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Photophysical and structural investigation of PyA-modified adenine cluster: its potential use for fluorescent DNA probes exhibiting distinct emission color changes†

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In this study, we found PyA-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 PyA pairs in the center aligned in an antiparallel manner. Spectral analysis of the A-Cluster revealed remarkable redshifted fluorescence with a large Stokes shift (~195 nm) and long life-time constant (31 ns), originated from exciton states formed by PyA 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 PyA 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.
bathochromic shifts and unique reddish fluorescence of polydeoxyadenylates containing a series of PyA residues positioned in 1,4 relationships. 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 PyA-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 PyA-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 PyA residues to display a large Stokes shift in a polydeoxyadenylate. We expected that the degree of stabilization of excited PyA residues 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 PyA-modified triad, pentad, or larger adenine sequence in the middle (Fig. 1, from A to A4).

The syntheses of PyA and all of the oligonucleotides were performed using previously reported procedures; 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 PyA residues.

As a result, we observed a reddish fluorescence signal from the duplex A3+4A4 (Fig. 1B), whereas the fluorescence of A+4A0 (at 520 nm) and A1+2A2 (at 465 nm) exhibited a small bathochromic shift (65 and 10 nm, respectively), originated from stacking interactions between the two PyA residues. This fluorescence (at 580 nm) also was clearly differentiated from that of single strand (Fig. 1C). These results implied newly
formed excited states of two stacked PyA residues and they could be controlled by base being apart from the two stacked PyA 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 PyAs was a minimum component, for unique reddish fluorescent emission, which we called an “A-Cluster.”

To identify basic photophysical properties of the A-Cluster, values of quantum yield (QY), λabs and λemi, and their ratios of fluorescence intensities in the blue to red regions were listed (Table S1). A-Cluster (A3+A4) showed λabs at 385 and 415 nm and major λemi 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). The QY of the reddish fluorescence of the A-Cluster was observed as 0.11-0.12, leading to clear fluorescent 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 PyA 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 PyA residue being shifted by only one base in the duplex. We suspected that the PyA residue of A3 would more readily stack with the PyA 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 PyA 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 NnA, n-A, or PyA (containing 1-ethynynaphthalene, 9-ethynylanthracene, and 3-ethynylpyrene, respectively, see Fig. S6†). Accordingly, stacking of PyA 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 PyA 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 fluorescence 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 λabs of 385 nm, rather than 415 nm. Moreover, we observed the decreased intensity at 415 nm and increased intensity at
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 isomeissive 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-plane depolarization of the fluorescence. Thus, the low value of 0.04 suggested the involvement of other electronic transitions between nucleobases in the A-cluster scaffold, inducing out-of-plane polarization, not limited to simple excitation of PyA.

We used Time-Correlated Single Photon Counting (TCSPC) to identify each of the states of A3A4. 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). The fluorescence signals at 450 nm may have resulted from single PyA state in the oligonucleotides. It was also evidenced by their almost same anisotropy values (0.2 for A3A4 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. 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. This indicates that the reddish fluorescence of the PyA-polydeoxyadenylate has same origin with the A-Cluster.

Notably, our observations implied that interaction geometries (e.g., intra or intermolecular interaction) of the PyAs in the A-Cluster or PyA-polydeoxyadenylate are not the only factor affecting the exciton state. Even though all A+A0, A1+A2, and A3A4 formed the exciton states based on intermolecular interaction, they exhibited clearly different fluorescence emission. Moreover, in fluorescence lifetime measurements, A+A0, containing flanking G-C pairs, exhibited relatively short fluorescence lifetime (18 ns), while both A1+A2 and A3A4 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. This interaction between the PyA 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, A1A2, A3A4, A17A18, and A19A20), as a result of strong electronic coupling among the pyrenes and numerous adenine moieties in the A-Cluster. Besides, the adenine-nonad system, A19A20 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 PyA state and exciton state formed by electronic interactions of stacked two PyAs 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 Tm and thermodynamic parameters were calculated from the UV melting curves according to general methods (Table S3†). Firstly, we examined the structural stability of the adenine-triad system. The A1A2, containing a stacked PyA pair, exhibited the high value of Tm (60.0 °C), as would be expected because of its stacking interaction. On the other hand, interestingly, despite five mismatches the A-Cluster A3A4 displayed both higher value of Tm and structural stability (Table S3†, 61.4 °C and ~90.7 kcal/mol, respectively) than that of A1A2. It also exhibited the lowest value of ΔSt (~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 PyA residues. In particular, the stacking of PyA residues increased the structural
energy between stability by 10.3 kcal/mol in the A-Cluster (i.e., the difference in the stability of the A-Cluster. Regardless of the position of the C residue, we observed structural destabilization ($T_m$) correlated with the stabilities of the clusters: the A- and G-clusters featured strong reddish fluorescence, whereas those of the C- and T-clusters did not (Fig. 4C). This behavior depended significantly on the position of the $^9A$ residue (Fig. 1D). The fluorescence of A$^3$A6 and A$^3$A7 would be same if the two $^9A$ planes of A$^3$A4 were located in same plane for the B-form duplex stabilized through hydrogen bonds.

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 $^9A$ residue (Fig. 1D). The fluorescence of A$^3$A6 and A$^3$A7 would be same if the two $^9A$ planes of A$^3$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 $^9A$ 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 A$^3$A11 contains one more stable A–T matched pair in place of an A–A mismatched pair, A$^3$A4 was more stable than A$^3$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 $^9A$ residues. A$^3$A21 and A$^3$A22 exhibited remarkably diminished reddish fluorescence (Fig. 4A). In addition, they exhibited the lowest values of $T_m$ (Table 5; 56.7 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, or T bases ($ΔH° = −76.3, −77.2, −79.8$ 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. (vi) The A-Cluster could be stabilized by series of purine bases rather than pyrimidine bases. We prepared other types of clusters incorporating C, G, or T sequences, so-called C- 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
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 PyA residues in longer polyadenylates. Firstly, we synthesized six A-Cluster-forming sequences, A29–A34 (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 broad fluorescence emission patterns (Fig. 6A). This behavior suggested that one A-Cluster disrupted another when their PyA 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, I2/I1 = 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–A37, having one or two C interrupters on the 3’ sides of the PyA 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 PyA-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. As our target molecule, we chose a biologically important microRNA, miR-21, associated with a wide variety of cancers and diseases. We synthesized and tested four
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, \( \frac{I_{455, MB}}{I_{455, MB}} \). 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 PyA 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.8 (ii) The sensitivity of an A-Cluster to positional changes of a single nucleotide or PyA 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, nanodevices26, and switches27 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-deazadenine, and other purines).

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Notes and references


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 MgCl2; 15 °C without annealing.