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The dual aptamer approach: rational design of a high-affinity FAD aptamer†

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A design strategy for high-affinity aptamers of complex biomolecules is presented. We developed an RNA with FAD-binding properties by combining known ATP- and FMN-aptamers. Cooperative binding of FAD was shown by SPR spectroscopy and fluorescence assays. The strategy should be transferable to several other biomolecules.

Flavin adenine dinucleotide (FAD) is an important redox cofactor involved in crucial metabolic pathways. Several flavoenzymes utilize flavins for metabolic reactions for example during glycolysis, the citric acid cycle, and fatty acid synthesis and oxidation.[1] Furthermore, FAD plays a role as cofactor in numerous proteins not involved in redox processes.[2,3] FAD contains a riboflavin (vitamin B_2) moiety, which forms from a tricyclic heteronuclear isoalloxazine and the polyalcohol ribitol. Riboflavin is a precursor of FAD and forms flavin mononucleotide (FMN) when a phosphate group is present at the ribitol moiety. Addition of an adenosine monophosphate to FMN results in FAD (Fig. 1A).

Flavins have been the target of RNA aptamers since the early days of the SELEX technology.[4–6] Aptamers are short, single-stranded nucleic acids able to form complex three-dimensional structures. They specifically recognize small molecules with low molecular weight[7] and display high affinity as well as selectivity for their targets.[8,9] The first aptamers were obtained using SELEX in the early 1990s.[4,10] Aptamers specifically binding FAD would have the potential to interfere with metabolic pathways. So far, such aptamers were originally isolated for FMN: they recognize the isoalloxazine ring and reportedly do bind FAD as well, albeit with lower affinity (0.5 *vs.* 0.7 μM in a buffer with 5 mM $MgCl_2$).[11] The secondary structure of FMN-aptamers isolated by Burgstaller and Famulok[11] consists of a highly conserved internal loop that is flanked by two stabilizing stems. Chain reversal is achieved by incorporation of a hairpin loop. The NMR structure of this aptamer showed that the binding is based on a specific intercalation of the isoalloxazine ring to the helix in the internal loop. The rest of the molecule, especially the adenosine part of FAD, is not involved in the binding to the aptamer and is therefore freely accessible.[12] In addition, FAD aptamers isolated by other groups only showed interaction with the isoalloxazine moiety.[13] In our study, we aimed at the creation of a more specific, high-affinity FAD aptamer. For this purpose we combined two known aptamers to enable them to bind FAD in a cooperative manner. The concept of linking two aptamers together has been explored before, for example a bi-partite aptamer has been constructed by linking two DNA aptamers that recognize two different surface receptor proteins.[14] Dual aptamer constructs recognizing a single molecule where realized targeting CD28[15] and thrombin proteins.[16] In this work we target for the first time a compound of comparable low molecular weight with two different aptamers.

The two targeted substructures of FAD, the isoalloxazine ring and the adenosine moiety, are connected *via* a ribitol residue and two phosphate groups, resulting in a distance we deemed sufficient for targeting both groups separately by a combined dual aptamer sequence consisting of two separate, specific aptamers against the isoalloxazine and the adenosine part. This strategy of multivalent binding to achieve higher affinities has already been reported for other binders such as lectins and antibodies.[17–19] In our design, we chose to combine the ATP-aptamer by Sassanfar and Szostak[10] with the FMN-aptamer by Burgstaller and Famulok.[11] The respective structural models based on NMR data for both aptamers indicated that the phosphate group of AMP binding to the ATP-aptamer as well as the phosphate group of the FMN binding to the FMN-aptamer were not covered by the aptamer structures. Hence a simultaneous binding of both aptamers to FAD seemed possible. In order to assess the optimal linkage between the two aptamers and to determine the actual affinity of both the single aptamers and the combined aptamer

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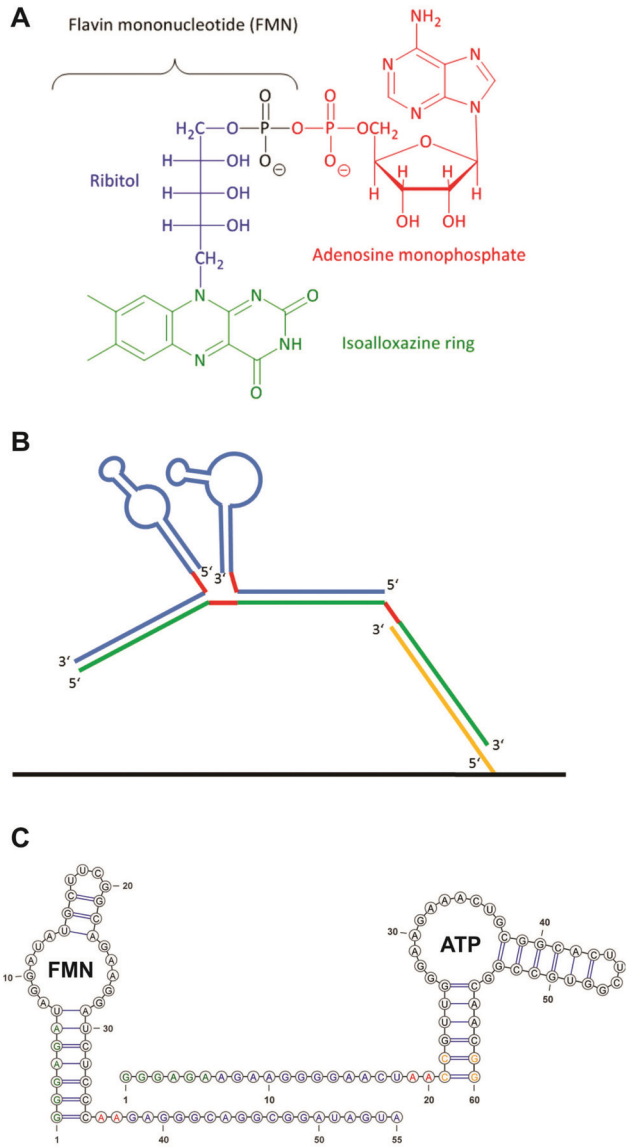

Fig. 1 Constructs and analytes. (A) Structure of FAD. The isoalloxazine ring (green) is connected by a ribitiol (blue) and phosphate (black) with an adenosine monophosphate (red). (B) Scheme of the used aptamer constructs; aptamer with overhang (blue) hybridizes with DNA-linker (green), which basepairs with the immobilized DNA anchor on the chip (yellow). Single-stranded spacer regions are depicted in red. (C) Sequences of the FMN-2 and the ATP-2 aptamers. The 18 nt overhang (blue), spacer regions (red), T7-promoter (green) and changed basepairs in comparison to the original aptamer (yellow) are shown.

construct, we initially conducted surface plasmon resonance (SPR) spectroscopy. In our design both RNA aptamers were hybridized to a DNA linker *via* 18 nt overhangs (green, Fig. 1B). The linker DNA was immobilized by hybridization to a 24 nt poly(T) anchor DNA (yellow, Fig. 1B) covalently linked to the Biacore CM5-chip *via* a 5′-aminohexyl-functionalization.[20]

A complementary 24 nt poly(A) overhang was attached to the DNA linker that is able to hybridize with the poly(T) overhang immobilized on the Biacore chip (Fig. 1B or C). The RNA aptamers were synthesized by *in vitro* transcription and contained a complementary sequence at the 3′-end for hybridization to the chip surface. Furthermore, single-stranded spacer sequences were included at different positions: (1) in the region between the aptamers and the overhangs and (2) within the DNA linker (Fig. 1B, red). This design enabled us to analyse a number of constructs with different spacer lengths (see Table 1). In our studies we combined three different ATP aptamers (ATP-1, ATP-2, ATP-4, numbers indicate spacer length: incorporation of one, two, or four adenosine residues) with 3 different FMN aptamers (FMN-1, FMN-2, FMN-4) and two linker sequences (Linker-2, Linker-4).

The RNA was purified by polyacrylamide gel-electrophoresis (PAGE) and hybridized with the DNA linker before immobilization by hybridization to the anchor DNA on the Biacore CM5 chip was performed. SPR spectroscopy was carried out for the single aptamers as well as for the combined constructs. Concentration series of the respective analyte (FMN, ATP or FAD) were injected at a high flow rate of 30 μL min^{-1} to avoid mass transfer effects, see ESI† for sensograms. The affinities determined by association and dissociation kinetics differed significantly for the investigated constructs. While the ATP-4 aptamer displayed a K_d of only 7.7 μM and the FMN-2 a K_d of 1 μM, the combination of both aptamers with Linker-4 showed the highest affinity to FAD with a K_d of 170 nM (see Table 2). The K_d values obtained from the kinetic evaluation were in accordance with affinities determined by steady state analysis (Table S1 and Fig. S1†).

Next, we systematically analysed all possible combinations of the ATP and FMN aptamers with different spacer lengths and DNA linkers, as listed in Table 1. Intriguingly, we found that all constructs with both an ATP and an FMN aptamer connected by a DNA linker showed a significantly higher affinity for FAD than the separate ATP and FMN aptamers (Table 2). Except for two constructs (Linker-4/ATP-1/FMN-1 and Linker-4/ATP-2/FMN-2) the affinities of the combined aptamer constructs for FAD were all in the same range between 120 nM and 290 nM. We conclude that the spacer lengths do not significantly influence the affinity of the aptamer constructs to FAD.

For applications of the designed high-affinity FAD binding sequences it would be advantageous if both aptamers could be transcribed in a single RNA sequence. In order to ensure that our findings can be transferred to a single molecule construct, we investigated a second design: instead of joining together the two aptamers by hybridization they were transcribed from a single DNA strand. Consequently, the whole construct – including two variable single-stranded spacer regions and an overhang for the chip hybridization – was encoded in one RNA molecule (Fig. S2†). We investigated 4 constructs of this type (Table S2†) and tested their affinity to FAD (Table S3†): we found K_d values between 380 nM and 500 nM for FAD, displaying again significantly higher FAD affinities compared to the single aptamer sequences. In general, the FAD affinity of these constructs was slightly lower compared to the constructs in

Table 1 DNA sequences of the different constructs. The non-transcribed part in the 5′ region of the DNA-aptamer sequences is shown in italics and the spacer regions are highlighted in bold

Construct name	DNA-sequence
FMN-1	*TAATACGACTCACTATA*GGGAGATAGGATATGCTTCGGCAGAAGGATCTCCC**A**GAGGGCAGGCGGATAGTA
FMN-2	*TAATACGACTCACTATA*GGGAGATAGGATATGCTTCGGCAGAAGGATCTCCC**AA**GAGGGCAGGCGGATAGTA
FMN-4	*TAATACGACTCACTATA*GGGAGATAGGATATGCTTCGGCAGAAGGATCTCCC**AAAA**GAGGGCAGGCGGATAGTA
ATP-1	*TAATACGACTCACTATA*GGGAGAAGAAGGGGAACT**A**CCGTTGGGAAGAAACTGCGGCACTTCGGTGCCGGCAACGG
ATP-2	*TAATACGACTCACTATA*GGGAGAAGAAGGGGAACT**AA**CCGTTGGGAAGAAACTGCGGCACTTCGGTGCCGGCAACGG
ATP-4	*TAATACGACTCACTATA*GGGAGAAGAAGGGGAACT**AAAA**CCGTTGGGAAGAAACTGCGGCACTTCGGTGCCGGCAACGG
Linker-2	TACTATCCGCCTGCCCTC**AA**AGTTCCCCTTCTTCTCCC**AAAAAAAA**AAAAAAAAAAAAAAAAAAAAAA
Linker-4	TACTATCCGCCTGCCCTC**AAAA**AGTTCCCCTTCTTCTCCC**AAAAAA**AAAAAAAAAAAAAAAAAAAAAAAA

Table 2 K_d values of different aptamer-linker constructs with FAD. K_d values and standard deviations were calculated from at least three independent Biacore measurements with FAD

Linker	ATP-aptamer	FMN-aptamer	Average K_d st.st. [μM]
Linker-2		FMN-4	0.99 ± 0.05
Linker-2		FMN-2	1.13 ± 0.09
Linker-2		FMN-1	1.17 ± 0.14
Linker-2	ATP-4		7.74 ± 1.4
Linker-2	ATP-2		9.41 ± 1.59
Linker-2	ATP-1		11.23 ± 2.72
Linker-2	ATP-4	FMN-4	0.29 ± 0.15
Linker-2	ATP-4	FMN-2	0.25 ± 0.05
Linker-2	ATP-4	FMN-1	0.27 ± 0.07
Linker-2	ATP-2	FMN-4	0.28 ± 0.14
Linker-2	ATP-2	FMN-2	0.25 ± 0.14
Linker-2	ATP-2	FMN-1	0.13 ± 0.04
Linker-2	ATP-1	FMN-4	0.28 ± 0.15
Linker-2	ATP-1	FMN-2	0.13 ± 0.03
Linker-2	ATP-1	FMN-1	0.16 ± 0.17
Linker-4		FMN-4	0.97 ± 0.06
Linker-4		FMN-2	1.07 ± 0.05
Linker-4		FMN-1	1.03 ± 0.10
Linker-4	ATP-4		6.28 ± 1.59
Linker-4	ATP-2		7.29 ± 1.75
Linker-4	ATP-1		7.21 ± 1.15
Linker-4	ATP-4	FMN-4	0.17 ± 0.09
Linker-4	ATP-4	FMN-2	0.23 ± 0.25
Linker-4	ATP-4	FMN-1	0.12 ± 0.09
Linker-4	ATP-2	FMN-4	0.27 ± 0.05
Linker-4	ATP-2	FMN-2	0.70 ± 0.24
Linker-4	ATP-2	FMN-1	0.25 ± 0.04
Linker-4	ATP-1	FMN-4	0.27 ± 0.05
Linker-4	ATP-1	FMN-2	0.23 ± 0.05
Linker-4	ATP-1	FMN-1	0.51 ± 0.15

which both aptamers were connected *via* a DNA linker. A potential reason for the slightly lower affinities could be a reduced flexibility of the constructs created from one RNA molecule compared to the DNA-hybridized linker constructs (Fig. S2†).

Next, we investigated the specificity of the dual aptamer constructs. As expected, we found that the ATP aptamer had no affinity to FMN, and neither had the FMN aptamer to ATP. In general, we showed that the introduction of the linker sequence did not influence the aptamers' affinities for the respective analyte. However, the affinities to FMN of a FMN aptamer and a combined ATP/FMN-aptamer were in the same range (K_d of 163 nM *versus* K_d of 186 nM, Table S4†). We therefore concluded that the combined aptamer constructs are not highly specific for FAD, but are also able to bind FMN with approximately the same affinity.

One disadvantage of SPR spectroscopy is the possibility of intermolecular interactions: the single constructs could be bound on the chip in close proximity to each other, which might facilitate intermolecular binding of FAD to two aptamers that are not part the same dual construct. Such an avidity effect would as well result in enhanced affinity. This hypothesis could explain similar affinities found for most of the analysed dual constructs and only marginal influences of spacer lengths. In order to exclude intermolecular binding/avidity contributions we verified the obtained results by carrying out fluorescence-based binding studies in solution.

For this purpose, we used the inherent fluorescence of FAD and its quenching upon binding to nucleic acids. In contrast to SPR experiments this method did not require immobilization of the aptamer constructs or FAD. Measurements were carried out with dilution series of PAGE-purified aptamer constructs. Intriguingly, we found that the resulting K_d values were in the same range as the values obtained by the SPR experiments (Table S5†). For the single ATP aptamer no fluorescence quenching was observed because the adenosine and not the fluorescent isoalloxazine system is bound by the RNA. Interestingly, the fluorescence measurements indicate that spacer length variation has a larger influence on binding affinities than previously noted. For example, the comparison of ATP and FMN aptamers with and without a DNA linker revealed an approximately 3- to 6-fold higher affinity for the more flexible aptamers joined together by the DNA linker (Table S5†). Although single FMN aptamers had binding affinities in the low μM range for FAD as well, our SPR and fluorescence measurement assays showed a significant decrease of the K_d for the combined constructs in comparison to the single aptamers.

Conclusions

We developed a platform to easily design and investigate high-affinity-aptamers. Taken together we showed that high-affinity FAD aptamers can be created by combining ATP and FMN

aptamers in a dual aptamer construct. In a comprehensive study we systematically investigated dual aptamer constructs connected by different spacer lengths on a DNA linker sequence. Using this design strategy, a number of other aptamers could potentially be combined for the creation of high-affinity aptamers. For example, recently further examples of cyclic di-nucleotides (CDNs) were discovered as biological signalling molecules.[21] By combining aptamers for adenosine and guanosine, the three compounds c-di-GMP, c-di-AMP, and c-AMP-GMP could be sensed by designing recognition sequences in a highly modular fashion. Furthermore, we demonstrated the basic principle of immobilizing the aptamer constructs to a Biacore CM5 Chip by hybridization to a complementary and covalently bound DNA strand. We obtained K_d values for the single aptamers that were in good agreement with reported values obtained by other techniques.[10,11] The affinity and specificity properties of aptamers show great potential for diagnostic, therapeutic, and analytical applications.[22] They are interesting candidates for sensors in the fields of environmental monitoring and medical diagnostics.[23,24] Moreover, in the context of riboswitches, they show important regulative biological functions.[25–27] New high-affinity aptamers could potentially be used as drugs or as ligand-binding domains in synthetic riboswitches in order to control gene expression.[28,29]

Notes and references

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