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Photophysical and structural investigation of ^{Py}A-modified adenine cluster: its potential use for fluorescent DNA probes exhibiting distinct emission color changes†

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Ki Tae Kim, Wooseok Heo, Taiha Joo* and Byeang Hyean Kim*

In this study, we found ^{Py}**A**-modified adenine cluster (A-Cluster), a minimum fluorescent unit for significant emission wavelength changes, and investigated its photophysical and structural properties. The basic A-Cluster unit was an adenine-pentad duplex containing stacked ^{Py}**A** pairs in the center aligned in an antiparallel manner. Spectral analysis of the A-Cluster revealed remarkable reddish fluorescence with a large Stokes shift (~195 nm) and long life-time constant (31 ns), originated from exciton states formed by ^{Py}**A** pairs and neighboring adenines. Structurally, the exciton state of A-Cluster exhibited unusually high stability, relative to that of other five-mismatched duplexes, as a result of stabilization through strong stacking interactions (zipper-like structure) of the mismatched A-A and ^{Py}**A** pairs, rather than through traditional Watson-Crick base pairing. These spectral and structural properties of the A-Clusters were specific to the adenine bases and highly disturbed by introducing other bases (T, G, and especially C) or an abasic site into the A-Cluster, whereas they were enhanced through synergistic effects in systems containing multiple A-Clusters. As a minimum unit for these unique properties, finally, the A-Cluster were exploited as a fluorescent probing system for specific nucleic acid sequences, such as miR-21, accompanying distinct fluorescence color changes from blue to red. These findings indicated potential utility of the A-Cluster as a part of fluorescent probes exhibiting clear signaling upon micro-environmental changes.

Introduction

Ever since the development of modification techniques for nucleic acids began, fluorescent variants have played important roles in probing single nucleotide polymorphisms (SNPs);[1,2] modified nucleosides;[3] specific nucleic acid sequences[2,4] and structures;[2,5] interactions with other biomolecules;[2,6] and microenvironments.[2,7] Furthermore, recently, in the continuing quest to understand biological events, many fluorescent probes for in vivo detection or visualization have been developed from novel fluorescent oligonucleotides and high-functioning fluorescent nucleic acid systems.[8] Accordingly, the creation of new appropriately functionalized fluorescent nucleic acids systems will expand the application for probing specific biological molecules or events.

One of the requirements for a promising fluorescent probe is clear signaling, such as a remarkable shift in the fluorescence wavelength, upon a micro-environmental change in nucleic acids (e. g. SNPs and local structures). In various probing systems, shifts in the fluorescence wavelength have generally been achieved through excimer/exciplex formation.[9] Despite its attractive features, it is still suffering from small Stokes shifts and partially overlapped fluorescence signal, potentially leading to false signal readout, and demanding reddish fluorescence signal, which is an important factor for biological applications. Therefore, the development of novel fluorescent nucleic acid probes exhibiting large Stokes shifts might enable the design of more efficient fluorescent probes capable of complementing previous systems.

The fluorescence of a fluorescent nucleoside residue in an oligonucleotide is strongly dependent on the nature of its flanking and complementary bases; a fluorophore can form an exciplex with either its nearest fluorophores or nucleobases to give shifted fluorescence.[9b,m] Interestingly, in specific sequences, such as polyadenylates, their excitation and emission patterns are affected by the number of adenine residues.[10] Even though the fluorescence of natural polyadenylates is very weak, they can exhibit unique long-lived excited states and large bathochromic shifts in fluorescence as a result of electronically strong interactions in the excited state among the adenine residues.[10a,11] Those reports led us to focus on the design of fluorescent nucleic acid systems displaying large Stokes shifts, beyond excimer/exciplex emission, based on other possible interactions within the polyadenylates, including fluorophore-fluorophore, fluorophore-base, and base-base interactions. Previously, our group observed large

Department of Chemistry, BK School of Molecular Science, Pohang University of Science and Technology (POSTECH), Pohang 790-784, South Korea. E-mail: bhkim@postech.ac.kr; thjoo@postech.ac.kr

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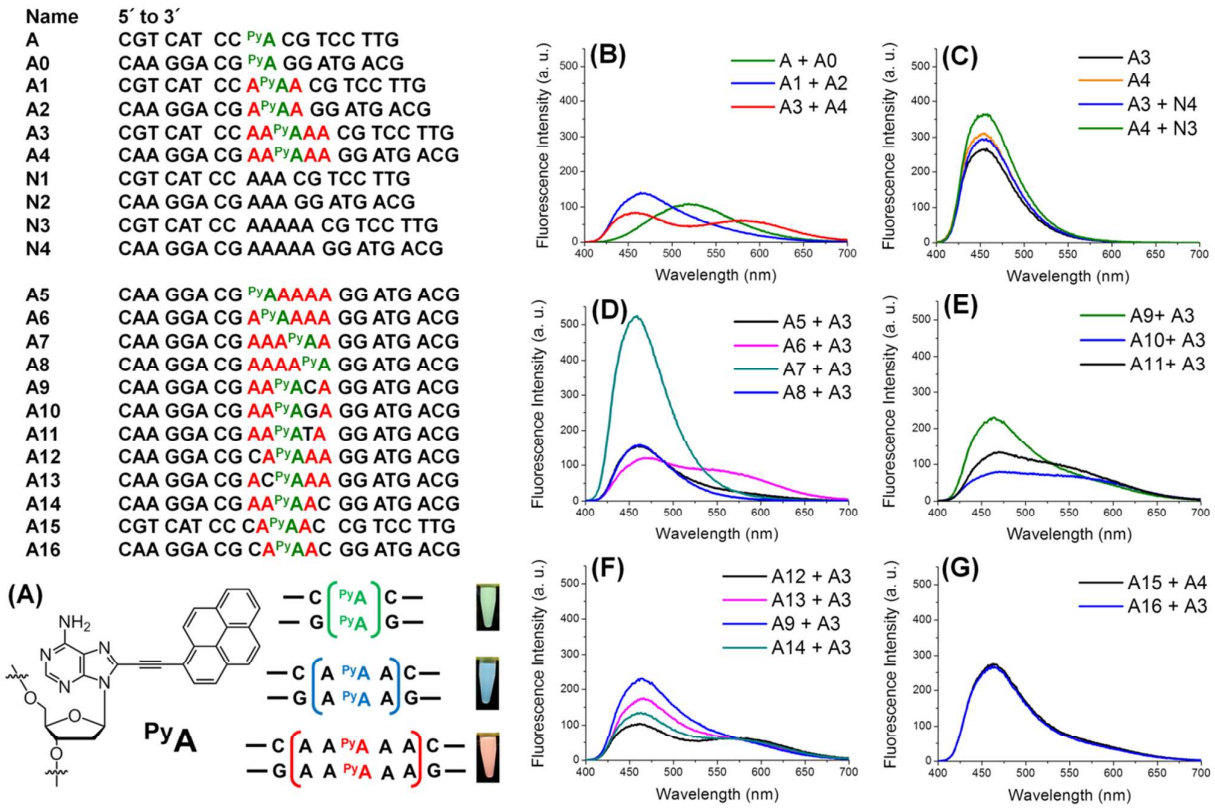


Fig. 1 ODNs synthesized to investigate A-Clusters. (A) Basic duplex scaffolds featuring different numbers of adenine units and (B) their effects on fluorescence. (C) Fluorescence spectra of adenine-pentad systems. (D-G) Changes in fluorescence of the A-Cluster according to (D) the position of the ^{Py}**A** unit, (E) variance of a single-nucleotide, (F) variance of the position of a single C residue, and (G) variance of a number of C units. Conditions: 1.5 μM of duplex; total volume: 1 mL; buffer: 50 mM trizma, 100 mM NaCl, 10 mM $MgCl_2$ (pH 7.2); 25 °C; λ_{ex}: 385 nm; $slit_{ex}$: 2.5 nm; $slit_{em}$: 5 nm.

bathochromic shifts and unique reddish fluorescence of polydeoxyadenylates containing a series of ^{Py}**A**s positioned in 1,4 relationships.[12] In spite of promising properties, however, a number of drawbacks, including the low synthetic yield, long sequence unit, and difficulties in analysis and further modifications, limited their applications for fluorescent DNA systems. Moreover, detailed spectral and structural properties of ^{Py}**A**-polydeoxyadenylates were not fully investigated in previous results.

In this present study, we established the smallest system that could induce a large Stokes shift: the so-called "A-Cluster." We tested their spectral and structural properties in different sequence contexts to gain valuable information regarding the behavior of the A-Cluster as well as ^{Py}**A**-polydeoxyadenylates. Furthermore, with the unique properties of the A-Cluster scaffold, we finally designed fluorescent beacon systems, accompanying significant fluorescence color change from blue to red, for detection of specific nucleic acid sequences. This demonstrated usability of the A-Cluster as a smallest unit for the reddish fluorescence in various types of fluorescent systems.

Results and discussion

Design, synthesis, and basic features of the A-Cluster

Firstly, we wished to find the minimum components necessary for the fluorescence of ^{Py}**A** residues to display a large Stokes shift in a polydeoxyadenylate. We expected that the degree of stabilization of excited ^{Py}**A**s would depend on the number of neighboring adenine residues rather than on 1,4 relationships. Accordingly, we designed and synthesized a model system, which is an anti-parallel duplex containing two Watson–Crick base sequences at the ends and a ^{Py}**A**-modified triad, pentad, or larger adenine sequence in the middle (Fig. 1, from **A** to **A4**). The syntheses of ^{Py}**A** and all of the oligonucleotides were performed using previously reported procedures[13]; their structures were confirmed using MALDI-TOF mass spectrometry (Table S5†). We recorded their UV absorption (Fig. S1†) and fluorescence emission spectra of model duplexes to determine the necessary factors for a large Stokes shift of the ^{Py}**A** residues.

As a result, we observed a reddish fluorescence signal from the duplex **A3+A4** (Fig. 1B), whereas the fluorescence of **A+A0** (at 520 nm) and **A1+A2** (at 465 nm) exhibited a small bathochromic shift (65 and 10 nm, respectively), originated from stacking interactions between the two ^{Py}**A** residues. This fluorescence (at 580 nm) also was clearly differentiated from that of single strand (Fig. 1C). These results implied newly

Name	5′ to 3′
A17	CGT CAT CC AAA^{Py}AAAA CG TCC TTG
A18	CAA GGA CG AAA^{Py}AAAA GG ATG ACG
A19	CGT CAT CC AAAA^{Py}AAAAA CG TCC TTG
A20	CAA GGA CG AAAA^{Py}AAAAA GG ATG ACG

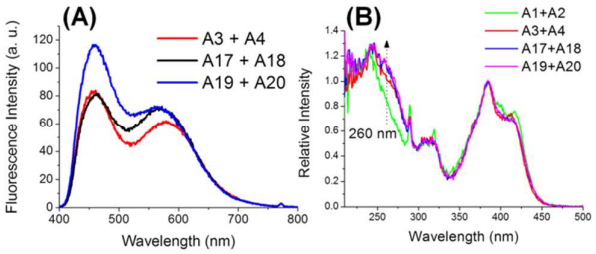


Fig. 2 (A) Fluorescence emission and (B) normalized excitation spectra (emission at 580 nm) of adenine-heptad and -nonad systems. Conditions: 1.5 μM of duplex; total volume: 1 mL; buffer: 50 mM trizma, 100 mM NaCl, 10 mM $MgCl_2$ (pH 7.2); 25 °C; λ_{ex}: 385 nm; $slit_{ex}$: 2.5 nm; $slit_{em}$: 5 nm.

formed excited states of two stacked ^{Py}**A** residues and they could be controlled by base being apart from the two stacked ^{Py}**A** residues (distant bases) as well as flanking bases. This reddish fluorescence was also monitored in adenine-heptad and adenine-nonad systems (Fig. 2A). Accordingly, we suspected that adenine-pentad anti-parallel duplex containing two stacked ^{Py}**A**s was a minimum component, for unique reddish fluorescence emission, which we called an "A-Cluster."

To identify basic photophysical properties of the A-Cluster, values of quantum yield (QY), λ_{abs} and λ_{emi}, and their ratios of fluorescence intensities in the blue to red regions were listed (Table S1†). A-Cluster (**A3+A4**) showed λ_{abs} at 385 and 415 nm and major λ_{emi} at 580 nm, and the absorbance was higher at 385 nm than at 415 nm (Fig. S1†). Thus, A-Cluster exhibited ~195 nm of Stokes shift, which is much larger than that of pyrene excimer generally used in fluorescent system (130 nm).[9h] The QY of the reddish fluorescence of the A-Cluster was observed as 0.11-0.12, leading to clear fluorescence signal easily recognized by the naked eye under the UV irradiation (Fig. 1A).

Photophysical behavior of the A-Cluster under various sequences

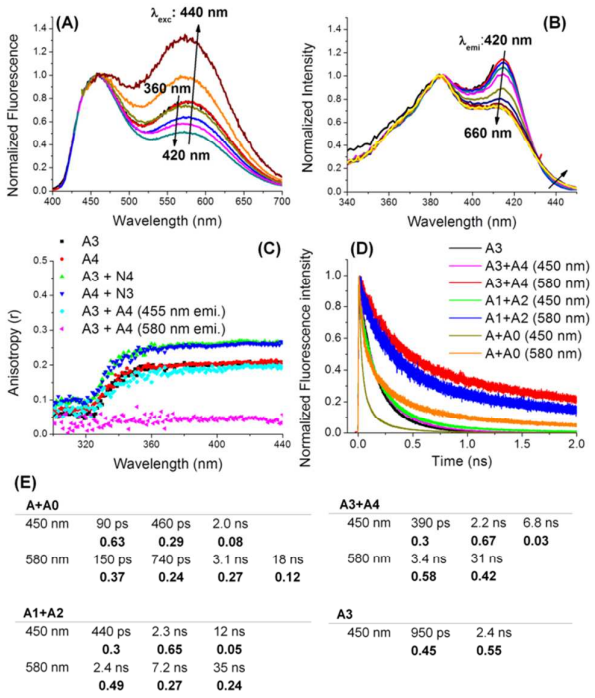
To understand behaviour of the A-Cluster, we monitored its photophysical properties under specific sequences (Table S1†). First, we synthesized **A5-8** to investigate the changes in fluorescence upon varying the position of the ^{Py}**A** residues and their stacking states. As a result, we observed that only **A6+A3** had a fluorescence pattern similar to that of **A4+A3**, while **A7+A3** exhibited a very strong signal only at 460 nm (Fig. 1D), even though they differed by the position of the ^{Py}**A** residue being shifted by only one base in the duplex. We suspected that the ^{Py}**A** residue of **A3** would more readily stack with the ^{Py}**A** residue of **A6**, positioned at the 5′ side, than with that of **A7** at 3′ side. As evidence for stacking interactions in **A4+A3** and **A6+A3**, we observed weakening of the absorptions at 415 nm of their ^{Py}**A** residues and higher thermal stability relative to those of the other duplexes (Fig. S1 and Table S3†). However, the red-shifted fluorescence was not maintained only by stacking interaction of fluorophores. We observed no significant fluorescence shift of the A-cluster system based on ^{Na}**A**, ^{An}**A**, or ^{Pe}**A** (containing 1-ethynylnaphthalene, 9-ethynylanthracene, and 3-ethynylperylene, respectively, see Fig. S6†). Accordingly, stacking of ^{Py}**A** residues is an important factor for the emission of reddish fluorescence near 580 nm.

Next, we examined the sequence-dependency of the A-Cluster. To do so, we introduced additional single-sequence variances, including A, C, G, and T residues (**A9+A3**, **A10+A3**, **A11+A3**). As expected, only **A4+A3**, having an intact A-Cluster, exhibited distinguishable reddish fluorescence; none of the others did (Fig. 1E), implying that reddish fluorescence is a characteristic feature of an adenine-pentad in the A-Cluster. Notably, the C base strongly disrupted the interactions among the adenine residues. Based on this, we examined the effect of the position of the interrupting C residue in the A-Cluster. We synthesized three additional oligonucleotides, **A12**-**14**, in which the single variance was located at all possible positions in the A-Cluster-forming sequence (from 5′ to 3′ end: **A12**, **A13**, **A9**, and **A14**). Compared with **A4+A3**, each of the other A-Clusters containing a single C variance displayed relatively weak fluorescence in the red region (Fig. 1F). The reddish fluorescence was significantly weakened when the C residue was located near the two stacking ^{Py}**A** residues at the core of the A-Cluster (**A9+A3** and **A13+A3**). Interestingly, this weakening was most effective in **A9+A3**, where the C variance of **A9** was on the 3′ side. Again, we observed structural asymmetry of the A-Cluster.

Furthermore, we could examine the fluorescence properties of A-Clusters having two, three, or four interrupter Cs by using **A15** and **A16**. Even for **A15+A4** and **A16+A3**, where the A-Cluster had only two interrupters at the end (outside of the A-cluster), the reddish fluorescence was diminished significantly (Fig. 1G). These phenomena were maintained in the triply and quadruply modified A-Clusters (Fig. S2†). Accordingly, adenine-pentad is a prerequisite for reddish fluorescence of the A-Cluster. These results implied significant sequence dependency (adenine specificity) of A-Cluster and its potential as environmental-sensitive probes.

Origin of two major fluorescence emissions of the A-Cluster

Our next work was to determine the origin of the fluorescent properties of the A-Cluster. Notably, we found that the A-Cluster featured only two fluorescence emission states in equilibrium, as evidenced by an isoemissive point at 572 nm (Fig. 1F). It seems that the ratios of the two emission states are mainly influenced by the interrupter C. For more details, we recorded fluorescence emission spectra at various excitation wavelengths (from 360 to 440 nm) and normalized them for better comparison. Upon increasing the excitation wavelength from 360 to 420 nm, the intensity of the reddish fluorescence diminished, whereas increasing the excitation wavelength from 420 to 440 nm increased it again (Fig. 3A). We suspected that the reddish fluorescence originated mainly from a value of λ_{abs} of 385 nm, rather than 415 nm. Moreover, we observed the decreased intensity at 415 nm and increased intensity at



(E)

A+A0				
450 nm	90 ps	460 ps	2.0 ns	
	0.63	**0.29**	**0.08**	
580 nm	150 ps	740 ps	3.1 ns	18 ns
	0.37	**0.24**	**0.27**	**0.12**

A3+A4				
450 nm	390 ps	2.2 ns	6.8 ns	
	0.3	**0.67**	**0.03**	
580 nm	3.4 ns	31 ns		
	0.58	**0.42**		

A1+A2			
450 nm	440 ps	2.3 ns	12 ns
	0.3	**0.65**	**0.05**
580 nm	2.4 ns	7.2 ns	35 ns
	0.49	**0.27**	**0.24**

A3		
450 nm	950 ps	2.4 ns
	0.45	**0.55**

Fig. 3 Normalized fluorescence (A) emission, (B) excitation, and (C) excitation anisotropy spectra of the A-Cluster, **A3+A4**. (D) Time-correlated single photon counting results and (E) time constants of various duplexes. Conditions: 1.5 μM of duplexes; total volume, 1 mL; 50 mM trizma buffer (pH 7.2), 100 mM NaCl, 10 mM $MgCl_2$; 25 °C.

440 nm in normalized fluorescence excitation spectra of the A-Cluster (Fig. 3B). During emission wavelength change from 420 to 660 nm, we observed an isoemissive point near 430 nm. Again, this indicated that there were two major independent states in the A-Cluster system, and one of them can be excited mainly by 385 nm and 420-440 nm light to emit reddish fluorescence. Excitation anisotropy spectra and the anisotropy value (r = 0.2 and 0.04 for 455 and 580 nm emission, respectively) also supported that two fluorescence emissions at 455 nm and 580 nm have different origins (Fig. 3C and Fig. S3†). Notably, for emission at 580 nm, the anisotropy (r = 0.04) was less than 0.1; this value can be considered as in-plain depolarization of the fluorescence.[14] Thus, the low value of 0.04 suggested the involvement of other electronic transitions between nucleobases in the A-cluster scaffold, inducing out-plane polarization[11b], not limited to simple excitation of ^{Py}**A**.

We used Time-Correlated Single Photon Counting (TCSPC) to identify each of the states of **A3+A4**. For fluorescence at 450 nm, we observed a major fluorescence lifetime of 2.2 ns, which is very similar to that of single stranded **A3** (Fig. 3E).[15] The fluorescence signals at 450 nm may have resulted from the single ^{Py}**A** state in the oligonucleotides. It was also evidenced by their almost same anisotropy values (0.2 for **A3+A4** and **A3**, Fig. 3C). In the 600 nm region, on the other hand, we monitored a very long fluorescence lifetime (31 ns, Fig. 3D, E and Table S2†), which is similar with the previous result.[12] Moreover, we also observed the absence of rise in the up-conversion and TCSPC during the monitored time range, even at a time resolution of 100 fs. It implied simultaneous emissions at 450 and 580 nm; such behavior is not representative of the excimer formation, but can be explained by considering an exciton state.[12,16] This indicates that the reddish fluorescence of the ^{Py}**A**-polydeoxyadenylate has same origin with the A-Cluster.

Notably, our observations implied that Interaction geometries (e.g., intra or intermolecular interaction) of the ^{Py}**A**s in the A-Cluster or ^{Py}**A**-polydeoxyadenylate are not the only factor affecting the exciton state. Even though all **A+A0**, **A1+A2**, and **A3+A4** formed the exciton states based on intermolecular interaction, they exhibited clearly different fluorescence emission. Moreover, in fluorescence life time measurements, **A+A0**, containing flanking G-C pairs, exhibited relatively short fluorescence lifetime (18 ns), while both **A1+A2** and **A3+A4** with flanking A exhibited long lifetime (Fig. 3E, >31 ns). It indicated that neighboring bases is the major factor affecting the exciton state, and neighboring adenines are involved in exciton formation to generate more red-shifted and long fluorescence emission.[11,17] This interaction between the ^{Py}**A** and adenines was further supported by excitation spectra (emission at 580 nm) of the A-Clusters containing different lengths of adenines. At 260 nm where natural bases absorb light most strongly, we observed enhanced signal upon increasing the number of adenines (Fig. 2B, **A1+A2**, **A3+A4**, **A17+A18**, and **A19+A20**), as a result of strong electronic coupling among the pyrenes and numerous adenine moieties in the A-Cluster.[15] Besides, the adenine-nonad system, **A19+A20** exhibited the highest ratio (1.18) of the fluorescence intensity under 260 nm to 385 nm excitation (Fig. S4†). Accordingly, we found that two major emission states existed in the A-Cluster; fluorescence at 450 and 580 nm are originated from single ^{Py}**A** state and exciton state formed by electronic interactions of stacked two ^{Py}**A**s and neighboring adenines, respectively.

Unusual high stability of the A-Cluster based on interstrand stacking interactions between adenine residues

Next, we examined the structural stability of the A-Cluster to determine the structural factors that affected the fluorescence emission. We used UV absorption data to obtain melting curves of the duplexes (Fig. S9†). The values of T_m and thermodynamic parameters were calculated from the UV melting curves according to general methods (Table S3†).[18] Firstly, we examined the structural stability of the adenine-triad system. The **A1+A2**, containing a stacked ^{Py}**A** pair, exhibited the high value of T_m (60.0 °C), as would be expected because of its stacking interaction. On the other hand, interestingly, despite five mismatches the A-Cluster **A3+A4** displayed both higher value of T_m and structural stability (Table S3†, 61.4 °C and –90.7 kcal/mol, respectively) than that of **A1+A2**. It also exhibited the lowest value of ΔS° (–243 cal/mol K), suggesting that the A-Cluster system may have a stable rigid structure. Relative to the adenine-triad, the adenine-pentad systems seemed to offer more favorable environments for stacking interactions between two ^{Py}**A** residues. In particular, the stacking of ^{Py}**A** residues increased the structural

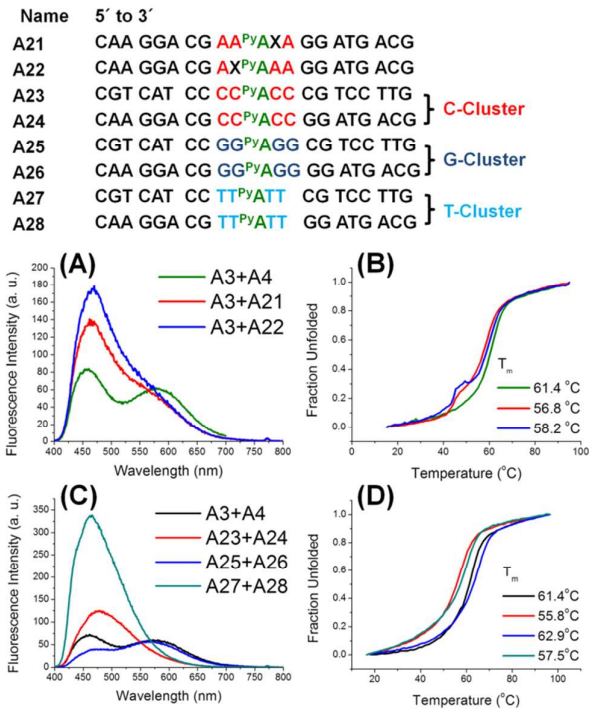


Fig. 4 (Top) Sequences of ODNs tested for the effect of stacking in the A-Cluster. (A) Fluorescence emission spectra and (B) UV melting curves of A-Clusters containing a single abasic site. (C) Fluorescence emission spectra and (D) UV melting curves of A-, C-, G-, and T-Clusters. Conditions: 1.5 μM of duplexes; total volume, 1 mL; 50 mM trizma buffer (pH 7.2), 100 mM NaCl, 10 mM $MgCl_2$; 25 °C; λ_{ex} = 385 nm; $slit_{ex}$ = 2.5 nm; $slit_{em}$ = 5 nm.

stability by 10.3 kcal/mol in the A-Cluster (i.e., the difference in energy between **A3+A4** and **N3+N4**)–a degree of stabilization greater than that of two general base pairs.[19]

Furthermore, we checked the effect of variance on the stability of the A-Cluster. Regardless of the position of the C residue, we observed structural destabilization (T_m = 58.0-59.3 °C; ΔH° from –76.3 to –84.6 kcal/mol) for **A12**,**13**,**9**, and **14+A3**. Moreover, this negative effect was enhanced when the C residue was located near the ^{Py}**A** pair or upon increasing the number of C residues. Furthermore, all examples of variance with C, T, and G residues induced significant destabilization (T_m = 58.7–59.1 °C; ΔH° from –76.3 to –79.8 kcal/mol for **A9**,**10**, and **11+A4**). From these results, we suspected that the emission state of the reddish fluorescence was strongly related to structural stability.

According to our observations, we anticipated that the A-Cluster formed a unique structure, distinct from that of the original B-form DNA structure, showing red-shifted fluorescence. Notably, the natural duplexes of **N3+N4** (T_m = 52.2 °C) were not that unstable, even though they featured five mismatch A–A pairs. From this finding, we deduced the strong interstrand interactions among the adenine residues. Previously, Tinoco and co-workers reported that two mismatched adenine residues having an anti-conformation could be intercalated in a duplex without the formation of hydrogen bonds.[20a] Furthermore, the structure of the self-duplex of d(GCGAAAGCT) was confirmed through X-ray crystallography to feature the mismatched adenine bases intercalated to form a zipper-like duplex.[20b,c] In addition, such a structure could also be formed within a duplex featuring four mismatched A–A pairs.[23d] Thus, in the context of their similar sequences, it was possible that our A-Cluster system, the adenine-pentad duplexes, could also adopt highly organized stacked structures similar to those of adenine-zipper structures.

We obtained several pieces of evidence to support the presence of such structures. (i) The A-Cluster exhibited strong stabilization in the antiparallel duplex, a feature of a zipper-like structure. (ii) The reddish fluorescence of the A-Cluster depended significantly on the position of the ^{Py}**A** residue (Fig. 1D). The fluorescence of **A3+A6** and **A3+A7** would be same if the two ^{Py}**A** planes of **A3+A4** were located in same plane for the B-form duplex stabilized through hydrogen bonds. Their fluorescence behavior was, however, totally different, consistent with only the two ^{Py}**A** residues pairing in a non-coplanar manner. (iii) The A-Cluster exhibited higher structural stability (–90.7 kcal/mol) than those of the modified A-Clusters containing C, G, or T bases (ΔH° = –76.3, –77.2, –79.8 kcal/mol, respectively). Even though **A3+A11** contains one more stable A–T matched pair in place of an A–A mismatched pair, **A3+A4** was more stable than **A3+A11**, implying the loss of a major hydrogen bonded pair and strong stacking interactions in this structure. (iv) The A-Cluster was significantly destabilized in the presence of an abasic site X. We introduced X residues in **A21** and **A22** (Fig. 4) to evaluate the effect of the absence of a base moiety on the fluorescence and stability of the A-Cluster. **A21** and **A22** contained X sequences in the 3′ and 5′ positions, respectively, relative to the stacked ^{Py}**A** residues. **A3+A21** and **A3+A22** exhibited remarkably diminished reddish fluorescence (Fig. 4A). In addition, they exhibited the lowest values of T_m (Table S3; 56.8 and 58.2 °C, respectively) and ΔH° (Table 2; –67.5 and –68.2 kcal/mol, respectively). Notably, we observed an additional T_m transition point near 44°C (Fig. 4B) that resulted from collapse of the A-Cluster part in the low-temperature region as a result of the destabilizing effect of the abasic sites. These results confirmed that stacking interactions had important effects on stabilizing the A-Cluster system. (v) The A-Cluster could be stabilized by series of purine bases rather than pyrimidine bases. We prepared other types of clusters incorporating C, G, and T sequences, so-called C-, G-, and T-Clusters, respectively (Fig. 4), to test whether other types of mismatches could also result in high stability. We observed that the fluorescence emission spectra of the A- and G-clusters featured strong reddish fluorescence, whereas those of the C- and T-Clusters did not (Fig. 4C). This behavior correlated with the stabilities of the clusters: the A- and G-Clusters (Fig. 4D, T_m = 61.4 and 62.9 °C, respectively) had much higher thermal stabilities than did the C- and T-Clusters (T_m = 55.8 and 57.5, respectively). The order of the values of T_m matched well with those previously reported (i.e., that purine mismatches generally exhibit higher stacking propensities).[21] Accordingly, the stability of the A-Cluster must have originated from interstrand stacking Interactions. We also performed molecular modeling to obtain greater perspective about the

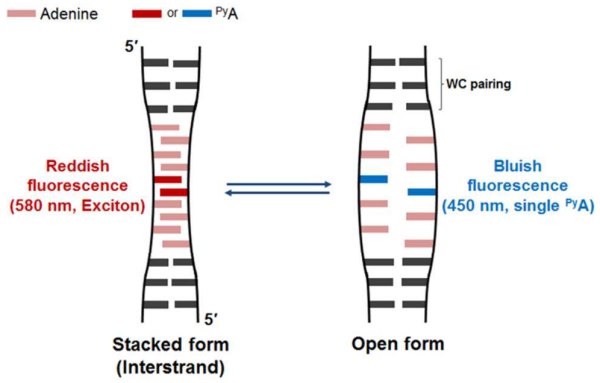


Fig. 5 Proposed spectral and structural behavior of the A-Cluster. Stacked and open states of the A-Cluster exhibit reddish and bluish fluorescence, respectively. One ^{Py}**A** residue would more readily stack with the other ^{Py}**A** positioned at the 3′ side.

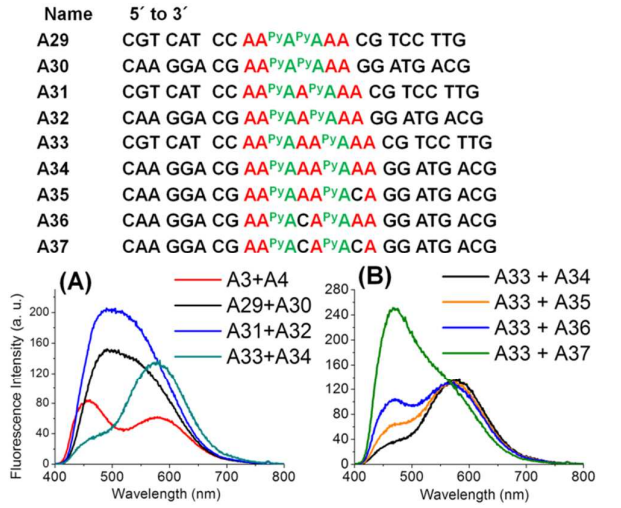


Fig. 6 (Top) Sequences of tested multi–A-Cluster systems. (A) Fluorescence spectra of two–A-Cluster systems with ^{Py}**A** residues at different distances. (B) Fluorescence spectra of two–A-Cluster systems with single or double C-variance at the 3′ positions of the ^{Py}**A** residue. Conditions for fluorescence spectra: 1.5 μM of duplexes; total volume, 1 mL; 50 mM trizma buffer (pH 7.2), 100 mM NaCl, 10 mM $MgCl_2$; 25 °C; λ_{ex} = 385 nm; $slit_{ex}$ = 2.5 nm; $slit_{em}$ = 5 nm.

structural features of the A-Cluster (see Fig. S5†). As a result, in the energy-minimized structure, we observed well-stacked adenine residues and two pyrene moieties in the A-Cluster (Fig. S5C and D†). Finally, we suggest the overall spectral and structural features of the A-Cluster, illustrated in Fig. 5. To obtain additional evidence for their real structures, we are currently performing NMR spectroscopic analyses of our A-Cluster.

Enhanced properties of the multi-A-Cluster systems

Having determined the properties of single A-Clusters, we investigated the interactions between A-Clusters to understand the fluorescence behavior of ^{Py}**A** residues in longer polyadenylates. Firstly, we synthesized six A-Cluster-forming sequences, **A29-34** (Fig. 6), as dual-A-Cluster systems having three different distances between the cores of the A-Clusters. Only the duplex **A33+A34**, which featured a distance of two base pairs between its fluorescent moieties, exhibited strong reddish fluorescence; in contrast, the duplexes **A29+A30** and **A31+A32** exhibited complex and broad fluorescence emission patterns (Fig. 6A). This behavior suggested that one A-Cluster disrupted another when their ^{Py}**A** residues were too closely located (separated by one or no base pairs). Consistent with this hypothesis, the thermal stability of **A33+A34** was the highest (64.3 °C; Table S3 and Fig. S9†) among our tested model duplexes. Furthermore, when compared with **A3+A4**, **A33+A34** displayed a significant enhancement in fluorescence in the long-wavelength region (Table S1, I_S:I_L = 6:94), implying that consecutive A-Clusters had a synergistic effect, presumably through stabilization of the first A-Cluster structure by the other. This finding also suggested that the formation of one A-Cluster was facilitated by another A-Cluster, rather than a natural DNA duplex.

Our next goal was to investigate the effect of a C interrupter on the fluorescence of two–A-Cluster systems and to determine whether each A-Cluster could act independently or not. We synthesized **A35-37**, having one or two C interrupters on the 3′ sides of the ^{Py}**A** residues. In the cases of **A33+A35** and **A33+A36**, which featured a C interrupter on the 3′ side, we observed decreases in the reddish fluorescence relative to that of **A33+A34** (Fig. 6B). Nevertheless, the degrees of decrement were smaller than those of the single A-Cluster systems, suggesting a synergistic effect of the two A-Clusters. This effect diminished significantly when introducing two C residues (Fig. 6B, **A33+A37**). On the other hand, however, dual-A-Cluster systems having C residues on the 5′ side exhibited small changes in fluorescence. Despite introducing two C residues into these duplexes, the reddish fluorescence was not fully quenched (Fig. S7A†). This sensitivity to C residues on the 3′ side is very similar to the behavior described above for the one–A-Cluster systems. These patterns and synergistic effects of the A-Cluster were also maintained in four-A-Cluster systems, the ^{Py}**A**-modified polyadenylate (see Fig. S7 and 8†), implying the modularity of the A-Cluster for enhanced fluorescent and structural properties in fluorescent nucleic acid systems.

Potential uses of the A-Cluster in fluorescent DNA probe: detection of specific sequences by distinct emission color changes

As a minimum unit for reddish fluorescence, the A-Cluster was expected to be useful tools for fluorescent probing systems due to its high susceptibility to changes in sequences and structures. To evaluate potential of the A-Cluster, we have tried to design and explore fluorescent probing systems for specific nucleic acid sequence. One of straightforward approaches for this was using formation of the A-Cluster scaffold in the presence of target sequences. To do so, we utilized a three-way junction type molecular beacon (MB, Fig. 7) having the A-Cluster as a reporting part. This type of binary probe was expected to have high sensitivity to change in sequences.[9j] As our target molecule, we chose a biologically important microRNA, miR-21, associated with a wide variety of cancers and diseases.[22] We synthesized and tested four

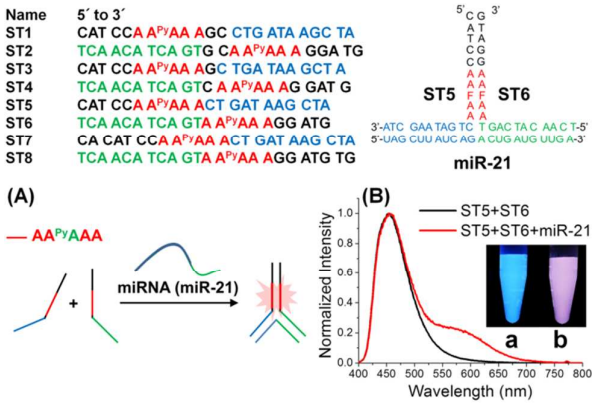


Fig. 7 (A) Fluorescent molecular beacons based on the A-Cluster for miR-21 detection. (B) Normalized fluorescence spectra of **ST5+ST6** in the absence or presence of miR-21. Inset: Fluorescence of **ST5+ST6** under irradiation with UV light in the (a) absence and (b) presence of miR-21. Conditions: 1.5 μM of MB and miR-21; total volume, 1 mL; 50 mM trizma buffer (pH 7.2), 100 mM NaCl, 10 mM $MgCl_2$; 15 °C without annealing.

different molecular beacon systems (**ST1-8**), having different stem lengths, for optimization. All the MBs exhibited increased reddish fluorescence in the presence of miR-21 and the corresponding DNA sequence, and their efficiency were evaluated by discrimination factor (Table S4 †). The discrimination factor was defined by an equation, $(I_{455}/ I_{600})_{MB}$ / $(I_{455}/ I_{600})_{MB+Target}$. As a result, we found that a MB formed by **ST5** and **ST6** exhibited the highest discrimination factor in the presence of the miR-21 (9.4) and the corresponding DNA sequence (10.5), leading to the significant fluorescence change from blue to red (Fig. 7B, inset). This difference was originated from structural differences between A- and B-form helices. Furthermore, we observed that the MB (**ST5+ST6**) exhibited low discrimination factor in the presence of single mismatched DNA targets, **M1-6**. Especially, it exhibited the lowest discrimination factor of 2.7 in the presence of **M1** where a single mismatch T was located near the three-way junction region. This value led to bluish signal differentiated from reddish fluorescence of fully matched three-way junction (Table S4†). We anticipated that the mismatch T effectively disrupted the A-Cluster scaffold by a paring between the mismatched T and A of the A-Cluster.[23] Taken together, these results demonstrate utility of the A-Cluster as a reporting part of fluorescent probing systems sensitive to alterations in DNA/RNA sequences or structures.

Conclusions

We have investigated a fluorescent DNA scaffold, the so-called "A-Cluster," that displays many interesting spectral and structural properties, including large Stokes shifts beyond the excimer; significant adenine-dependency in fluorescence (not only on flanking bases but also distant bases); position-dependency of two ^{Py}**A** residues; high stability even in five-mismatched duplexes; and synergistic effects in multiple A-Clusters. We suspect that as a minimum unit for distinct fluorescence emission changes, the A-Clusters provide extended scope of interactions among fluorophores and nucleobases in polyadenylate scaffolds, and might be useful for the creation of novel fluorescent DNA systems that function complementarily to previously developed systems. Let us consider four possible examples. (i) A-Clusters could be introduced into molecular beacon systems as replacements for fluorophore and quencher units or excimer pairs to provide significant color changes while avoiding autofluorescence.[8f] (ii) The sensitivity of an A-Cluster to positional changes of a single nucleotide or ^{Py}**A** should provide efficient scaffolds for the detection of various polymorphisms, as structural probes for DNA/RNA,[24] and as aptasensors for biomolecules.[25] (iii) The unique fluorescence properties of A-Clusters are based on interactions among their adenine residues, providing additional evidence to explain the photo-behavior of polyadenylates. (iv) Systems containing multiple A-Clusters are possible candidate materials for the preparation of DNA nanostructures, nanodevices[26], and switches[27] that function with accompanying fluorescence changes because they form stable duplexes through stacking interactions, distinct from canonical Watson–Crick base pairing. For realization of these and other possible applications, the spectral and structural properties of the A-Clusters, such as high quantum yield, brightness, more red-shifted fluorescence, and stability, should be optimized through modifications of their fluorophores and conditions and further investigated under various sequences (e.g., inosine, 2-aminopurine, 7-deazaadenine, and other purines).

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