black) and with 100 nM of various antibiotics in the presence of 100 mM NaCl: ampicillin (red), cephalexin (blue), nafcillin (orange) and chloramphenicol (green).



^{1}H NMR (700 MHz, D_2O) δ 7.44 (aromatic CH, 14-16), 5.47 (CH, 7 and 8), 4.17 (CH, 3), 3.34 and 3.17 (impurities), 1.50 (CH_3, 2), 1.43 (CH_3, 1), 1.27, 1.15 and 0.67 (impurities).
^{13}C NMR (126 MHz, D_2O) δ 174.64 (C_q, 10), 174.47 (C_q, 5), 174.33 (C_q, 6), 138.21 (C_q, 13), 129.09 (aromatic CH, 15), 128.67 (aromatic CH, 16), 127.05 (aromatic CH, 14), 73.09 (CH, 4), 66.51 (CH, 8), 64.17 (C_q, 3), 57.85 and 57.83 (CH, 7 and 11), 30.06 (CH_3, 2), 26.31 (CH_3,1).

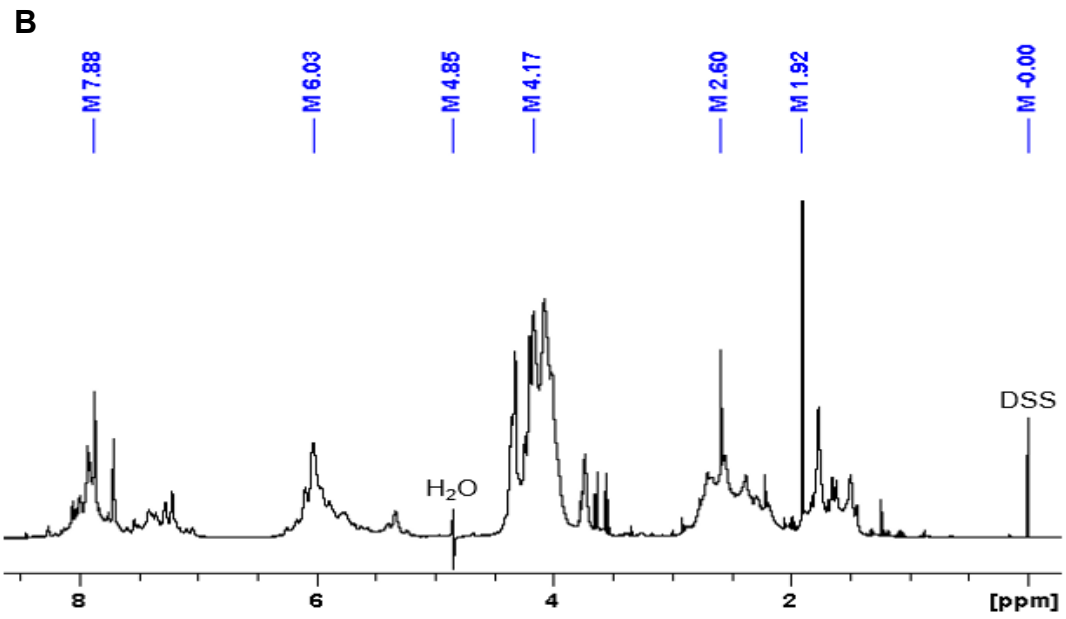


Figure S.2. A) 1D ^{1}H spectrum of pure ampicillin in D_2O with integration regions and values (red) indicating the number of hydrogen atoms and their assignment (black labels) match the labelled positions in the depicted structure (right). B) 1D ^{1}H spectrum of pure AMP17 in D_2O.

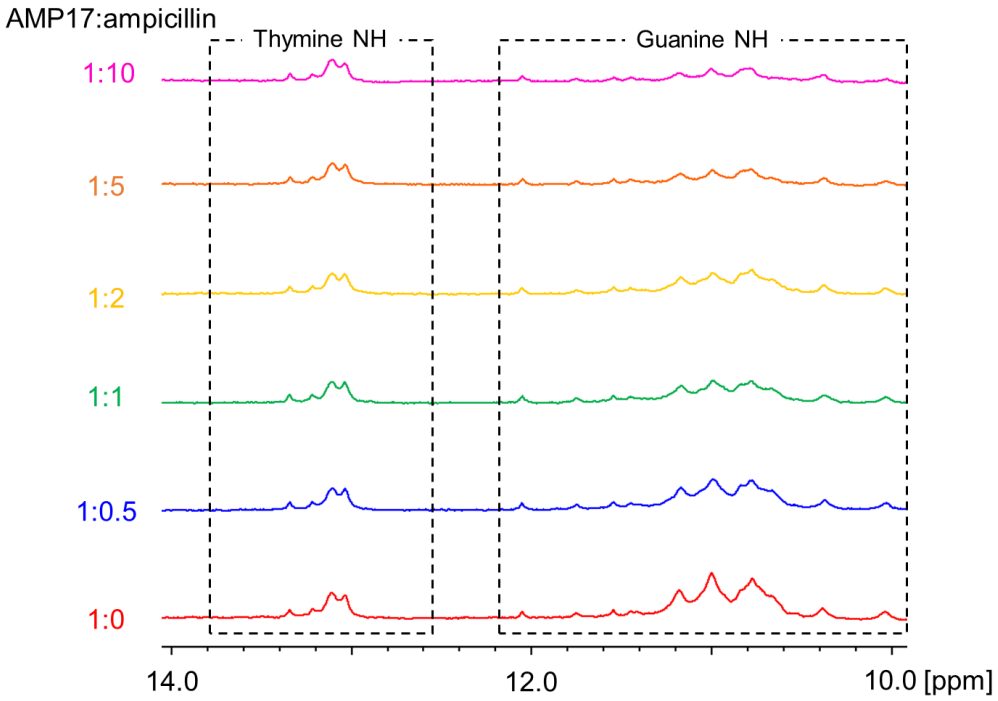
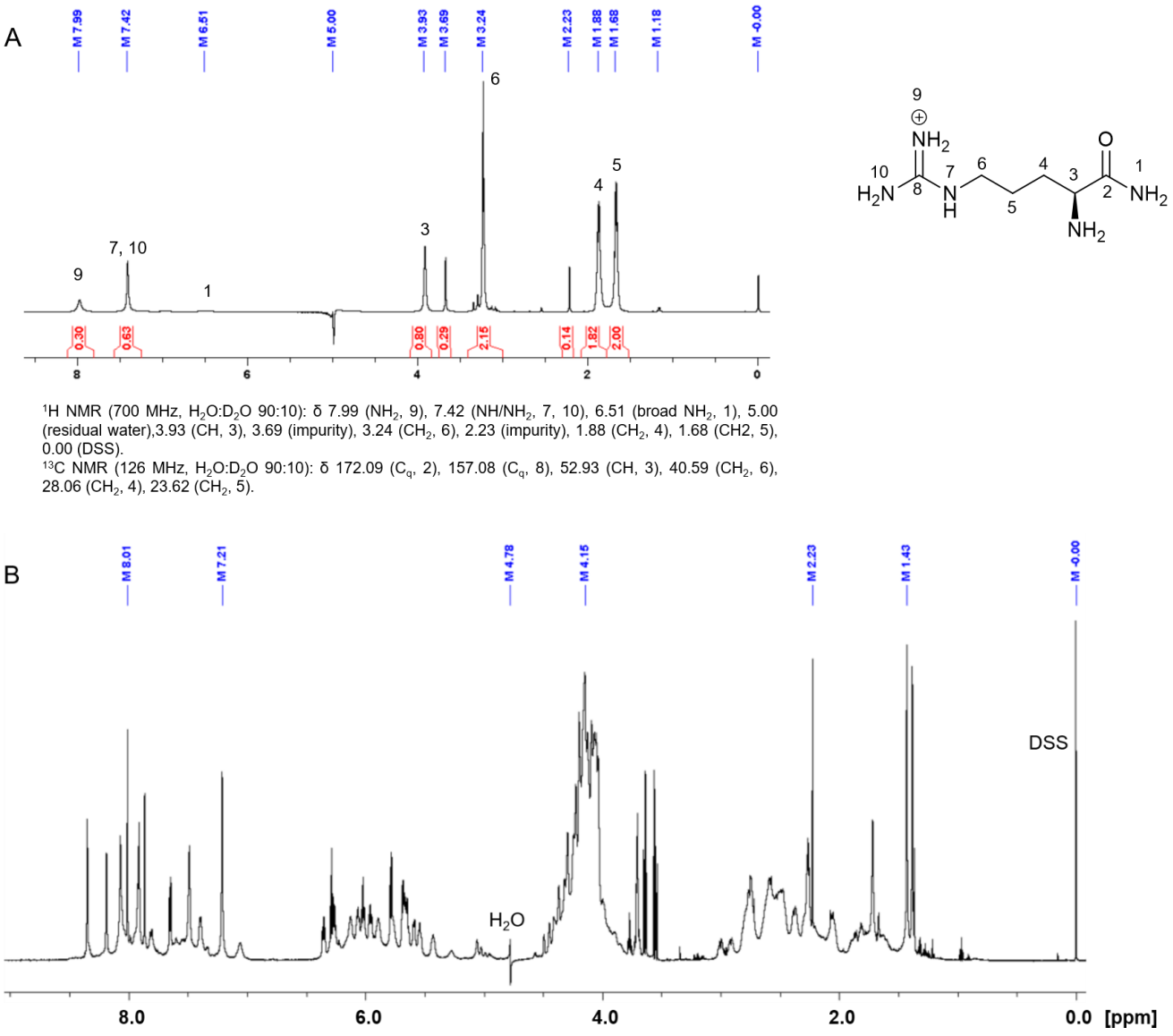


Figure S.3. Overlay of the imino region of the AMP17 aptamer during titration with ampicillin, ratios are indicated (0.4 mM AMP17, H_2O/D_2O 90:10, pH 7, 700 MHz).



^{1}H NMR (700 MHz, H_2O:D_2O 90:10): δ 7.99 (NH_2, 9), 7.42 (NH/NH_2, 7, 10), 6.51 (broad NH_2, 1), 5.00 (residual water),3.93 (CH, 3), 3.69 (impurity), 3.24 (CH_2, 6), 2.23 (impurity), 1.88 (CH_2, 4), 1.68 (CH2, 5), 0.00 (DSS).
^{13}C NMR (126 MHz, H_2O:D_2O 90:10): δ 172.09 (C_q, 2), 157.08 (C_q, 8), 52.93 (CH, 3), 40.59 (CH_2, 6), 28.06 (CH_2, 4), 23.62 (CH_2, 5).

Figure S.4. A) 1D ^{1}H spectrum of pure L-argininamide in H_2O:D_2O 90:10 with integration regions and values (red) indicating the number of hydrogen atoms and their assignment (black labels) match the labelled positions in the depicted structure (right). B) 1D ^{1}H spectrum of pure 1OLD in D_2O.

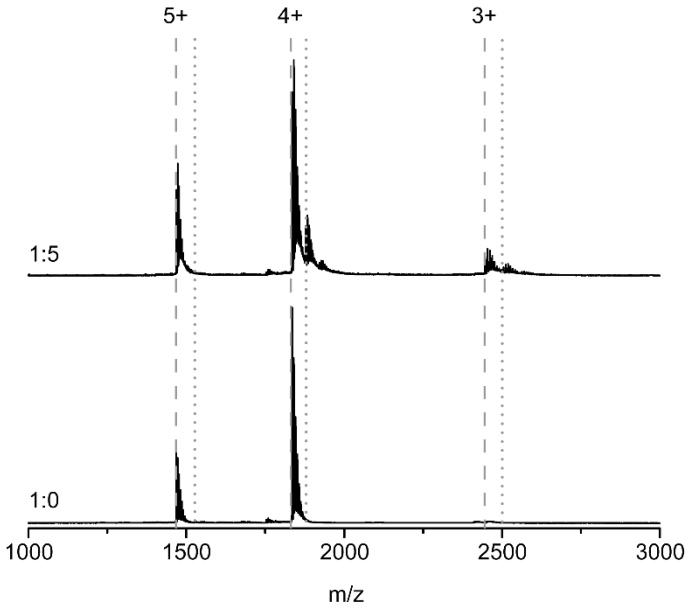


Figure S.5. Native nESI-MS of the 1OLD aptamer with and without L-argininamide incubated at a 1:5 aptamer:L-argininamide ratio in 150 mM AmAc (pH 6.8). Theoretical peaks of the apo form (dashed lines) and complex (dotted lines) are indicated for the 5+, 4+ and 3+ charge state.

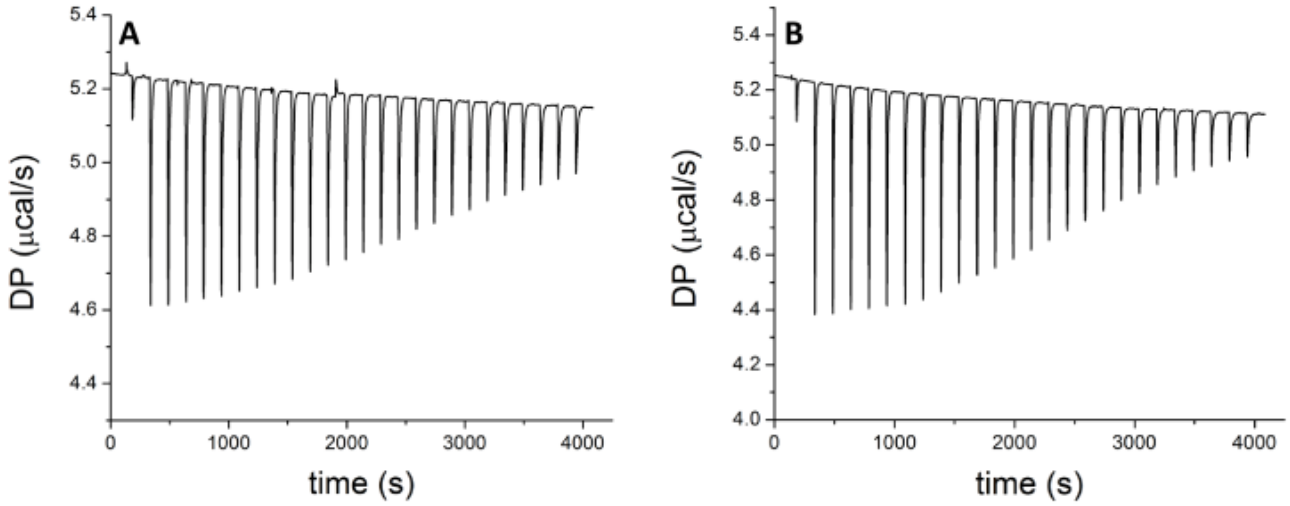


Figure S.6. Thermograms of the titrations with AuNPs; A) 1 µM AMP17 in the presence of 4 nM AuNPs, titrated with 1 mM AMP in MilliQ water, B) titration of 4 nM AuNPs with 1 mM AMP in MilliQ water.

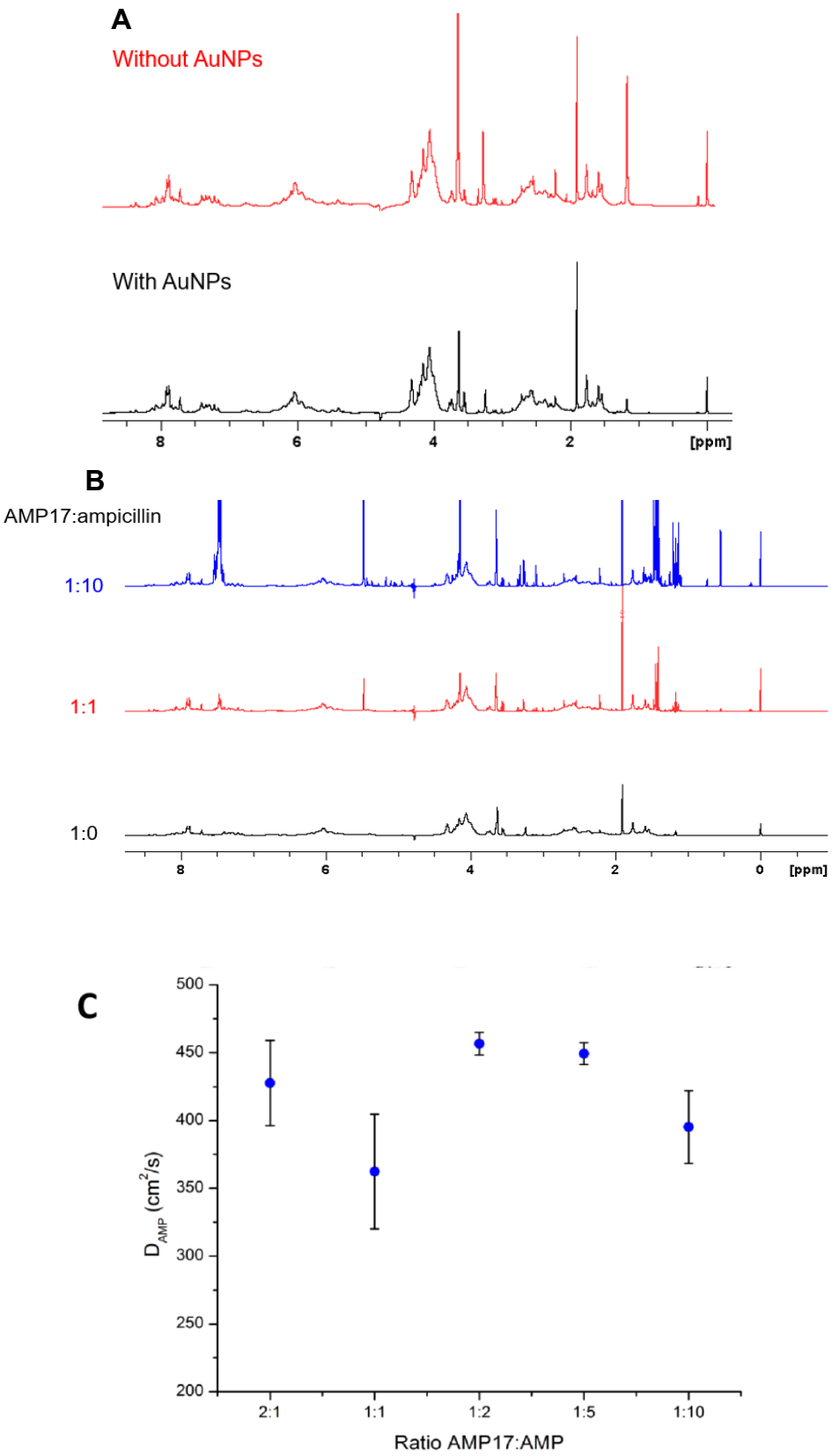


Figure S.7. A) Overlay of 1D ^{1}H spectra of AMP17 with (black) and without (red) AuNPs; B) Overlay of the titration experiment, AMP17:ampicillin ratios are indicated, in the presence of AuNPs; C) Diffusion coefficient of ampicillin upon titration into a solution of AMP17 in presence of AuNPs.

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