Supplementary Information

Table S1. Kinetic parameters of an ATP-aptamer, a FMN-aptamer and a combination of both with FAD.

Linker	ATP-Aptamer	FMN-Aptamer	k_a [1/Ms]	k_d [1/s]	K_d (kinetic) [µM]
Linker-4		FMN-2	5.4E+04	5.5E-02	1.02
Linker-4	ATP-4		1.1E+04	9.0E-02	8.18
Linker-4	ATP-4	FMN-2	8.1E+04	1.4E-01	0.17

Table S2. DNA sequences of aptamer constructs consisting of one RNA.
The non-transcribed part of the 5' region of the DNA-aptamer sequences are shown in italics and the spacer regions are highlighted in bold.

Construct Name	DNA-Sequence
FMN/ATP-1	*TAATACGACTCACTATA*GGGAGATAGGATATGCTTCGGCAGAAGGATCTCCC**A**CCGTTGGGAAGA AACTGCGGCACTTCGGTGCCGGCAACGG
FMN/ATP-2	*TAATACGACTCACTATA*GGGAGATAGGATATGCTTCGGCAGAAGGATCTCCC**AA**CCGTTGGGAAG AAACTGCGGCACTTCGGTGCCGGCAACGG
FMN/ATP-4	*TAATACGACTCACTATA*GGGAGATAGGATATGCTTCGGCAGAAGGATCTCCC**AAAA**CCGTTGGGA AGAAACTGCGGCACTTCGGTGCCGGCAACGG
FMN/ATP-6	*TAATACGACTCACTATA*GGGAGATAGGATATGCTTCGGCAGAAGGATCTCCC**AAAAAA**CCGTTGG GAAGAAACTGCGGCACTTCGGTGCCGGCAACGG

Table S3. K_d values of different aptamer constructs consisting of one RNA with FAD. K_d values and standard deviations were calculated from at least three independent Biacore measurements with FAD

Construct	Average K_d st.st. [µM]
FMN/ATP-6	0.38 ± 0.09
FMN/ATP-4	0.34 ± 0.02
FMN/ATP-2	0.48 ± 0.03
FMN/ATP-1	0.50 ± 0.01

Table S4. K_d values of different aptamer constructs with different analytes.
K_d values and standard deviations were calculated from at least three independent Biacore measurements.

Linker	ATP-Aptamer	FMN-Aptamer	Analyte	Average K_d st.st. [µM]
Linker-4	ATP-4		ATP	1.08 ± 0.13
Linker-4		FMN-2	ATP	no binding
Linker-4	ATP-1		FMN	no binding
Linker-4		FMN-2	FMN	0.163 ± 0.004
no linker	FMN/ATP-4		FMN	0.186 ± 0.006

Table S5. K_d values of different aptamer constructs for FAD or FMN.
K_d values and standard deviations were calculated from at least three independent fluorescence-binding measurements with FAD or FMN.

Linker	ATP-Aptamer	FMN-Aptamer	Ligand	Average K_d [µM]
	ATP-4		FAD	no quenching
		FMN-2	FAD	0.38 ± 0.04
		FMN-2	FMN	0.22 ± 0.01
		FMN-1	FAD	0.26 ± 0.06
Linker-4	ATP-4	FMN-2	FAD	0.14 ± 0.02
	ATP-4	FMN-2	FAD	0.40 ± 0.24
Linker-2	ATP-4	FMN-1	FAD	0.10 ± 0.04
	ATP-4	FMN-1	FAD	0.59 ± 0.26
	FMN/ATP-6		FAD	0.45 ± 0.27
	FMN/ATP-4		FAD	0.17 ± 0.04
	FMN/ATP-4		FMN	0.13 ± 0.02

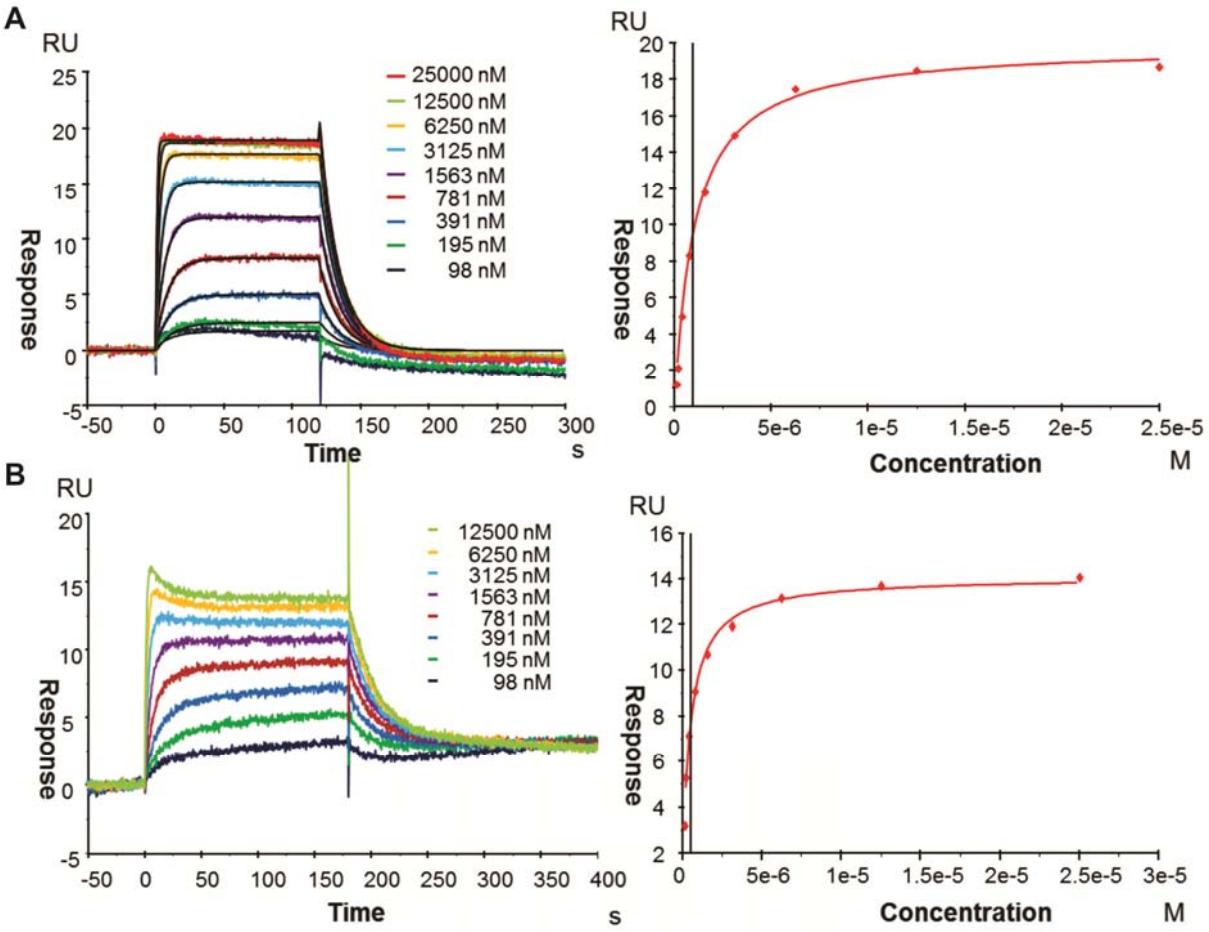

Figure S1. Biacore sensograms and steady state evaluation with FAD as analyte.
A Left: Biacore sensogram of the FMN-2 aptamer with the Linker-4; the black curves show the kinetic Langmuir 1:1 fit. Right: the steady state evaluation fit (red line) with the vertical black line indicating the K_d value. **B** Left: Biacore sensogram of the ATP-4 and the FMN-2 aptamers with the Linker-4. Right: the steady state evaluation fit (red line) with the vertical black line indicating the K_d value.

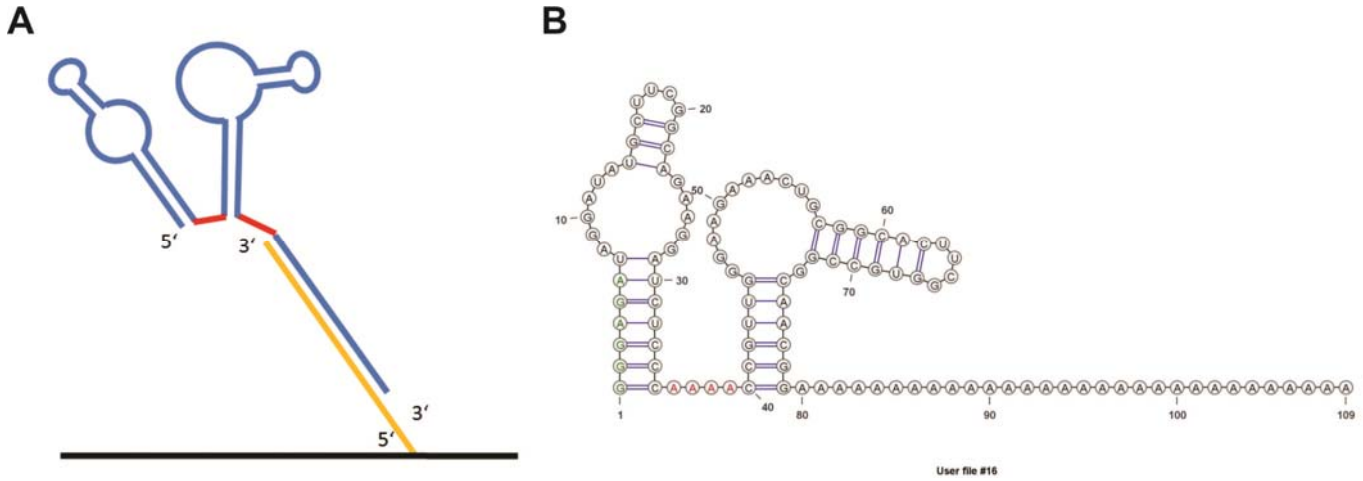

Figure S2. Design of combined aptamer within one RNA molecule. **A** Schematic illustration of the aptamer construct. Connected aptamer molecules with overhangs (blue) hybridize with the DNA immobilized on the chip (yellow). Spacer regions of variable lengths are depicted in red. **B** Sequence of the combined FMN/ATP-4 Aptamer. Single stranded spacer nucleotides are shown in red and the T7 promoter sequence is depicted in green.

Experimental Procedures

Aptamer Constructs

DNA oligonucleotides were purchased from Sigma Aldrich. Whereas the immobilized strand and the linker strand were DNA single strands and could be purchased directly, the aptamers needed to consist of RNA. In order to obtain double-stranded DNA as a substrate for the *in vitro* transcription (T7 promoter) and to amplify the DNA, a PCR with Phusion Hot Start II polymerase was performed according to standard procedures. The double stranded DNA was purified via Agarose-gel-extraction and concentrated with EtOH precepitation. *In vitro* transcription was perfomed with T7 RNA polymerase for 2h at 37°C. RNA was purified via 6% preparative PAGE and concentrated with EtOH precipitatation. For hybridization oligonucleotides were mixed in equimolar ratios and incubated at 95°C for 5 min in HBS-N/$MgCl_2$ buffer (150 mM NaCl, 10 mM HEPES, 5mM $MgCl_2$, pH 7.4). The temperature was slowely cooled down for 8h. For hybridization of the linker constructs, the two aptamer sequences were used in a 1.5-fold excess to the linker to assure that all linker molecules were hybridized with the two different aptamer sequences.

SPR spectroscopy

All SPR measurements were perfomed with a Biacore T100 instrument at 25°C. Prior to the measurements either a 5'-aminohexyl- modified 24-mer poly (T) sequence ([AmC6F]TTTTTT TTTTTTTTTTTTTTTTT) or a 3'-amino C7 modified DNA sequence containing a complementary T7 promoter sequence (CGTCGCAGATCGTGTCTTCC[AmC7]) were covalently bound to a Biacore CM5 chip by NHS/EDC coupling. The coupling reaction was performed at 25°C in HBS-N buffer as running buffer. Each of the four flow cells were treated equally but separately. For the activation of the carboxyl groups of the carboxymethylated dextran matrix on the chip, a freshly prepared 1:1 mixture of EDC and NHS solution was applied to the chip for 7 min at a flow rate of 10 µL/min. After the activation a solution of Amino-modified DNA (20 µM) and CTAB (0.6 mM) in 10 mM HEPES was injected for 1 hour at a flow rate of 2 µL/min. Finally, a 1 M ethanolamine solution was injected at a flow rate of 10 µL/min for 7 min in order to react with the remaining non-reacted succinimidyl esters.[77]

Prior to every Biacore measurement, the system was primed three times with running buffer, which was either HBS-N+$MgCl_2$ buffer in case of the experiments with the aptamers. The different oligonucleotides, containing a specific overhang which is complementary to the immobilized DNA on the chip, were diluted to a 5 µM solution in the running buffer and injected at a low flow rate of 2 µL/min for 6 min. To flow cell 1, either no oligonucleotides were hybridized or the DNA linker sequences without aptamers, since it was used as reference flow cell. After hybridization of the aptamer sequences onto the chip, a concentration series of the analyte (e.g. FAD) diluted in running buffer, was injected to all flow cells at a high flow rate of 30 µL/min for 2 min. After the dissociation time of 10 min a complete dissociation could be

reached. The range of the concentration series was chosen based on the estimated K_D value. To remove the hybridized oligonucleotides from the chip surface, a 25 mM NaOH solution was injected over all flow cells for 2 min at a flow rate of 10 µL/min. The resulting raw data were processed and evaluated with the Biacore T100 evaluation software.

After each run a desorb procedure was performed and every two weeks or if it seemed to be necessary a superclean procedure was performed.

Fluorescence binding measurements

To a dilution series of RNA or DNA a diluted FAD or FMN solution was added to reach a final concentration of 100 nM FAD or FMN. For all dilutions the same buffers were used as for SPR experiments (HBS-N+$MgCl_2$ buffer). For the aptamer sequences 2-fold dilutions ranging from 10 µM to 19.5 nM were used. 50 µL of each sample was then transferred to a black 384-well plate and the fluorescence was measured with an excitation wavelength of 450 nm and an emission wavelength of 525 nm with a Tecan infinite M200 reader. The obtained data were plotted and fitted with the sigmoidal Hill1 fit with n=1 in Origin.