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PERSPECTIVE

Targeting DNA base pair mismatch with artificial nucleobases. Advances and perspectives in triple helix strategy

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This review, divided into three sections, describes the contribution of the chemists' community to the development and application of triple helix strategy by using artificial nucleic acids, particularly for the recognition of DNA sequences incorporating base pair inversions. Firstly, the development of nucleobases that recognise CG inversion is surveyed followed secondly by specific recognition of TA inverted base pair. Finally, we point out in the last section recent perspectives and applications, driven from knowledge in nucleic acids interactions, in the growing field of nanotechnology and supramolecular chemistry at the border area of physics, chemistry and molecular biology.

Introduction

DNA is the biomolecule of life since it plays a key role in the storage and transfer of genetic information of most organisms. The expression of DNA operates through complexes formed with other biomolecules in the cell and involves non-covalent and reversible interactions. Therefore, based on these complex macromolecular interactions, efforts have been devoted to finding efficient ways to control gene expression and regulation by creating ligands that can bind selectively to specific DNA sequences.

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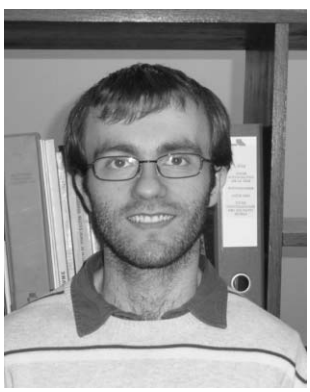
Now, it is well established that hybridization of two complementary strands gives rise to the familiar DNA antiparallel helix according to Watson–Crick base pairing, where strands have opposite 5′ to 3′ polarity. The resulting canonical B-DNA helix has two obvious grooves in its structure known as the major and the minor groove, driven from the geometry of the sugar–base arrangement and base–pair interaction. Each groove has distinct hydrogen bonding faces at the edges of the DNA base pairs (Fig. 1), and its geometry is highly dependent on the nature of nucleobase composition.

Based on this 3D DNA arrangement and inspired by nature, two main approaches have been developed to specifically target DNA double helix: the minor and the major groove binders. In this review we will only focus on the development of triplex forming



Vincent Malnuit

Vincent Malnuit received his engineering degree (2007) from the National Polytechnic Institute of Chemistry (ENSIACET, Toulouse, France) with an exchange at the National Engineering School of Chemistry (ENSCM, Montpellier, France). He carried out his engineering degree dissertation project on the synthesis of activators and inhibitors of angiogenesis at Sanofi-Aventis. He is currently finishing a PhD degree under the supervision of Dr Rachid Benhida at the University of Nice Sophia-Antipolis (Nice, France). His current research focuses on the synthesis of new RNA ligands, bioactive nucleosides and post-synthetic labeling of oligonucleotides.

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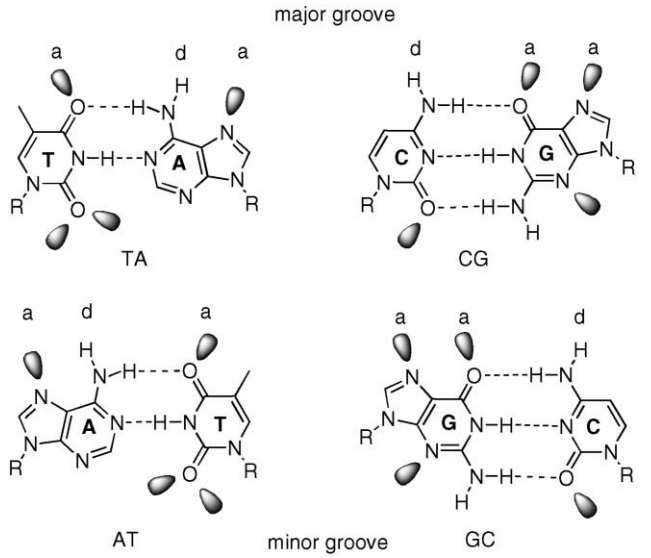

Fig. 1 Schematic representation of the four base pair combinations and the free donor (d) and acceptor (a) sites.

oligonucleotides known as major groove binders. Especially, the molecular recognition of DNA base pairs by both natural and synthetic nucleobases will be surveyed, in a non-exhaustive way.

DNA recognition by TFOs

Triplex forming oligonucleotides (TFOs) represent a class of sequence specific DNA ligands with the ability of regulating gene expression, thus having a great potential in therapy.[1–4] The ability of oligonucleotides to form triplexes was first discovered in 1957 by Felsenfeld *et al.*, who demonstrated that polyuridylic acid and polyadenylic acid strands were capable of forming a stable complex.[5] Thirty years later, Helene's group (Paris) and Dervan's group (Pasadena) demonstrated that short oligonucleotides can bind in the major groove of the DNA duplex to form a triple-helical structure (Fig. 2) and induce DNA cleavage at a specific site, thus

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Rachid Benhida is currently Director of research at CNRS. He received his Ph.D. from University of Paris XI, Orsay (1992). After two post-doctoral positions: Hoechst/Roussel-Uclaf (1992-93) and "Institut Curie-Paris" with Prof. Claude Monneret (1994), he joined the "Institut de Chimie des Substances Naturelles", ICSN, as ANRS post-doc award and as Chargé de recherche CNRS in the same year (1995). He moved from ICSN to the University of Nice Sophia Antipolis in 2002. His current research is focused on nucleic acids chemistry, the development of novel synthetic methods and their applications in the synthesis of bioactive molecules

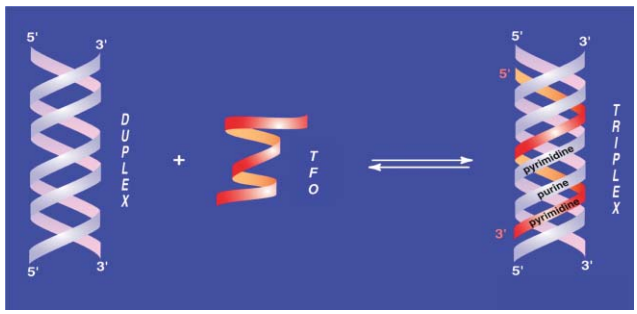

Fig. 2 TFO hybridization with formation of DNA triplex.

showing the huge potential of these molecules in controlling gene expression.[6–7] So far, triplex formation has been shown to prevent protein binding to DNA,[8–11] to inhibit DNA replication,[12–13] to alter gene expression,[14–19] to direct site-specific DNA damage,[20–22] to enhance recombination[23–25] and to induce mutagenesis, which has been used to knock out target genes in cultured cells and in animals.[26–28]

While triplex formation is straightforward under controlled conditions *in vitro*, the nuclear environment of living cells presents substantial obstacles. The third strand must be nuclease resistant, overcome the charge repulsion between the third strand phosphates and those of the duplex target, form a triplex at physiological pH, and overcome entropic barriers leading to the formation of a structure imposing constraints on both members of the complex. Furthermore, triplex formation is limited to oligopyrimidine·oligopurine ds-DNA targets and any single TA or CG base pair interruption reduces strongly the stability of the triplex.[29–31] Base and sugar modifications that address all these issues have been described.[32–34] In this review we will focus on the application of artificial nucleobases designed to overcome sequence limitations caused by single base mismatch.

In the last few years, two main approaches, which involve a number of chemical modifications, have been proposed to overcome this sequence limitation. One of these strategies consists of the conjugation of intercalating agents to the 5′- or the 3′-end or to internal positions of the TFO in order to stabilise the triplex containing base-pair interruptions in the purine motif. An alternative and original approach is the specific base strategy which calls for the synthesis of new modified bases able to form hydrogen bonds with one or both partners of the TA or the CG Watson–Crick inverted base pairs in the major groove. A summary of recent years discoveries in the field are reported.

Triplex formation and structure

As illustrated in Fig. 3, the bases of the third strand form hydrogen bonds (Hoogsteen or reverse Hoogsteen) with the purine bases already involved in Watson–Crick base pairs thus forming base triplets. Triplex formation obeys precise rules imposed by several structural constraints.[35] Intermolecular triplex formation requires a purine-rich stretch of duplex DNA to which a single-stranded TFO can bind *via* Hoogsteen hydrogen bonding in either a parallel or an antiparallel orientation. Antiparallel triplexes are typically formed by purine (GA) or mixed (GT) TFOs forming canonical G.GC, A.AT and T.AT triplets through reverse Hoogsteen hydrogen bonding. Parallel triplexes are formed by pyrimidine TFOs

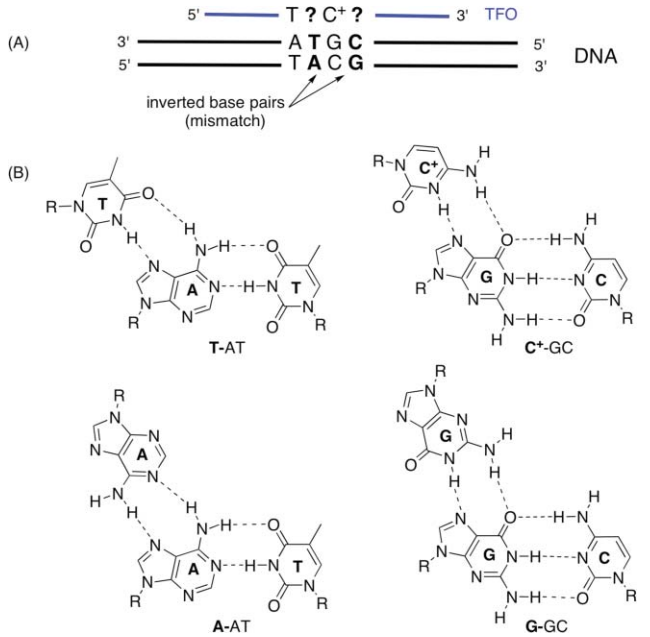

Fig. 3 (A) Triplex (TFO–DNA) with the inverted base pairs in bold. (B) Canonical triplexes involving Hoogsteen and reverse-Hoogsteen base pairing.

forming canonical C^+.GC (C^+ represents *N3*-protonated cytosine) and T.AT triplets resulting from Hoogsteen hydrogen bonding (Fig. 3).[36,37] Optimal target sequences must harbor consecutive purines on the same strand since only purine bases are able to establish two Hoogsteen or reverse-Hoogsteen hydrogen bonds in the major groove of DNA: this is the main restriction to the repertoire of potential target sites. The 3D structures of Hoogsteen and reverse-Hoogsteen triplexes are shown in Fig. 4.[38,39]

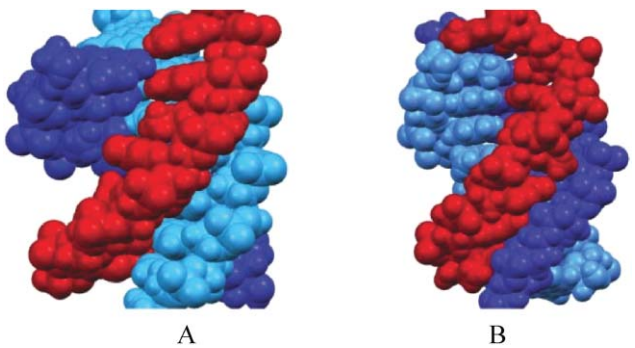

Fig. 4 Structures of parallel (A) and anti-parallel (B) triplexes (third strand in red) (PDB, ref. 38, 39).

I. Recognition of CG base pair inversion

I.1. Modified bases designed to form one Hoogsteen hydrogen bond

In order to circumvent the sequence limitations in the recognition of ds-DNA by TFOs, Dervan proposed 2′-deoxynebularine (**N**, Fig. 5) to bind CG base pair by one hydrogen bond at physiologically relevant conditions (pH and temperature). This compound interacts with the CG base pair with an affinity comparable to the T.CG triplet, but with a better selectivity than thymidine within a purine.purine.pyrimidine (Pu.Pu.Py) triplex.[40]

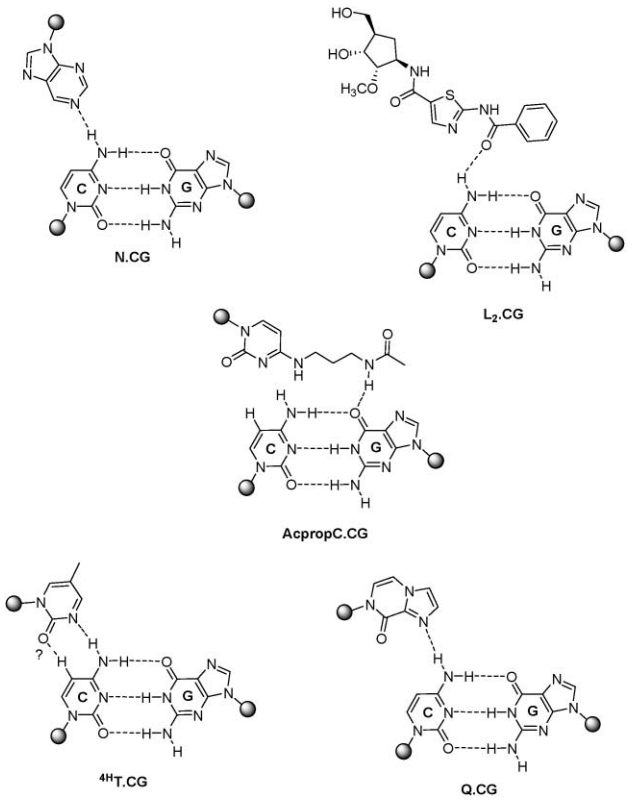

Fig. 5 One hydrogen-bond designed nucleobases for CG base pair recognition.

Lehmann *et al.*[41] reported on the synthesis of two carbocyclic ribonucleosides with extended aromatic moietys attached *via* amide bonds ($\mathbf{L_1}$, Fig. 6, and $\mathbf{L_2}$, Fig. 5). Those new nucleobases appeared to bind preferentially to pyrimidine bases by a sequence-specific intercalating mode.

Furthermore, Miller and coworkers studied how *N*4-substitution in cytosine nucleobase influences triplex formation. In particular, they demonstrated that N^4-(3-acetamidopropyl)-cytosine **AcpropC** was able to form a triplet selectively with a CG base pair inversion on the target duplex (Fig. 5).[42]

Prevot-Halter and Leumann reported the incorporation of 5-methylpyrimidin-2-one ($^{4H'}$**T**, Fig. 5) into oligonucleotides and demonstrated its affinity and selectivity for a CG base pair.[43] This new nucleobase is thought to bind *via* (i) a hydrogen bond between N3 of pyrimidinone and NH_2 of cytosine and (ii) a non-conventional CH–O hydrogen bond between H–C5 of cytosine and O2 of pyrimidinone.[44]

Finally, Leumann's team reported on the synthesis of a new imidazopyrazinone nucleoside **Q** (Fig. 5).[45] This latter appeared to be less selective than 5-methylpyrimidin-2-one and did not distinguish between CG and GC base pairs. Furthermore, the triplex stability was found to be highly dependent on the nature of the neighbour bases around the target site.

I.2. Modified bases designed to form two Hoogsteen hydrogen bonds

To improve the stability of triplex containing a pyrimidine–purine inversion, Dervan earlier reported on the synthesis of the

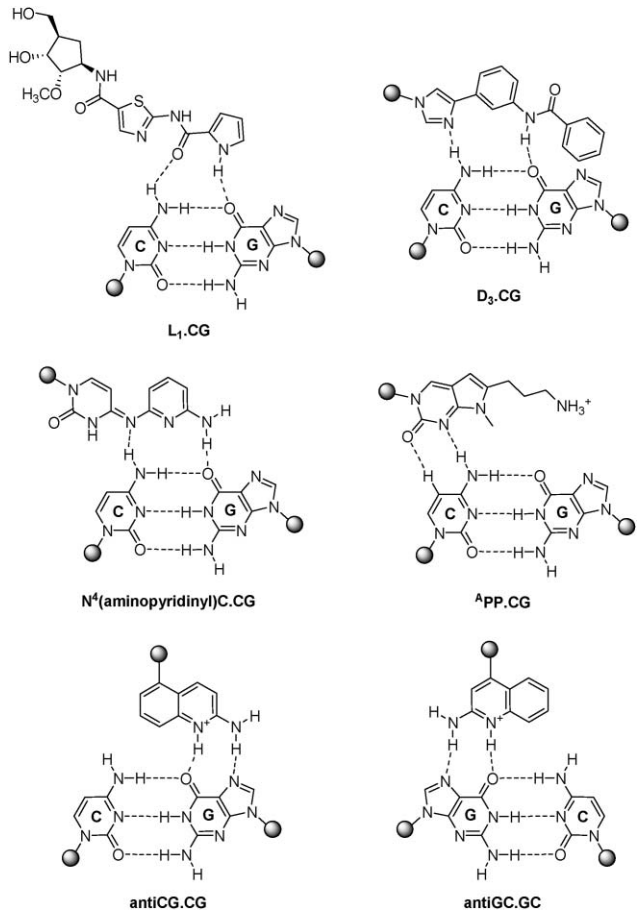

Fig. 6 Two hydrogen-bonds designed nucleobases for CG base pair recognition.

non-natural deoxyribonucleoside 1-(2-deoxy-β-D-ribofuranosyl)-4-(3-benzamidophenyl)imidazole (**D_3**, Fig. 6).[46] This modified base was selective for TA and CG inversions probably through the formation of one hydrogen bond between N3 of imidazole and NH_2 of cytosine as well as between NH of amide and O6 of guanosine. Affinity-cleaving analysis showed that **D_3** binds almost equivalently with TA and CG base pairs. Further NMR experiments demonstrated that **D_3** recognises pyrimidine–purine base pairs mostly by a sequence-specific intercalating mode.[47]

Miller reported the synthesis and incorporation of N^4-(6-aminopyridinyl)-2′-deoxycytidine into oligonucleotides (Fig. 6).[48] UV and circular dichroism studies showed that this cytidine analogue did not distinguish between CG and AT base pairs.[49] Concerning CG recognition, *T*m values are comparable to those obtained with C^+.GC triplet at neutral pH. It was also assumed that this analogue was involved in H-bonding with O6 of guanosine and N4 amino group of cytosine *via* the imino-tautomer (Fig. 6).

In a similar way, Doronina and Behr reported on the synthesis of 4-guanidinocytidine analogues designed to form two hydrogen bonds with guanine in CG or GC base pairs depending on the anomeric configuration. Indeed, the β-anomer was designed to be GC selective while the α-anomer should recognise the CG base pair.[50] Unfortunately, the incorporation of these new nucleosides into triplex forming oligonucleotides was problematic and led to TFOs with limited selectivity.[33]

More recently, Li *et al.* developed an interesting series of four *C*-nucleoside analogues designed for recognition of the four Watson–Crick base pairs.[51–53] They were designed to perform Hoogsteen base pairing selectively with the purine base of the duplex. For instance, 5-substituted 2-aminoquinoline was proved to be efficient for the formation of stable triplex with sequences bearing a CG inversion, while 4-substituted 2-aminoquinoline is specific for GC base pairs (**antiCG** and **antiGC**, Fig. 6).[53]

Substituted 3*H*-pyrrolo[2,3-*d*]pyrimidin-2(7*H*)-one nucleoside analogues were incorporated into TFOs and were found to selectively bind CG inversions with a greater affinity than T (**^{A}PP**, Fig. 6).[54–56] The best results were obtained with 3-aminopropyl derivative.

I.3. Modified bases designed to form three Hoogsteen hydrogen bonds

A promising way to further improve the stability and the specificity of triplexes directed on sequences containing a pyrimidine·purine base pair inversion is to perform Hoogsteen hydrogen bonds with all free acceptor and donor sites. For this purpose, the nucleobase has to crossover the major groove to interact with both Watson–Crick bases.

An example of the application of this strategy was reported by Sasaki *et al.* in 1995 using a newly synthesized benzaminoimidazole-glycyl (**BIG**) nucleobase.[57] The authors demonstrated, using 1D and 2D-NMR experiments, that this modified nucleobase was able to selectively recognise a CG base pair by forming three Hoogsteen hydrogen bonds (Fig. 7).

Lengeler and Weisz showed the ability of various free substituted phthalimide-derived nucleosides to recognise a CG Watson–Crick base pair (**Phth**, Fig. 7).[58] NMR experiments demonstrated the formation of two hydrogen bonds in CD_2Cl_2 and a third one for ureido-substituted nucleoside analogues. However, these interactions were not observed when these nucleosides were incorporated into oligonucleotides.[59]

Zimmerman and co-workers proposed an ureido methyl-naphthimidazole nucleoside (**Z**, Fig. 7) to bind a CG base pair in a selective way.[60] NMR studies of the free nucleobase in $CDCl_3$ demonstrated the formation of three Hoogsteen hydrogen bonds with both bases of CG. Unfortunately, the incorporation of this new nucleoside analogue into TFOs did not lead to the effective recognition of CG.

Analogously, Sun et co-workers proposed ureido benzimidazole and benzoxazole analogues (**HB**, Fig. 7).[61] NMR studies proved the ability of free nucleosides to perform three Hoogsteen hydrogen bonds with CG base pair.

In order to improve the formation of triplex with this type of nucleoside analogue, Mertz *et al.* synthesized an ureido isoindolin-1-one able to form three Hoogsteen hydrogen bonds with CG base pair (**isoI**, Fig. 7).[62] Incorporation into oligonucleotides was successful but the triplet with CG was not robust and little stabilization of triplex formation was observed, demonstrating that despite favorable recognition in model studies, the artificial base did not effectively recognise duplex DNA to form py·pu·py type triple helices.[63]

Weisz and co-workers also improved the previously described analogue **D_3** by the synthesis of the ureido-analogue **D_4** (**D_4**, Fig. 7).[64] The free nucleoside **D_4** was found to recognise CG base

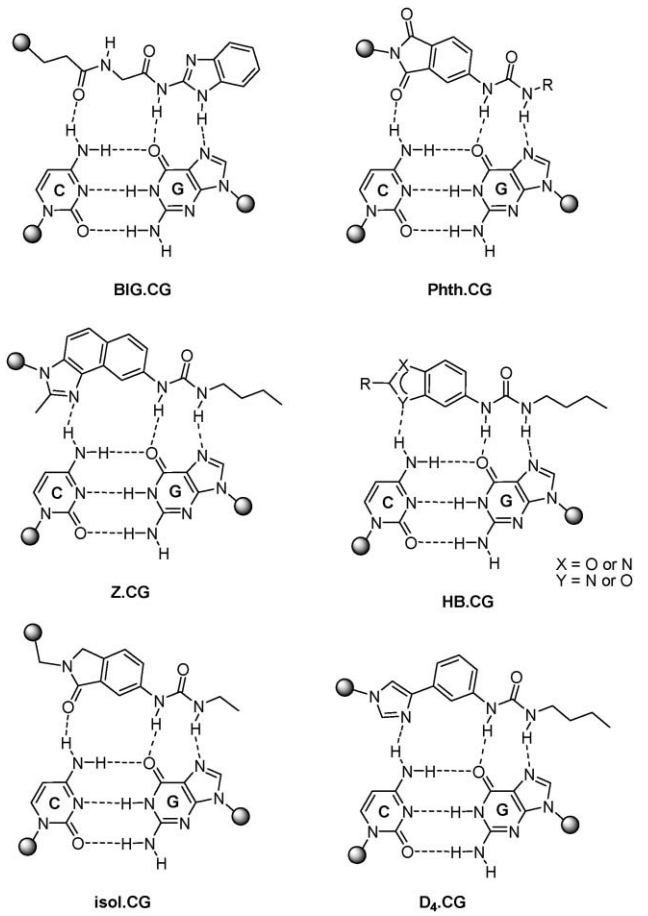

Fig. 7 Three hydrogen-bonds designed nucleobases for CG base pair recognition.

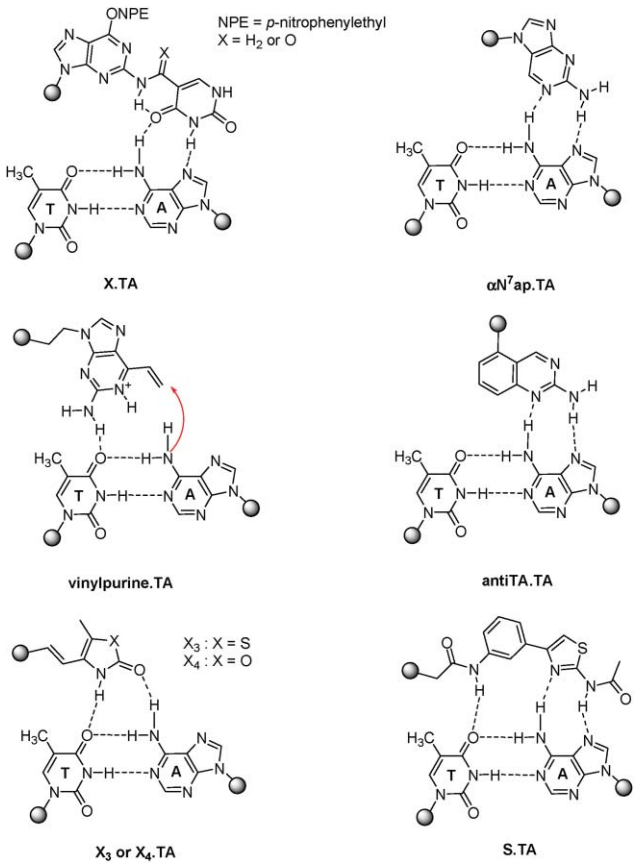

Fig. 8 Nucleobases designed for TA base pair recognition.

pairs and involved a similar mode of interaction as the other ureido analogues listed above. Furthermore, incorporation of $\mathbf{D_4}$ into TFOs showed that this nucleoside acts as a universal base.[65]

II. Recognition of TA base pair inversion

Targeting TA base pair inversion in oligopurine·oligopyrimidine dsDNA to form triplex complexes faces even more difficulties than CG recognition. In fact, the steric hindrance that occurs with the 5-methyl group of thymidine constitutes a further limitation for the accessibility to the donor and acceptor sites. For this reason, only a few examples of modified nucleobases were reported to target TA base pair compared to CG.

For example, Orson *et al.* reported on the incorporation of 3-nitropyrrole into a 15-mer oligonucleotide bearing an acridine intercalator at the 5′-end.[66] It was found that substitutions with 3-aminopyrrole closer to the intercalator end of the TFO had a more deleterious effect on the dissociation constant than those further away. Molecular modeling demonstrated that 3-nitropyrrole.TA triad is isomorphous with the known A.AT triad, which explains its selectivity for TA base pair inversion.

In the same year, Saito and Kuroda proposed a new nucleoside analogue designed to cross-over the major groove of dsDNA and form two hydrogen bonds with N7 and NH_2 of adenine in TA base pairs (**X**, Fig. 8).[67] NMR studies showed the ability of **X** to bind with adenine in $CDCl_3$ but incorporation into oligonucleotides was not reported.

Parel and Leumann reported on triplex formation in the antiparallel binding motif using modified oligonucleotides containing N^9-and N^7-2-aminopurine deoxynucleosides.[68] They studied the influence of the stereochemistry of the anomeric position on the selectivity and affinity towards all four base pairs. Among these new analogues, α-N^7-2-aminopurine was found to be specific and bind to TA rather than the other base pairs, although with moderate affinity (**αN^{7}ap**, Fig. 8).

Sasaki and co-workers reported on the incorporation into TFOs of 2-amino-6-vinylpurine bearing an ethyl spacer from the 2-deoxyribose unit.[69] It was demonstrated that it is possible to achieve selective cross-linking with the adenine of the TA base pair within the triple helix, thus enhancing the stability of the triplex at neutral pHs (**vinylpurine**, Fig. 8).

Li et *al.*, analogously to antiGC and antiCG described in the previous section (Fig. 6),[51–53] reported the selective recognition of TA base pair with 5-substituted 2-aminoquinazoline *via* two hydrogen bonds with adenine (**antiTA**, Fig. 8).

More recently, Rothman reported on the synthesis of heterocyclic ethenyl *C*-nucleosides designed for the recognition of TA base pair within triplex by formation of two Hoogsteen hydrogen bonds ($\mathbf{X_3}$ and $\mathbf{X_4}$, Fig. 8).[70,71] However, the incorporation of those nucleosides into TFOs was not described.

Some of us previously reported a new 6-(thiazolyl-5)-benzimidazole nucleobase for the recognition of TA base pair inversion.[72,73] This extended aromatic nucleoside analogue, bearing

a rigidified nucleobase, was designed to perform three Hoogsteen hydrogen bonds with both bases of TA doublet. However, UV/Vis study showed a moderate affinity of this nucleoside towards the four base pairs.

The same authors also reported on the synthesis and incorporation into TFOs of a new aminophenyl-thiazole nucleobase designed to form three Hoogsteen hydrogen bonds with both bases of TA pair (**S**, Fig. 8).[74,75] UV/Vis study showed that **S** nucleobase has good selectivity for TA inverted base pair with *T*m values close to those of canonical triplexes. To our knowledge, **S** is still one of the best nucleobases that could be used for recognition of TA base pair interruption.

Fox and co-workers later reported on the incorporation of **S** nucleobase into TFOs, in combination with other modified nucleosides (like ^{A}**PP** discussed above) to perform the complete recognition of all four base pairs in dsDNA at physiological pH.[56,76] They demonstrated successful triplex formation at a 19-mer oligopurine sequence containing two CG and two TA interruptions. The authors also pointed out the moderate selectivity of **S** between TA and CG base pairs.

Finally, Van Craynest *et al.* reported on the synthesis of new nucleosides as extended guanine analogues derived from aminobenzimidazole and thymine or 5-substituted uracil.[77] These nucleosides were designed to form three hydrogen bonds with TA base pairs. The properties of TFOs containing these modified nucleobases were not reported.

III. Other additional modifications

Many studies on the effect of modifications of the sugar units or phosphodiester backbone on the stability of triplex have been carried out. The main ideas were (i) to introduce positive charges in the sugar or phosphodiester backbones in order to decrease the anionic character of the third strand and to prevent charge repulsions with dsDNA, (ii) to synthesize oligonucleotides with constrained conformations and (iii) to use intercalating agents in order to increase triplex stability. The most promising work in recognizing base pair inversions and, at the same time, in stabilizing triplex formation in oligopurine·oligopyrimidine dsDNA came from dual recognition by the use of artificial nucleobases (described in the previous sections) and modified sugar or phosphodiester backbone.

III.1. Aminoalkyl substitution on the sugar unit

With the aim of stabilizing triplex formation by making interactions with phosphodiester backbone of dsDNA, Cuenoud and co-workers reported 2′-aminoalkyl modified TFOs.[78] From different *T*m value measurements, it appeared that 2′-aminoethoxy group was the best modification, with an increase of the *T*m values by 3.5 °C per modification. A complete NMR spectroscopy study showed the dual recognition with dsDNA by contacting both bases and phosphodiester backbone.[79] It turned out that the 2′-aminoethoxy side chain is in a perfect conformation for optimum electrostatic interaction with the *pro-R* oxygen of the phosphodiester belonging to the DNA second strand. The unique biophysical properties of these 2′-aminoethoxy-modified TFOs combined with their resistance to enzyme cleavage make them highly promising for biological applications. A few years later, Puri *et al.* demonstrated the *in vivo* ability of this class of TFOs to form stable triplexes on targeted genes.[80]

In line with these results, Brown and co-workers synthesized a 2′-aminoethoxy modified nucleoside bearing a 5-(aminopropargyl)uridine nucleobase, for the specific recognition of AT base pairs (**BAU** triplet, Fig. 9).[81–82] This new nucleoside is able to form two hydrogen bonds with the adenine of AT base pair and bears two positive charges able to make additional contact with the phosphodiester backbone of dsDNA. These modifications induced a large increase in triplex stability even at pH 7.5.[83]

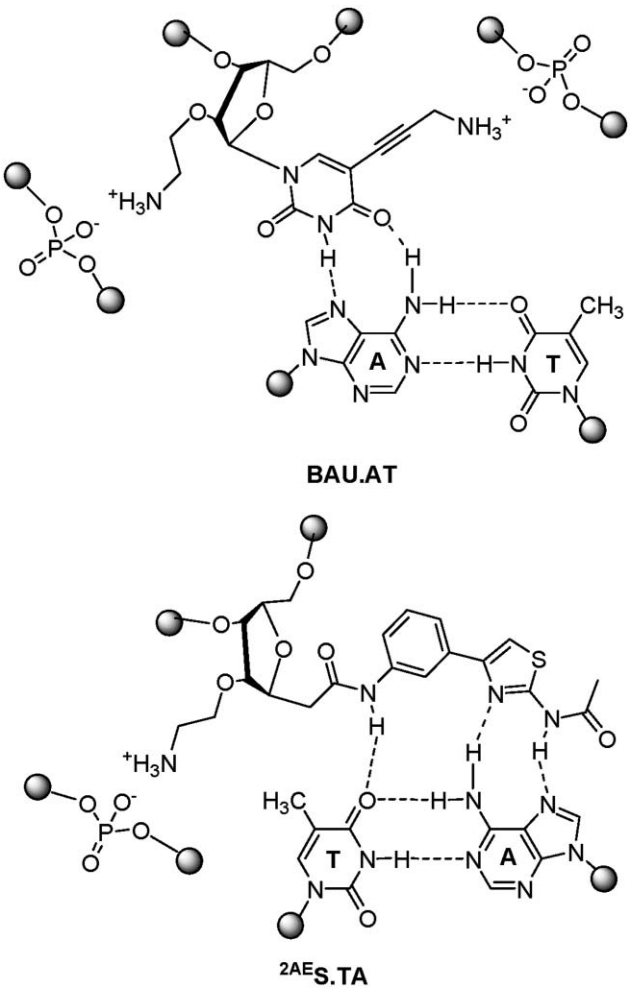

Fig. 9 Structure of the 2′-aminoethoxy-modified TFOs.

In similar way, Buchini and Leumann reported the 2′-aminoethoxy-modified analogue of 5-methyl-1*H*-pyrimidin-2-one nucleoside (^{4H}**T**, Fig. 5).[84–85] It was proved that the modification on the sugar unit led to a large increase of the triplex stability, providing a great tool for the specific recognition of CG base pair interruptions. The authors also reported the high ability of fully 2′-aminoethoxy-modified TFOs to recognise multiple CG inversions.[86]

Fox and co-workers recently used the 2′-aminoethyl modification on the nucleoside **S** reported by Benhida and co-workers for the recognition of TA base pair interruption (^{2AE}**S**, Fig. 9).[74–76,87] Fluorescence, UV/Vis and DNase footprinting

experiments showed that ^{2AE}**S** has greater affinity than **S** for TA interruption. The combination of **S** and 2′-aminoethoxy modifications make this nucleoside the best one described so far for the recognition of TA base pair inversion.

Atsumi *et al.* reported the synthesis and the thermal stability of triplexes containing 4′α-*C*-aminoalkyl-2′-deoxynucleosides.[88] These nucleosides led to stable triple helixes with dsDNA when incorporated into TFOs. Moreover, the authors showed that this class of TFOs was stable to the cleavage by DNase I and snake venom phosphodiesterase.

III.2. Contribution of conformationally constrained nucleosides

Sugar units in oligonucleotides are able to adopt two conformations, *N*-type conformation and *S*-type conformation (Fig. 10). Imanishi and co-workers investigated the properties in triplex forming ability of conformationally constrained nucleosides. They synthesized 2′-*O*,4′-*C*-methylene bridged nucleic acids (**BNA**, Fig. 10).[89,90] Triplex study showed an increase in *T*m values of 4 to 5 °C per modification and the binding constant was at least 300-fold higher than that of natural oligonucleotides. It has been reported that BNA-oligonucleotides inhibited the NF-kB transcription factor (p50)-target dsDNA. This class of TFOs was found to be nuclease resistant and is highly promising for *in vivo* studies.

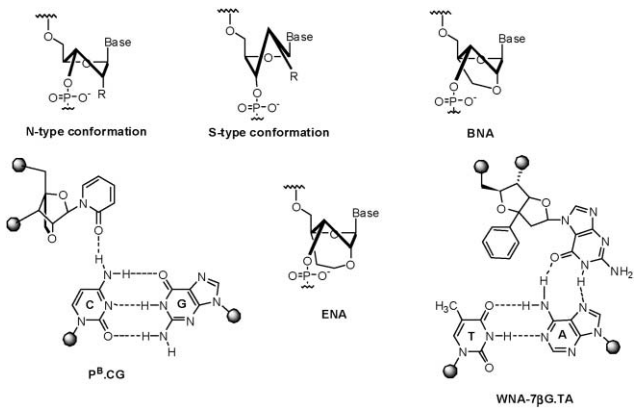

Fig. 10 Conformationally constrained nucleosides.

Another example was reported by Obika *et al.* using bridged nucleosides featuring a modified 2-pyridone nucleobase (**P^{B}**, Fig. 10).[91,92] The authors demonstrated the ability of this new nucleoside to recognise CG base pair when incorporated into TFOs, even at physiological conditions. The BNA modification led to an increase of 9 °C in *T*m values compared to that of the unmodified 2-pyridone nucleoside. **P^{B}** represents one of the best analogues for the recognition of CG inversion.

The same authors also developed the BNA containing 1-isoquinolone as nucleobase and found similar binding constants for CG, compared to **P^{B}**.[93] Intending to recognise TA base pair inversion, the authors developed BNAs containing 2- and 3-hydroxybenzene and indole as nucleobase analogues that were able to bind the targeted TA base pair inversion.[94,95] In similar way, Savy *et al.* investigated the ability of 3′-*O*,4′-*C* and 2′-*O*,4′-*C* bridged uridine to form triplexes and observed that only the former led to an increase in *T*m values.[96]

However, one of the major limitations to the use of BNAs is that oligonucleotides fully modified with 2′,4′-BNA do not form triplexes. To circumvent this problem, Imanishi and co-workers reported the ability of TFOs fully modified with 2′-*O*,4′-*C*-*ethylene*-bridged nucleic acids (**ENA**, Fig. 10) to make triplexes at physiological pH.[97] These nucleic acids have a torsion angle that is more suitable for triplex formation.

Maeda and co-workers reported in 2001 the synthesis of a W-shaped bicyclic nucleic acid (**WNA-7βG**, Fig. 10). This latter is composed of three parts, a benzene ring providing a stacking motif, an heterocyclic ring for the formation of Hoogsteen hydrogen bonds, and a bicyclic skeleton to hold these two parts. For the recognition of TA inversion, they used a WNA bearing a 7β guanine nucleobase that forms stable triplexes.[98,99]

Later the βT and βC WNA analogues were described for the specific recognition of TA and CG inversion sites, respectively.[100,101] Triplexes formed with these nucleosides turned out to be even more stable than canonical triplexes, even at low cationic concentrations. Nevertheless, triplex formation by these analogues depends on their neighbouring bases within the TFO. For instance, the WNA bearing a β-3-aminopyrazole (WNA-β3AP) instead of β-cytosine proved to be highly efficient for stabilizing triplex when incorporated into the 3′-G(WNA-β3AP)G-5′ sequence.[102,103] Therefore, recognition of base pair inversions in other relevant sequences is still to be established.

III.3. Non-(deoxy)ribonucleotide nucleic acids

An alternative approach for targeting dsDNA by triplex formation is to synthesize non-(deoxy)ribonucleotide nucleic acids, by replacing sugar units or phosphodiester backbone with neutral or cationic skeletons in order to minimize the DNA charge repulsion.

Häberli and Leumann reported on the synthesis and incorporation into TFOs of pyrrolidino *C*-nucleosides (**dpΨU**, Fig. 11).[104,105] They firstly proposed pyrrolidino *C*-pseudonucleosides bearing

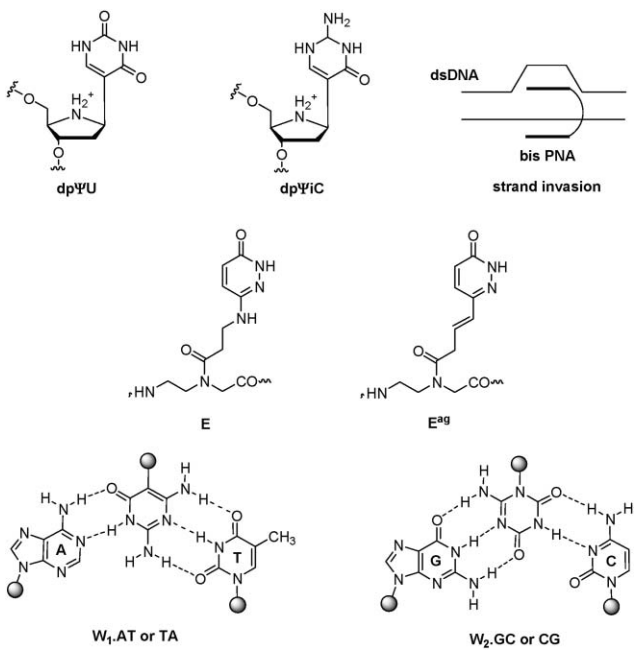

Fig. 11 Dual recognition with non-natural nucleic acids.

uracil and *N*-1-methyluracil for replacement of thymidine in TFOs. However, this modification destabilized the triplex by –13 °C to –1 °C in *T*m values per modification. Interestingly, incorporation of pyrrolidino *C*-pseudo isocytidine into TFOs in replacement of C showed substantial increased triplex stability compared to unmodified TFOs (**dpΨiC**, Fig. 11).[106] This increase was probably due to the possible charge at the pyrrolidino unit, able to interact with the *pro-R* oxygen of DNA phosphate. Similar *T*m values were obtained with pyrrolidino *C*-nucleosides bearing pyridin-2-one and 2-aminopyridine in replacement of T and C, respectively.[107]

One of the most important series of molecules designed to target dsDNA is peptide nucleic acids (PNAs). The skeleton is constructed of a chain of polyamide linked *N*-(2-aminoethyl)glycine units.[108] Being neutral, the backbone is less likely to generate charge repulsion with the anionic phosphodiester backbone of dsDNA. The central amino group bears a nucleobase to provide Hoogsteen hydrogen bonds with bases in the target duplex. However, while TFOs normally recognise dsDNA through major groove triplex formation, PNAs may bind dsDNA exploiting different predictable modes of recognition depending on the PNA and DNA sequence as well as the ambient conditions. In fact, at low ionic strength, high homopyrimidine PNA oligomer concentrations, or long reaction times, triplex invasion complexes dominate instead of conventional triplex formation.

In their efforts to recognise TA base pair inversion, Nielsen and co-workers reported on the great stability in triplex formation obtained for PNAs incorporating two pyridazinone monomers (**E** and **E**^{ag}, Fig. 11).[109,110] Thermal stability studies demonstrated that E·TA triplet was considerably more stable than G.TA triplet, although it was significantly less stable than the canonical C$^+$·GC and T.AT triplets. The use of 3-nitropyrrole and 5-nitroindole in PNAs as universal bases was also reported.[111,112] Those monomers form stable triplexes without base pair discrimination.

Hansen *et al.* clearly demonstrated the competition between duplex invasion over triplex formation by studying PNAs incorporating pseudoisocytidine in replacement of C (Fig. 11).[113] The ratio between the two processes is critically dependent on PNA concentration, oligomer length, and composition. Therefore, the application of PNA in triple helix strategy is limited by the competitive strand invasion process.

Taking advantage of the PNA's ability to make strand invasion, McLaughlin and co-workers described the formation of triplex with modified nucleobases connected on PNA backbone. Indeed, those PNAs bearing diaminopyridinone and/or aminotriazindione nucleobases were shown capable of disrupting duplex oligonucleotides in order to perform hydrogen bonding with Watson–Crick faces of both bases (**W**$_1$ and **W**$_2$, Janus-Wedge DNA complex, Fig. 11).[114,115]

Recent applications of TFOs

Another attractive feature of artificial nucleic acids is their high potential application in the growing field of nano-biotechnology, since the pioneering work by Seeman group on DNA nanotechnology.[116–118] Indeed, the self-assembly of DNA and TFOs is a versatile and attractive tool for the construction of nano-materials. The Watson–Crick and Hoogsteen pairing rules in nucleic acids can be used for the rational design of nanostructures with defined topologies, geometries and properties. Different applications have been recently reported for the construction of highly nano-structured and specifically modified nano-materials based on TFOs. Some examples are given to illustrate, in a non-exhaustive way, the potential of TFOs in the growing field of nucleic acids-based nano-biotechnology.

Richert and co-workers recently reported an attractive approach in single-walled carbon nano-tubes (**SWCNT**, Fig. 12) technology using TFO hybridization principle.[119] They clearly showed the potential of DNA and labelled TFOs to produce highly structured carbon nano-tubes when treated with SWCNTs. The authors used SWCNT and DNA hairpin to form complexes in which the hairpin wrapped around a nano-tube. These complexes were then specifically recognized with labelled TFOs at the stem portion of the hairpin. It is worth noting that in this reported work the non-covalent Watson–Crick and Hoogsteen interactions did not perturb the electronic properties of the SWCNT–TFO nano-materials, extending therefore the potential application of TFOs in nucleic acids-based nano-structuration.

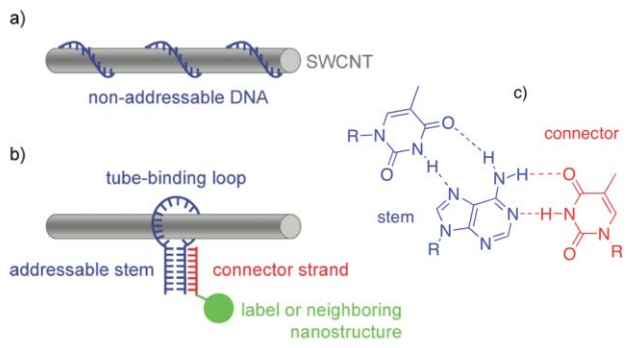

Fig. 12 SWCNT complexed with DNA (a) single-stranded oligonucleotide helically wrapped around a nanotube, (b) hairpin DNA (blue) binding both to a SWCNT and a complementary third strand (connector, red), (c) T.AT triplet (Reprinted with permission from ref. 119. Copyright 2010 American Chemical Society).

Mirkin and co-workers reported a new calorimetric method based on DNA-gold nanoparticles for the screening of triple helix binders (Fig. 13).[120–121] This method seems to be highly sensitive due to the specific optical properties of Au-TFO nanoparticles that induced enhanced binding properties between ligand and

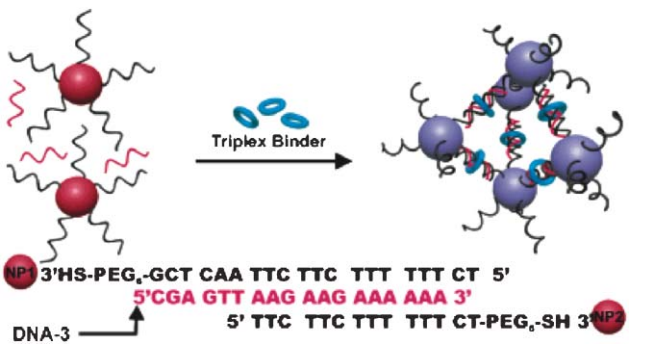

Fig. 13 Representation of the structure and color change of nanoassembly in the presence of triplex binder at room temperature (Reprinted with permission from ref. 120,121. Copyright 2006, American Chemical Society).

TFOs and an increased resolution of the melting transitions. In addition to the high sensitivity, the reported data clearly attested to the high selectivity with an improved analytical discrimination between DNA and triple helix binders, compared to the classical methods (gel electrophoresis and UV–Vis melting experiments). The interaction was also monitored by the color change of the aggregated nano-structures. Indeed, the authors observed that, upon complexation with TFO binders, the triplex structures are stabilized and evolved, after aggregation, to structured nanoparticles, characterized by a red-to-blue band shift and a concomitant decrease in absorbance at 520 nm.

Another application of TFOs in nanoconstruction was recently reported by Nordén's group.[122] The authors developed DNA nanostructures that could be exploited as an information storage device based on pH-driven triplex strand formation or nanoscale circuits based on electron transfer. The obtained nanoconstructs contained two original pseudo-hexagonal units and were characterized using gel electrophoresis, atomic force microscopy and fluorescence spectroscopy. The triple helix principle was used to address pH-dependent nano-structuration since pyrimidine-rich TFOs could be controlled by a pH change of the system (cytosine protonation). Indeed, protonation of cytosine is required in order to establish two hydrogen bonds with guanine (Fig. 14). Therefore, pyrimidine TFO motif is highly stable at low pH and the DNA-third strand binding could be reversed when increasing the pH of the system. In this study, the authors used 2-aminoethoxy-thymidine (^{A}**T**) to recognize AT base pairs and 3-methyl-2-aminopyridine (^{Me}**P**) to recognize GC pairs. ^{Me}**P**, previously developed by Leumann's group, is an interesting analogue of cytosine that can be protonated near physiological pH.[84,123] ^{A}**T** nucleobase was reported by Fox and Cuenoud groups for AT base pair recognition.[76,124]

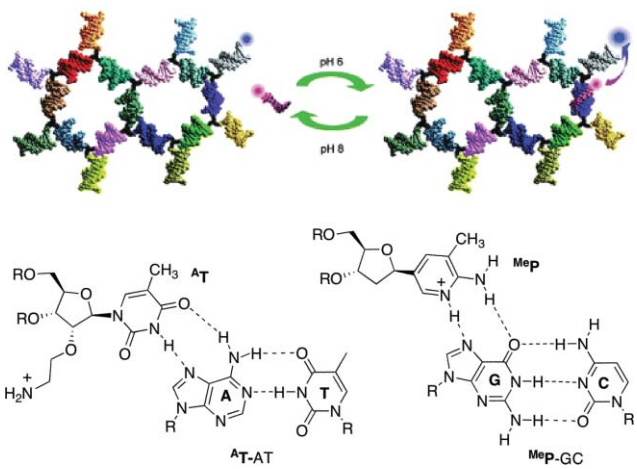

Fig. 14 Schematic representation of the nanostructures. Each ten-mer side is composed of a unique sequence, orthogonal to all the other sequences, as indicated by the color-coding. ^{A}**T**.AT triplet (left) and ^{Me}**P**.GC triplet (right). The TFO bases are shown in blue (Reprinted with permission from ref. 122. Copyright 2007 American Chemical Society).

In similar way, this special pH-dependent characteristic of TFOs' pyrimidine motif was also exploited for the generation of DNA motors, in which the acid source works as a fuel.[125] This DNA nanomachine was constructed using three DNA strands (Fig. 15): long strand L (red) and two short strands (black) in

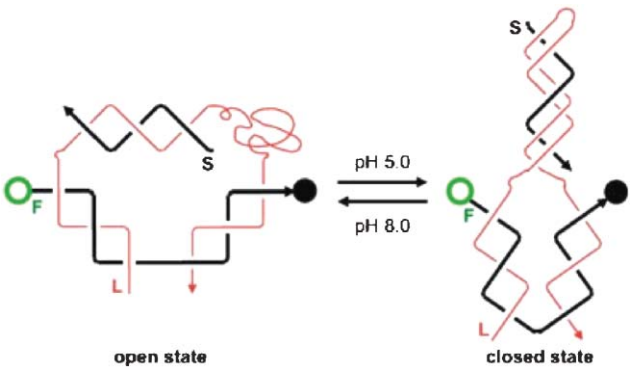

Fig. 15 DNA nanomachine consisting of three strands: a strand with a fluorescent label (F), a long strand (L), and a short strand (S). The open and solid circles represent rhodamine green and black hole quencher-1 (BHQ-1), respectively. A DNA triplex involving the S and L strands forms and dissociates reversibly (Reprinted with permission from ref. 125. Copyright 2004 Wiley).

an open and closed state. Under pH 8, the double helix is the major form. Interestingly, upon decreasing the pH of the system, a duplex to triplex transition takes place and the system evolves to the closed state. This transition was demonstrated by using fluorescence resonance energy transfer spectroscopy (FRET) and polyacrylamide gel electrophoresis. The FRET technique allows a real time monitoring of this original DNA motor based on TFOs.[125]

Conclusion

In this review we attempted to highlight the recent contribution of chemistry to the development of artificial nucleosides and nucleic acids and their application in the triple helix strategy. The extension of the DNA recognition repertoire by TFOs and modified nucleic acids will undoubtedly open the way for other rational applications in the field of nucleic acids chemistry and biology. Furthermore, it is clear that the base pairing code in DNA and TFOs offers high possibilities in nano-biotechnology compared to other molecules. The complementarity rules allow for rational design of nucleic acids-based nano-materials in a precise and specific fashion. Moreover, nucleic acids could be chemically modified using pre- or post-synthetic transformations at specific positions allowing therefore for other applications for this promising class of molecules.

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