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This is an **author produced version** of a paper published in:

Organic and Biomolecular Chemistry 15.36 (2017): 7558-75655

DOI: <http://dx.doi.org/10.1039/C7OB01930K>

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# Dye-conjugated Complementary Lipophilic Nucleosides as Useful Probes to Study Association Processes by Fluorescence Resonance Energy Transfer†

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Modern supramolecular chemistry relies on the combination of diverse analytical techniques that can afford complementary information on complex self-assembly landscapes. Among them, resonance energy transfer, monitored by fluorescence emission spectroscopy, arises as a sensitive and convenient phenomenon to report binding intermolecular interactions. The use of molecular probes labelled with suitable complementary energy-transfer pairs can provide valuable information about the thermodynamics, kinetics and self-sorting characteristics of a particular self-assembled system. The objective of this work is the generation of a set of nucleoside FRET probes that can be reliably employed to prove and analyse quantitatively H-bonding interactions between complementary Watson-Crick pairs. We first describe the preparation of a set of lipophilic nucleosides that are linked to a  $\pi$ -conjugated functional fragment. The bases include guanosine, 2-aminoadenosine as purine heterocycles, and cytidine, and uridine as complementary pyrimidine bases. The  $\pi$ -conjugated moiety comprises either a short phenylene-ethynylene oligomer, a bithiophene, or a BODIPY dye. We then demonstrate that the last two chromophores constitute an energy donor-acceptor couple and that donor emission quenching can be related to the ratio of molecules bound to the complementary acceptor pair. Hence, fluorescence spectroscopy in combination with resonance energy transfer, are shown here to be useful tools to study and quantify the association and self-sorting events between complementary and non-complementary nucleosides in apolar aromatic solvents, where binding strength is considerably high and sensitive techniques that employ low concentrations are demanded.

## Introduction

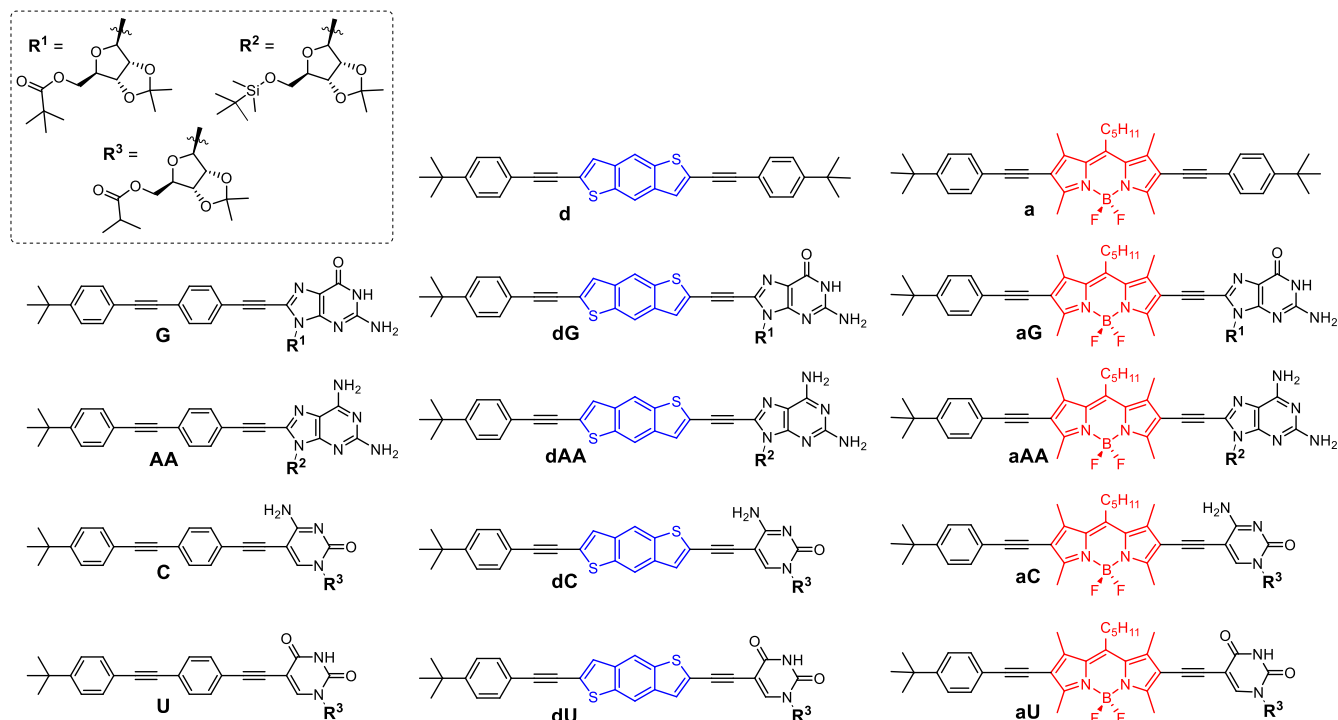
The design of heterocyclic moieties having specific H-bonding patterns of donor and acceptor groups<sup>1</sup> has been exploited by supramolecular chemists to construct a wide variety of self-assembled systems.<sup>2</sup> Inspired by the DNA double strand association, one of the most commonly employed sets of H-bonding modules are the nucleobases. A plethora of synthetic assemblies, that range from discrete complexes to soft and polymeric materials have been developed by making use of complementary Watson-Crick H-bonding interactions.<sup>3</sup> In order to achieve large binding constants ( $> 10^4$ - $10^5$  M<sup>-1</sup>) and therefore high self-assembly fidelities in organic solvents, chemists have to rely on cooperative effects,<sup>4</sup> reduce the solvent H-bonding competing ability, or increase the number of H-bonding donor and acceptor groups in the heterocyclic fragment to three or even four.<sup>5</sup> However, as the supramolecular systems increase in complexity due to a large number of participating entities and/or to the establishment of multiple competing equilibria, their full understanding becomes likewise more complicated. The typical methods to quantify such strong associations,<sup>6</sup> like <sup>1</sup>H NMR, become no longer reliable and more sensitive techniques,<sup>7</sup> that require lower concentrations and that can afford complementary information are demanded.

Among these methods, Förster resonance energy transfer (FRET), measured by fluorescence spectroscopy, is a particularly useful phenomenon in which the energy of a photoexcited donor fluorophore (d) is conveyed to an energy-accepting unit

(a) through long-range dipole-dipole interactions.<sup>8</sup> This excitation energy transference exhibits a strong dependence on the spectral overlap of donor emission and acceptor absorption, as well as on their relative orientation and distance, covering the dimensions of most (bio)molecular complexes (1-10 nm). These attributes, in combination with the inherent characteristics of fluorescence emission, makes FRET a potent tool to probe molecular interactions and dynamic changes in supramolecular chemistry.<sup>9</sup> After the conceptual work of Sessler and Harriman on noncovalently linked photosynthetic model systems based on nucleobase pairing,<sup>10</sup> the groups of Rebek<sup>11</sup> and Diederich<sup>12</sup> provided some of the first examples on the use of FRET to study exchange kinetics and time-resolved conformational switching in supramolecular complexes. Donor-acceptor energy transfer probes have also been used as a tool to investigate diverse features of discrete self-assembled systems, such as: isomer distribution of dimeric cyclic peptides;<sup>13</sup> guest inclusion in G-quadruplexes;<sup>14</sup> protein assembly and activity with synthetic supramolecular elements;<sup>15</sup> or rotaxane,<sup>16</sup> foldaxane<sup>17</sup> and coordination-driven self-assembly<sup>18</sup> dynamics.

The objective of this work, which is integrated in a wider research programme, is the generation of a set of nucleoside FRET probes that can be reliably employed to prove and analyse quantitatively H-bonding interactions between complementary Watson-Crick pairs. Firstly, we report on the synthesis and characterization of the set of molecular probes displayed in Figure 1. They comprise non-binding reference compounds (**d** and **a**), and nucleosides (guanosine (**G**), cytidine (**C**), 2-aminoadenosine (hereafter abbreviated as **AA**) and uridine (**U**)) having a non-chromophoric p-phenylene moiety (**G**, **C**, **AA**, **U**), an energy donor benzo[1,2-b:4,5-b']dithiophene unit<sup>19</sup> (**dG**, **dC**, **dAA**, **dU**), or an acceptor BODIPY<sup>20</sup> block (**aG**, **aC**, **aAA**, **aU**). To

were substituted with bulky lipophilic groups so as to increase solubility and prevent stacking interactions. Furthermore, with this family of molecular probes, we evaluate FRET processes as a tool to monitor binding interactions between complementary and non-complementary lipophilic nucleosides. We demonstrate that donor emission quenching can be related to the ratio of molecules bound to the complementary acceptor pair. In this way, titration experiments allowed us to measure their association strength in apolar aromatic solvents, where binding strength is considerably high and sensitive techniques that employ low concentrations are demanded. Moreover, this family of nucleoside FRET probes are employed to assess the



the best of our knowledge, this is the first time these dyes are employed as a FRET pair. The ribose units at the nucleobases

occurrence of self-sorting events between complementary and non-complementary nucleobases.

**Fig. 1** Structure of reference compounds **d** and **a**, and nucleosides **G**, **A**, **C**, **U**, **dG**, **dAA**, **dC**, **dU**, **aG**, **aAA**, **aC** and **aU**.

## Results and discussion

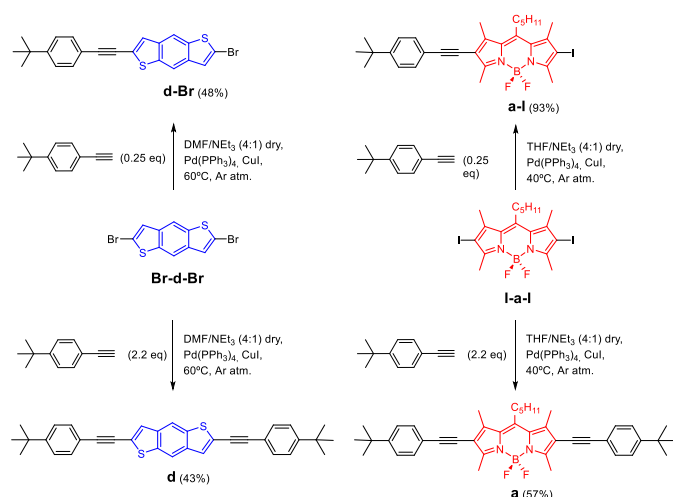
### Synthesis of Lipophilic Nucleosides

Nucleosides **dG**, **dC**, **dAA**, **dU**, **aG**, **aC**, **aAA** and **aU**, as well as reference compounds **d**, **a**, (Figure 1) were synthesized by consecutive palladium-catalyzed Sonogashira reactions between the corresponding dihalogenated central blocks, which carry the energy donor (bithiophene) and acceptor (BODIPY) functions, and 5- (pyrimidines) or 8- (purines) ethynyl-substituted nucleosides equipped with lipophilic riboses (**G1**, **C1**, **AA1** and **U1**). The synthesis and characterization of the latter, as well as of reference nucleosides **G**, **C**, **AA** and **U** was reported previously by us.<sup>21</sup>

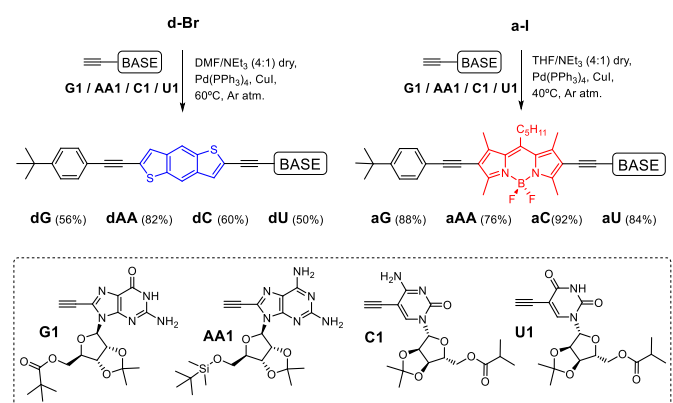
Scheme 1 shows the synthesis of the donor and acceptor references **d** and **a**, as well as monohalogenated intermediates

**d-Br** and **a-I**. The substitution products can be obtained in the same reaction and subsequently separated by column chromatography. In order to optimize their relative abundance in the reaction crude according to our needs, we adjusted the 4-*tert*-butyl-phenylacetylene / dihalogenated block ratio (see Scheme 1 and the experimental section at the Supporting Information). In general, the yields obtained in these Sonogashira reactions range between 40-95%, and are higher and required milder conditions when the iodinated BODIPY central block was employed.

Functional donor **dG**, **dC**, **dAA** and **dU**, and acceptor **aG**, **aC**, **aAA** and **aU** nucleosides were then respectively prepared from **d-Br** and **a-I** in a single step by Sonogashira coupling with the corresponding 8-ethynylpurine (**G1**, **AA1**) and 5-ethynylpyrimidine (**C1**, **U1**), as shown in Scheme 2.



**Scheme 1.** Synthesis of compounds **d**, **a**, **d-Br** and **d-I** from dibromobithiophene **Br-d-Br** and diiodobODIPY **I-a-I**.



**Scheme 2.** Synthesis of nucleosides **dG**, **dAA**, **dC**, **dU**, **aG**, **aAA**, **aC** and **aU** via Sonogashira coupling reaction between **d-Br** or **a-I** and ethynyl-nucleobases **G1**, **AA1**, **C1** and **U1**.

## Determination of the fundamental electrochemical and photophysical properties

Previous to the analysis of their binding properties, we performed a series of absorption, fluorescence emission and cyclic voltammetry (CV) measurements in order to determine the most important electrochemical and photophysical parameters of the reference compounds, as well as of the mono- and dinucleosides monomers. These include the maximum absorption wavelength ( $\lambda_{\max}^{\text{ab}}$ ), the extinction coefficient at that wavelength ( $\epsilon_{\max}$ ), the maximum emission wavelength ( $\lambda_{\max}^{\text{em}}$ ), the fluorescence quantum yield ( $\Phi_f$ ), and the oxidation and reduction wave potentials ( $E_{\text{ox}}$  and  $E_{\text{red}}$ ). All the measured values are shown in Table 1. Further details, as well as cyclic voltammograms (Figure S1) and absorption and emission spectra (Figure S2) of the reference compounds can be found in the Supporting Information of this article.

Reference nucleosides **G**, **C**, **AA** and **U** display absorption and emission maxima around 330-360 nm and 365-425 nm, respectively. Due to their larger  $\pi$ -system, purines **G** and **AA** have red-shifted absorption and emission features with respect to pyrimidines **C** and **U**. Emission quantum yields are also larger

for the purine molecules. Compounds equipped with the donor bithiophene **d** unit display absorption maxima in the 360-400 nm range, while the emission is characterized by two maxima in the 410-460 nm range. BODIPY-containing energy-accepting **a** molecules show absorption maxima around 570 nm, whereas emission maxima are centered in the 600-615 nm range. Again, the presence of the purine heterocycle produces slightly larger red-shifts in absorption and emission maxima than pyrimidines. On the other hand, the base attached does not have a pronounced impact on the emission quantum yields of bithiophene donors, which always range between 0.35 and 0.50. They reduce, however, the emission quantum yield of the BODIPY dyes, especially in the case of purines **aG** and **aAA**. This partial quenching might be ascribed to the participation of a photoinduced electron transfer event from the purines, which exhibit the lowest oxidation potentials among the nucleobases (*ca.* 0.8 V vs  $\text{Fc}/\text{Fc}^+$ ), to the BODIPY acceptor, whose reduction potential is close to -1.5 V.

**Table 1.** Most relevant photophysical and electrochemical parameters of the reference compounds and nucleosides employed in this work.

Compound	$\lambda_{\max}^{\text{ab}}$ nm <sup>[a]</sup>	$\epsilon_{\max}$ M <sup>-1</sup> cm <sup>-1</sup> <sup>[a]</sup>	$\lambda_{\max}^{\text{em}}$ nm <sup>[a]</sup>	$\Phi_f$ [a]	$E_{\text{ox}}$ V <sup>[b]</sup>	$E_{\text{red}}$ V <sup>[b]</sup>
<b>d</b>	363	68700	405, 430	0.36	0.84	-[c]
<b>a</b>	564	60800	600	0.85	0.70	-1.57
<b>G</b>	351	61000	399, 418	0.65	0.85	-[c]
<b>AA</b>	360	49300	426	0.87	0.77	-[c]
<b>C</b>	331	61700	366, 384	0.25	-[c]	-[c]
<b>U</b>	330	40400	368, 389	0.25	-[c]	-[c]
<b>dG</b>	392	43600	427, 454	0.44		
<b>dAA</b>	394	45500	435, 458	0.40		
<b>dC</b>	368	51400	411, 435	0.49		
<b>dU</b>	366	53200	417, 443	0.36		
<b>aG</b>	568	33800	614	0.33		
<b>aAA</b>	572	40000	610	0.35		
<b>aC</b>	564	44700	609	0.64		
<b>aU</b>	568	36000	602	0.59		

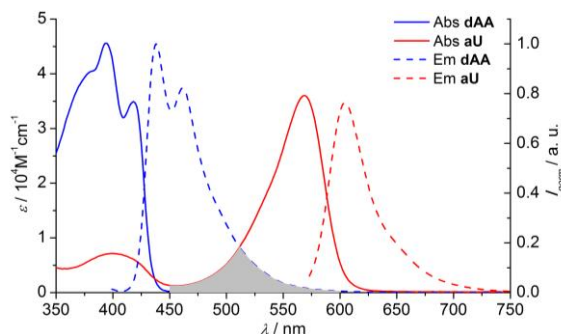
<sup>[a]</sup> Data in toluene. <sup>[b]</sup> Data in  $\text{CH}_2\text{Cl}_2$  vs  $\text{Fc}/\text{Fc}^+$ . <sup>[c]</sup> No redox process was detected within the -1.8 to 1.5 V scan window (see Figure S1).

Bithiophene and BODIPY dyes **d** and **a**, display absorption and emission maxima that are separated by about 200 nm and the donor emission partially overlaps with acceptor absorption in the 450-550 nm region (see Figure 2 as an example), which is a requirement for achieving high FRET efficiencies.

## Analysis of the FRET effect between energy donor and acceptor pairs

The energy transfer effect was then analysed in appropriate donor-acceptor complementary and non-complementary 1:1 mixtures. A set of experiments were designed that included recording: 1) Absorption spectra of the donor, the acceptor and the 1:1 mixture; 2) Emission spectra of the donor, the acceptor

and the 1:1 mixture at diverse excitation wavelengths, one of them corresponding to the maximum donor/acceptor absorption ratio (in the 350-400 nm region, see Figure 2, in order to maximize the FRET effect); 3) Excitation spectra of the donor, the acceptor and the 1:1 mixture at different emission wavelengths, namely the donor and acceptor emission maxima; 4) Temperature-dependent emission spectra of the 1:1 mixture within the -5–95 °C range. Furthermore, each of these experiments was performed at two concentrations in toluene:  $5 \cdot 10^{-5}$  M and  $10^{-5}$  M.



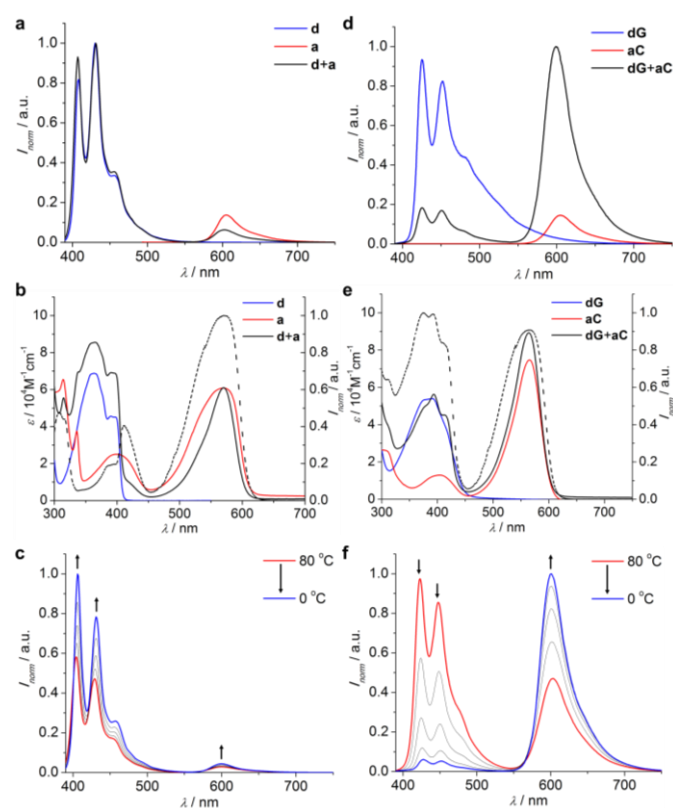
**Fig. 2** Absorption and emission spectra of **dAA** ( $\lambda_{\text{exc}} = 438$  nm) and **aU** ( $\lambda_{\text{exc}} = 602$  nm) in toluene at  $1 \cdot 10^{-5}$  M, showing the partial spectral overlap (grey area) between donor emission and acceptor absorption.

We first analysed the **d+a** 1:1 mixture. We wanted to have a reference situation where no specific H-bonding takes place, since none of these compounds is substituted by nucleobases, and discard energy transfer effects caused by intermolecular association *via* other non-specific supramolecular interactions, like  $\pi$ - $\pi$  stacking of donor and acceptor molecules. The results with this pair of compounds are shown in Figures 3a-c. Both absorption and emission spectra of the **d+a** 1:1 mixture at the two concentrations are basically a superimposition of the spectra measured for **d** and **a** separately, indicating that no FRET occurs in this non-interacting mixture (Figure 3a). Excitation experiments supported this conclusion, since the excitation spectrum recorded at the acceptor emission maximum displayed only the absorption features of the **a** molecule, and no significant contribution of the donor **d** was detected (Figure 3b). Moreover, in temperature-dependent experiments, both **d** and **a** emission intensity moderately increased with decreasing temperature (Figure 3c), a commonly observed phenomenon that is attributed to the planarization of the  $\pi$ -system and that further discards any interaction between these molecules.

This situation was then compared with mixtures of donor-acceptor pairs bearing complementary nucleobases, i.e. **dG+aC**, **dC+aG**, **dAA+aU**, and **dU+AA**. We show in Figure 3d-f the results obtained with the **dG+aC** combination, while the other mixtures are displayed in the S.I. (Figure S2).

When mixing the **dG+aC** FRET couple, the spectra obtained manifest a drastic change with respect to the isolated **dG** and **aC** solutions and the results are markedly different with respect to the previous **d+a** 1:1 mixture. First of all, fluorescence emission of the donor **dG** component is strongly quenched in

the mixture, while **aC** acceptor emission is enhanced (Figure 3d). Moreover, the excitation spectra showed now a significant contribution of the **dG** chromophore to the **aC** emission band (Figure 3e). These features are characteristic of an energy transfer event from donor to acceptor that, in view of the almost complete **dG** emission quenching measured at  $5 \cdot 10^{-5}$  M, we can define as virtually quantitative. Energy transfer is hence observed because of the strong G-C binding between donor and acceptor components, which are held at close distances in the complex. As a matter of fact, and in sharp contrast to the previous **d+a** situation, the FRET effect decreases considerably with increasing temperature (Figure 3f), which is a direct consequence of the dissociation of the **dG+aC** complex. Similar results were obtained by exchanging donor and acceptor components in the **dC+aG** FRET couple, and **dC** emission was again quenched in the 1:1 mixture (Figure S2B).



**Fig. 3** (a-b, d-e) Emission ( $\lambda_{\text{exc}} = 353$  nm) (a,d) absorption (solid lines) and excitation (dashed lines;  $\lambda_{\text{em}} = 612$  nm) (b,e) spectra of compounds **d** and **a** (a,b) or **dG** and **aC** (d,e) and their 1:1 mixtures **d+a** or **dG+aC**. (c, f) Emission spectra ( $\lambda_{\text{exc}} = 353$  nm) of the **d+a** (c) or **dG+aC** (f) 1:1 mixtures as a function of temperature. Arrows indicate the evolution of donor and acceptor emission maxima when decreasing temperature in the 80-0 °C range. In all cases the concentration of donor and acceptor compounds was set at  $C = 1 \cdot 10^{-5}$  M in toluene.

Donor emission deactivation by energy transfer was in contrast much less efficient using the **dAA+aU** or **dU+AA** FRET pairs or mixtures of non-complementary bases (see Figure S2). Table 2 collects the donor fluorescence emission quenching ratios measured in toluene solutions of 1:1 mixtures of donor-acceptor chromophores. The use of the AA-U binding interaction led to FRET efficiencies that are negligible at  $10^{-5}$  M

and not higher than 0.4 at  $5 \cdot 10^{-5}$  M. This was obviously ascribed to a lower population of H-bound donor-acceptor pairs in solution due to the weaker association constant between complementary nucleobases, when compared to the G-C pair (*vide infra*). On the other hand, the use of non-complementary purine-pyrimidine donor-acceptor FRET couples like, for instance **dAA** + **aC** or **dG** + **aU** (Figure S2), led to small or null energy transfer efficiencies, which resembles the results obtained with the non-interacting **d+a** 1:1 mixture.

**Table 2.** Emission quenching ratios and FRET efficiencies obtained in 1:1 donor-acceptor mixtures, and calculated association constants ( $M^{-1}$ ) between complementary nucleosides in toluene.

	aG	aC	aAA	aU
dG		0.86/0.99 <sup>[a]</sup> 5.0·10 <sup>5</sup> <sup>[b]</sup> 1.2·10 <sup>5</sup> <sup>[c]</sup>		0.05/0.12 <sup>[a]</sup>
dC	0.56/0.84 <sup>[a]</sup> 3.0·10 <sup>5</sup> <sup>[b]</sup> n.d. <sup>[c]</sup>			
dAA		0/0.04 <sup>[a]</sup>		0.03/0.26 <sup>[a]</sup> 2.0·10 <sup>3</sup> <sup>[b]</sup> 7.8·10 <sup>3</sup> <sup>[c]</sup>
dU			0.10/0.37 <sup>[a]</sup> 1.6·10 <sup>3</sup> <sup>[b]</sup> n.d. <sup>[c]</sup>	

<sup>[a]</sup> FRET efficiency at  $10^{-5}$  M /  $5 \cdot 10^{-5}$  M concentration calculated as:  $E_{\text{FRET}} = 1 - (I_{\text{DA}}/I_{\text{D}})$ , where  $I_{\text{D}}$  and  $I_{\text{DA}}$  are the fluorescence emission intensities of the donor molecule in the absence or presence of the corresponding acceptor, respectively. <sup>[b]</sup> Association constants ( $M^{-1}$ ) calculated from emission data and fitted with ReactLabTM EQUILIBRIA. <sup>[c]</sup> NMR data obtained from titration experiments with molecules **G**, **C**, **AA**, **U** and fitted with the Matlab® scripts developed by P. Thordarson. <sup>[23]</sup> n.d.: not determined (see text)

#### Analysis of the 1:1 binding between complementary mononucleosides. Determination of the G-C and AA-U association constants ( $K_a$ ) in toluene.

Guanine-cytosine and adenine or 2-aminoadenine-uracil binding in organic solvents, usually  $\text{CHCl}_3$ , has already been studied by a number of authors by means of  $^1\text{H}$  NMR titrations.<sup>18,24,25</sup> However, association constants between complementary nucleobases have, to the best of our knowledge, never been reported before in solvents of lower polarity, like toluene, despite aromatic solvents are often used in nucleobase self-assembled systems. The reasons are manifold. On one hand, the strong self-association of the nucleobases (in particular G) at typical (relatively high) NMR concentrations in apolar solvents results in low solubilities and produce rather broad and poorly resolved  $^1\text{H}$  NMR spectra. On the other, the increase in association strength of the complementary 1:1 complexes results in binding isotherms that saturate quite rapidly, avoiding an accurate fitting of the equilibrium data. All these problems were evident when trying to determine the association constants in  $^1\text{H}$  NMR titration experiments in toluene- $\text{D}_8$ . The bithiophene and BODIPY mononucleosides could not be employed here due to their low solubility and broad spectrum features in toluene- $\text{D}_8$ . We then

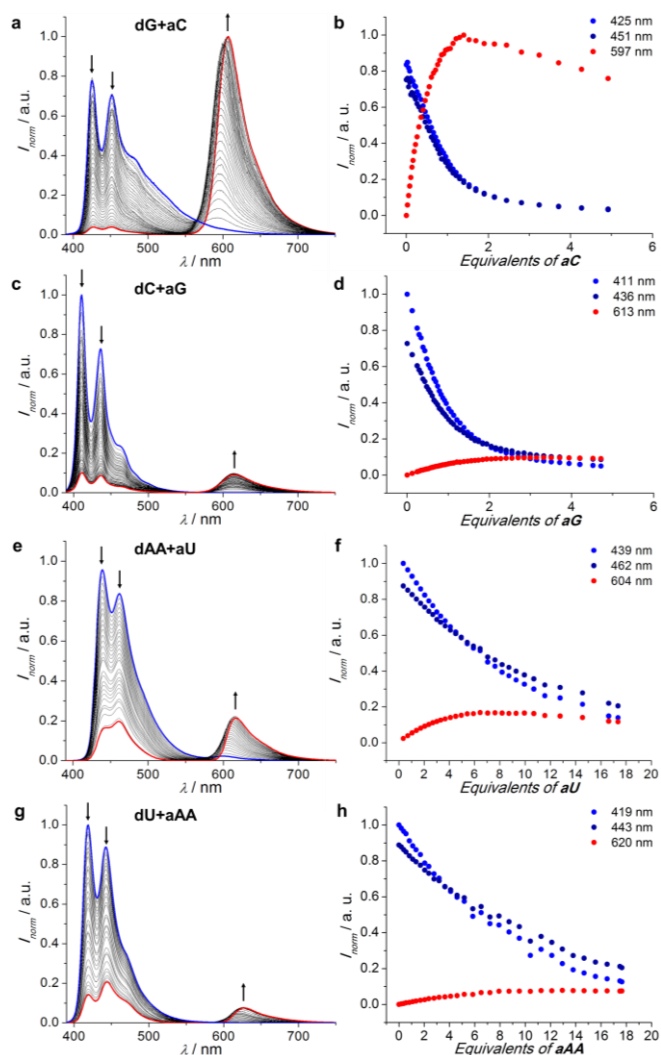
tried with compounds **G**, **C**, **AA** and **U**, but still the NMR features appeared broad and poorly resolved, even at relatively low concentrations for NMR ( $1 \cdot 10^{-4}$  M), so only a few of these titrations could be fitted properly (Table 2; see also Figure S3B and Table S1).

However, the previous experiments demonstrate that the donor emission can be efficiently deactivated in the presence of the acceptor due to an energy transfer process and that the extent of emission quenching depends on the population of H-bound donor-acceptor pairs. Therefore, emission spectroscopy, which employs lower analyte concentrations, can be employed here to determine the association constant ( $K_a$ ) between complementary nucleosides by titration experiments. These measurements report through binding isotherms the changes in a physical observable (in this case, emission intensity) experienced by the donor upon varying the concentration of the acceptor.

Increasing amounts of the corresponding acceptor were added to toluene solutions of the donor, whose concentration was kept constant along the experiment. We evaluated G-C and AA-U binding in 4 titration experiments: **dG+aC**, **dC+aG**, **dAA+aU**, and **dU+aAA**. Figure 4 shows the evolution of the emission spectra along these titrations in toluene and the representative binding isotherms obtained for the G-C and AA-U pairs. The measured binding constants between complementary bases in toluene, displayed in Table 2 (see also Figure S3), are on the order of *ca.*  $K_{\text{G-C}} = 3\text{-}5 \cdot 10^5 M^{-1}$  and *ca.*  $K_{\text{AA-U}} = 2 \cdot 10^3 M^{-1}$ . These values are significantly higher, about one order of magnitude, than those reported in  $\text{CHCl}_3$  ( $K_{\text{G-C}} = 3 \cdot 10^4 M^{-1}$  and  $K_{\text{AA-U}} = 3 \cdot 10^2 M^{-1}$ ),<sup>21-23</sup> as expected in view of the lower H-bonding competing ability of this apolar aromatic solvent. Unfortunately, owing to lack of data from the literature, we could not evaluate if the association constants calculated from our modified nucleosides are likely to be different for other lipophilic nucleosides with different substitution patterns. In any case, we deem that the association constants determined here in toluene can be taken as a useful standard value for further studies of nucleobase H-bonded systems in this nonpolar solvent.

These equilibrium constant values are actually within the lower limits to be determined by fluorescence spectroscopy.<sup>6</sup> This is evident when analysing the differences between the binding isotherms generated along the titrations (see Figure 4). In the case of the G-C pair, whose association constant is higher than the AA-U pair, as explained by the Jorgensen model of secondary H-bonding interactions,<sup>22a</sup> saturation (*i.e.* full binding) was reached after adding *ca.* 3-4 equivalents of complementary acceptor nucleoside in the **dG+aC** or **dC+aG** titrations at  $10^{-5}$  M concentration. However, for the weaker **dAA+aU** or **dU+aAA** combinations, more than 15 equivalents of acceptor component were required to reach saturation, even at a higher  $10^{-4}$  M concentration.



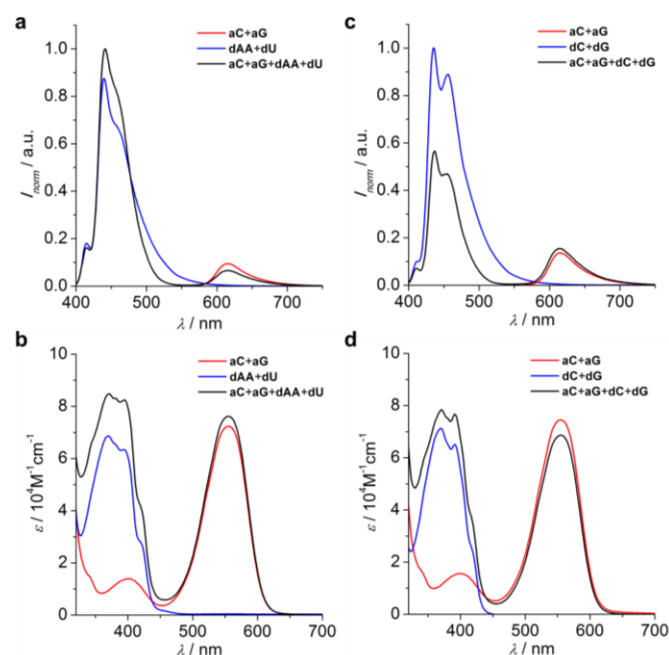


**Fig. 4** (a,c,e,g) Emission spectra (from the initial blue to the final red spectra) and (b,d,f,h) evolution of the emission intensity of the donor and acceptor fluorophores at different wavelengths obtained along the titration experiments between (a,b) dG+aC, (c,d) dC+aG, (e,f) dAA+aU and (g,h) dU+aAA with increasing concentration of the corresponding acceptor compound.  $K_{G-C}$  and  $K_{AA-U}$  values were calculated by fitting the donor emission decay (blue points).

Our family of H-bonding FRET probes can also be used to assess self-sorting events in mixtures of complementary or non-complementary nucleosides. In order to prove self-sorting phenomena between non-complementary G-C and AA-U pairs, we analysed the fluorescence emission changes experienced by 1:1 dAA+dU and 1:1 aG+aC combinations when mixed together, thus yielding a 1:1:1:1 dAA+dU+aG+aC quaternary mixture. As shown in Fig. 5a, the ratio between the emission intensities in the 400-550 nm region (where the bithiophene nucleosides emit) and the 550-700 nm region (corresponding to the emission of the BODIPY nucleosides) remain virtually unchanged when the quaternary mixture is generated. This indicates that energy transfer events are insignificant in this mixture and thus that dAA/dU donors do not bind to aG/aC acceptors.

The same experiment was then performed with a quaternary mixture of complementary nucleosides. More

specifically, a dG+dC 1:1 mixture was added over an aG+aC 1:1 mixture and the emission spectra were recorded before and after mixing at the same  $5 \cdot 10^{-5}$  M concentration. As can be seen in Fig. 5b, the 450-550/550-700 nm emission ratio is considerably diminished by 40%. This is a result that denotes the absence of self-sorting events in this mixture, and that all possible dG-dC, aG-aC, dG-aC and aG-dC complexes are statistically formed when the four nucleoside probes are mixed in  $5 \cdot 10^{-5}$  M toluene solutions. In the last two complexes, where energy donors and acceptors are combined in the same Watson-Crick pair, resonance energy transfer events can take place, therefore leading to a significant donor emission quenching and a slight acceptor emission enhancement.



**Fig. 5** (a-c) Emission ( $\lambda_{\text{exc}} = 369$  nm,  $\lambda_{\text{exc}}$  (aC, aG) = 554 nm) and (b-d) absorption spectra of the 1:1 mixtures aC+aG, dAA+dU (a-b), aC+aG, dC+dG (c-d) and their 1:1:1:1 mixtures aC+aG+dAA+dU (a-b) and aC+aG+dC+dG (c-d). In all cases the concentration of each molecule was set at  $C = 5 \cdot 10^{-5}$  M in toluene.

## Conclusions

A set of novel nucleosides, substituted with bulky lipophilic groups at the ribose and featuring bithiophene donor and BODIPY acceptor FRET dyes, has been synthesized in this work. Together, they constitute a relevant collection of synthetic probes useful to study their self-assembly in solution by means of fluorescence resonance energy transfer. Our molecular design consists in linking the lipophilic purines and pyrimidines through their 8- or 5- positions, respectively, to the donor/acceptor functional fragments *via* an ethynylene group. This structural features allowed us to form H-bonded ensembles where the chromophores are rigidly arranged in an exact angle and  $\pi$ -conjugated to the nucleobases, which enhances energy transfer within the resulting complexes. This is therefore a design in which the nucleobase is part of the FRET

dye, and not simply appended to it through a flexible, non-conjugated linker, which should increase FRET sensitivity upon binding. As a matter of fact, we demonstrated that donor emission can be efficiently deactivated in the presence of the complementary acceptor due to an excitation energy transfer process, and the extent of emission quenching depends on the population of H-bound donor-acceptor couple. In this way, titration experiments allowed us to calculate the association constants between G-C ( $K_{G-C}$ ) and AA-U ( $K_{AA-U}$ ) Watson-Crick pairs. The utility and potential interest of our set of nucleoside FRET probes was also proven in self-sorting experiments where the non-complementary H-bonding nature of the G-C and AA-U pairs is demonstrated. Only when energy donors and acceptors do not bind in solution their relative fluorescence intensities remain unchanged upon generation of the quaternary mixtures.

Current studies in our group employ these FRET mononucleoside probes to examine and understand more complex self-assembly scenarios where supramolecular polymerization processes occur in competition with macrocyclization processes.

## Acknowledgements

Funding from the European Research Council (ERC-Starting Grant 279548 PROGRAM-NANO) and MINECO (CTQ2014-57729-P) is gratefully acknowledged. E.F. would like to thank the Sharif University of Technology of Iran for financial support.

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- ReactLab™ EQUILIBRIA. *Jplus Consulting Pty Ltd*. This software offers the possibility of global fitting, meaning that all wavelengths in the whole spectra are fitted simultaneously. In addition, both host and guest nucleoside dimerizations can be included in the fitting as competitive processes to the binding between nucleobase pairs.
- The Matlab® scripts developed by P. Thordarson (see also ref. 6b) offer the possibility of fitting several wavelengths simultaneously, thus enhancing the quality of the fitting procedure.