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Bis-phenanthridinium–adenine conjugates as fluorescent and CD reporters for fine structural differences in ds-DNA/RNA and ss-RNA structures

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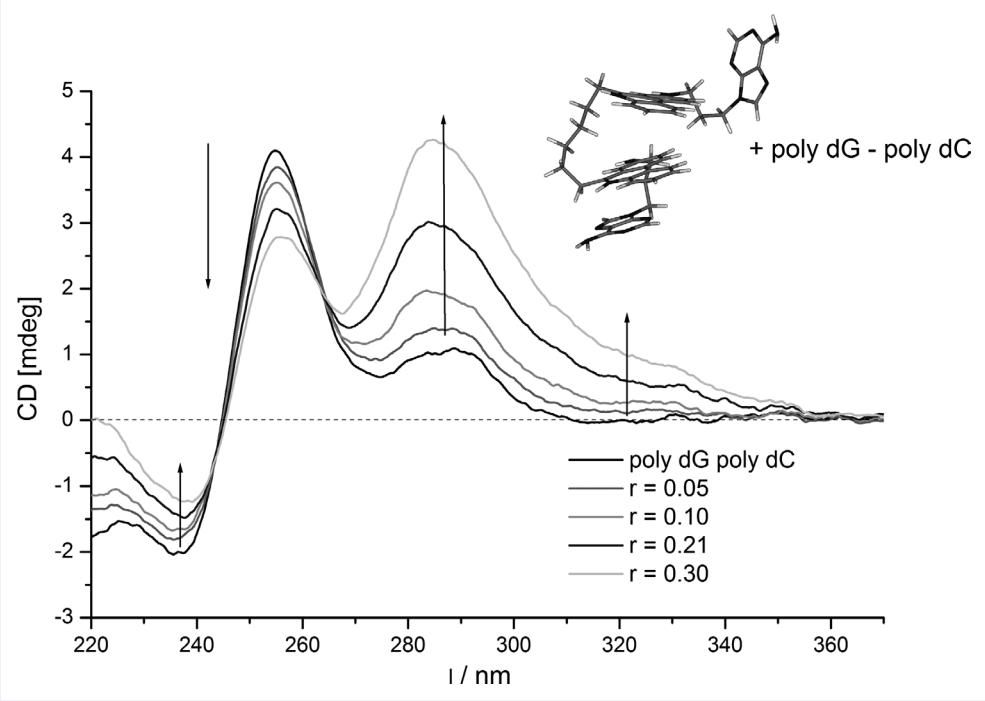
ABSTRACT

Two structurally similar bis-phenanthridinium–adenine conjugates (equipped with one or two adenines, respectively), exhibiting strong ds-DNA/RNA groove binding, revealed ratiometric fluorescent recognition of alternating AT-DNA with respect to other ds-DNA/RNA and ss-RNA. Further, CD spectra pattern of adenine–bis-phenanthridinium conjugate/polynucleotide complexes strongly depended on polynucleotide secondary structure. Attached adenine was essential for spectrophotometric recognition response, ds-DNA stabilisation and orientation of compounds regarding chiral DNA helix.



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DNA recognition; Phenanthridine–adenine conjugates; fluorimetric titration; CD titration

Introduction

The majority of natural and artificial applications involving formation of supramolecular small molecule–DNA/RNA complex depend on several non-covalent binding modes to double-stranded (ds) DNA/RNA: intercalation, minor or major groove binding and external electrostatic binding (*1*). However, small molecules can rely only on a limited number of interacting groups, which can control selectivity of binding within DNA/RNA binding sites.

Therefore, it is highly challenging task to design single molecule able to selectively bind to various DNA and RNA structures and to show different response upon binding using sensitive and simple methods.

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One of the many approaches to this aim comprises synthesis of hybrid, multifunctional molecules. Such small molecule could contain the following: (a) one or more intercalator units capable for π–π stacking interaction with DNA/RNA bases, (b) nucleobase to provide Watson–Crick or non-canonical hydrogen bonding with DNA/RNA bases and (c) linker between intercalator and nucleobase (*2–10*).

Combinations of one or two intercalators, one or two linked nucleobases and variations in linker rigidity and charge caused surprisingly different behaviour upon binding to polynucleotides. That included different activity for specific secondary structures, for example poly rAH$^+$–poly rAH$^+$ regions and/or consecutive poly A regions (*8, 9*), as well as different spectroscopic response upon binding (*5, 11, 12*).

In many of these cases, structural pre-organisation of dyes in free state proved to be very important for their DNA/RNA binding properties since polynucleotide sequences differ significantly in secondary structure and binding properties of available binding sites (Table S1)†. However, the binding event usually requires adjustment of both dye and polynucleotide conformation, to achieve optimal electrostatic and/or shape sensitive host–guest interaction.

As an extension of our earlier investigation, here is the study report of interactions in aqueous solutions of previously synthesised bis-phenantridinium compounds linked to one or two adenines (**1** and **2,** respectively, Figure 1, Scheme S1†) (*7*) with ds-DNA/RNA and ss-RNA. The aim was to examine influence of one or two adenine moieties as hydrogen bond donors/acceptors and compare with previous results of reference bis-phenanthridinium **3** (*5, 7*). Conjugate **1** revealed high (log Ks = 6.9) and selective affinity towards complementary nucleotide (UMP), accompanied with specific change in the UV/Vis spectrum of phenanthridine subunits (*7*).

Results and discussion

Interactions with ds-DNA, ds-RNA and ss-RNA

Interactions of **1** and **2** (*7*) **(**Figure 1**)** with ds-polynucleotides and ss-poly U in water were examined by means of fluorimetric titrations, thermal melting experiments, CD titrations and isothermal titration calorimetry (ITC) titrations. Low solubility of compounds prevented NMR study and viscometry experiments. Examined compounds **1** and **2** and reference compound **3** were previously fully characterised in aqueous medium (*7*), whereby their improved solubility and interactions with DNA/RNA at weakly acidic conditions (pH 5) should be stressed due to the phenanthridine nitrogen protonation.

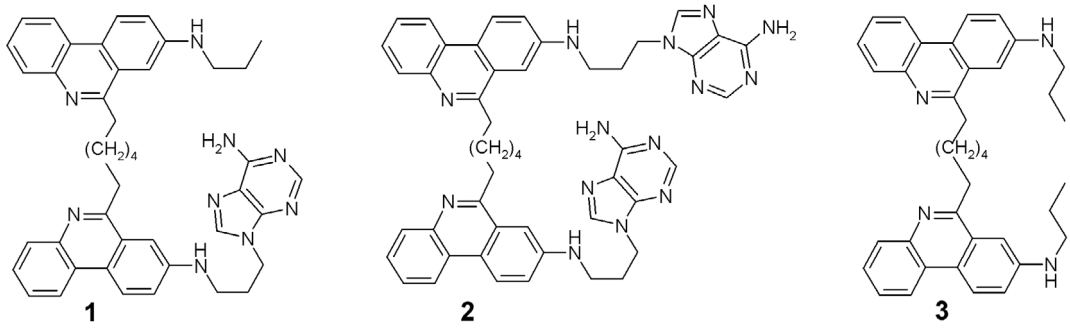

Figure 1. Examined compounds **1** and **2** and reference compound **3** (*7*).

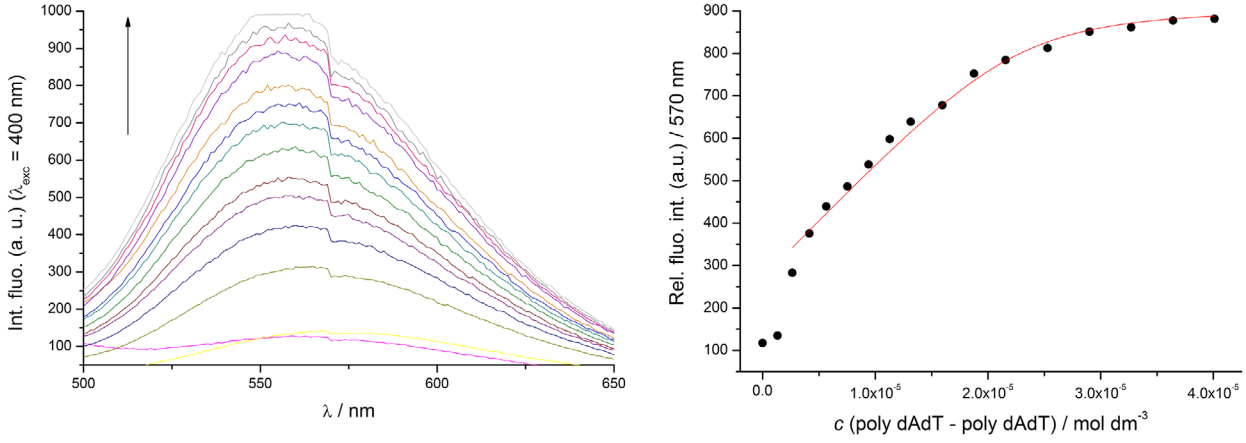

Figure 2. (Colour online) Changes in the fluorescence of **2** ($c = 2 \times 10^{-6}$ mol dm^{-3}) upon titration with poly dAdT–poly dAdT (citrate buffer, pH = 5.0, $c = 0.03$ mol dm^{-3}; $\lambda_{exc} = 400$ nm).

All experiments were done in water at weakly acidic conditions (pH 5). Phenanthridine protonation also improved recognition of nucleotide/DNA/RNA targets (*9, 13*). It should also be noted that poly rA is partially protonated at pH 5 and forms double-stranded poly AH^+–poly AH^+ (*5, 14*).

Fluorimetric studies

Conjugates **1** and **2** exhibited strong fluorescence increase upon addition of any polynucleotide (Figure 2), at variance to only slight emission increase of reference bis-phenanthridinium **3** (*5*). Although affinities of **1** and **2** (Table 1) compared with **3** (*5*) (Table S2[†]) were similar, for analogue polynucleotides, pronounced difference of spectroscopic changes pointed the impact of adenines covalently attached to phenanthridinium.

For both **1** and **2**, addition of any DNA/RNA initiated small hypsochromic shifts of emission maxima ($\Delta\lambda_{em}$ = 5–10 nm). By far the strongest emission increase of both **1** and **2** was observed upon addition of alternating AT-AT DNA and poly AH^+-poly AH^+, while other ds-DNA and ds-RNA enhanced emission of **1** and **2** to a significantly lower extent and even weaker enhancement was observed for ss-RNA. (Figure 3).

Fluorescence titration data were processed by the Scatchard equation (*15*) to get binding constants and ratios $n_{[\text{bound compound}]/[\text{polynucleotide}]}$ (Table 1). For calculations of stability constants, we used data at high excess of polynucleotide over studied compound ($r < 0.3$). These results were compared with stability constants which were calculated from ITC measurement (see chapter *ITC measurements*). For both fluorimetric and ITC titrations, values of log Ks were recalculated according to Scatchard equation for fixed value n = 0.1 for easier comparison. The results are presented in (Table 1).

Generally, adenine conjugates **1** and **2** showed similar affinity towards all studied ds-DNA, and order of magnitude lower affinity to ds-RNA. These affinities of **1** and **2** were comparable to the affinity of reference compound **3** (*5*). Based on our previous results (*4, 7, 9*) some selectivity was expected of phenanthridine–adenine conjugates towards single-stranded complementary polynucleotides, for instance poly U. While mono-adenine conjugate **1** showed very strong binding affinity and selectivity for nucleotide UMP (*7*), this behaviour did not reflect on binding to poly U (Table 1). Most intriguingly, bis-adenine conjugate **2** revealed almost two orders of magnitude higher affinity towards poly U (log Ks 4.6) in comparison with mono-adenine conjugate **1** or reference compound **3** (log Ks < 3) (*5*). Increased binding affinity could be explained

Table 1. Stability constants (log K_s)[a,b] for complexes of **1** and **2** at pH 5 with ss- and ds-polynucleotides calculated according to fluorimetric and ITC titrations.

	1		**2**	
	[a]fluorescence	[b]ITC	[a]fluorescence	[b]ITC
poly dA–poly dT	>5[c]	5.6	5.4	[d]ca. 6
poly dAdT–poly dAdT	6.3	5.8	7.1	[d]ca. 6
poly dG–poly dC	6.0	6.3	6.1	[e]
poly dGdC–poly dGdC	6.6	5.3	6.8	[e]
poly rA–poly rU	5.0	5.4	5.2	[e]
poly rAH^+–poly rAH^+	5.4	[f]	5.2	[f]
poly U	>3[g]	[f]	4.6	[f]

[a]Processing of titration data by means of Scatchard equation (*15*) gave values of ratio $n = 0.1 \pm 0.05$ for most complexes; for easier comparison, values of log Ks are recalculated for fixed n = 0.1; correlation coefficients for all calculated Ks > 0.99; estimation of error of stability constant (Ks) and n is ±20%.
[b]Stability constants calculated from ITC titrations; for easier comparison, values of log Ks are recalculated for fixed n = 0.1.
[c]Small fluorescence changes hampered accurate calculation of binding constant.
[d]Estimated value due to low heat changes, allowing collection of only limited number of points.
[e]Small heat changes did n'ot allow collections of enough data points for calculation of binding constant.
[f]Not measured.
[g]Estimated value due to less than 20% of complex formed.

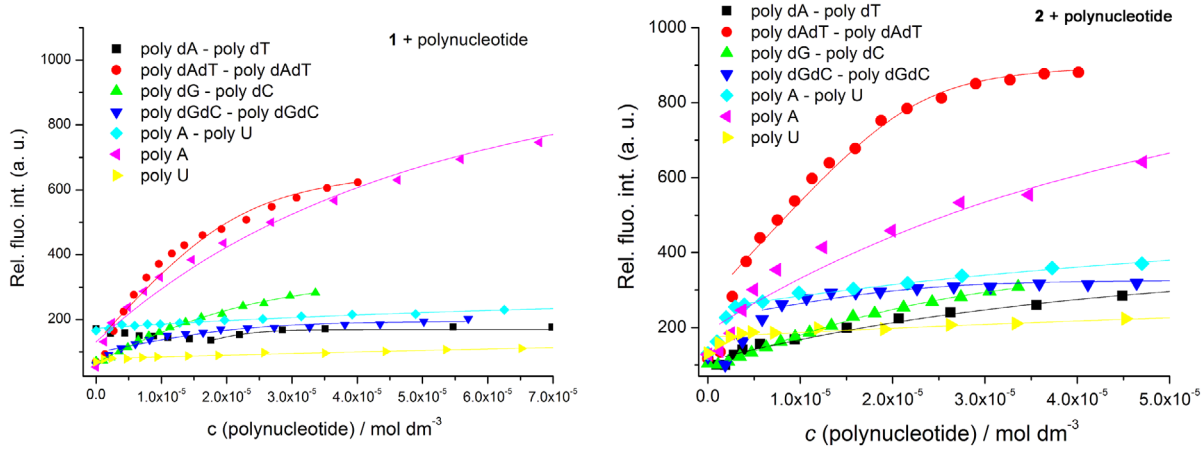

Figure 3. (Colour online) Changes in fluorescence of **1** (left) and **2** (right) ($c = 2.2 \times 10^{-6}$ mol dm^{-3}) upon addition of polynucleotides (pH = 5.0; λ_{exc} = 400 nm).

by additional interactions (possible hydrogen bonding) of two adenines of **2** with uraciles besides intercalation of phenanthridinium units into poly U.

Taking into account calculated stability constants (Table 1), particular fluorescence selectivity (Figure 3) towards AT-AT DNA and AH^+-AH^+ RNA with respect to all other DNA/RNA was not based on difference in binding affinity. Furthermore, the fluorescence of phenanthridinium intercalators is not sensitive to DNA or RNA sequence (AT-; GC- and AU-base pairs offer similar aromatic stacking possibilities) (*16*). Only the change of phenanthridinium binding mode (*17*) or interactions controlled by DNA minor groove (*18*) can cause significantly different emission response. Therefore, fluorescence sensitivity of **1** and **2** on differences in DNA/RNA structure (as well as thermal denaturation experiments) suggest binding into DNA/RNA grooves.

Closer comparison of polynucleotide structures revealed that just those polynucleotides inducing the strongest fluorescence enhancement (AT-AT DNA and AH^+-AH^+ RNA) had adenines in both strands. Moreover, fluorescence selectivity was significantly more pronounced for bisadenine conjugate **2** than for monoadenine **1**, while reference **3** showed no selectivity (*5*), which pointed towards important contribution of appended adenine(s) on fluorimetric response.

Thermal melting studies

Thermal denaturation experiments (Table S3†) revealed that **1** and **2** more efficiently stabilise alternating AT-DNA in comparison with homo AT-DNA, while ds-RNA stabilisation was negligible. Such difference between ds-DNA and ds-RNA does not support intercalative binding mode. No stabilisation of any DNA/RNA was observed for **3** (*5*), stressing the impact of appended adenines on **1** and **2**.

ITC measurements

For comparison purposes, stability constants were additionally determined by ITC titrations of polynucleotides with **1** and **2**. Despite the efforts to optimise the titrations (solubility of compound/polynucleotide complexes not allowing higher concentrations), observed heat changes during titration were quite low for **1** and even lower for **2**. For latter derivative, limited number of data were collected only for two AT-DNA polynucleotides, allowing only estimation of binding constants. Nonetheless, for the most of experiments, it was possible to calculate stability constants (Table 1, Table S4†, Supp. Info.†) which agreed well with fluorimetric results (almost within the same order of magnitude). Groove binding as the binding mode was supported by positive enthropy change (Table S4†, Supp. Info.†), which was probably consequence of the release of structured water upon complex formation (*19, 20*).

CD spectroscopic measurements

To elucidate the structural parameters of **1** and **2**/polynucleotide complexes, more structurally informative methods were necessary. We relied on the CD spectropolarimetry as a highly sensitive method towards conformational changes in the secondary structure of polynucleotides (*21*). Furthermore, achiral small molecules like **1** and **2** with no intrinsic CD spectrum could eventually acquire induced CD spectrum (ICD) upon binding to polynucleotides, which could give useful information about modes of interaction (*21, 22*).

Addition of conjugates **1** and **2** resulted for most DNA and RNA in decrease of CD bands intensity within the 230–265 nm range, attributed to the partial chirality loss of the double-stranded DNA/RNA as well as ss-RNA helices (Figures 4 and 5, Figures S3–S7†, Supp. Info.†), while reference bis-phenanthridinium **3** mostly did not change CD

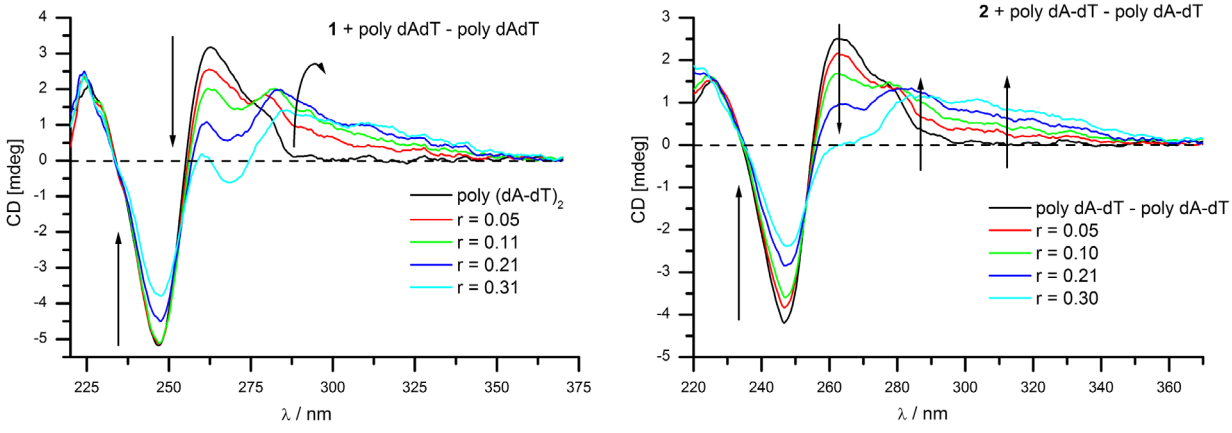

Figure 4. (Colour online) CD titration of **1** (left) and **2** (right) with poly dA-dT–poly dA-dT ($c = 2.5 \times 10^{-5}$ mol dm^{-3}) at molar ratios r = [compound]/[polynucleotide], (citrate buffer, pH = 5.0, $c = 0.03$ mol dm^{-3}).

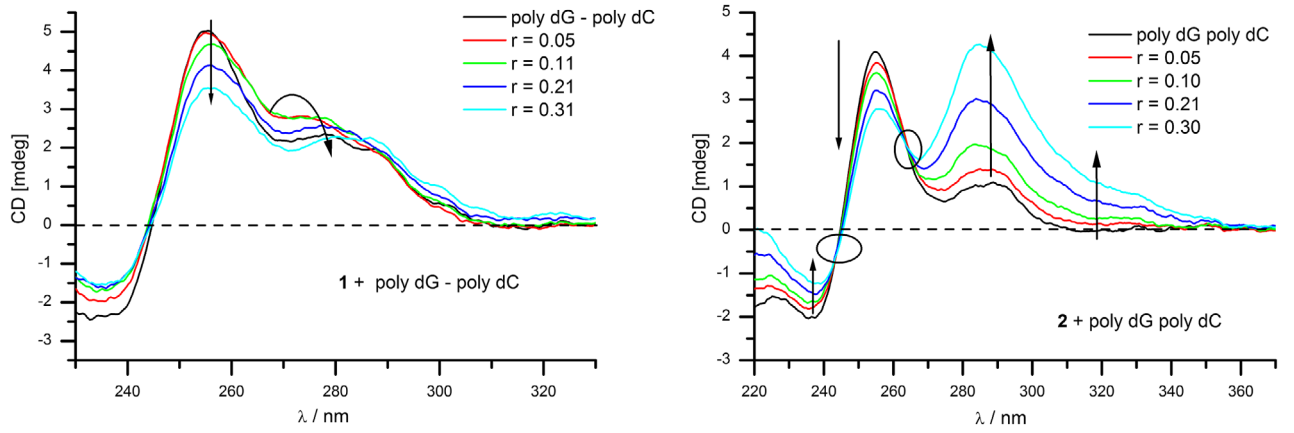

Figure 5. (Colour online) CD titration of **1** (left) and **2** (right) with poly dG–poly dC ($c = 2.5 \times 10^{-5}$ mol dm^{-3}) at molar ratios r = [compound]/[polynucleotide], (sodium citrate/HCl buffer, pH = 5.0, $c = 0.03$ mol dm^{-3}).

spectra of polynucleotides (*5*). The 265–290-nm range in CD spectra comprises the contributions of several chromophores (polynucleotide's nucleobases, adenines linked to conjugate and phenanthridinium), which are likely to mutually interact and therefore observed changes are hard to interpret. However, the intensity of changes in CD spectra was negligible for **3** (*5*), somewhat larger for mono-adenine conjugate **1** and significantly stronger for bis-adenine conjugate **2**. Such tendency is in agreement with previously noted CD spectra changes of uracil conjugates (*5*), stressing the important impact of covalently attached adenine to the uniform orientation of bis-phenanthridine chromophore within ds-polynucleotide binding site.

Changes in the CD spectra >300 nm can be attributed to the induced (I)CD bands of phenanthridinium chromophore, where DNA/RNA does not absorb. Since ICD spectra are very sensitive to mutual orientation of ligand within DNA, it indicates uniform orientation of dye molecules. The closer inspection of ICD bands at $r_{[\mathbf{1}\text{ or }\mathbf{2}]/[\text{polynucleotide}]} \leq 0.3$ (at higher ratios aggregation was observed) revealed that each DNA is characterised by different ICD bands pattern. Namely, for complexes of **1** and **2** with poly dA–poly dT (Figure S4†) and poly dGdC–poly dGdC (Figure S3†), no ICD bands >300 nm were observed†. For example, although **2,** unlike **1**, changed CD spectra of poly dA–poly dT significantly (Supp. Info., Figure S4†), absence of obvious ICD signal addressed to non-uniform binding of **2** with respect to chiral polynucleotide's backbone.

On the other hand, binding of **1** and **2** to poly dAdT–poly dAdT and poly dG–poly dC caused positive ICD bands >300 nm (Figures 4 and 5), whereby isoelliptic points (marked by ellipses) supported one type of complex.

For both compounds, significant change of CD spectra could be noticed at λ 230–260. Pronounced change of CD spectra was caused by change of DNA chiral backbone (Figure 4). The appearance of ICD bands >300 nm could be correlated to the steric properties of corresponding DNA minor groove (Table S1)†. For instance, poly dAdT–poly dAdT minor groove width is ideal for small molecules binding even for aromatic dimers (*23, 24*), while by half narrower minor groove of poly dA–poly dT (*25*) cannot accommodate folded structure of **1** and **2** without significant structural rearranging (*24*).

Width of poly dGdC–poly dGdC minor groove is broader than poly dAdT–poly dAdT but exocyclic guanine amino groups protruding from both DNA strands into minor groove sterically hinder small molecule insertion, thus almost completely abolishing positive ICD band >300 nm (see Supp. Info.†). However, poly dG–poly dC guanine amino groups are aligned along only one side of minor groove, the dC-side obviously available for insertion and uniform orientation of phenanthridine chromophore (*14, 26*). For instance, it was shown that acceptors that are sterically able to accommodate wider groove and to form hydrogen bonds with guanine amino group will be favourable to bind to GC sequences (*27*). The wider groove width of GC sequences can also favour the formation of stacked heterocyclic complexes within the groove (*19, 28*), while nitrogen atoms as H-bond acceptors are proposed to be useful in recognising important minor groove elements of GC-rich regions (*27*).

Comparison of CD titrations of poly dG–poly dC with **1** and **2** points that two similar compounds, that differ in one adenine moiety, cause considerably different change of CD spectra of homo-polymer pdG-pdC (Figure 5). Structural difference resulted in distinctively different influence on structure and chirality of this DNA: mono-adenine compound **1** caused weak ICD signal >300 nm, while bis-adenine compound **2** induced very strong positive ICD signal, that suggested again synergistic impact of two adenines of **2** on the uniform orientation of phenanthridine chromophores with respect to the DNA helical axis. Conjugate **2** probably adopts appropriate geometry for wider poly dG–poly dC minor groove, whose shape offers possibility

for binding of stacked molecules. Further, it is known that non-canonical GA pair can stabilise RNA tertiary structures, especially at pH values below 7 (*29, 30*). Since adenine moieties of **2** were not stacked inside of phenanthridinium lypophilic cavity, they could be available for additional interaction, possibly non-canonical hydrogen bonding that could stabilise DNA–dye complex.

Interaction of **1** and **2** with ds-RNA (poly rA–poly rU) showed decrease of band at 265 nm much more pronounced for **2** than for **1**, and only **2** induced weak negative ICD band >300 nm (Figure S5†). Similar changes of the band at 265 nm were observed for poly AH^+–poly AH^+ (Figure S6†). The interaction of **1** and **2** with poly rU, ss-polynucleotide complementary to adenines attached to studied compounds, revealed decrease of polynucleotide CD spectrum (Figure S7†), that was more pronounced for **2**. Since both species, RNA and dye, absorb at 275 nm, it is not possible to distiguish decrease of poly U CD spectra from eventual negative ICD signal; however, observed changes could result from the intercalation (*22, 31, 32*) of phenanthridinium unit.

Conclusions

Selective spectroscopic responses of conjugates **1** and **2** on various DNA/RNA depend on the attached adenine(s) that is additionally stressed by very poor spectrophotometric response of reference **3** in all experiments with DNA/RNA. Furthermore, **1** or **2** spectrophotometric response depends on two essential parameters: (a) the convenient size and steric property of minor groove (positive ICD bands observed only for alternating AT- and homo-GC-DNA) and (b) the possibility of **1-** and **2**-attached adenine(s) to form additional interactions within grooves (yielding ratiometric fluorescence recognition of only alternating AT-DNA, or pAH^+-pAH^+ with respect to much weaker emission increase for homo-GC-DNA and other polynucleotides). Compound **2** showed stronger signal response (ICD, fluorescence) then **1,** thus additionally stressing the adenine interaction contributions. In this line is also stronger affinity of bis-adenine conjugate **2** towards complementary ss-RNA (poly rU), in comparison with mono-adenine **1** and reference **3**, indicating synergistic interaction of two attached adenines of **2** with uracils.

These properties support further studies on **1** and **2** and their analogues with the aim of dual-method probe development, relying on fluorimetry and CD measurements. For instance, new probe could simultaneously detect the presence of alternating AT-AT DNA and homo-GC-DNA along the mixed ds-DNA sequence (of known number of base pairs) by ICD bands >300 nm. Further, it would be possible to deduce the ratio between alternating AT-AT sequence occurrence with respect to all other types of sequences used in this study by analysis of fluorescence increase intensity with respect to DNA-base pair composition. Since readability of both emission and CD signal for **1** and **2** is currently in submicromolar range, chromophore improvement planned in our future research could additionally decrease detection threshold, approaching the nanomolar sensitivity of the most used fluorimetric probes.

Experimental

Materials

Studied **1** and **2** were prepared according to published procedures (*7*).

Polynucleotides were purchased as noted: poly dGdC–poly dGdC, poly dAdT–poly dAdT, poly dG–poly dC, poly dA–poly dT, poly A–poly U, poly A and poly U (Sigma) and dissolved in sodium cacodylate buffer, I = 0.05 mol dm^{-3}, pH = 7. Polynucleotide concentration was determined spectroscopically (*33*) as the concentration of phosphates. The concentration of the stock solution (10 mM) of single-stranded poly A at pH 7 was determined by UV absorbance measurement at 258 nm using a molar extinction coefficient (ε) value of 9800 M^{-1} cm^{-1}, and it was expressed as the concentration of phosphates. It is important to note that at experimental conditions (pH = 5), poly A was protonated and formed double helix (*5, 14*). The double-stranded conformation of poly (A) was obtained by lowering the pH value from the initial value of 7.0 to 5.0 and its concentration directly derived from the concentration (ss-poly A). The formation of ds-poly A was confirmed by CD and thermal melting experiments (*34, 35*).

Procedures

Thermal melting studies

Thermal melting experiments were performed on a Varian Cary 100 Bio spectrometer in quartz cuvettes (1 cm). The measurements were done in aqueous buffer solution at pH 5 (sodium citrate/HCl buffer I = 0.03 mol dm^{-3}). Thermal melting curves for ds-DNA, ds-RNA and their complexes with **1** and **2** were determined by following the absorption change at 260 nm, a function of temperature (*36, 37*). Absorbance of the ligands was subtracted from every curve, and the absorbance scale was normalised. T_m values are the mid-points of the transition curves determined from the maximum of the first derivative and checked graphically by the tangent method. The ΔT_m values were calculated subtracting T_m of the free nucleic acid from T_m of the complex. Every ΔT_m value here reported was the average of at least two measurements. The error in ΔT_m is ±0.5 °C.

Fluorimetric studies

Fluorescence spectra were recorded on Varian Cary Eclipse fluorimeter in quartz cuvettes (1 cm). The measurements were performed in aqueous buffer solution at pH 5 (sodium citrate/HCl buffer, $I = 0.03$ mol dm^{-3}). In fluorimetric experiments, excitation wavelengths at $\lambda_{max} = 400$ nm were used in order to avoid absorption of excitation light by added polynucleotides. Fluorimetric titrations were performed by adding portions of polynucleotide solution ($c = 2\times10^{-6}$ mol dm^{-3}) into the solution of the studied compound. After mixing polynucleotides with studied compounds, it was observed in all cases that equilibrium was reached in less than 120 s. In following 2–3 h, fluorescence spectra of complexes remained constant. Emission was collected in the range $\lambda_{em} = 500$–650 nm. Fluorescence spectra were collected at $r < 0.3$ (r = (compound)/(polynucleotide)) to assure one dominant binding mode. Data that were processed by means of Scatchard equation (*15*) gave values of ratio $n_{[\text{bound compound}]/[\text{polynucleotide}]}$ in the range 0.07–0.15, but for easier comparison, all Ks values were re-calculated for fixed $n = 0.1$. Calculated values for Ks have satisfactory correlation coefficients (>0.99).

CD spectroscopic measurements

CD spectra were recorded on JASCO J815 spectrophotometer at room temperature using appropriate 1 cm path quartz cuvettes in sodium citrate/HCl buffer ($I = 0.03$ mol dm^{-3}) with scanning speed of 200 nm/min. Buffer background was subtracted from each spectra, while each spectrum was result of five accumulations. Examined **1** and **2** are achiral and therefore do not possess intrinsic CD spectrum. CD experiments were performed by adding portions of compound stock solution into the solution of polynucleotide ($c = 2\times10^{-5}$ mol dm^{-3}).

ITC measurements

ITC experiments were performed on a MicroCal VP-ITC microcalorimeter (MicroCal, Inc., Northampton, MA, USA). Origin 7.0 software, supplied by the manufacturer, was used for data analysis. The reference cell was filled with ultrapure water. In all titration experiments, stock solutions of **1** or **2** were injected from rotating syringe (307 rpm) into the isothermal cell, equilibrated at 25.0 °C, containing 1.4406 mL of polynucleotide.

In the titration experiments, aliquots of the compounds **1** and **2** (10–20 µL, $c = 22$–160 µM) were injected from a rotating syringe (307 rpm) into the calorimeter reaction cell containing 1.4406 mL of the polynucleotide ($c = 13$–100 µM).

The time spacing between each injection was 180 s in all experiments with the compound **1** and 240 s for the experiments with the compound **2**. Initial delay before first injection was 600 s in all experiments. All solutions used for ITC experiments were degassed prior to use under vacuum (0.3 bar, 10 min) to eliminate air bubbles. Also, blank experiments were done to determine the heats of dilution.

Electronic Supplementary Information (ESI)† available

Table with structural properties of DNA and RNA, stability constants data for reference **3**, ITC data, CD spectra can be found online here: http://dx.doi.org/10.1080/10610278.2015.1099655

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