3. Methods

3.1 Oligonucleotide Synthesis and Purification

Following the chemical synthesis on a Perseptive Biosystems Expedite Synthesizer 8900, oligonucleotides were cleaved from the solid support and deprotected in 1 ml concentrated ammonium hydroxide. The deprotection reaction was conducted at 55°C for 8 h or at 80°C for 1 h to fully remove base-protecting groups. Samples were centrifuged, and the supernatant was butanol precipitated and dried under vacuum. Dried residues were resuspended in urea gel loading buffer. Denaturing PAGE was used to remove impurities and to recover the desired-length nucleic acids. Samples were loaded onto polyacrylamide/8M urea gels (1xTBE) and electrophoresed for 1 h at 20 W. Nucleic acids were visualized by UV shadowing and the desired band was excised. Gel containing the nucleic acid was electroeluted for 1 h at 300 V and ethanol precipitated with 300 mM KCl, pH 5.5 and 2.5 vol. of absolute alcohol. Yields were assessed by UV absorbance (OD_{260}). For the determination of DNA and RNA concentration, the Biopolymer Calculator (Schepartz et al., Yale University: http://paris.chem.yale.edu/extinct.html) was used.

3.2 *In Vitro* Transcription and Removal of DNA

The *in vitro* T7 transcription reaction (Milligan, et al., 1987) contained 50 nM template, 10 mM DTT, 10 mM NTPs (except for GTP (18 mM) to account for the G-rich abortive initiation products), 35 mM $MgCl_2$, 0.01% Triton-X-100, 10 mM spermidine, approximately 4000 U/ml T7 RNA polymerase, and 40 mM Tris-HCl, pH 8.0. Reactions were incubated at 37°C over night. RNAs were typically labeled in 200 µl reactions with 1 µCi [α-^{32}P]UTP. When a radiolabeled NTP is used, it is usually desirable to modify the reaction conditions to optimize the incorporation of this component. Unlabeled UTP was therefore lowered 10-fold in the transcription reaction.

If single-stranded DNA was used for transcription, an oligo with the complementary sequence of the T7 promoter was annealed together with the template DNA by heating to 80°C and cooling to room temperature in order to introduce the double-stranded T7 promoter for the T7 polymerase.

Following gel purification, any DNA contaminating the transcripts was removed in a 200 µl DNase reaction that contained 10 Units of enzyme and was incubated at 37°C for 1 h. The DNA-free transcripts were then phenol:chloroform extracted and ethanol precipitated with 300 mM KCl prior to selection.

3.3 Reverse Transcription

Selected RNA was ethanol precipitated, resuspended in H_2O and reverse transcribed with a large molar excess of reverse transcription primer. The RNA-molecules were hybridized for one minute at 70°C with 2 µM reverse transcription primer, 1 x reverse transcription buffer, 1 µM of each dNTP, 20 mM DTT in a 100 µl final volume, then incubated at 42°C for 2 min before addition of 1 U/µl Superscript Reverse Transcriptase, which initiated the reverse transcription for 50 min at 42°C. At the end, the enzyme was inactivated by a 70°C step for 15 min.

3.4 Polymerase Chain Reaction (PCR)

Single-stranded library DNA or reverse-transcribed RNA was PCR-amplified to yield transcriptionally competent double-stranded DNA. When necessary, the T7 promoter was introduced via a primer containing the appropriate sequence.

Single-stranded DNA molecules were amplified in a 100-µl PCR reaction. Reactions contained 50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton-X-100, 1.5 mM $MgCl_2$, 200 µM dNTPs and were started with approximately 50 nM extendable template and 0.5 µM of the appropriate 5’ and 3’-primers. Templates and primers were initially denatured with a 5-minute 95°C step, and then the reactions were cycled using optimal conditions for the respective sequences, usually as follows: 1-minute denaturation at

95°C, 1-minute annealing at 55°C, and 1-minute extension at 72°C. *Taq* DNA polymerase (5 Boehringer Mannheim unit equivalents) was added directly prior to the first extension. The high concentration of enzyme and modified hot start protocol increased PCR efficiency. Care was taken to not skew the distribution of enriched molecules by over-amplification.

3.5 Functional-Domain Mapping by Alkaline Hydrolysis

To determine the functional region of each aptamer sequence, 3'-end mapping was performed as described by Pan and Uhlenbeck (1992) and Turner (1997). A synthetic oligodeoxynucleotide corresponding to each minimal sequence was synthesized and transcribed to generate RNA. Partially hydrolyzed 5'-^{32}P-labeled RNA was passed over a GTP column, washed, and eluted as in the selection. The flow-through and GTP-eluted RNA were analyzed by PAGE to determine the 3'-end of the region required for GTP binding. Carrier tRNA (10 μg) was added to the flow-through and elution fractions, and the samples were desalted on a Bio-Spin 30 Chromatography Column. Salt (300 mM KCl) was added, and the samples were ethanol precipitated. Elution ("1") and flow-through ("2") fractions were run on a denaturing polyacrylamide gel along digested ("3") and undigested ("4") controls to map the boundaries (see Fig. 2). Following electrophoresis, the glass plates were separated and the gel was lifted onto 3M blotting paper and dried under vacuum on a gel drier. Dried gels were placed in cassettes and exposed to the phosphor storage screen over night. Images were read using a Molecular Dynamics 400 Series PhosphorImager, and analyzed.

5'-end mapping was performed with unlabeled partially hydrolyzed RNA, which was subjected to affinity enrichment for GTP as above, then reverse transcribed with 5'-end-^{32}P-labeled primers. The resultant cDNA represented the lengths of the RNA in each fraction and was analyzed in a similar manner to the 3'-end mapping. The K_d of each minimal aptamer, transcribed from a synthetic oligonucleotide, was determined to make certain that no portion of the sequence contributing to GTP binding had been deleted.

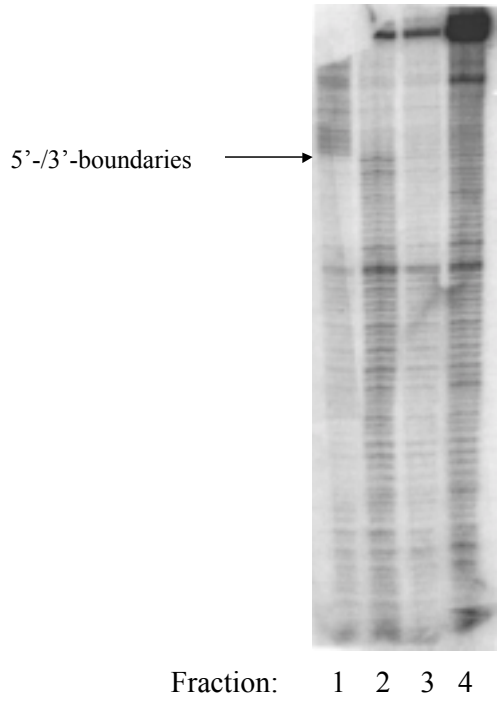

Fig. 2: Functional Domain Mapping of Aptamer Class II by Alkaline Hydrolysis. Comparison of the elution (1) and wash (2) fractions with a digested (3) and undigested (4) control indicated clear 5' and 3' boundaries (marked by an arrow) for the functional domain when being analyzed by denaturing PAGE.

3.6 Library Design and Synthesis

The libraries for re-selection carried a random domain of consecutive deoxyribonucleotides positioned between flanking hybridizing arms of constant sequence of which one contained the T7 promoter. Degenerate oligodeoxynucleotide templates were generated using an equimolar phosphoramidite mixture (A:C:G:T ratio of 3:3:2:2) to achieve approximately equal incorporation of each base (Bartel and Szostak, 1993a) and thus the desired amount of mutagenesis ("doping") ($21 \pm 3\%$, confirmed by sequencing). One-micromole syntheses of these templates were performed on a Perseptive Biosystems Expedite Synthesizer 8900 according to the manufacturer's instructions. Pools were sequenced prior to the re-selection to confirm the correct

incorporation ratio. Following the chemical synthesis and isolation, the pool molecules existed only as single copies of single-stranded DNA. Libraries of approximately 10^{12} different random-sequence RNA molecules were then produced by PCR and *in vitro* T7 transcription of the DNA templates and purified by denaturing PAGE.

3.7 Immobilizing GTP-γ-S on Thiopropyl Sepharose 6B

Thiopropyl sepharose beads were swelled in approximately 3 volumes of 10x TE buffer. After vigorous vortexing, the beads were allowed to settle and then poured into a new tube in order to separate out large beads that could block the column. After a 1h-incubation at 4ºC, the beads were spun down, the supernatant was discarded and fresh 10x TE buffer was added. After repeating this procedure three times, the beads were left at 4ºC for another 30 min. After spinning and discarding the supernatant again, 25 mg GTP-γ-S were added to 4 ml beads, incubated at 4ºC and washed extensively before use in a selection.

To quantify the amount of nucleotide that was immobilized, a small amount of the beads was incubated with 20 mM DTT for 10 min. The supernatant was then applied to a TLC plate together with a positive control of GTP of known concentration. After the TLC plate had run in a chamber with 1.5 M formic acid and 1.5 M lithium fluoride, the concentration of the immobilized GTP could be calculated based on the intensity of the spots observed by UV shadowing.

Alternatively, the amount of immobilized GTP can be determined by measuring the absorption of GTP-γ-S at 252 nm and 343 nm. Based on the following equation, the concentration of the immobilized GTP on thiopropyl sepharose beads can thus be determined:

$$\text{GTP-}\gamma\text{-S [mM]} = (A_{252} - 0.496 \times A_{343}) / 13.149$$

3.8 *In Vitro* Re-Selection Procedure

^{32}P-labeled RNAs were selected on GTP-γ-S-thiopropyl-sepharose columns with approximately 0.5 mM GTP immobilized. A thiopropyl-sepharose pre-column prior to each new round of selection filtered out molecules that bound the thiopropyl sepharose itself or that recognized parts of the spacer arm linking GTP to the thiopropyl sepharose (Ellington and Szostak 1992; Famulok and Szostak, 1992). Column volumes were equal to the swelled volume of the thiopropyl sepharose, and the pre-column volume was one-half that of the selection column volume. The selection buffer contained 200 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, 50μM EDTA, pH 6.2. The free magnesium concentration was also kept constant throughout the washing and elution portions of the selection by adding additional Mg^{2+} to the elution buffer (approximately 1.5 Mg^{2+}/GTP; Storer and Cornish-Bowden, 1976) to account for the intrinsic binding of Mg^{2+} by GTP at these salt and buffer conditions.

10 column volumes of selection buffer were used to equilibrate both the GTP- and thipropyl-sepharose columns. RNA was dissolved in water and denatured by heating to 80°C for 2 min. An equal amount of 2x selection buffer was added, and the RNA allowed to equilibrate for 1 h before application to the pre-column. After nutating the pre-column for 10 minutes, the RNA was washed onto the selection column with 3x1 pre-column volume washes. The selection column was then nutated for 24 h. Unbound RNA was eluted from the column by washing it with a sufficient amount of selection buffer. Bound RNAs were then eluted with 6x1 column volumes of elution buffer by affinity elution with free GTP (Sassanfar and Szostak, 1993), nutating for 15 min each. The elution buffer contained 5 mM GTP in selection buffer adjusted to constant free magnesium. All fractions were Cerenkov counted to determine the amount of RNA that was selected (Famulok, 1994). The elution fractions were combined for amplification. Pre-elution fractions were not included in reverse transcription and amplification.

Pool DNA obtained after the final round was cloned and sequenced as described previously (Famulok, 1994; Jenne and Famulok, 1998).

3.9 TOPO Cloning, Preparation of Plasmid DNA and Sequencing

All cloning was performed using the TOPO TA cloning kit. PCR was ended with a polymerization step (10 min at 70°C) to introduce an A at the end of each DNA sequence necessary for TOPO cloning. This product was purified for sequencing using the Qiaquick PCR purification kit, which removed unincorporated nucleotides and primers. After TOPO Cloning according to the protocol of the manufacturer, colonies containing the cloned libraries were selected, and the DNA for sequencing was produced by miniprep.

For small-scale preparation of plasmid DNA, a 5-ml culture of LB medium containing the appropriate antibiotic (ampicillin, 100 □g/ml) was inoculated with a single bacterial colony using a sterile pipet tip. The culture was placed at 37ºC in a rotating incubator for 16 hours. The preparation of the plasmid DNA was conducted using the Miniprep Kit from Qiagen following the instructions of the manufacturer. Sequencing reactions were performed by the DNA Sequencing Core Facility of the Massachusetts General Hospital.

The primary structure of the aptamers was analyzed using the Seqlab program of the GCG (Genetic Computer Group) package to discover regions of similar sequence.

3.10 Determination of Aptamer Secondary Structures and Their Minimum Free Energy

The program mfold, version 3.1 was used for secondary structure prediction of aptamer RNA (Zuker, 2003).

Minimum free energy of aptamer secondary structures was computed using the program RNAfold from the ViennaRNA Package version 1.4, a dynamic programming search method for finding minimal-free energy secondary structures based on empirically derived thermodynamic parameters (Hofacker et al., 1995). The software was obtained

from http://www.tbi.univie.ac.at/~ivo/RNA and compiled under Intel Linux. Sequences were folded at 37°C using default settings.

3.11 Spinfiltration and Equilibrium Dialysis: Methods for Determining K_d Values and Fraction of Correctly Folded Aptamer

3.11.1 Determining the Apparent K_ds of GTP Aptamers

To assess aptamer binding to GTP, four separate spinfiltration assays for each aptamer were performed to determine their apparent dissociation constants (K_ds) (Jenison et al., 1994). These involved the initial examination of RNA concentrations over several orders of magnitude (typically 0.5 nM - 50 μM) and more detailed testing of three different concentrations of RNA in the region where approximately 40-60% (0.67-1.5xK_d) of the ligand were bound (Martin et al., 1991).

Without performing thermal melting curves, the secondary structure of the RNA can introduce an error of up to 20% when determining the concentration. To determine the exact concentration of the stock RNA from which the experimental dilutions were made, thermal melting curves were performed on a Cary spectrophotometer (heating from 15ºC to 95ºC at a rate of 5ºC/min, holding at 95ºC for 1 min, cooling from to 95ºC to 15ºC at a rate of 5ºC/min, holding at 15ºC for 1 min; repeated 3 times (3 "legs", see Fig. 3) and holding at 25ºC at the end) in 1 ml 1x binding buffer (a temperature-stable phosphate buffer). The RNA was diluted to a concentration that yielded an absorption of approximately 0.5 when measuring it at λ=260 nm. The A_{260} values at 95ºC when the RNA is completely denatured were averaged for calculating the exact RNA concentration while excluding the first value that is usually obtained with incorrectly folded RNA. A slight hyperchromic shift was observed caused by decomposition of the RNA, but due to the low pH of the binding buffer (pH=6.2) observed degradation was negligible.

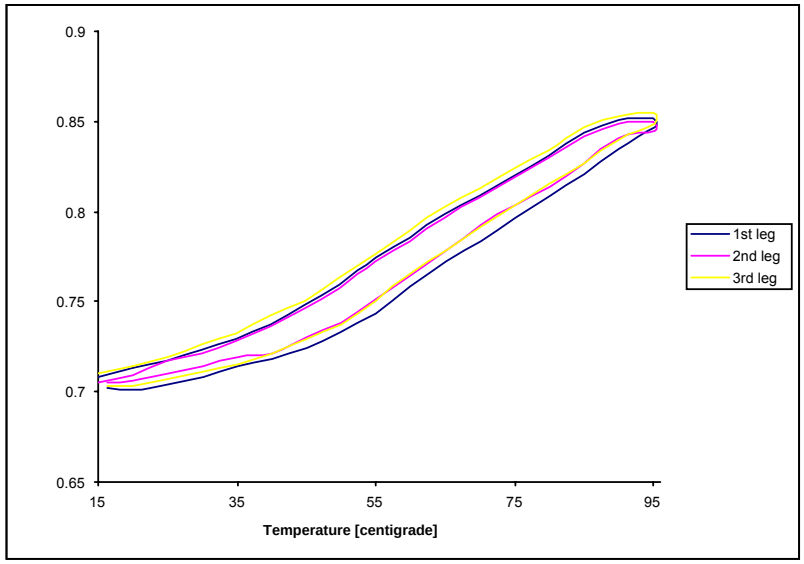

Fig. 3: Thermal Melting Curve. Three consecutive thermal melting experiments were performed with aptamer 10-6 and the mean of the absorption at 95ºC was used to calculate the RNA concentration.

After the appropriate experimental RNA dilutions had been prepared in a 100 µl volume, the mixture was heated to 80ºC for 5 min and 100 µl of 2x binding buffer containing 0.22 nM [α-^{32}P]GTP were added. The samples were equilibrated over night at room temperature and then transferred into the top chamber of a Microcon YM-30 spin filter. The tubes were centrifuged at 13,000 g for 5 sec to saturate the membrane and the filters were placed into new collection tubes and again spun at 13,000 g for 20 sec, allowing 25 µl of solution to flow through the membrane. Similar to equilibrium dialysis, the solution that remained above the molecular-weight cutoff membrane ("top" (T) fraction) contained free GTP ($[L]_f$), free RNA ($[R]_f$), and RNA-bound GTP ([RL]); the filtrate ("bottom" (B) fraction) contained only free GTP ($[L]_f$). A 20-µl sample was removed from each side of the filter, and the radioactivity was measured by scintillation counting for 1 min (see Fig. 4).

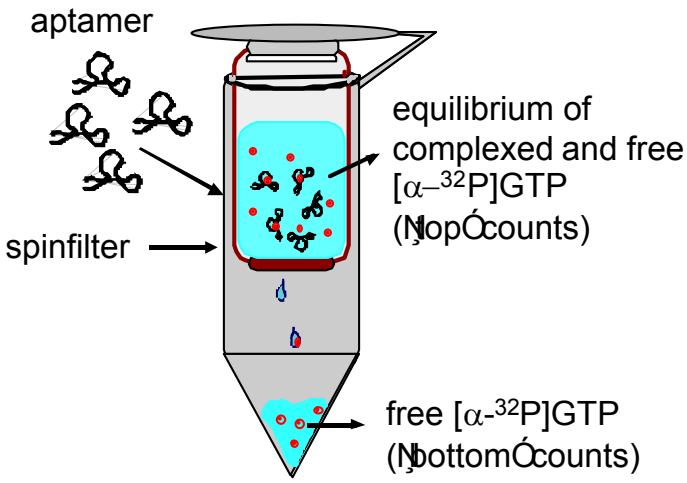

Fig. 4: Spinfiltration Method for Determining K_ds (Courtesy of Rosa Larralde).

Aggregates such as self-association of GTP monomers into G-quartets inhibit the flow of free GTP through the membrane. In order to ensure that samples were obtained at equilibrium, particularly for aptamers with fast on- and off-rates, the sample from the "top" fraction was taken prior to spinning, the sample from the "bottom" fraction after the spin. In this context, it is imperative to use a large sample volume and never to spin through more than 10% in order not to distort the equilibrium. Also, it is important to perform the spin as fast as possible and therefore to use membrane cut-off sizes that are large while preventing any RNA from flowing through. In addition, variables were introduced when determining the dissociation constants of the aptamers to account for non-specific binding of labeled GTP to the filter membrane or the RNA ("b") and the maximum fraction of labeled GTP that can be bound ideally ("c") (see equations (16) and (30) in the derivation below).

3.11.2 Derivation of the Equation Used for K_d Determination

(The methods described in the following chapters were developed in collaboration with James Carothers, Glen Cho, Rosa Larralde and Anthony Keefe.)

The dissociation constant (K_d) of a complex is defined by the following equation:

$$K_d = \frac{[R]_f * [L]_f}{[RL]} \quad (1)$$

where:

$[R]_f$: concentration of free RNA

$[L]_f$: concentration of free ligand (GTP in this case)

[RL]: complex of RNA bound to its ligand

The total RNA concentration consists of the concentration of free RNA and the concentration of ligand-bound RNA, and the same is true for the ligand as well:

$$[R]_f = [R]_t - [RL] \quad (2)$$

$$[L]_f = [L]_t - [RL] \quad (3)$$

with:

$[R]_t$: total RNA concentration

$[L]_t$: total ligand concentration

Therefore, when substituting the variable for the free ligand concentration in equation (1) with equation (2):

$$K_d = \frac{[R]_f * ([L]_t - [RL])}{[RL]} \quad (4)$$

Re-arranging of equation (4) results in the Langmuir-Hill equation (9) (Langmuir, 1918):

$$K_d * [RL] = [L]_t * [R]_f - [RL] * [R]_f \quad (5)$$

$$K_d * [RL] + [RL] * [R]_f = [L]_t * [R]_f \quad (6)$$

$$[RL] * (K_d + [R]_f) = [L]_t * [R]_f \quad (7)$$

$$[RL] = [L]_t \frac{[R]_f}{K_d + [R]_f} \quad (8)$$

$$\frac{[RL]}{[L]_t} = \frac{[R]_f}{K_d + [R]_f} \quad (9)$$

Based on equations (2) and (3):

$$[RL] = [R]_t - [R]_f = [L]_t - [L]_f \quad (10)$$

Therefore:

$$[R]_f = [R]_t - [L]_t + [L]_f \quad (11)$$

When replacing the variable for $[R]_f$ in equation (9) with equation (11), the resulting equation representing the fraction of bound ligand results in:

$$\frac{[L]_{bound}}{[L]_{total}} = \frac{[RL]}{[L]_t} = \frac{[R]_t - [L]_t + [L]_f}{K_d + [R]_t - [L]_t + [L]_f} \quad (12)$$

Due to the experimental difficulty in measuring the amount of free RNA, it is crucial for the following assumption to be valid for accurately determining the K_d using the amount of total RNA in the calculation:

At large ratios of aptamer to ligand concentration, the total concentration of the ligand can be regarded as sufficiently small to be ignored (e.g. at a 25-fold excess of aptamer, the error would be 2% and decreasing at larger ratios). Therefore:

$$\text{if } [L]_t << [R]_t \text{ , then } [R]_t - [L]_t + [L]_f \sim [R]_t \quad (13)$$

and thus:

$$\frac{[L]_{bound}}{[L]_{total}} = \frac{[R]_t}{K_d + [R]_t} \qquad (14)$$

Therefore, at $[R]_t = K_d$:

$$\frac{[L]_{bound}}{[L]_{total}} = \frac{1}{2}$$

(see Fig. 5)

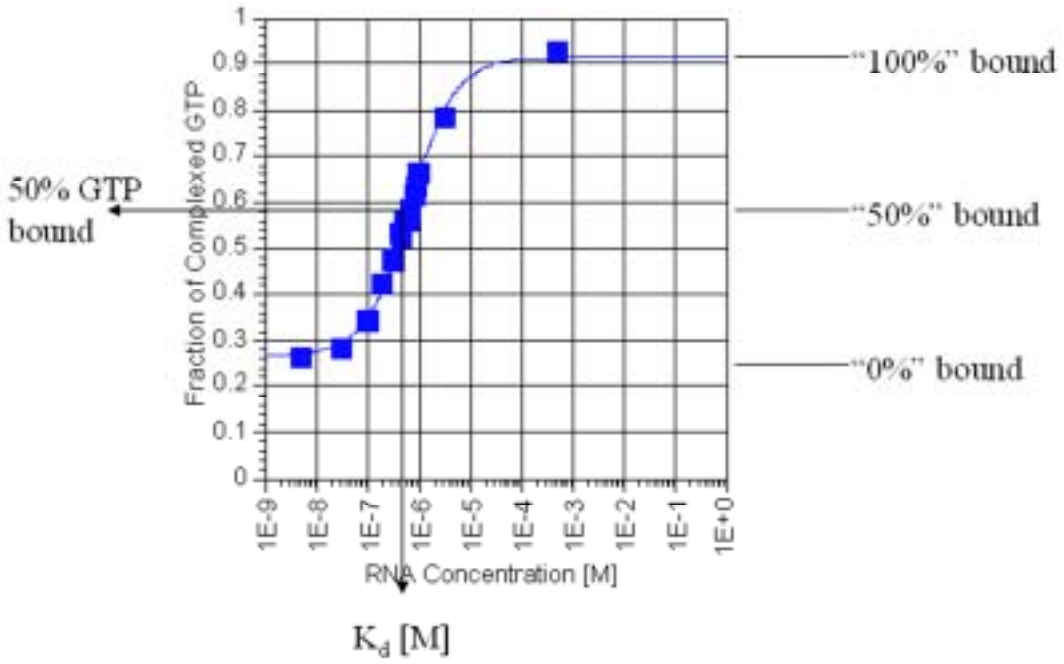

Fig. 5: Example of Equation (16) Fitted for K_d Determination (K_d marked).

The ratio of bound to total aptamer concentration was measured by spinfiltration or equilibrium dialysis using [α-^{32}P]GTP as the ligand. Data were gathered over a range of aptamer concentrations and plotted against the fraction of GTP bound to RNA, which had been calculated in the following way:

$$\frac{T-B}{T} = \frac{[RL]+[L]_f-[L]_f}{[RL]+[L]_f} = \frac{[RL]}{[RL]+[L]_f} \qquad (15)$$

B: "bottom" counts obtained by scintillation counting of a 20-µl sample from the filtrate after spinfiltration

T: "top" counts obtained by scintillation counting of a 20-µl sample taken from the fraction in the top chamber of the filter membrane before spinfiltration

In order to determine the K_d, this equation was then fitted, following equation (14) and using the program DeltaGraph:

$$\frac{[T-B]}{[T]} = b + \left(c * \left(\frac{[R]_t}{[R]_t + K_d} \right) \right) \qquad (16)$$

b: baseline response (non-specific binding of labeled GTP to the filter membrane or the RNA; defined as 0% binding)

c: maximum response (maximum fraction of labeled GTP that can be bound ideally; defined as 100% binding)

$[R]_t$: total RNA concentration

$[RL]/[L]_t$: fraction of labeled GTP bound

b, c and K_d are parameters fitted through non-linear regression using the program DeltaGraph (see Fig. 6).

The K_ds were averaged to give the overall K_d.

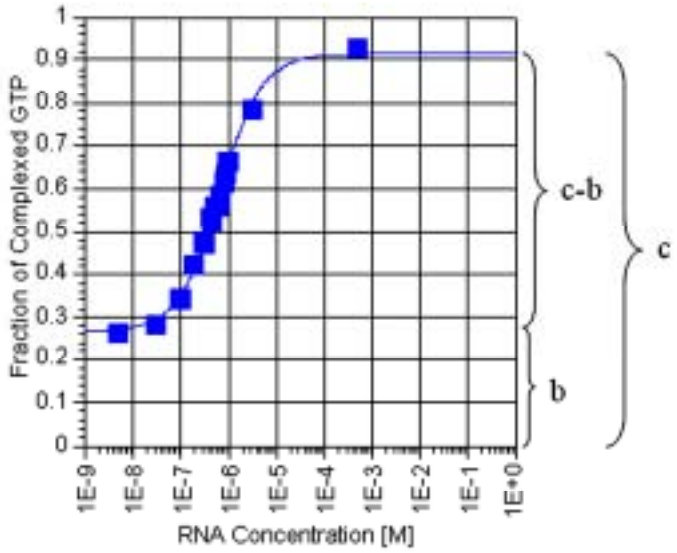

Fig. 6: Example of Equation (16) Fitted for K_d Determination (variables "b" and "c" are marked).

3.11.3 Competitor Experiments

For the correct determination of K_ds with the method explained previously, it is crucial that the ligand concentration is substantially lower than the aptamer concentration and therefore negligible. When conducting competition experiments in the way described above, this premise is violated. Therefore, these experiments lead to the determination of IC50s rather than K_ds. An IC50 is defined as the concentration of the competitor required to compete for half of the specific binding. If the affinity of the RNA for the competitor is high, its IC50 will be low. This value then has to be standardized by dividing the resulting IC50s by the one obtained with unlabeled GTP used as a competitor to the [α-^{32}P]GTP included in the binding buffer.

In order to determine the IC50 for GTP and some of its analogs for the different aptamers, the appropriate concentration of competitor was prepared in 2x binding buffer. The $MgCl_2$ concentration was adjusted to 1.5x competitor concentration and the pH to 6.2. RNA was prepared at a concentration of 4xK_d (2xK_d = final concentration) in 100 µl

H_2O and heated to 80°C for 5 min. Samples were equilibrated over night at room temperature, 80 µl 2x binding buffer containing 0.22 nM [α-^{32}P]GTP were added and another over night equilibration followed. For a no-competitor control, 100µl 2x binding buffer containing 0.22 nM [α-^{32}P]GTP were added. The samples were subsequently transferred to Microcon YM-30 spin columns and treated as described in the previous chapter for K_d determination.

When performing competitor experiments, the fraction of labeled GTP bound to RNA is plotted against the varied competitor concentration while the RNA concentration remains constant at roughly 2xK_d. The basic equation for determination of K_ds (16) is then employed, but the variables in the equation signify different parameters (see Fig. 7):

$$\frac{[T-B]}{[T]} = b + \left(c * \left(\frac{[R]_t}{[R]_t + IC50} \right) \right) \qquad (16)$$

b: baseline response or total initial binding (non-specific binding of labeled GTP to the filter membrane or the RNA; a function of RNA concentration, the quality of the labeled GTP, the K_d of the aptamer with GTP and non-specific binding; defined as 0% binding)
c: maximum decrease in binding (maximum fraction of labeled GTP that can be bound in the absence of the competitor; defined as 100% binding)
$[R]_t$: total competitor concentration
$[RL]/[L]_t$: fraction of labeled GTP bound (= not displaced by the competitor)
b, c and K_d are parameters fitted through non-linear regression using the program DeltaGraph.
Ideally, b=-c, the difference signifies the amount of unspecific binding.

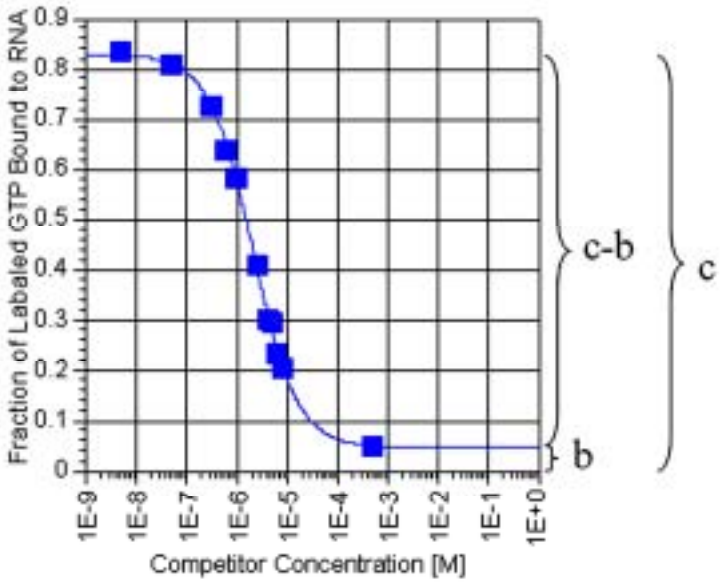

Fig. 7: Example of Equation (16) Fitted for IC50 Determination (variables "b" and "c" are marked).

3.11.4 Determining the Real K_d and Percentage of Correctly Folded Aptamer

Conventional methods for K_d determination only measure the apparent K_d without accounting for the dependence of this value on the percentage of the aptamer correctly folded. Competitor experiments with unlabeled GTP had thus to be performed.

As described previously, the following equation is used for K_d determination:

$$\frac{[RL]}{[L]_t} = \frac{[R]_t}{[R]_t + K_d} \qquad (17)$$

$[R]_t$: total RNA concentration

$[RL]/[L]_t$: fraction of labeled GTP bound

K_d is fitted through non-linear regression using the program DeltaGraph.

Rearranging equation (17) leads to the following equation:

$$\frac{[RL]}{[L]_t} = \frac{1}{1 + \frac{Kd}{[R]_t}} \tag{18}$$

When employing a GTP competitor, some of the RNA is complexed by the competitor. This amount ([RI]) therefore has to be subtracted from $[R]_t$, the value representing total RNA concentration:

$$\frac{[RL]}{[L]_t} = \frac{1}{1 + \frac{K_d}{[R]_t - [RI]}} \tag{19}$$

In order to determine [RI], the following factors have to be taken into account: RNA (R) and competitor (inhibitor I) in solution are in the following equilibrium:

$$[R]_f + [I]_f \leftrightarrow [RI] \tag{20}$$

Based on this equilibrium, the apparent K_d of the competitor can be calculated in the following way:

$$K_{d(I)} = \frac{[R]_f * [I]_f}{[RI]} \tag{21}$$

$K_{d(I)}$: K_d of the competitor

$[R]_f$: concentration of free RNA

$[I]_f$: concentration of free inhibitor

[RI]: concentration of RNA complexed by inhibitor

Assuming:

$[R]_f = [R]_t - [RI]$

$[I]_f = [I]_t - [RI]$

with:

$[R]_t$: total RNA concentration

$[I]_t$: total inhibitor concentration

Including these variables into equation (21) results in the following equation:

$$K_{d(I)} = \frac{([R]_t - [RI]) * ([I]_t - [RI])}{[RI]} = \frac{[R]_t[I]_t - [I]_t * [RI] - [R]_t * [RI] + [RI]^2}{[RI]} \quad (22)$$

Transformation of the equation above and setting it equal to 0 results in the following equation:

$$0 = [RI]^2 - [RI]([I]_t + [R]_t + K_{d(I)}) + [R]_t[I]_t \quad (23)$$

This equation resembles a quadratic equation of the form:

$$0 = ax^2 + bx + c \quad (24)$$

with:

$a = 1$

$b = -([I]_t + [R]_t + K_{d(I)})$

$c = [R]_t[I]_t$

This equation can be solved by applying the following equation:

$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \quad (25)$$

$$a \neq 0$$

To account for misfolding of the RNA, a factor (f) is introduced in the following equation describing the total available RNA aptamer concentration:

$$[R] = [R]_t * f \tag{26}$$

Inserting the variables from equations (23) and (26) into the quadratic equation (25) results in the following equation (27), which is solved only when subtracting the square root. The variable for the apparent K_d of the competitor ($K_{d(I)}$) can now be replaced with the one for the real K_d of the competitor:

$$[RI] = \frac{\left([R]_t * f + [I]_t + K_d\right) \pm \sqrt{\left(\left([R]_t * f + [I]_t + K_d\right)^2 - 4 * [R]_t * f * [I]_t\right)}}{2} \tag{27}$$

[RI]: amount of RNA complexed by inhibitor
$[R]_t$: total RNA concentration
f: fraction of the RNA folded correctly
$[I]_t$: total inhibitor concentration

Before substituting the variable for [RI] in equation (19) with equation (27), equation (19) was normalized by dividing it by equation (18) (equation for calculating the K_d without inhibitor):

$$\frac{[RL]}{[L]_t} = \frac{\dfrac{1}{1 + \dfrac{K_d}{[R]_t - [RI]}}}{\dfrac{1}{1 + \dfrac{K_d}{[R]_t}}} \tag{28}$$

This equation can be re-arranged in the following way:

$$\frac{[RL]}{[L]_t} = \frac{1 + \frac{K_d}{[R]_t}}{1 + \frac{K_d}{[R]_t - [RI]}} \qquad (29)$$

When replacing [R] with $[R]_t$*f (26), inserting the solution of the quadratic equation for [RI] (27) into (29) and adding a factor ("b") that accounts for the fact that the labeled GTP is never completely replaced by the competitor, the final equation results in:

(30)

$$\frac{[RL]}{[L]_t} = b + \frac{1 + \left(\frac{K_d}{([R]_t * f)} \right)}{1 + \frac{K_d}{[R]_t * f - \left[\frac{([R]_t * f + [I]_t + K_d) - \sqrt{(([R]_t * f + [I]_t + K_d)^2 - 4 * [R]_t * f * [I]_t)}}{2} \right]}}$$

b: total initial binding (due to non-specific binding of labeled GTP to the filter membrane or the RNA; defined as 0% binding)

K_d: real K_d

$[R]_t$: total RNA concentration

f: fraction of RNA correctly folded

$[I]_t$: total inhibitor concentration

$[RL]/[L]_t$: fraction of labeled GTP bound

K_d and f are parameters fitted through non-linear regression using the program DeltaGraph.

In these experiments, only the labeled GTP is traceable, therefore the data is gathered as described previously and then the fraction of labeled GTP displaced by competitor is plotted against the competitor concentration.

3.11.5 Determination of GTP Background Binding

Instead of including a negative control in each series of measurements, which would itself be subject to random fluctuations and systematic errors, the mean amount of GTP that was retained by the membrane of the Microcon YM-30 spinfilters under these assay conditions was determined by performing three series of measurements: 200µl of 1x binding buffer with 0.11 nM GTP were equilibrated over night and samples were then collected and counted as described above.

The mean background GTP binding was determined as (8.41%±0.69%) with a 95% confidence interval of 6.32% to 10.50%. One half of the mean count retention (4.205%) was thus added to the bottom counts and one half (4.205%) subtracted from the top counts.

3.11.6 Alternative Methods for K_d Determination

K_d measurements can also be conducted by Biacore, equilibrium dialysis, gelshift assay and ultracentrifugation. More than one of these methods should be used to confirm the values obtained. Therefore equilibrium dialysis was also employed to measure binding of the aptamers to GTP and yielded dissociation constants equivalent to those determined by equilibrium filtration.

At the start of the equilibrium dialysis experiment, the aptamer and ligand were present only in one of the two cells of the dialysis chamber while the other side was filled with reaction buffer. The cells were separated by a semipermeable membrane through which only the ligand could pass. The dissociation constant was determined after over night incubation when an equilibrium was established. Aliquots from both sides were taken and measured according to the method described above.