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Characterization of TectoRNA Assembly with Cationic Conjugated Polymers

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Specific long-range RNA−RNA interactions stabilize and define the fully compacted, biologically active structures of RNA molecules.[1] Many of these structural motifs, originally identified in natural RNAs by comparative sequence and structural analysis,[2] have led to the design of "tectoRNAs": artificial molecular units that self-assemble through well-defined tertiary interactions to form supramolecular architectures.[3] TectoRNAs are also useful molecular units to obtain insight into the thermodynamics, and thus the autonomous assembling properties, of supramolecular RNA−RNA recognition.[3b] Understanding RNA−RNA assemblies should ultimately lead to the rational design and shape prediction of RNA-based macromolecules.

These considerations provide motivation for developing new solution methods that probe dynamic equilibria in libraries of RNA molecules. Widely used techniques for examining RNA association, such as gel shift mobility assays,[4,3b] surface plasmon resonance techniques,[5] and UV melting curves analysis[6] are time-consuming, require large RNA quantities, or do not probe solution processes in situ.

Herein, we report the characterization of tectoRNA assembly through tetraloop/receptor interactions[3a,b] (**TRI**s) by using fluorescence resonance energy transfer (FRET) from water-soluble cationic conjugated polymers (CCPs).[7] The electronic structure of the CCPs coordinates the action of a large number of absorbing units and delivers excitations to reporter chromophores, resulting in amplification of fluorescence signals.[8] The tectoRNA/CCP technique is amenable for examination of RNA libraries and can be used to evaluate dissociation equilibrium constants (K_d).

The approach in this study begins with a fluorophore-labeled probe RNA (RNA-F*) which is treated with a target structure (RNA_T). Heterodimer formation, (RNA_T/RNA-F*), increases the total negative charge on the F*-bearing macromolecule and reduces the number of negatively charged molecules (relative to unbound RNA_T + RNA-F*). On the basis of electrostatic interactions, we anticipated more effective binding between CCP and (RNA_T/RNA-F*), a reduction of the average CCP- - -F* distance, and more effective FRET.

To test this approach, we investigated the assembly properties of tectoRNAs that interact via two different GNRA **TRI**s (N: any nucleotide; R: purine, Figure 1): a highly specific GAAA **TRI**, that was kept invariant, and a GRAA **TRI**, originally selected by in vitro selection.[9] Specifically, RNA**1** (Figure 1B) was tested for its ability to dimerize with three different targets, RNA**2**, RNA**3**, and RNA**4**. RNA**1** contains a GAAA tetraloop (in red) that can be specifically recognized by the 11nt-motif receptor (in green) of RNA**2**−**4**, and a GRAA receptor (in blue) with affinity for a GGAA tetraloop and to a lesser extent GAAA and GUAA tetraloops. All RNA molecules were synthesized by in vitro transcription of PCR-generated DNA templates using T7 RNA polymerase.

Nondenaturing polyacrylamide gel electrophoresis (see insets, Figure 2) was used to determine the association of ^{32}P-radiolabeled

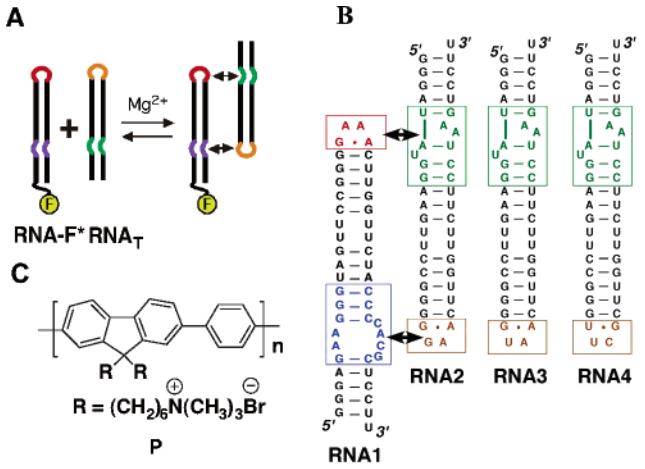

Figure 1. (A) Dimerization of tectoRNA molecules based on specific interactions between tetraloops (red and orange) and tetraloop receptors (green and blue).[3a,b] (B) RNAs used in this study. RNA**1** and RNA**2** are shown as a dimer. The color code is the same as in (A). (C) Structure of **P** (n = 20∼30).

RNA**1** with the other RNAs in the presence of 15 mM $Mg(OAc)_2$ at 15 °C. RNA**1** does not self-dimerize in the range of concentrations tested. Measured K_d's are consistent with the expectations based on the prebuilt **TRI**s (K_d = 10 ± 2 nM for RNA**1**/RNA**2**, GGAA loop, and K_d = 150 ± 30 nM for RNA**1**/RNA**3**, GUAA loop). When UUCG serves as the tetraloop (RNA**4**), this mutation completely abolishes binding even at high [RNA], as the GGAA receptor is unable to recognize tetraloops other than GNRA loops.[3a,b]

RNA**1** with fluorescein attached to the 5′ end (RNA**1**-F*) was used as the probe structure. Fluorescein was chosen since its absorption overlaps the emission of the CCP (**P**) displayed in Figure 1C ($\lambda_{abs\mathbf{P}}$ = 380 nm, $\lambda_{em\mathbf{P}}$ = 424 nm, $\lambda_{abs\mathbf{F*}}$ = 490 nm, $\lambda_{em\mathbf{F*}}$ = 514 nm).[10] Irradiation at 380 nm selectively excites **P**.

Figure 2 shows a series of fluorescence spectra as a function of [RNA]. The FRET ratio (F* intensity/**P** intensity) normalizes fluctuations in overall intensity.[11] At [RNA**1**-F*] = 5 nM, the FRET ratio is equal for RNA**2**−**4**.[12] Within this concentration range, there is no association, and the three target RNAs behave similarly. On the basis of the gel-determined K_d values, at [RNA**1**-F*] = 100 nM, RNA**1**-F*/RNA**2** is nearly all in the dimer state, RNA**1**-F*/RNA**3** forms ∼30% of dimer, and there is no association with RNA**4**. FRET ratios in Figure 2B are consistent with this trend. Furthermore, the integrated F* emission from RNA**1**-F*/RNA**2**, is ∼15 times greater than by direct excitation at its absorption maximum in the absence of **P**, indicating signal amplification by the conjugated polymer structure. When [RNA**1**-F*] = 740 nM, the probe concentration is high enough to obtain RNA**1**-F*/RNA**2** and RNA**1**-F*/RNA**3** as dimers, while RNA**1**-F* + RNA**4** remain monomeric, consistent with the spectra in Figure 2C. Figure 2 demonstrates that the CCP-based approach responds to the level

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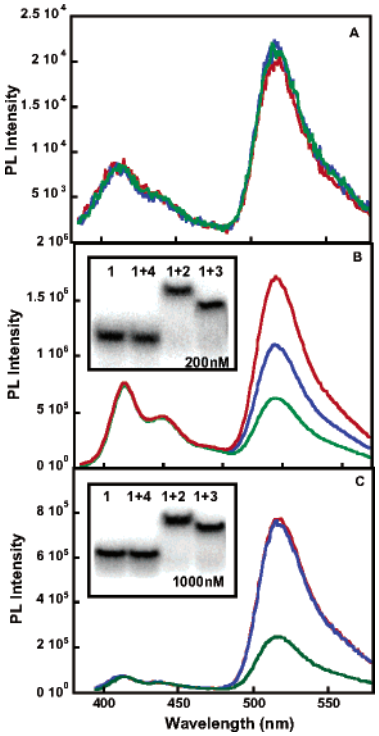

Figure 2. Fluorescence spectra for RNA**1**-F*/RNA**2**−**4**/**P** solutions in 25 mM HEPES buffer, $[Mg(OAc)_2] = 15$ mM, at 15 °C. (A) [RNA**1**-F*] = 5nM, at the presence of $[\mathbf{P}] = 1.5 \times 10^{-7}$M; (B) [RNA**1**-F*] = 100 nM, at the presence of $[\mathbf{P}] = 4 \times 10^{-6}$M; (C) [RNA**1**-F*] = 740 nM, in the presence of $[\mathbf{P}] = 2.3 \times 10^{-5}$M. The excitation wavelength is 380 nm. Emission intensities were normalized relative to the **P** emission. Red: RNA**1**-F* + RNA**2**; Blue: RNA**1**-F* + RNA**3**; Green: RNA**1**-F* + RNA**4**.

of tectoRNA dimerization in solution by increasing the FRET ratio and provides the benefits of optical amplification.

Quantitative information can be obtained. Specifically, solution K_d's can be determined by monitoring F* intensity changes as a function of [RNA-F*] and the ratio $(I_T - I_{NB})/I_{NB}$, where I_T and I_{NB} are the F* intensities in the presence of the target RNA (RNA_T) and a nonbinding RNA (RNA_{NB}), respectively, while keeping [**P**] constant. Normalization relative to RNA_{NB} is required since [F*] increases linearly with [RNA-F*]. By focusing on $(I_T - I_{NB})/I_{NB}$ one can detect the concentration range wherein increased fluorescence is the result of dimerization. Figure 3 shows a plot of $(I_T - I_{NB})/I_{NB}$ against [RNA-F*] with [RNA-F*] = [RNA**1**-F*], RNA_T = RNA**2**, and RNA_{NB} = RNA**4**. The $(I_T - I_{NB})/I_{NB}$ value increases in the [RNA-F*] range from 5 to 10 nM, and it saturates at 30 nM. From this optical titration one obtains a K_d for RNA**1**/RNA**2** of 6 ± 2 nM, within the same range of gel-determined values that have been obtained in similar experimental conditions.

In summary, we report an assay that interfaces RNA association with the light-harvesting properties of CCPs. Electrostatic interactions bring the macromolecules into close proximity to ensure FRET. FRET differences between bound and unbound RNAs can be used to estimate dissociation constants in solution. As shown here, RNA−RNA interactions are sufficiently robust to avoid

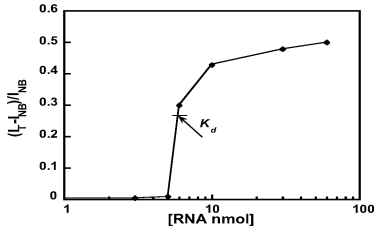

Figure 3. Plot of $(I_T - I_{NB})/I_{NB}$ against [RNA-F*]. Concentration dependence dimerization between RNA**1**-F* and RNA**2** at 15 mM $Mg(OAc)_2$ in HEPEs buffer ([HEPES] = 25 mM) in the presence of $[\mathbf{P}] = 4 \times 10^{-6}$ M. $\lambda_{exc} = 380$ nm.

perturbation from the CCP, within the time scale of the measurement. Optimization of **P** properties with a better understanding of the factors that control the association between **P** and RNAs should yield practical detection of RNA−RNA tertiary interactions among RNA libraries.

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Supporting Information Available: Details for RNA synthesis and FRET experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) The estimated error in [RNA] is ∼20%.

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