Role of Mg$^{2+}$ ions in flavin recognition by RNA aptamer

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The role of Mg$^{2+}$ ion in flavin (flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)) recognition by RNA aptamer has been explored through steady state and time-resolved fluorescence, circular dichroism (CD), thermal melting (TM) and isothermal titration calorimetry (ITC) studies. A strong quenching of flavin emission is detected due to stacking interaction with the nucleobases in the mismatched region of aptamer, and it enhances manifold with increasing Mg$^{2+}$ concentrations. A comparatively lower binding affinity toward FAD compared to FMN is attributed to the presence of intramolecular 'stack' conformer of FAD, which cannot participate in the intermolecular stacking interactions with the nucleobases. CD and TM studies predict that flavin detection causes structural reformation of RNA aptamer. ITC results indicate that flavin detection is thermodynamically feasible and highly enthalpy driven.

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1. Introduction

Aptamers are defined as small sequence of nucleotides which are engineered through repeated rounds of in vitro systematic evolution of ligands by exponential enrichment (SELEX) and can sense specific molecular targets with high selectivity [1–4]. RNA aptamers are characterized by unpaired or mismatched bases which help to fold the RNA in distinct secondary and tertiary structures containing loops and bulges. These loops and bulges are suitable for selective interactions with small molecules, proteins, nucleic acids, cells, tissues and organisms [5–11]. Although the importance of RNA aptamer as small molecular sensor has been portrayed in a number of reports [12], a little has been focused on flavoprotein cofactor binding aptamers. Flavins are the most ubiquitous cofactor [13–16] in nature is known to catalyze a huge number of redox reactions. From recent past flavin binding RNA aptamers had been highlighted as point of interest. Burgstaller et al. first isolated aptamer sequences, which were able to recognize flavin co-factors [17,18]. NMR results concluded that isoalloxazine ring of flavin mononucleotide (FMN, Scheme 1) can intercalate between G-G mismatch and G-U-A base triple, which results the selective affinity for flavins [19]. Moreover, this recognition specificity is associated with hydrogen bonding of the uracil like edge of isoalloxazine to Hoogsteen edge of adenine at the interaction site. This structural stabilization had been employed to the design allosteric ribozyme whose self-cleavage and self-ligation reactions were enhanced nearby 300-fold in presence of FMN [20,21]. Later Lauhon et al. isolated riboflavin and nicotinamide detecting RNA aptamer which upon binding with riboflavin resulted a two-tiered G-quartet structure [22]. Although selective to redox co-factors, aptamers never found to differentiate between oxidized and reduced form of 5-deazariboflavin derivative, which is a structural analog of riboflavin [22]. SELEX method was also used to identify RNA aptamers that recognizes flavin adenine dinucleotide (FAD, Scheme 1) but unable to distinguish FAD from FADH$_2$ [23]. Through experimental and computational methodologies Anderson et al. detected 14-mer RNA aptamer which was selective to flavins (riboflavin, FMN and FAD) and showed better interaction with FMN compared to others [24]. The electron density on the phosphate group was speculated to partially delocalize on the flavin ring, which strengthened hydrogen bonding and $\pi$-stacking interactions between isoalloxazine and nucleobases [24]. Notably, literatures lack any detailing about higher selectivity toward FMN as well as structural information about FAD-aptamer complexation. The major complexity of FAD compared to FMN is the existence of FAD in variable conformations (namely, ‘stack’, ‘partially stack’ and ‘unstack’) in water, as it constituted by a fluorophore (isoalloxazine) and a quencher (adenine) [25–27]. Stack conformer of FAD is more stable than unstack the conformer due to the $\pi$-$\pi$ stacking interaction between flavin and adenine moiety. This stacking interaction results a quenched lifetime of about 9 ps due to the excited state intramolecular electron transfer from adenine to flavin moiety [25–27]. In this context, it seems interesting to explore particularly which conformer of FAD is appropriate for the interaction with aptamer. Since the dinucleotide cofactors have special evolutionary importance, it is worth to have a better insight about their interactions with RNA aptamer (Scheme 1). We have...
and NaH$_2$O$_2$ (Molecular biology grade) were procured (biograde, Aldrich and Na$_2$HPO$_4$ and Na$_2$HPO$_4$ (Molecular biology grade) were procured from Sisco Research Laboratories (SRL-India). NaCl (Bioxtra purity ≥ 99.5%) and EDTA (BioUltra purity ≥ 99%) were purchased from Sigma Aldrich. RNA aptamer (100 nano-moles synthesis scale, HPLC purified) was brought from integrated DNA technology (IDT). Phosphate buffer (Na$_2$HPO$_4$ + Na$_2$HPO$_4$, 10 mM) of pH 7.0 (containing 150 mM NaCl, 0.1 mM EDTA) was used for all sample preparations, dilutions and experiments. The Mg$^{2+}$ ion concentration was maintained by addition of concentrated stock of MgCl$_2$ (biograde, Sigma–Aldrich, purity ≥ 97%). All the samples and buffer preparation were done in autoclaved Milli-Q water (18.2 μS cm$^{-1}$).

For all the spectroscopic studies, concentrations of FAD and FMN were kept at ~5 μM to avoid molecular aggregation. RNA concentration was varied from 1 μM to 50 μM for steady state and time-resolved fluorescence measurements. The molar extinction coefficient (347,200 M$^{-1}$ cm$^{-1}$, from IDT) at 260 nm was used for preparation of RNA solution. Before using RNA, it was annealed up to 90 °C in phosphate buffer saline (PBS) with respective Mg$^{2+}$ ion concentration and kept overnight at 4 °C to have a stable tertiary structure of the RNA. All the spectroscopic and calorimetric data collections were done at 298 K, unless otherwise mentioned.

2.2. Instrumentations

Solution pH was measured by pH-1500 (Eutech Instruments, USA) and verified by silicon micro sensor pocket sized pH meter (isfetcom. Co. Ltd., Japan). Absorption spectra were recorded in Evolution 300 UV–Visible spectrophotometer (Thermo Fisher Scientific, USA). Thermal melting (TM) studies were performed in CARY-300 Bio UV–Vis spectrophotometer (Agilent U.S.A.). RNA melting studies in absence and in presence of flavin were done by heating the sample from 20 °C to 90 °C followed by slow cooling up to room temperature. The concentration of flavin (10 μM) and RNA aptamer (2 μM) were kept fixed in TM studies.

Steady state fluorescence spectra were collected in Fluorolog-3 (Horiba JobinYvon, U.S.A.). Fluorescence lifetimes were collected from time-correlated single photon counting (TCSPC) set-up from IBH Horiba JobinYvon (U.S.A.) using 444 nm diode laser. The detail description of the instrument is mentioned elsewhere [32,33]. Analysis of lifetime data was done by IBH DAS6 software. The lifetime data were fitted with a minimum number of exponential. Quality of each fitting was judged by $\chi^2$ values and the visual inspection of the residuals. The value of $\chi^2 \approx 1$ was considered as best fit for the plots.

Circular dichroism (CD) spectra were recorded on a J-815 CD spectro-polarimeter (JASCO, USA). Each CD profile is an average of 5 scans of the same sample collected at a scan speed of 20 nm/min. During CD measurement, RNA concentration was kept fixed and the concentrations of flavin was increased steadily. For CD studies a fixed concentration of RNA aptamer (~2 μM) was titrated with increasing flavin concentrations (up to ~10 μM for FAD and FMN). CD spectrum of an identical concentration of blank flavin (~10 μM for FAD and FMN) was collected and subtracted from CD spectrum of flavin containing RNA aptamer.

2. Experimental section

2.1. Materials and methods

FAD (HPLC grade, purity ≥ 96%) was purchased from Sigma Aldrich and FMN was bought from Fluka (HPLC grade, purity ~90%). Both the flavins were used without further purification. Na$_2$HPO$_4$ and Na$_2$HPO$_4$ (Molecular biology grade) were procured from Sisco Research Laboratories (SRL-India). NaCl (Bioxtra purity ≥ 99.5%) and EDTA (BioUltra purity ≥ 99%) were purchased from Sigma Aldrich. RNA aptamer (100 nano-moles synthesis scale, HPLC purified) was brought from integrated DNA technology (IDT). Phosphate buffer (Na$_2$HPO$_4$ + Na$_2$HPO$_4$, 10 mM) of pH 7.0 (containing 150 mM NaCl, 0.1 mM EDTA) was used for all sample preparations, dilutions and experiments. The Mg$^{2+}$ ion concentration was maintained by addition of concentrated stock of MgCl$_2$ (biograde, Sigma–Aldrich, purity ≥ 97%). All the samples and buffer preparation were done in autoclaved Milli-Q water (18.2 μS cm$^{-1}$).

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Thermodynamics of the binding of flavin to RNA aptamer was estimated using isothermal titration calorimeter (Microcal, ITC 200, USA). A fixed concentration of RNA stock solution (20 μM) was kept in titration cell as a titre and was titrated by flavins (FAD and FMN individually) having a concentration of 120 μM. Each experiment consisted of 20 injections (each containing 2 μL titrant volumes except the first injection as its volume was 0.5 μL) with a successive time gap of 200 s between two injections. For proper mixing the solutions, the stirring speed was maintained at 1000 rotations/min (RPM). Cell temperature was kept fixed at 298 K and nearly 3 successive titrations were used to confirm the ITC curves and were fitted using ‘single site binding’ model. A blank titration of identical flavin concentration (4/24120 μM) with blank PBS containing respective Mg²⁺ ion concentration was subtracted from the sample data in order to correct the dilution enthalpy.

3. Results and discussions

3.1. Steady state absorption and emission results

FAD absorption in water and in presence of RNA aptamer (containing 10 mM Mg²⁺) is shown in Fig. 1a and the absorption spectra at lower Mg²⁺ concentrations (2 mM and 4 mM) are provided in supporting information (Fig. S1). Flavin absorption in water is characterized by two intense II–II' transition bands originated from isoalloxazine chromophore corresponding to $S_0 \rightarrow S_1$ ($\lambda_{abs} \sim 450$ nm) and $S_0 \rightarrow S_2$ ($\lambda_{abs} \sim 375$ nm) transitions (Fig. 1). With increasing concentration of RNA aptamer, absorption at both of $\sim 375$ nm and $\sim 450$ nm bands of FAD decreases, which becomes more prominent at higher Mg²⁺ concentrations. Moreover, a prominent red shift is detected especially for $S_0 \rightarrow S_1$ band at higher RNA concentrations. Interestingly this shift does not depend over Mg²⁺ concentrations. FMN, a structural analog of FAD, exhibits comparable decrease in absorbance with increasing RNA concentration (Fig. 1b). A prominent red shift is also detected for FMN along with a peeping hump at $\sim 480$ nm,
which may originate from lower energy transition of RNA-bound isoalloxazine chromophore. The red shift in absorption spectra for both FMN and FAD may be attributed to the H-bonding interaction between flavins and aptamer [37]. Mg$^{2+}$ concentration dependent decrease in absorbance along with a red shift are preliminary indications toward the interaction between flavins and aptamer.

Flavins (FAD/FMN) exhibit an unstructured emission at ~530 nm in buffer (PBS, pH 7.2), which originates from isoalloxazine ring [26,27,32,35,36,38]. To explore the effect over flavin fluorescence upon binding to aptamer, a fixed concentration of FAD/FMN in buffer is titrated by rising concentration of RNA aptamer (with 10 mM Mg$^{2+}$) (Fig. 2). Emission spectra at lower Mg$^{2+}$ concentrations are shown in supporting information (Fig. S2). The emission intensity of both FMN and FAD decreases sharply with increasing RNA concentration. The extent of quenching for FMN is higher than FAD at any Mg$^{2+}$ concentrations and is clearly visible from Stern–Volmer plots shown in Fig. 2c (a detailed description of SV plots and results is given in Note S1 and Fig. S3). Moreover, FMN offers a more prominent hypochromic shift in emission spectra compared to FAD (Fig. 2, insets). The blue shift and a strong quenching infer effective binding interaction between flavins and RNA aptamer. The difference of quenching between FAD and FMN is attributed to different extent of affinity and selectivity of the aptamer toward flavins. A relatively lower extent of quenching for FAD can be attributed to its conformational flexibility ('stack' and 'unstack'). The 'stack' conformers of FAD being intramolecularly involved in stacking interaction (flavin ring is already involved in stacking interaction with adenine moiety of FAD) cannot participate in the inter-molecular stacking interaction with RNA aptamer. Hence, FAD offers a lesser extent of quenching. The binding interaction between FAD/FMN and aptamer is further confirmed from the changes observed in steady state anisotropy (Fig. 3a). Anisotropy dictates the extent of depolarization mainly contributed by the rotational diffusion process [27,32]. The molecular association process restricts the rotational diffusion of the fluorophore, and thereby leads to an increase in anisotropy. Therefore, an increase in steady state anisotropy infers that the flavins experience rigid environment when they bind with RNA aptamer.

Next, when we focus on probable mechanism(s) of quenching, and two major possibilities come into the picture. Either resonance energy transfer between RNA nucleobases and flavin can quench the fluorescence or electron transfer from nucleobases to flavin may also lead to the quenching. Since there is no overlap between the emission spectra of flavin (donor) and the absorption spectrum of any of the nucleobases (acceptor) the possibility of energy transfer can easily be ruled out. Therefore, plausible reason for quenching is the electron transfer between nucleobases and isoalloxazine ring, where nucleobases (e.g. adenine $E_{ox} = 1.5$ eV [39])/adenosine $E_{ox} = 1.46$ eV [39,40], uracil ($E_{ox} = 0.88$ eV [41]), cytidine ($E_{ox} = 1.6$ eV [39,40]), and guanosine ($E_{ox} = 1.29$ eV [39,40])) act as potential electron donor to photoexcited flavin ring ($E_{red} = -0.24$ eV [42]), and results a heavily quenched fluorescence of flavin. Notably, similar quenching behavior of FAD and FMN inside the protein nanocavity was recently reported by us and we proposed a possible ultrafast electron transfer from aromatic amino acid to FMN/FAD inside the binding pocket as a reason for observed quenching effect [32]. As the absorption spectra of flavins (FMN/FAD) are also getting affected by the RNA aptamer, we cannot rule out the possibility of static quenching or ground state complex formation, which may also contribute to the quenching process, and can be verified by the lifetime results discussed in the later part of the manuscript.

Next obvious question is the probable binding location of flavins in RNA aptamer. Among various possibilities, we first focus intercalation mode of binding and groove binding mode of interaction in the RNA aptamer (Scheme 1). To verify these possibilities, we have checked the interaction scenario of flavin with 18-mer inter-complementary RNA [(AU)$_{18}$]. Being completely double stranded, (AU)$_{18}$-flavin interaction is possible only through intercalation or groove binding. We have found both FMN and FAD do not show any alteration in emission intensity/position with increasing (AU)$_{18}$ concentration (Fig. S4a). The above observations indicate that no interaction is going on between (AU)$_{18}$ and flavin. On the basis of these evidences, we have inferred that FAD/FMN is not intercalating or groove binding in the stem region (double stranded region) of the aptamer. Therefore, the observed changes in intensity as well as in absorption profiles infer that FMN/FAD is interacting with the mismatch sequences (bulge region) of RNA aptamer (Scheme 1). It is well known that several ligands and small molecules bind to the bulge and loop segments of RNA [5–11]. Previously, NMR followed by MD simulation showed isoalloxazine ring of FMN involves in stacking interaction with the flanking G-G pair and G10.U12.A25 triple in bulge segment of RNA aptamer [19]. In summary, steady state results indicate that flavin ring of both FMN and FAD interacts with mismatch bases, like G9.G27, G10-U12-A25 triple and A13-G24 in the bulge segment of RNA aptamer, and stacking interactions between the nucleobases and flavin ring lead to ultrafast electron transfer from nucleobases to flavin.

A very indulging care has been taken to the fluorescence study with variable concentrations of Mg$^{2+}$, as it is known that tertiary structure of RNA strongly depends on Mg$^{2+}$ concentration [28,29].
We found with increasing Mg²⁺ concentration (from 2 mM to 10 mM), the relative quenching for both of FAD and FMN enhances significantly (Fig. 2c). Moreover, the quenching is higher for FMN compared to FAD (Fig. 2c). To verify that FAD dynamics is not affected by Mg²⁺, we have titrated a fixed concentration of FAD with increasing Mg²⁺ under identical experimental condition, and interestingly we found no variation in intensity or emission position (Fig. S4b). Hence, variable effect in emission intensity at higher Mg²⁺ concentrations appears due to structural stabilization of flavin bound RNA aptamer. It is well established that Mg²⁺ involving in stacking interactions with the mismatch nucleobases is obtained from Scatchard plot (Note S2, Fig. S5) [43]. Mg²⁺ and to check the reversibility of the binding process, temperature increases to 37°C. We noticed the emission intensity of flavins reverts back to its natural, RNA aptamer structure collapses and aptamer-bound flavin has additional conformational flexibility.

To confirm the binding interaction of flavins with RNA aptamer and to check the reversibility of the binding process, temperature induced fluorescence measurement is performed (Fig. 3b). We have noticed the emission intensity of flavins reverts back to its initial value with increasing temperature. With increasing temperature, RNA aptamer structure collapses and aptamer-bound flavin gets released into buffer. As temperature induced denaturation of RNA aptamer results the retrieval of flavin intensity, it can be inferred that flavins are subjected to stacking interactions with the nucleobases of the aptamer. This observation also corroborates well with the conclusion that flavin ring of both FAD and FMN involves in stacking interactions with the mismatch nucleobases in the bulge region of aptamer.

3.2. Association behavior of flavins-aptamer interaction

A quantitative estimation of binding affinity between flavins and aptamer is obtained from Scatchard plot (Note S2, Fig. S5)[43]. Mg²⁺ concentration dependent studies show a monumental impact over binding constant of flavin with RNA aptamer. FAD at 2 mM Mg²⁺ concentration offers a binding constant 1.40 × 10⁴ M⁻¹, which enhances ~100 fold at maximum Mg²⁺ concentration (10 mM) (Table 1). A more intriguing results is observed for FMN, which shows a 10 fold higher association constant compared to FAD at 2 mM Mg²⁺ concentration and rises up to 2.0 × 10⁷ M⁻¹ at 10 mM Mg²⁺ concentration. The high binding affinity of FMN/FAD in presence of higher Mg²⁺ is attributed to the formation of a more stable tertiary structure of RNA aptamer. Moreover, Mg²⁺ favor the interaction between nucleobase and isooxazoline by screening the negative charge of backbone phosphates. These results closely resemble to the binding constant determined from fluorescence assay performed by Anderson et al. and it was shown that FMN exhibits three times higher binding affinity than that of FAD [24]. Although the reason of lower binding affinity was not addressed in the previous literature but in present work we do. We have shown that conformational flexibility of FAD may be intriguing factor for the lesser binding affinity as well as selectivity toward the RNA aptamer. Binding constants along with the free energy changes (Table 1) infer that the detection of flavin by the aptamer is highly energy favored and Mg²⁺ ion dependent process.

3.3. Impact over RNA structure; CD and thermal melting study

CD signal is sensitive to minute structural changes in bio-molecules like polynucleotides (DNA/RNA) or proteins during interaction with ions, ligands or macromolecules [32,44,45]. Hence, the impact over aptamer structure due to Mg²⁺ as well as flavin binding is elucidated from circular dichroism studies (Fig. 4a). RNA aptamer alone in PBS exhibits two dips (at ~210 nm and ~235 nm) along with a peak at ~265 nm. A reasonable number of previous reports already characterized this features as a signature of A-form of RNA, where the base pairs are tilted relative to helix axis [46–50]. Note that most of the RNA structures found in nature are abundant in A from containing bulges and/or loops, which are able to selectively trap small molecules and ligands [46–50]. The CD spectra of aptamer show dependence on Mg²⁺ concentration (as the peak at 265 nm is increased by ~20%, while moving from 2 mM to 10 mM of Mg²⁺ concentration), which infers that aptamer structure is gaining more stability at higher Mg²⁺ concentration. Here it is relevant to mention that Jack et al. also observed similar changes in CD spectra of an antiterminator model RNA, when the Mg²⁺ concentrations increased from 0 to 15 mM. They attributed this small changes to the increased base stacking interaction in presence of higher Mg²⁺ concentration [50]. In presence of flavin (either FAD or FMN) a detectable decrease in CD signal is noticed at ~265 nm (Fig. 4a). Moreover, a clear blue shift at ~265 nm peak is observed in CD spectrum due to FAD or FMN binding. The decreasing CD signal along with blue shift infers about the interaction of FAD/FMN with RNA aptamer. Similar changes for FMN and FAD in CD spectra confirm that isooxazoline ring is responsible for the interaction with RNA aptamer. Douthart et al. previously reported intercalation of ethidium bromide in ds-RNA through CD spectroscopy, where a visible increase at ~265 nm CD signal was concluded as an outcome of intercalation to RNA [46]. Therefore, the descending CD signal at ~265 nm for FAD and FMN infers about structural or conformational alteration of RNA aptamer due to flavin binding to the bulge region of the aptamer. Interestingly we did not notice any induced CD signal for FAD/FMN binding.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>[Mg²⁺] = 2 mM</th>
<th>[Mg²⁺] = 4 mM</th>
<th>[Mg²⁺] = 10 mM</th>
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<tr>
<td><strong>FAD</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>From fluorescence</td>
<td>K</td>
<td>1.40 × 10⁴ M⁻¹</td>
<td>1.20 × 10⁵ M⁻¹</td>
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<tr>
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<td>ΔG°</td>
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<td>−28.84 kJ/mol</td>
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<tr>
<td>From ITC</td>
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<tr>
<td></td>
<td>ΔH</td>
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<td>−(6.56 ± 0.16) × 10¹ J/mol</td>
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<tr>
<td></td>
<td>ΔS</td>
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<td>−102.83 J/mol/deg</td>
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<td></td>
<td>ΔG°</td>
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<td>−34.862 kJ/mol</td>
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<tr>
<td><strong>FMN</strong></td>
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<tr>
<td>From fluorescence</td>
<td>K</td>
<td>6.50 × 10⁷ M⁻¹</td>
<td>1.07 × 10⁸ M⁻¹</td>
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<tr>
<td></td>
<td>ΔG°</td>
<td>−33.022 kJ/mol</td>
<td>−34.28 kJ/mol</td>
</tr>
<tr>
<td>From ITC</td>
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<td>(8.60 ± 2.23) × 10⁹ M⁻¹</td>
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<tr>
<td></td>
<td>ΔH</td>
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<tr>
<td></td>
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<td>ΔG°</td>
<td>−32.64 kJ/mol</td>
<td>−40.05 kJ/mol</td>
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Induced CD for a polynucleotide–ligand interaction appears in case of either intercalation or groove binding phenomenon. The absence of induced CD signal is actually inferring that flavins are not intercalating or binding to the groove regions of RNA aptamer rather interacting with the mismatch regions of RNA, which also supports the previous conclusion regarding interaction between flavins and aptamer.

The structural stability of aptamer during flavin binding is also verified through thermal melting study. The aptamer without flavin shows two distinct sigmoidal regions showing melting at 42 °C and 72 °C. The lower melting temperature (42 °C) may appear due to the opening of small stem-loop structure from U14 to A23, whereas higher temperature (72 °C) may be attributed to the global melting of the RNA structure. The global melting temperature matches well with the value checked from IDT under identical salt concentrations. In presence of FAD and FMN, the melting temperature of small stem-loop structure is found to be varied from 42 °C for RNA alone to 46 °C for FAD-bound RNA and 47 °C for FMN bound RNA at 10 mM Mg²⁺ concentration. Interestingly, no such prominent change is observed for global melting temperature by interaction with either of the flavins. This observation might indicate that flavin binding stabilizes the stem-loop region of aptamer but does not perturb global melting of aptamer. These changes also infer that the isoxazol ring does not intercalate to the complimentary strand of aptamer as intercalation between base pairs is known to alter global melting temperature to a higher extent.

3.4. Isothermal titration calorimetry (ITC): a thermodynamic glimpse

For biomolecule–ligand interaction thermodynamic parameters play crucial role, as they provide detailed information about binding interaction, structural reformation and feasibility of the ligand-macromolecular association [51–56]. ITC profiles of flavins–aptamer interaction are shown in Fig. 5 and the results are depicted in Table 1. Each enthalpy burst in upper panel represents individual RNA injections, and the amount of heat liberated by the successive additions is plotted against the molar ratio of flavin to aptamer in the lower panel. A standard nonlinear least-square regression binding model (single site) is employed to fit the results. Binding stoichiometries at low Mg²⁺ ion concentration are determined to be 1.15 and 1.0 for FAD and FMN, respectively. At higher Mg²⁺ ion concentrations (10 mM) the ‘n’ (the molar ratio of ligand to macromolecule) values appear to be 0.7 and 0.67 for FAD and FMN, respectively and it suggests that each flavin molecule binds to single site of RNA aptamer, which is in agreement with our steady state and NMR [19] results, predicting 1:1 interaction between flavin and aptamer. The association constant obtained for FMN (8.60 × 10⁶ M⁻¹) is almost four times higher in magnitude compared to FAD (1.30 × 10⁶ M⁻¹) in presence of RNA (at 10 mM of Mg²⁺ concentration), which corroborates well with steady state emission results under similar experimental condition. The difference of association constant between FAD and FMN may appear due to conformational flexibility of FAD, which is not present in case of FMN. Table 1 offers a high negative heat of deflection for flavins binding to RNA aptamer, which indicates an exothermic nature of binding. We have already anticipated from steady state emission, CD and melting studies that isoxazol ring of FMN/FAD interacts with mismatch nucleobases in the bulge segment of the aptamer. Therefore, we believe the high negative enthalpy values obtained from ITC studies are resulted from the above mentioned interactions, and it suggest the bulge is sufficiently flexible and permits an optimal interaction with flavin. Interestingly, the change in entropy during flavins binding to aptamer is found to be negative for all cases, irrespective to Mg²⁺ concentrations. A negative change in entropy can be attributed to the favorable electrostatic interactions between ligands and RNA [55,57]. Note that the resultant entropy change appeared during flavins–aptamer binding is the combined effect of drug entropy change, RNA entropy change as well as entropy change of water during binding process [58]. It is obvious that drug entropy will decrease while binds to aptamer, and the flexibility of RNA decreases during the interaction with drug or protein [59]. On the other hand, water entropy generally increases when a drug binds to DNA/RNA, as water molecules are coming out from the surrounding of ligand binding location in RNA [58]. Therefore, the negative entropy is originated by the cancellation of positive entropy change for water by the negative entropy change of RNA as well as flavins. Moreover, few previous studies report about a large negative TAS value, which is likely to reflect the ligand assisted refolding of RNA aptamer [60]. The CD results also dictate some kind of conformational change of aptamer during binding interaction with flavins. The standard free energy changes calculated from enthalpy as well as entropy contributions are well corroborative with steady state fluorescence measurements at 298 K. Finally, the negative values of standard free energy changes conclude that the binding of flavins to aptamer is highly spontaneous in nature.

3.5. Time resolved measurements

Time resolved fluorescence measurement is highly sensitive to the excited-state interaction between the probe and RNA, and hence is a unique technique to depict the environment around the fluorophore in excited state. Thus, fluorescence lifetime
measurements are performed to understand the interaction scenario between flavins and RNA aptamer. FMN exhibits single exponential decay at pH 7 having a lifetime \(4.3 \text{ ns}\) (Fig. 6). In presence of RNA, a new short-lived component of \(200 \text{ ps}\) appears for FMN, and average lifetime reduced (Fig. 6). Unlike FMN, FAD exhibits three distinct lifetime components at pH 7, a \(10 \text{ ps}\) component (appears from stack conformer of FAD), \(2.2 \text{ ns}\) (arises ‘partially stack’ conformer) and \(4 \text{ ns}\) lifetime (appears from open conformer of FAD) \[26,27,61\]. Note that due to limited time response of TCSPC (\(40 \text{ ps}\)) instrument, we are not able to detect the lifetime of ‘stack’ conformer. Hence, we detect a bi-exponential decay for FAD with two distinct lifetimes of \(4.3 \text{ and } 2.3 \text{ ns}\) having relative contributions of 55% and 45%, respectively (Fig. 6, Table S1). The \(4.3 \text{ ns}\) component represents the ‘unstack’ conformer, whereas the \(2.3 \text{ ns}\) component corresponds to ‘partially stack’ conformer of FAD in which the isoalloxazine moiety does not stack but interacts with the other parts of the molecule \[26,27\]. With the gradual increase in RNA concentration, the lifetime of the two conformers remains same but offers a decreasing contribution of the long component. Moreover, a new component with a reduced lifetime of around \(200 \text{ ps}\) appears in presence of RNA. The reduced lifetime appeared in the decay profiles of both FMN and FAD (Fig. 6, Table S1) is an outcome of the quenching effect of isoalloxazine fluorescence by the nucleobases. In a nutshell, the lifetime results are in accordance with our steady state fluorescence results, where we have also detected quenching for flavin at higher concentration of RNA aptamer. Here it is pertinent to mention that the extent of quenching (90%) observed in the steady state results are not reflected in the time-resolved data both for FMN and FAD. This observation tempted us to conclude that

Fig. 5. Isothermal Titration Calorimetry profiles of (a) FAD (in 4 mM Mg\(^{2+}\)) and (b) FMN (in 4 mM Mg\(^{2+}\)) with increasing concentration of RNA aptamer, whereas (c) and (d) represents ITC of FAD and FMN with RNA aptamer in presence of 10 mM Mg\(^{2+}\) ion concentration, respectively. The upper panels in all cases represent change in enthalpy at each injection, whereas the lower panels show the overall variation in enthalpy for the process.
significant population of flavins are involved in static quenching or
ground state complexation (we have already mentioned in steady
state section) and those molecules do not take part in fluorescence;
as a result, they do not contribute to the measured lifetime.

4. Conclusion

Present work describes the detailed biophysical insight of flavin
(FAD and FMN) recognition by RNA aptamer. Steady state and
time-resolved fluorescence results depict quenching of flavin due
to stacking interactions with mismatch bases like G9.G27,
G10-U12-A25 triple and A13-G24 in the bulge region of aptamer.
Circular dichroism (CD) and thermal melting (TM) studies predict
that flavin binding causes structural reformation, majorly in the
small stem-loop segment of the aptamer. Isothermal titration
calorimetry (ITC) study shows flavin detection is mainly enthalpy
driven process. The negative changes in standard Gibb’s free
energy conclude about the feasibility of interaction. We have also
observed that FAD shows relatively lower binding affinity to apt-
amer compared to FMN, which is attributed to the conformational
flexibility of FAD. As ‘stack’ conformer of FAD remains silent during
binding process due to the involvement of intramolecular stacking
interaction between isoalloxazine ring and adenine moiety, a rea-
sonable population of FAD conformers cannot participate to further
external stacking interaction with nucleobases of aptamer.
Moreover, we have noticed that the feasibility of the recognition
process is highly Mg$^{2+}$ ion concentration dependent, as variation of Mg$^{2+}$
concentrations alters the stability of RNA aptamer.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in
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