Chapter 5

Light-Regulated RNA–Small Molecule Interactions
5.1 Introduction

The ability to regulate key properties of chemical or biological systems by external stimuli holds much promise for the fields of material and life sciences. One of the most attractive stimuli is light, because it can be applied with very high spatiotemporal resolution, with a wide range of intensities and wavelengths and without invasive effects on living organisms.\textsuperscript{1,2} For example, a light-switchable gene expression system could precisely control spatiotemporal gene expression in multicellular organisms.\textsuperscript{3} One way to achieve this is to exploit photochromic molecules that show distinctive changes in their properties upon irradiation. Among the photoswitches, azobenzenes are most widely used for biological studies, due to their easy availability and the considerable geometrical differences between trans and cis isomer.\textsuperscript{4} So far, UV light is the mostly applied source to induce photoisomerization of azobenzenes. However, photons in this region exhibit high energy, which is associated with high phototoxicity. Moreover, UV light does not penetrate deeply into tissue. These disadvantages greatly limit its application in the biological context. A straightforward solution to circumvent these limitations is to use light in the visible and/or near-infrared (NIR) region that shows deeper tissue penetration and lower phototoxicity.\textsuperscript{5} For this purpose, the group of Hecht rationally designed redshifted azobenzene derivatives by introducing electron-withdrawing fluoro substituents.\textsuperscript{6} This molecular engineering yielded bistable molecular switches with extremely long thermal half-life in both trans and cis form.

In this context, a series of photoswitchable aminoglycosides containing azobenzene moieties were synthesized in Chapter 4. It was found that one paromomycin dimer showed different antibiotic activities against bacteria carrying resistance genes before and after visible light irradiation. Encouraged by these results, in this chapter we decided to study the interactions between these photoswitchable dimers and a RNA aptamer for paromomycin. For this purpose, a well characterized aptamer sequence was chosen. It was found that this aptamer bound to the trans form of a fluorinated azobenzene paromomycin dimer (F-dimer) 100 times stronger than to the corresponding cis isomer of F-dimer. Since the binding event can be switched by visible light, this result provided a promising first-step towards the development of an artificial riboswitch that regulates gene expression by light.

5.2 Results & Discussion

5.2.1 Design and synthesis of photoswitchable aminoglycosides
During the past years, riboswitches have been developed as regulatory elements of messenger RNA (mRNA) molecules that binds small molecules and this binding event results in a change in translation of the proteins encoded by the mRNA. Our group recently designed a riboswitch which is comprised of a sensor that recognizes Neomycin B as a small molecule input signal, an actuator that controls the output signal and a transmitter that channels the signal from the sensor to the actuator. In order to achieve photocontrol of protein production at the RNA level, here we describe a novel artificial photoresponsive gene expression system based on the aptameric sensor in our riboswitch that can selectively bind with only one isomer of the photoswitchable small signaling molecule (Figure 5.1). Upon binding with this isomer, the riboswitch undergoes a structural rearrangement which can unveil the ribosome binding site and hence activate the downstream gene expression. To this end, the first-step is to find a suitable aptamer-photoswitchable small molecule pair in which only one isomer can specifically recognize the aptamer with high binding affinity.

**Figure 5.1.** Control of bacterial gene expression by aptamers against photoswitchable small molecules as genetic control elements. In the present case, the aptamer recognizes only one isomer of the photoswitchable target, represented as the green circle.

Figure 5.2(b) shows the structures of both the “trans” and “cis” isomers of photoswitchable azobenzenes that we employed as photoresponsive building blocks. Ortho-fluoroazobenzene has been selected due to their high photoconversion efficiencies as well as excellent thermal stabilities of both of its isomers. We designed three photoswitchable dimeric aminoglycoside bearing azobenzene and ortho-fluoroazobenzene called H-dimer, F-dimer and F-dimer-azide (Figure
5.2c), and envisioned that the trans and cis isomer would bind RNA target with different affinity, therefore providing the possibility to further control the transcription and gene expression using visible light.

**Figure 5.2.** Molecular structures of photoswitchable paromomycins. (a) Structure of paromomycin and the pKa values of the amino groups. (b) Schematic of azobenzene trans-cis isomerization. The photoisomerization of azobenzene (R=H) is traditionally triggered by UV and visible light irradiation. Irradiation at 365 nm leads to conversion of the trans isomer to the cis isomer and visible-light switches the cis isomer back to the trans form. The cis to trans isomerization occurs also thermally at ambient temperature, albeit at a slower rate than the photoisomerization. In ortho-fluorooazobenzenes (R=F), visible light is used for both trans to cis (λ > 500 nm) and cis back to trans (λ = 450 nm) isomerizations. (c) Molecular structures of H-dimer (1), F-dimer (2) and F-dimer-azide (3).

In general, H-dimer (2) and F-dimer (3) were synthesized from paromomycin and NHS activated ortho-fluorooazobenzene dicarboxylic acid / azobenzene-4, 4’-dicarboxylic acid which were prepared by an extension of the previously reported procedure. NHS activated azobenzene dicarboxylic acid and paromomycin in tenfold excess were utilized to provide paromomycin dimers with the same procedure as described in chapter 4. After purification from HPLC, the final compounds were
obtained as the trifluoroacetate salts with ~20% yield due to the possible steric hindrance between the two bulky aminoglycosides.

Scheme 5.1. Mono-functionalization at ring IV of paromomycin with azobenzene as a linker.

For further application in bacterial cells, 2 and 3 will be toxic due to their antibiotic characteristics. With this consideration in mind, we synthesized a F-dimer derivative, which was anticipated to keep the binding affinity with RNA aptamer but with reduced antibiotic activity. It was prepared from F-dimer (3) by a one-step diazo transfer reaction at the C3-position of ring I of paramomycin according to the procedure developed by our group before (Scheme 5.2). This synthetic shortcut is based on the application of the shelf-stable, non-explosive and water soluble diazo-transfer reagent, imidazole-1-sulfonyl azide 5 (Scheme 5.2), allowing regioselective azide introduction at the least basic amino group at the 2-DOS ring in F-dimer. After the synthesis, HPLC and NMR are used to purify and characterize the target compound F-dimer-azide. (see Figure 5.5)
Scheme 5.2. Regioselective azide introduction in C3 position of 2-DOS ring applying diazo-transfer reagent 7·HCl.

5.2.2 Identification of the structure of F-dimer-azide

The structure of H-dimer and F-dimer were verified and discussed in chapter 4. The HPLC fraction containing di-azido F-dimer derivatives were purified and characterized by 1D- and 2D-NMR spectroscopy to determine at which position the azide was introduced. As shown in Scheme 5.2, F-dimer was transformed regioselectively at the 2-DOS ring resulting in aminoglycoside derivative 4.

The HSQC spectrum of derivative 4 (Figure 5.3) shows that the amino group in C3 position of 2-DOS ring reacted regioselectively. In comparison to the 2D-spectrum of paromomycin F-dimer (Figure 5.3b) the \( J(C3-H) \) coupling shows a remarkable chemical shift to lower field proving the regioselective azide introduction at this position. As determined by \(^1\)H-NMR spectroscopy, the regioselectivity of the reaction is approx. 85%. The selective transformation of the amino group at the C3-position can be explained by the different basicity of the amino groups. While five out of six amino groups have a pKa value ranging from 7.55 to 8.8, the amine in C3 position of the 2-DOS ring is less basic with a pKa of 5.74 (Figure 5.2a).\(^6\) Therefore, only this amino group is mainly accessible for the reagent 7·HCl under the applied conditions, because all other amines are protonated and inactive for transformation.

Moreover, to avoid the use of aqueous buffer solutions, the reaction was performed in water in the presence of sodium hydroxide to adjust an initial pH of 6.4 in the reaction mixture. Here, 24 equivalents of imidazole-1-sulfonyl azide 7·HCl were applied to ensure high conversion.
5.2.3 Photochemical behavior and Thermal stability of the cis isomers

Overall, the following data show that these compounds undergo the isomerization in a reversible manner, establishing a set of photochromic RNA aptamer ligands with visible light responsive properties. UV-vis absorption spectra of the F-dimer-azide were measured in water. Photoisomerization was monitored by UV-vis spectroscopy, UPLC, and 1H NMR. This compound exhibits azobenzene-specific reversible photoisomerization (Figure 5.4), generating a cis isomer upon irradiation with green light (>500 nm). Reversion to the trans form is expected to occur upon irradiation with blue light (450 nm). The corresponding changes in the absorption spectra during irradiation are shown in Figure 5.4 as the other two switches H-dimer and F-dimer were described in chapter 4. They share the emergence of a broad absorption band between 230 and 600 nm with maxima at 316 nm, which is a typical strong $\pi \rightarrow \pi^*$ absorption band for trans isomer of ortho-fluoroazobenzenes. A weaker $n \rightarrow \pi^*$ band centered around 440 nm in water was also observed. The absorption maximum at 316 nm decreased within 7 hours of photo-irradiation. This result indicates that the azobenzene groups were slowly and quantitatively isomerized to the corresponding cis isomer. Two isosbestic points at 275 and 445 nm were also observed. This shows that the photochemical valence isomerization of F-dimer-azide occurred selectively without any side reaction under this irradiation condition.

The cis-isomer of F-dimer-azide was thermally stable at room temperature in the dark with a thermal half life time of around 8 days but can gradually isomerize to the trans-isomer when exposed to visible light (Figure s5.2). The trans/cis ratio of the F-dimer-azide at the photostationary state was determined by the peak area in UPLC analysis with UV detection at the isosbestic point (275 nm) (Figure s5.1) and was further confirmed with $^1$H NMR (Figure 5.5). As described in chapter 4, when
four fluoro substituents are appended onto the azobenzene, the separation of the n-π* bands between the trans and cis isomers is consistently around 42 nm and the PSS ratios are high. Photoirradiation at >500 nm for overnight led to the photostationary state, where the trans/cis ratio was 33:67. Irradiation of the cis-predominant mixture at 450 nm for overnight led to a trans/cis ratio of 85:15.

Figure 5.4. Changes in the absorption spectra of a solution of F-dimer-azide in MQ water (10 μM), a) upon irradiation at >500 nm for the given time periods; b) upon irradiation at 450 nm for the given time periods.

5.2.4 Binding affinity

The dimeric paromomycin derivatives were used with the following expectations: (1) the paromomycin groups in the dimer may bind to RNA not only through electrostatic interactions but also by hydrogen bonding to nucleotide bases; (2) the azobenzenes inserted between the two paromomycins may produce a major effect from their structural changes for the modulation of the spatial orientation of the two paromomycin groups; (3) the binding of photoresponsive aminoglycoside derivatives and their RNA aptamer pair provide a molecular switching system, which could be turned on and off by the visible light.10,11 In this study we applied the 23mer RNA aptamer (sequence: 5’-GGA CUG GGC GAG AAG UUU AGU CC-3’) for H-dimer, F-dimer and F-dimer-azide. This sequence was selected by SELEX and was well studied by Wallis and coworkers before.7
Figure 5.5. $^1$H-NMR (500MHz, D$_2$O) spectrum of F-dimer-azide. (a) Pure trans isomer after HPLC purification; (b) pure cis isomer after HPLC purification; (c) PSS after green light (>500 nm) irradiation; (d) PSS after blue light (450 nm) irradiation.

We used isothermal titration calorimetry (ITC) to study the binding of H-dimer, F-dimer and F-dimer-azide with the RNA aptamer sequence in PBS buffer (60 mM, pH 7.5) (see experimental part). Figure 5.6 shows the raw ITC profiles resulting from the titration of F-dimer (trans and cis isomer), H-dimer and F-dimer-azide (trans and cis isomer) into the solution of RNA aptamer (panels A-C), from which the integrated heats were fitted to a one-binding site model by substracting the corresponding dilution heats derived from the injection of identical amount of photoswitchable ligands into buffer alone.
Light-Regulated RNA-Small Molecule Interactions

a) F-dimer

Time (min)

μWatts

Pure Trans

Pure Cis

Molar Ratio

kJ mol$^{-1}$ of injectant

μWatts

Data: amo204415_NDH
Model: OneSites
Ch#2/DoF = 1.75165
N 0.666 ± 0.0910 Sites
K 1.076 ± 0.146 M$^{-1}$
ΔH -1.790E4 ± 1.164 kJ/mol
ΔS -129 [Joules/mol]deg

Molar Ratio

kJ mol$^{-1}$ of injectant

b) H-dimer

Time (min)

μcal/sec

Pure Cis

PSS @ 450 nm

PSS @ 365 nm

μcal/sec

Data: amo204415_NDH
Model: OneSites
Ch#2/DoF = 1.75165
N 0.666 ± 0.0910 Sites
K 1.076 ± 0.146 M$^{-1}$
ΔH -1.790E4 ± 1.164 kJ/mol
ΔS -129 [Joules/mol]deg

Molar Ratio

kJ mol$^{-1}$ of injectant
c) F-dimer-azide

Figure 5.6. Isothermal titration calorimetry results of photoswitchable ligands with RNA aptamer: a) F-dimer (trans and cis isomer); b) H-dimer (PSS mixture under 365 nm irradiation and 450 nm irradiation) and c) F-dimer-azide (trans and cis isomer). The top row shows the heats resulting from each injection of ligands into the aptamer solutions studied. In the bottom row, the integrated heats after correcting for the heat of dilution, as well as, the best-fitted function are shown.

All the thermodynamic parameters were summarized in the Table 5.1. It reveals that the interactions between photoswitchable ligands and RNA aptamer was driven by enthalpy and resulted in exothermic reactions, indicating the specific ligands and RNA aptamer interaction contribute strongly to the high complex stability. Moreover, each of these ITC profiles has one apparent phase, suggestive of one distinct binding event and the presence of one binding site within the RNA sequence. In the case of H-dimer, the steepness of the phases from the titration indicated a loose interaction in PSS mixture under 365nm irradiation (with an association constant, $K_a = 1.28 \times 10^5$ M$^{-1}$), which is 1.37 times higher than its PSS mixture under 450nm irradiation (with an association constant, $K_a = 9.31 \times 10^4$ M$^{-1}$). This small difference can be explained by the low photoconversions and the short thermal half-life. However, F-dimer reaches a photostationary state containing 78% cis isomer when exposed to green light (>500 nm). More importantly, with the long thermal half-life, cis isomer can be purified by HPLC and further be used for the ITC
experiments. As shown in figure 5.6a, the binding of F-dimer with aptamer are stronger than H-dimer, which might due to the different binding of the electron poor fluorine-substituted benzene rings compared to the non-fluorinated rings with RNA aptamer. More interestingly, the binding constant of the aptamer with F-dimer in trans form (with an association constant, $K_a^{trans} = 1.07 \times 10^6$ M$^{-1}$) is two orders of magnitude higher than that of the cis form (with an association constant, $K_a^{cis} = 1.36 \times 10^4$ M$^{-1}$). The low binding affinity of the cis F-dimer precluded us from accurately fitting the entire profile to obtain estimates of binding affinity and stoichiometry. Finally, for the F-dimer-azide, both of the two isomers' binding affinities are weak and show barely no difference. This might be due to the fact that the introduction of azide groups greatly reduces the interaction between the F-dimer-azide ligand and RNA aptamer. Altogether, from these ITC measurements it can be concluded that photoswitchable ligand F-dimer can bind to a single aptamer with high affinity and this RNA aptamer can distinguish between the two isomeric forms of F-dimer, providing a promising candidate for further applications of optocal regulation of biological systems.

**Table 5.1** Association constants ($K_a$), molar entropy ($\Delta S^o$) and molar enthalpy ($\Delta H^o$ for complexation of ligands H-dimer, F-dimer and F-dimer-azide with RNA aptamer.

<table>
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<th>compound</th>
<th>Binding site</th>
<th>$K_a / 10^4$ M$^{-1}$</th>
<th>$\Delta H^o$ / kcal mol$^{-1}$</th>
<th>$\Delta S^o$ / cal mol$^{-1}$ deg$^{-1}$</th>
</tr>
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<tbody>
<tr>
<td>H-dimer PSS under 365 nm</td>
<td>1</td>
<td>12.8</td>
<td>-12830</td>
<td>-4.3*10$^4$</td>
</tr>
<tr>
<td>H-dimer PSS under 450 nm</td>
<td>1</td>
<td>9.31</td>
<td>-8877</td>
<td>-2.98*10$^4$</td>
</tr>
<tr>
<td>F-dimer pure trans</td>
<td>1</td>
<td>107</td>
<td>-72.9</td>
<td>-129</td>
</tr>
<tr>
<td>F-dimer pure cis</td>
<td>1</td>
<td>1.36</td>
<td>-145.3</td>
<td>-4790</td>
</tr>
<tr>
<td>F-dimer-azide pure trans</td>
<td>1</td>
<td>3.37</td>
<td>-5.94</td>
<td>0.786</td>
</tr>
<tr>
<td>F-dimer-azide pure cis</td>
<td>1</td>
<td>3.95</td>
<td>-6.93</td>
<td>-2.83</td>
</tr>
</tbody>
</table>

**5.3 Conclution**

In summary, we have investigated the photo-regulated interactions between a RNA aptamer and three photoswitchable ligands. Their binding can be modulated by appropriate photoirradiation on azobenzene chromophores. It was found that the
selected aptamer can bind to the trans isomer of F-dimer 100 times stronger than its cis isomer. To date, only few photoswitchable ligands were found able to be distinguished by a RNA aptamer, recognizing only one of their two isomeric forms. Therefore, the light switchable small-molecule–aptamer system described in this chapter holds great potential to be developed into a photochemical riboswitch that can then be used to spatially and temporally regulate gene function. The best suited candidate can be used as a switch is F-dimer because it showed the largest affinity difference between its cis and trans form. Moreover, the respective isomers can be isolated and then applied in pristine form to achieve highest on-off ratio in gene regulation.

5.4 Experimental Section

5.4.1 Materials & Methods

NMR and heteronuclear single-quantum correlation (HSQC) spectra were recorded on a Varian Unity Inova (500 MHz for $^1$H-NMR, $^{13}$C-NMR and HSQC) NMR spectrometer at 25 °C. High resolution mass spectrometry (HRMS) was carried out on a LTQ ORBITRAP XL instrument (Thermo Scientific) employing electron impact ionization in positive ion mode (EI+). Chromatographic separations were carried out on a Shimadzu VP series HPLC modular system (DGU-14A3 Online Vacuum-Degasser, two LC-20 AT pumps, SIL-20A auto sampler, CTP-20 A column oven, RID-10 refractive detector, FRC-10 A fraction collector and Shimadzu LCsolution software). HPLC purification was performed with a Waters Spherisorb ODS-2 C18 analytical (250 x 4.6 mm) and semi-preparative column (250 x 10 mm) (spherical particles of 5 µm and 80 Å pore size) using isocratic elution at 30 °C. A pH-meter (Hanna Instruments pH 209) equipped with a glass combination electrode was used for pH adjustments of the reaction buffers. All chemicals and reagents were purchased from commercial suppliers and used without further purification, unless otherwise noted. Paromomycin sulfate salt (98 %) was purchased from Sigma Aldrich and used as received. For HPLC purification trifluoroacetic acid (TFA) (Sigma-Aldrich, HPLC grade) and acetonitrile (Sigma-Aldrich, HPLC grade) were used. Ultrapure water (specific resistance > 18.4 MΩ cm) was obtained by Milli-Q water purification system (Sartorius®). All compounds (including intermediates) were stored in a refrigerator at about +5 °C, unless otherwise stated.

5.4.2 General Procedures
5.4.2.1 Synthesis of diazo-transfer reagent Imidazole-1-sulfonyl azide hydrochloride

Sulfuryl chloride (1.6 mL, 20 mmol) was added dropwise to an ice-cooled suspension of sodium azide (1.3 g, 20 mmol) in acetonitrile (20 mL) and the mixture was stirred overnight at room temperature. Then imidazole (2.6 g, 38 mmol) was added portion-wise to the ice-cooled mixture and the resulting slurry was stirred for additional 3 h at room temperature. The reaction mixture was diluted with ethyl acetate (40 mL), washed twice with water (40 mL) and then twice with saturated aqueous NaHCO₃ solution (40 mL), dried over MgSO₄ and filtered. The filtrate was cooled in an ice-batch and a 3 M HCl methanolic solution (10 mL) was added dropwise to precipitate the product. Finally, the filter cake was washed three times with ethyl acetate (10 mL) to obtain 7 as colorless hydrochloride salt. Yield: 1.9 g (9.1 mmol, 45% yield).

**1H-NMR (500 MHz, D₂O, 25 °C, TMS):** δ = 9.53 ppm (s, 1H, H-2), 8.07 ppm (s, 1H, H-5), 7.67 ppm (s, 1H, H-4). **13C-NMR (100 MHz, D₂O, 25 °C, TMS):** δ = 137.6 ppm, 122.6 ppm, 120.18 ppm. HRMS (EI+) (m/z): found 174.0078 [M+H]+, calc. 174.0080 [M+H]+.

5.4.2.2 Synthesis of F-dimer-3-C-azido paromomycin

A solution of F-dimer (0.02 mmol) in water (20 mL) was combined with a solution of imidazole-1-sulfonyl azide hydrochloride (84 mg, 0.48 mmol, 24 eq.) in water (20 mL). A pH value of 6.4 of the reaction mixture was adjusted by addition of aq. 2M NaOH solution and stirred at room temperature for 40 h before washing twice with dichloromethane (40 mL) to remove the excess of diazo-transfer reagent. The aqueous solution was concentrated under reduced pressure to remove dichloromethane residue and freeze dried. The residue was resolved in water (2 ml) and filtered through 0.45 µm syringe filter. Each 100 µL-fraction was purified by HPLC using a Waters Spherisorb ODS-2C18 semi-preparative column. Solvent A: H₂O (HPLC grade) + 0.028% v/v trifluoroacetic acid (TFA); solvent B: H₂O/MeCN 2:1+ 0.028% v/v TFA; detection at 314 nm and 275nm. tₚ = 18.2 min (A/B 100:0–87:13 in 45 min) at a flow rate of 3 ml/min at 30°C to afford the azido antibiotic azobenzene derivative as trifluoroacetic acid salt for mass spectrometry.
and NMR analysis. $^1$H-NMR (500 MHz, D$_2$O, 25 °C, TMS): δ (ppm) = 7.65 (m, 4 H, azo), 5.32 (d, J = 3 Hz, 2H, 1-H’), 5.27 (s, 2H, 1-H’´), 5.21 (s, 2H, 1-H’”), 4.39 (d, J = 4 Hz, 2H, 2-H’”), 4.33 (t, J = 5.5 Hz, 2H, 3-H’”), 4.26 (m, 2H, 3-H”), 4.22 (m, 2H, 5-H”), 4.14 (m, 2H, 4-H”), 3.96 (dd, J = 17.5 Hz, J = 9.5 Hz, 2H, 6-Ha”), 3.89 (t, J = 10 Hz, 2H, 6-H), 3.84–3.78 (m, 4H, 5-Ha”’, 3-H”), 3.82–3.64 (m, 8H, 4-H”, 6-Hb”), 3.55 (s(br), 2H, 2-H”), 3.22–3.17 (m, 6H, 2-H’, 1-H, 4-H), 2.38 (dt, J = 12.5 Hz, J = 4Hz, 2H, 2-Heq), 1.69 (dd, J = 12.15 Hz, 2H, 2-Hax). $^{13}$C-signals based on HSQC (500 MHz, D$_2$O, 25 °C): δ (ppm) =111.98 (azo), 111.01 (C-1”´), 95.46 (C-1”), 94.50 (C-1””), 85.76 (C-5), 80.93 (C-4”), 77.44 (C-4), 74.49 (C-3”), 72.98 (C-5””), 72.9 (C-2”), 72.42 (C-5”), 72.33 (C-6), 69.28 (C-3”), 68.58 (C-4”), 67.63 (C-3”), 66.39 (C-4”), 61.1 (C-5”), 59.89 (C-6), 58.34 (C-3), 54.01 (C-2”), 50.82 (C-2””), 49.56 (C-1), 40.3 (C-6””), 29.21 (C-2). C$_{23}$H$_{43}$N$_7$O$_{14}$; HRMS (El+) (m/z): found 1589.64 [M+H]$^+$, calc. 1589.44 [M+H]$^+$.

5.4.3 Isothermal titration calorimetry (ITC)

Isothermal titration calorimetry was used to determine the binding constants between aptamer and photoswitchable ligands using a MicroCal ITC 200 Microcalorimeter (Northampton, MA). A 1 mM ligands solution was injected from a 40 µL rotating syringe into an isothermal sample chamber containing 200 µL of 7 µM aptamer solution. A TAE buffer (pH 8.0) was used to prepare both solutions, including the buffer to load the reference cell. The experiments were carried out at 25 °C and with a stirring speed of 750 rpm. Typically, injections of 1.2 µL of titrant were added into the cell with a 120 seconds delay between each injections, up to a aptamer/ligand ratio of 1:10. The effective heat of the ligand-aptamer interaction upon each titration step was corrected for dilution effect by subtracting the values obtained in the titration of ligands into buffer solution. Each injection generated a point for point heat curve (microcalories per second vs time) which was conveniently integrated in order to obtain the heats of the bimolecular interactions associated with those injections. Then the normalized heat signals were analyzed by using the ITC non-linear curve fitting functions for one binding site from MicroCal Origin 7.0 software (MicroCal, Inc.; Northampton, MA). The satisfactorily fitted cure was used to determine the molar enthalpy change for binding ($\Delta H^o$) and the corresponding binding constant (Ka). Fundamental thermodynamic equations were used to determine the molar free energy of binding, $\Delta G^o$, and the molar entropy change ($\Delta S^o$). Each ligand-aptamer titration was repeated at least three times in order to increase the accuracy of the thermodynamic parameters.
Author Contribution

In this chapter, Jingyi Huang performed the synthesis of the photoswitchable aminoglycosides and characterized photochemical behavior of the resulting photoswitchable aminoglycosides by nuclear magnetic resonance and UV-vis spectroscopy. Shuo Yang quantified the ratio of photoisomers at photostationary-state by UPLC. Avishek Paul investigated the binding affinity of the photoswitches to the RNA aptamer by isothermal titration calorimetry.
Chapter 5

Reference

Appendix

Figure S 5.1. UPLC traces (recorded at the corresponding isosbestic points) of **F-dimer-azide** in MQ water. a) pure trans isomer after HPLC purification (upper panel) and pure cis isomer after HPLC purification (lower panel); b) PSS mixtures after irradiation with different wavelengths.
**Figure S 5.2.** Determination of half-life for F-dimer-azide at 37 °C in water in the dark. First, PSS was reached upon green light (>500 nm) irradiation, after that the absorption was measured at $\lambda_{\text{max}} = 324$ nm. Line presents linear fit.

**Figure S 5.3.** HSQC (500 MHz, D$_2$O) spectrum of F-dimer-azide (4).