Enzymatic construction of metal-mediated nucleic acid base pairs

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Abstract

Artificial metal-base pairs have become increasingly important in nucleic acids chemistry due to their high thermal stability, water solubility, orthogonality to natural base pairs and low cost of production. These interesting properties combined with ease of chemical and enzymatic synthesis have prompted their use in several practical applications including the construction of nanomolecular devices, ions sensors and metal nanowires. Chemical synthesis of metal base pairs is highly efficient and enables the rapid screening of novel metal base pair candidates. However, chemical synthesis is limited to rather short oligonucleotides and requires rather important synthetic efforts. Herein, we discuss recent progress made for the enzymatic construction of metal base pairs which can alleviate some of these limitations. First, we highlight the possibility of generating metal base pairs using canonical nucleotides and then describe how modified nucleotides can be used in this context. We also provide a description of the main analytical techniques used for the analysis of the nature and the formation of metal-base pairs together with relevant examples of their applications.

Introduction

Metal-mediated nucleic acid base pairs are non-canonical nucleobase pairs composed of two ligand-type nucleoside analogues and a bridging metal ion.[1] In a metal base pair, the hydrogen bonds present in natural DNA duplexes are replaced formally by coordinative bonds mediated by specific metal cations.[2] Metal base pairs can be formed with natural nucleobases where soft metals are bound to the endocyclic nitrogen atoms[3, 4] or can be obtained with artificial nucleotides which act as ligands for a broader variety of metal cations.[5] Indeed, the use of modified nucleobases, which can be derivatives of natural ones or completely artificial, allows the formation of a higher diversity of metal base pairs when compared to those obtained with only natural nucleotides (**Figure 1**).[6-8] Metal-mediated base pairs are orthogonal to the natural Watson-Crick base pairs, possess a high thermal stability, are water soluble, often minimally absorb UV light, and usually induce little perturbations of the three dimensional structures of B-DNA duplexes.[5, 9]

The property of nucleic acids to assemble in a sequence-directed manner to form multidimensional structures can be exploited with metal base pairs to design functional molecules.[10] This consideration has introduced several applications of oligonucleotides-containing metal base pairs, such as metal nanowires,[11, 12] DNA-based logic gates[13-15], molecular magnets[16], ion sensors[17], charge transfer devices[18, 19] and polymerase modulators.[20, 21] For instance, metal nanowires formed by an uninterrupted one-dimensional mercury[11] or silver[12] arrays have been recently synthesized and crystallized. These nanowires may find application in the construction of electronic devices, such as high performance transparent conductive films.

Metal base pairs can also be applied in the construction of DNA-based logic gates, which can be thought of as an alternative to transistor-based logic gates to generate molecular computing. DNA-based logic gates could be applied to develop smart sensors for the detection of specific combinations of metal ions or they can allow the construction of proper DNA-based computers where the output of one gate is used as the input of another.[15]

Due to their inherent binding to specific metal cations, metal base pairs can also be used for the construction of DNA-based metal sensors. In this context, functional nucleic acids, i.e., aptamers and DNAzymes, are particularly adapted to sense metal cations.[22-24] Finally, an interest towards the electrical conductance of DNA strands containing metal base pairs has been raised[19] because intraduplex metal complexes could act as charge-carriers for electron transportation in DNA.

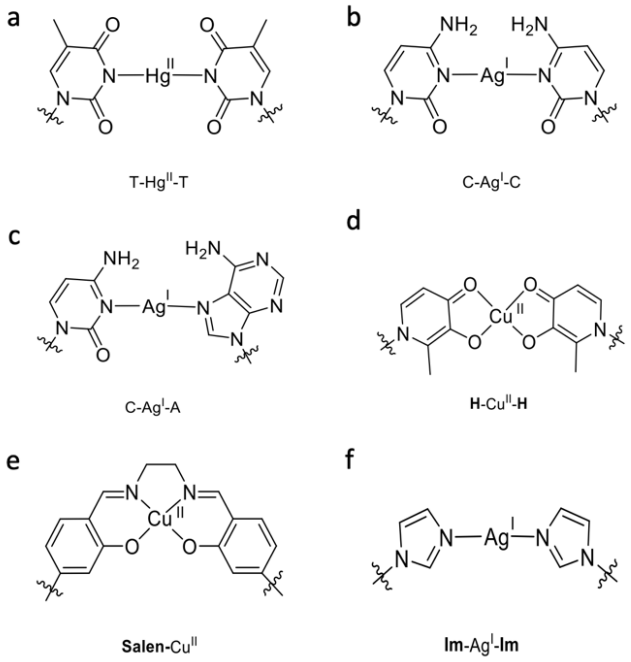


Figure 1. Recent and relevant examples of metal base pairs obtained with natural (**a**, **b**, **c**) and artificial (**d**, **e**, **f**) nucleobases.

The incorporation of metal base pairs in nucleic acids has been initially performed by application of a chemical strategy that involves the construction of oligonucleotides through automated solid-phase synthesis with phosphoramidite building blocks.[25, 26] The introduction of a metal ion into a specific pre-designed binding site can then be performed in aqueous solution by heating and annealing two single-stranded oligonucleotides that contain nucleotide mismatches in presence of cations responsible for the stabilisation of the structure. However, while this approach is convenient and has been employed for the investigation of numerous metal base pairs, it is restricted to rather short oligonucleotides and requires significant synthetic efforts. In addition, more sensitive functional groups such as aldehydes are not compatible with automated DNA synthesis and often require additional orthogonal protecting group strategies [27] therefore limiting the choice of chemistry that can be investigated in the context of metal base pair formation.[28]

An alluring alternative to chemical synthesis is the enzyme-catalyzed DNA synthesis through formation of metal ion mediated base pairs (referred to enzymatic construction or formation of metal base pairs in this article) which raises the possibility of forming long DNA metal nanowires and obtaining functional nucleic acids with an expanded genetic alphabet.[29] In the enzymatic approach, oligonucleotide templates are obtained by means of solid-phase synthesis. Subsequently, the triphosphate of interest is incorporated into DNA opposite the modified or natural templating nucleotide via the combined presence of DNA polymerases and metal cations.[30-32] This method can be applied to both natural and artificial nucleotides with the condition that polymerases accept the modified nucleotides as substrates and by-passes the metal base pair.[29-34]

The purpose of this review article is to discuss the recent progress made to generate metal-mediated nucleic acids base pairs using polymerase-mediated enzymatic synthesis. A first section of this review describes how this approach is applied to natural nucleobases and is followed by an extensive

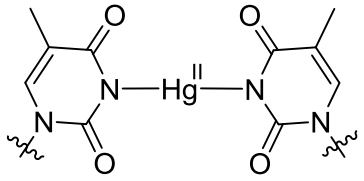
presentation of the enzymatic synthesis of metal base pairs using artificial nucleobases. Finally, a description of the most common analytical tools that are used to investigate the enzymatic formation of novel metal base pairs is reported.

Enzymatic construction of metal base pairs using natural nucleotides

Early interest[35-37] in the binding of metal to nucleic acids initiated a quest for potent artificial metal base pairs using solid phase synthesis and first successful examples were already reported in the late 1990s.[38-41] However, the preparation of metal base pairs by polymerase-mediated synthesis started much later when the possibility of using natural mismatches was discovered. The effect of metal ions on natural nucleotides was first investigated using small, synthetic oligonucleotides, which in turn prompted further studies to assess the possibility of their enzymatic construction. In this section, the most common and stable metal base pairs involving canonical nucleotides along with their enzymatic construction is presented.

T-Hg^{II}-T base pair

The first reports suggesting the formation of a stable metal base pair were published by Katz et *al*. in the early 1960 who showed the formation of a T-Hg^{II}-T base pair (**Figure 2**).[42, 43] Later, the stability of DNA duplexes equipped with internal T-T mismatches was studied by thermal denaturation experiments in the presence of various metal cations.[44] The presence of Hg^{II} cations substantially stabilized the duplexes (ΔT_m = +10°C) whereas none of the 14 other metal cations tested had an influence on the melting temperature.[44] The T-Hg^{II}-T base pair is therefore slightly more stable than the A-T natural pair (which displays a ΔT_m = +7°C compared to a T-T mismatch). Kondo and co-workers reported in 2014 the first crystal structure of a B-form DNA duplex containing two consecutive T-Hg^{II}-T base pairs (**Figure 3**).[45] In the absence of mercury cations, the DNA duplex was found to adopt a distorted non helical conformation. However, upon addition of Hg^{II} cations, the DNA duplex switched to the B-form. Mercury cations were found to be linked to the *N3* atom of thymine and the size and the position of the T-Hg^{II}-T base pair revealed to be similar to the natural Watson-Crick base pairs. These studies show that the T-Hg^{II}-T system does not induce any significant structural perturbation of a B-DNA duplex and might be compatible with the enzymatic construction of metal base pairs.



T-Hg^{II}-T

Figure 2. Schematic representation of T-Hg^{II}-T base pair.

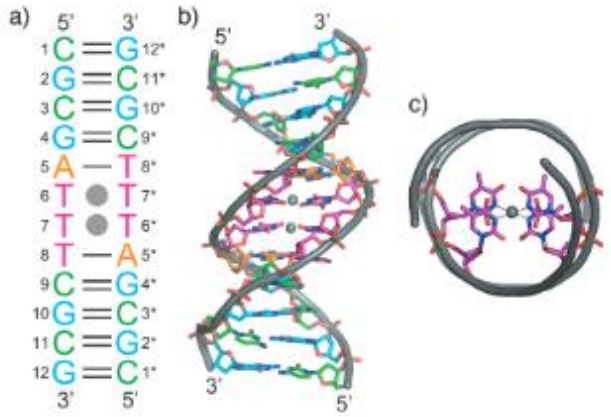
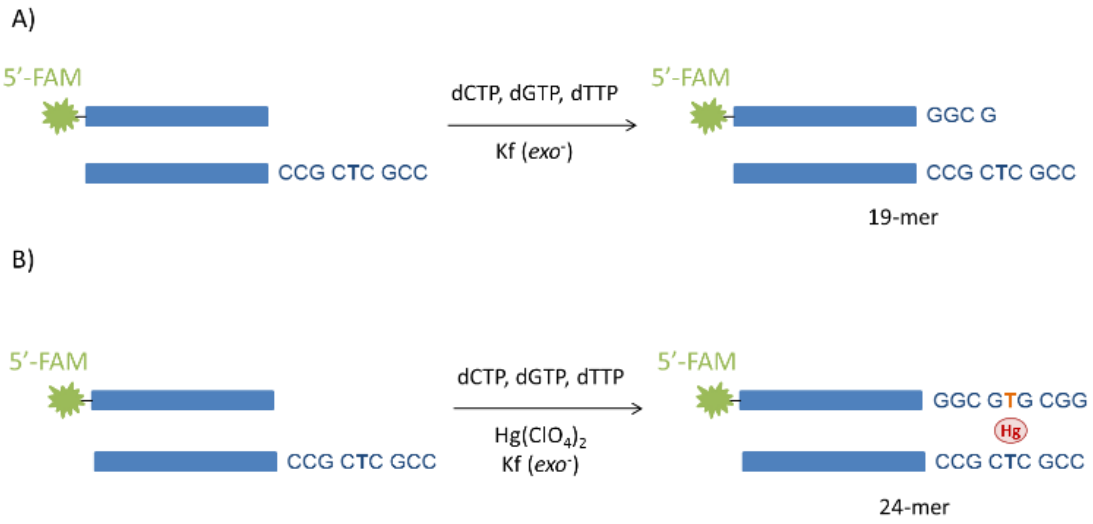


Figure 3. a) Secondary and b) crystal structure of a B-form DNA duplex in the presence of Hg^{II}; c) top-view of T-Hg^{II}-T base pair. Figure taken with permission from John Wiley and Sons.[45]

The first enzymatic construction of a metal-base pair involving natural nucleotides was reported by Urata and co-workers.[30] In this seminal work, the formation of the T-Hg^{II}-T base pairs was studied under primer extension (PEX) reactions.[30] PEX experiments were conducted using templates equipped with a nine-nucleotide region containing one T residue and no A base.[30] In the presence of dTTP, dCTP and dGTP and in the absence of dATP, the PEX reaction mediated by the Kf (*exo*$^-$) polymerase was terminated at the site opposite the T base in the absence of metal ions (**Scheme 1, A**). However, in the presence of low to moderate concentrations (10 to 100 μM) of Hg^{II}, full length products could be obtained (**Scheme 1B** and **Figure 4**). KOD Dash and Taq polymerases were also able to incorporate and by-pass the modified metal base pair. Nine other metal cations were tested under these conditions but none of them led to a full incorporation of dCTP, dGTP or dTTP nucleotides. Hence, the incorporation of a dTTP opposite a T base can be achieved by PEX reactions in the strict presence of Hg^{II} cations. Polymerases are also able to by-pass the modified base pair to give full length products.[30] Recently, Urata and co-workers challenged polymerases to construct a series of ten consecutive T-Hg^{II}-T base pairs.[46] Kf (*exo*$^-$) polymerase managed to incorporate and further by-pass three consecutive T-Hg^{II}-T pairs in the presence of mercury cations. However, the polymerase was not able to construct four or more T-Hg^{II}-T base pairs under the same experimental conditions. In order to decrease the polymerase fidelity, the PEX reactions were conducted in the presence of manganese cations (1 mM). Under these conditions, Kf (*exo*$^-$) polymerase incorporated and further by-passed up to seven dT nucleotides opposite templating dT residues in the presence of Hg^{II}. Therminator polymerase was also tested in the presence of the manganese cofactor and was able to incorporate a series of ten consecutive dTTPs in the presence of mercury cations. However, this polymerase could not add additional dA, dC, or dG nucleotides after the stretch of T-Hg^{II}-T base pairs. In order to complete the strand, dTTP was first added with mercury and manganese cations using Therminator polymerase. Other dNTPs were then added with Kf (*exo*$^-$) polymerase to afford the full-length oligonucleotide. This two-polymerase strategy therefore allows for the construction and by-pass of 10 consecutive mercury-mediated base pairs and could be used for the development of DNA-based nanowires.[46]



Scheme 1. PEX reaction using a 5'-FAM primer in the presence of dCTP, dGTP, dTTP using Kf (*exo*$^-$) polymerase and A) in the absence and B) in the presence of Hg^{II} cations.

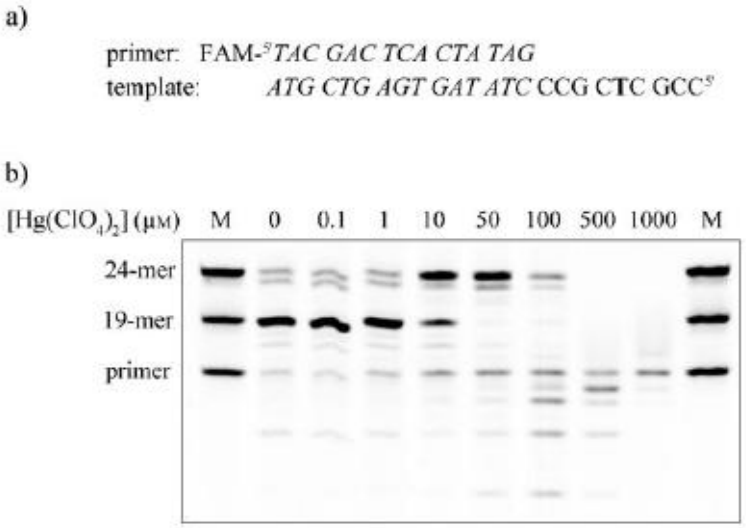


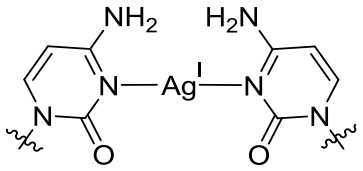
Figure 4. a) Primer and template sequences used for PEX reactions; b) PEX reactions using various mercury concentrations. Figure taken with permission from John Wiley and Sons from reference 32.

The PCR amplification of a system containing a terminal T mismatch was studied in parallel with Taq polymerase.[13] The presence of Hg^{II} cations was required to obtain amplified products. No amplification could be observed in the presence of other metal species.[13]

C-Ag^{I}-C base pair

Following the discovery of the T-Hg^{II}-T base pair, Ono and co-workers studied the effect of metal ions on duplexes containing a C-C mismatch.[47] Among the 14 metal cations that were tested, only Ag^{I} cations had an effect on the thermal transition profile. Indeed, upon the addition of one equivalent of Ag^{I}, the melting temperature increased by +8.8°C, whereas it had no effect on G-C or A-T bases. The stabilization

occurred between pH 5 and 9, due to the protonation state of the cytosine bases. An NMR spectroscopic study confirmed the formation of a C-Ag^{I}-C base pair (**Figure 5**) with the apparition of significant changes in the imino region. In 2015, Kondo and co-workers reported a crystal structure of an RNA duplex (**Figure 6**) containing a C-Ag^{I}-C base pair. This structure also confirmed the specific binding of the silver cation to the C-C mismatch in an *N3*-Ag^{I}-*N3* linear coordination. The metal base pair was shown to be structurally similar to the natural Watson-Crick base pairs and the presence of silver did not disturb the A-form RNA duplex.[48]



C-Ag^{I}-C

Figure 5. Structure of C-Ag^{I}-C base pair.

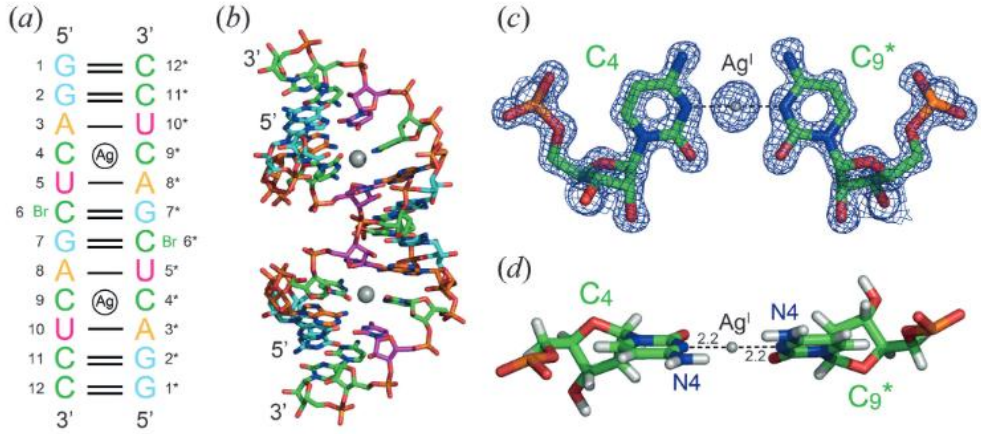
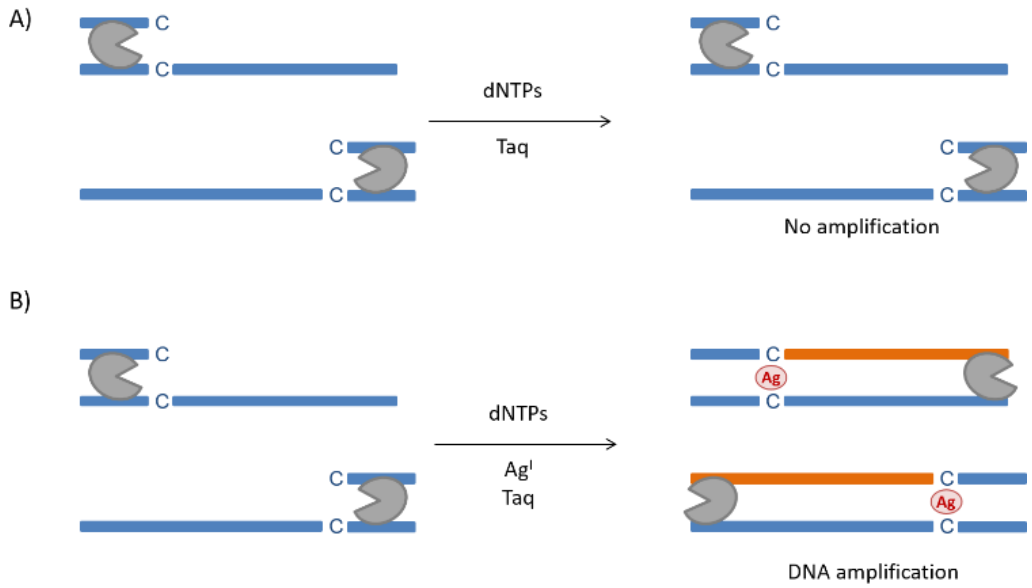


Figure 6. a) Secondary and b) crystal structure of an A-form RNA duplex in the presence of Ag^{I}; c) local structure of C-Ag^{I}-C base pair; d) side view of C-Ag^{I}-C base pair. Figure taken with permission from John Wiley and Sons.[48]

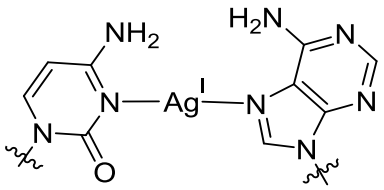
Based on these favourable properties, Park *et al.* used this base pair for the construction of a molecular logic-gate system based on PCR amplification. They designed forward/reverse primers containing a terminal C mismatch at their 3'-end. In the absence of silver cations, the presence of the mismatches prevents the elongation and therefore does not lead to DNA amplification (**Scheme 2**, **A**). However, upon the addition of silver ions the C-Ag^{I}-C base pair is formed (**Scheme 2**, **B**) and PCR amplicons could be obtained using the Taq polymerase.[13] Other metal cations were tested under these conditions but the results showed that only Ag^{I} led to the generation of the expected PCR products. No PCR amplification of the mismatch pair could be observed in the absence of silver cations. However, a few years later, PEX studies performed on this system revealed that a C-Ag^{I}-C base pair could not be formed.[49, 50]



Scheme 2. PCR amplification using a C-C mismatch A) in the absence of Ag^{I} cations and B) in the presence of Ag^{I} cations.

C-Ag^{I}-A base pair

In 2012, Urata and co-workers reported the unexpected enzymatic construction of a C-Ag^{I}-A base pair (**Figure 7**).[49] PEX reactions were carried out using the Kf (*exo*$^{-}$) polymerase in the presence and absence of Ag^{I} ions with a template containing a C nucleotide located immediately after the 3'-end of the complementary primer. In the absence of Ag^{I}, no incorporation could be observed with dATP, dCTP or dTTP. However, in the presence of silver cations, Kf (*exo*$^{-}$) misincorporated a dAMP opposite a templating C nucleotide. Even if the formation of the C-Ag^{I}-A base pair was not quantitative, formation of the metal base pair was confirmed by MALDI-TOF. Interestingly, dCTP incorporation opposite a templating dA nucleotide was not observed under the same conditions. PEX experiments were tested with 11 other cations displaying different oxidation states. Some metal cations such as Mn^{II}, Cu^{I} and Cu^{II} also promoted the formation of a similar metal base pair, albeit in much reduced yields. More interestingly, UV melting experiments with a 9-mer DNA duplex showed a better stabilization of the C-Ag^{I}-C base pair compared to the A-Ag^{I}-C base pair (ΔT_m = +8.3°C and +4.0°C, respectively).[49] The structural properties of the C-Ag^{I}-A base pair have not yet been investigated by NMR or X-ray crystallography.

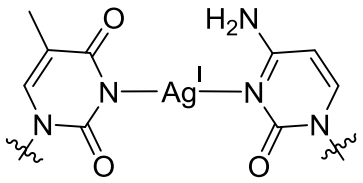


C-Ag^{I}-A

Figure 7. C-Ag^{I}-A base pair.

T-Ag^{I}-C base pair

Following the discovery of the possible enzymatic construction of a C-Ag^{I}-A base pair, the same group reported the enzymatic construction of a T-Ag^{I}-C base pair (**Figure 8**). Following the same methodology, Kf (*exo*$^{-}$) was found to readily incorporate a dTTP opposite a C-containing template in the strict presence of silver cations. The reverse reaction (incorporation of a dC nucleotide opposite a templating dT) also led to the formation of the expected n+1 product via formation of the T-Ag^{I}-C base pair. Metal specificity experiments showed that the use of either Hg^{II} or Ag^{I} ions could lead to full-length products when dTTP or dCTP were used opposite a templating dT nucleotide (thus forming T-Hg^{II}-T and C-Ag^{I}-T base pairs) but not opposite a templating dC nucleotide (despite the known stabilizing effect of silver on dC-dC mismatches). These results indicate that the incorporation of incoming dT and dC nucleotides can be programmed by the nature of the metal cation and the site on the DNA template.[50]



T-Ag^{I}-C

Figure 8. Representation of T-Ag^{I}-C base pair.

Based on all the different reports described in this section, the efficiency for the enzymatic construction of Ag^{I}-mediated base pairs with natural nucleotides appears to follow the trend A-Ag^{I}-C > T-Ag^{I}-C >> C-Ag^{I}-C. UV melting experiments were recently conducted[51] with 15-mer oligonucleotides that showed that DNA stabilization of mismatches via the addition of silver(I) follows the trend C-Ag^{I}-C (ΔT_m = +9.9°C) > T-Ag^{I}-C (ΔT_m = +6.2°C) ~ A-Ag^{I}-C (ΔT_m = +6.0°C).[51] The noticeable difference between DNA duplex stabilization and DNA enzymatic construction can be partially explained by the influence of the positive net charge of C-Ag^{I}-C, which might be unfavourable for polymerases. The shape complementarity of the base pairs as well as their stability in the absence of metal ions could also affect the incorporation efficiency by polymerases.[51] However, these studies indicate that DNA stabilization and enzymatic construction of metal base pair might not be driven by the same rules. Similar observations were made with modified nucleotides (*vide infra*).[33, 34]

Enzymatic construction of metal base pairs with modified nucleotides

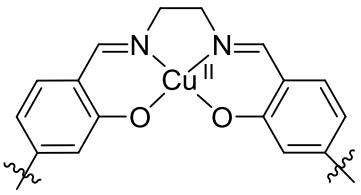
Even if natural nucleotides have been found to form metal base pairs, their coordination is limited to a small number or metal ions and do not display the level of orthogonality required for some applications such as the selection of aptamers.[52] In this context, the use of modified nucleotides able to coordinate other metal species could circumvent some of these limitations.

Indeed, the expansion of the genetic alphabet with the formation of artificial nucleotides has been a long-standing goal of Synthetic Biology and Xenobiology.[53] The past decade has seen the design of many

artificial base pairs whose interaction was mainly based on hydrogen bonding,[54, 55] shape complementarity[56, 57] or hydrophobic interactions.[58] However, some unnatural base pairs (UBPs) are prone to dephosphorylation[59, 60], and among them, cyclic π-conjugated analogues can be photoactivated by near-visible light, producing reactive oxygen species (ROS) which are highly toxic for DNA and cells.[61, 62] Therefore, metal base pairs characterized by modified nucleobases are arising as alternatives to these UBPs. Indeed, in metal base pairs, the use of modified analogs can convey orthogonality to the system which is not possible for natural nucleotides. To be correctly inserted, the non-canonical ligands should have dimensions that are comparable with the natural nucleosides, should be planar to interact with the adjacent units, their spatial arrangement should be compatible with the coordination geometry of the bridging metal ion and they should have affinity for the tested metal. Numerous modified nucleotides have been studied for their potential ability to create stable metal base pairs under thermal denaturation assays and have been recently reviewed.[1, 2, 63, 64] In this section, only the nucleotides that have been considered for the enzymatic construction of artificial metal base pairs will be discussed.

Salen-Cu^{II} base pair

The *N*,*N*'-bis(salicylidene)ethylenediamine (**Salen**) artificial metal base pair involves a different formation strategy. In a first step, two salicylic aldehyde moieties (**Sal**) are introduced into complementary strands of a DNA duplex by solid-phase chemical synthesis. Both aldehyde moieties can then react with an ethylene diamine additive to create the crosslinking **Salen** ligand via imine formation.[27, 65] The subsequent addition of a transition metal ion such as Cu^{II}, Mn^{II}, VO^{II}, Fe^{II} or Ni^{II} prevents the hydrolysis of the imines and leads to the formation of the artificial metal base pair (**Figure 9**).[65] This process is highly cooperative since ethylene diamine is strictly required for the formation of the diamine bridge and metal complexation prevents the spontaneous hydrolysis of the ligand. Melting temperature studies have shown that the DNA duplex was better stabilized in presence of Cu^{II} ions.[27] In the absence of metal cations but in the presence of ethylene diamine, crosslink between both strands was shown to increase the T_m by +5°C. If only Cu^{II} was added to the **Sal** ligands, a strong stabilization of the duplex was detected (ΔT_m = +15°C). However, upon addition of both ethylene diamine and one equivalent of Cu^{II}, an impressive ΔT_m of +42.5°C was observed.[27] Crystal structure of the modified DNA duplex inside the *Bst Pol I* polymerase was obtained (**Figure 10**). It revealed the coordination of the copper ions to the **Salen** ligand through a square planar geometry. Comparison with the A-T base pair showed that the **Salen** ligand induces only minimal perturbations to the DNA duplex.[65]



Salen-Cu^{II}

Figure 9. Schematic representation of **Salen**-Cu^{II} base pair.

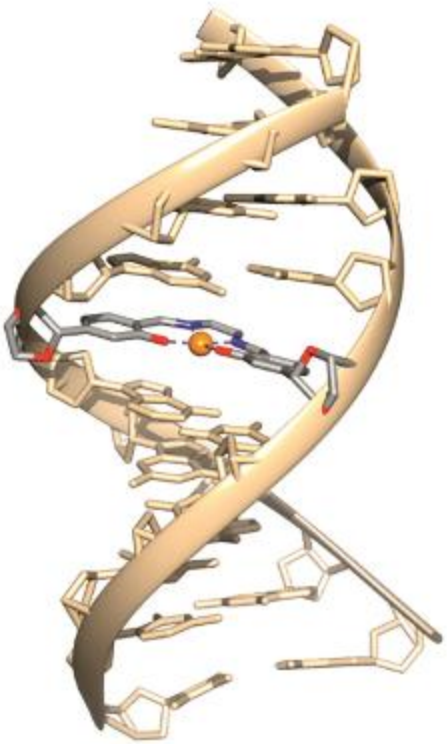
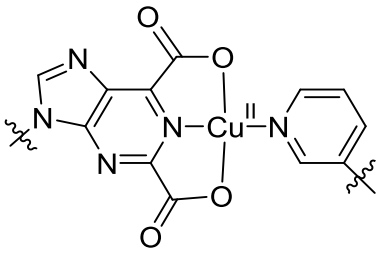


Figure 10. Crystal structure of modified DNA in *Bst Pol I*.[8] Figures taken with permission from Elsevier.

Carell et al. could take advantage of these favourable properties of the **Salen**-Cu^{II} base pair and reported the first enzymatic construction of a metal base pair involving modified nucleotides.[31] **Sal** was introduced into a 30-mer oligonucleotide by solid-phase synthesis and hybridized to a complementary primer ending one nucleotide downstream from the **Sal** modification. PEX reactions were conducted in the presence and absence of metal ions and ethylene diamine with the *Bst* polymerase. The latter was able to incorporate the **Sal** triphosphate with high efficiency, in the presence and the absence of the two additives. Specificity experiments revealed that dATP, dCTP, dTTP and to a smaller extend dGTP were also incorporated opposite the **Sal** modification in the absence of any additives. However, in presence of ethylene diamine and Cu^{II}, the incorporation of canonical nucleotides was strongly reduced. When **Sal** triphosphate was used, PEX reactions with unmodified oligonucleotides revealed its partial misincorporation opposite to A and T nucleotides in the absence of any additives and opposite to A, G and T in their presence. A competitive by-pass experiment based on the simultaneous use of dATP, dCTP, dGTP, dTTP and **Sal** triphosphates led to the formation of the full length oligonucleotide in the presence of ethylene diamine and Cu^{II}. In the absence of additives, only the **Sal** triphosphate could be incorporated.[31] PCR experiments were also conducted using a 153 base pair long oligonucleotide which includes three **Sal** modifications present in the 5'-G**Sal**T-3', 5'-A**Sal**C-3' and 5'-T**Sal**G-3' sequence environments. The same concentration of canonical and modified dNTPs was used with KOD XL polymerase. High amplification could be obtained only in the presence of ethylene diamine and Cu^{II}. LC-MS verification showed that the **Salen** content did not diminish during the PCR amplification.[31] To the best of our knowledge, the **Salen**-Cu^{II} represents the only artificial base pair fully compatible with enzymatic conditions.

Pur^{DC}-Cu^{II}-3Py base pair

The first example of the formation of a hetero base pair under enzymatic conditions using artificial nucleotides was reported by Switzer and co-workers in 2013.[32] The hetero base pair is composed of a planar tridentate ligand purine-2,6-dicarboxylate (**Pur^{DC}**) and a 3-pyridine-containing nucleoside (**3Py**). The **Pur^{DC}-3Py** system can coordinate cations through an unsymmetrical [3+1] coordination environment. Modified oligonucleotides were synthesized using the phosphoramidite building blocks of the purine-2,6-dicarboxylate (**Pur^{DC}**) and the 3-pyridine (**3Py**) analogs and used for thermal denaturation experiments.[32, 66] 11 metal ions were tested for their ability to stabilize the **Pur^{DC}-3Py** base pair (T_m = 13.5°C). This analysis revealed that Cu^{II} and Zn^{II} represented potent candidates for the formation of stable metal base pairs since the addition of 5 equivalents of those metal ions led to a massive stabilization of the duplexes by ΔT_m = +25.5°C and ΔT_m = +25.0°C, respectively. The increase of the melting temperature under addition of Cu^{II} was not observed with a control DNA duplex containing G and C in place of the modifications, or in the homo systems containing **Pur^{DC}-Pur^{DC}** and **3Py-3Py** mispairs. These preliminary experiments led to an enzymatic study for the formation of the **Pur^{DC}**-Cu^{II}-**3Py** base pair (**Figure 11**).[32]



Pur^{DC}-Cu^{II}-**3Py**

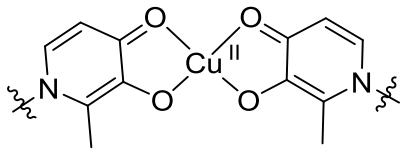
Figure 11. Structure of **Pur^{DC}**-Cu^{II}-**3Py** base pair.

After the synthesis of the **Pur^{DC}** triphosphate, PEX reactions were carried out to incorporate a **Pur^{DC}** nucleotide opposite a templating **3Py** moiety in the presence of Cu^{II} ions. Among the ten polymerases that were screened, five (*i.e.* Kf (*exo*$^-$), MMLV-RT, Vent (*exo*$^-$), Tth and Deep Vent) were found to efficiently incorporate the modified triphosphate with a clear metal concentration dependence. 50 µM of Cu^{II} were required for a complete formation of the metal base pair and no incorporation could be detected in the absence of copper (II). A by-pass experiment was also conducted. To do so, the artificial metal base pair was first formed, followed by the addition of natural dNTPs. Kf (*exo*$^-$) and Deep Vent led to the extension of the oligonucleotide and full-length products could be obtained. **Pur^{DC}**-Cu^{II}-**3Py** could be formed efficiently under enzymatic conditions by the incorporation of a **Pur^{DC}** triphosphate opposite a **3Py**.[32] However, the enzymatic incorporation of a **3Py** triphosphate face to a **Pur^{DC}** moiety and PCR amplification studies have not been reported yet. Even if the structure of a Dipic (pyridine-2,6-dicarboxylate)- Cu^{II}-**3Py** base pair has been reported,[67] the **Pur^{DC}**-Cu^{II}-**3Py** structure has not been solved yet.

H-Cu^{II}-H base pair

The 3-hydroxy-4-pyridone scaffold acts as a bidentate ligand able to form square planar 2:1 complexes with Cu^{II} cations.[68] Shionoya and co-workers attached a hydroxypyridone to the C1 position of a deoxyribose (called nucleoside **H**) and synthesized the corresponding phosphoramidite.[69] The latter was

incorporated into a 15-mer DNA duplex for metal dependence studies using UV melting temperature experiments. In the absence of metal ions, the **H**-**H** base pair was found to be destabilizing in respect to the natural A-T base pair (ΔT_m = -7.2°C). However, the addition of Cu^{II} ions led to a stabilization of the duplex by 13°C for the **H**-Cu^{II}-**H** base pair (**Figure 12**), whereas it had no influence on the melting temperature of a duplex containing a natural A-T pair.[69] An EPR analysis of the **H**-Cu^{II}-**H** base pair confirmed the square planar coordination of Cu^{II}.



H-Cu^{II}-**H**

Figure 12. Representative figure of **H**-Cu^{II}-**H** base pair.

The acceptance of an **H** triphosphate (d**H**TP) [21] by polymerases was first assessed with the TdT polymerase, which is known to incorporate a large number of natural or modified nucleotides at the 3'-end of single-stranded oligonucleotides in an untemplated manner.[70] In presence of 10 mM of Mg^{II} ions, a known cofactor of the TdT polymerase,[71] an average of 5 **H** nucleotides was appended at the 3'-end of a 20-mer oligonucleotide. The subsequent addition of Cu^{II} ions led to the formation of interstrand **H**-Cu^{II}-**H** base pairs, which was confirmed by UV measurements.[21] Interestingly, a reduction of the concentration Mg^{II} ions led to an increased incorporation of the modified triphosphate.[72] This could be explained by the complexation of Mg^{II} by the **H** nucleotides at higher magnesium concentrations, leading to the formation of an unfavorable secondary structure that prevents further incorporation of **H** nucleotides by the TdT polymerase.[72] So far, the possibility of incorporating **H** nucleotides with d**H**TP opposite templating **H** units in the presence of Cu^{II} has not been reported. On the other hand,the enzymatic incorporation of **H** nucleotides was investigated by PEX reactions with the Kf (*exo*$^-$) polymerase opposite templating natural nucleotides.[73, 74] The **H** nucleotide was quantitatively incorporated opposite templating A or T nucleotides, whereas only a small fraction of n+1 products was detected when the templating nucleotides were C or G. When templates contained two consecutive A or T nucleotides, only one **H** incorporation event could be achieved. Since the Kf (*exo*$^-$) polymerase was not able to by-pass the modified **H**-A or **H**-T mispair, a two-polymerase strategy was developed. After incorporation of an **H** nucleotide into DNA by the Kf (*exo*$^-$), Dpo4 polymerase was directly added with canonical nucleotides, resulting in the expected full-length products. This work enables the enzymatic construction of **H**-containing oligonucleotides in a controlled manned and was used to develop metal-responsive deoxyribozymes (or DNAzymes) which are controlled by the formation of the **H**-Cu^{II}-**H** base pair.[73] Similarly, a DNAzyme system exhibiting an AND logic gate response to both Cu^{II} and Ag^{I} cations was also developed using the **H**-Cu^{II}-**H** artificial base pair.[24] More recently, a similar strategy was pursued but with the translesion DNA polymerase Dpo4 which incorporates up to three **H** nucleotides opposite templating dT nucleotides in near quantitative yields. This feature was used to engineer allosteric DNAzymes that display a strong response and dependence to Cu^{II}.[74]

A structural investigation of the artificial metal base pair was performed in 2008. Meggers and co-workers reported the crystal structure of an (*S*)-GNA duplex containing two **H** ligands (**Figure 13**). Glycerol nucleic acid (GNA) is a nucleic acid analog in which the entire sugar-phosphate backbone of

DNA has been similarly substituted by an acyclic scaffold based on propylene glycol.[75] The self-complementary strands form a duplex in the presence of two equivalents of copper cations. The hydroxypyridone ligands were found to coordinate copper cations in a square planar geometry without causing distortions to the C_1-C_1 distance, compared to natural Watson Crick base pairs.[76]

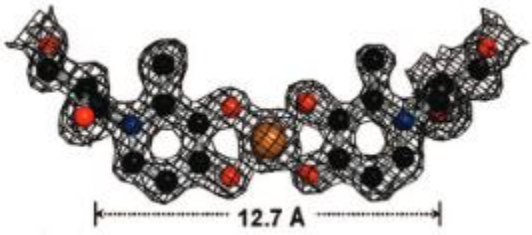
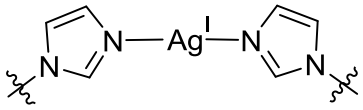


Figure 13. Electron density of the the **H**-Cu^{II}-**H** base pair with the terminal G-C base pair. Figure taken with permission from the American Chemical Society.[76]

Metal base pairs using nucleotides containing imidazole units

The imidazole moiety (**Im**) has been one of the most studied artificial homobase pair since it was shown to form very stable metal base pairs in the presence of Ag^I (**Figure 14**).[77] Melting temperature studies with complementary 26-mer oligonucleotides including one or two neighbouring **Im** modifications revealed that the presence of these **Im**-**Im** mispairs destabilized the DNA duplex (by -7.2°C and -12.2°C, respectively). Upon addition of Ag^I, an increase of the melting temperatures can be observed for systems with one and two **Im** base pair modifications (ΔT_m = +6.0°C and +11.0°C respectively).[77] Hence, the thermal stability of **Im**-Ag^I-**Im** is comparable to that of a canonical A:T base pair. An NMR structure study of a DNA duplex containing a series of three **Im**-Ag^I-**Im** base pairs revealed only minor distortions of the B-DNA duplex compared to canonical base pairs (**Figure 15**).[3]



Im-Ag^I-**Im**

Figure 14. Structure of **Im**-Ag^I-**Im**.

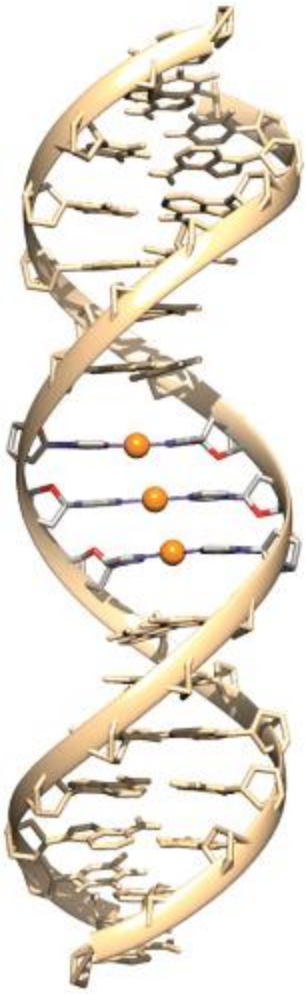


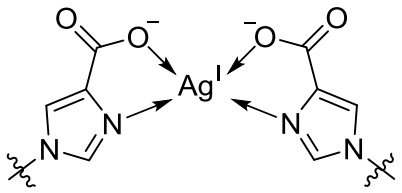
Figure 15. Structure of a DNA duplex containing a series of three **Im**-Ag^{I}-**Im** base pairs.[8] Figure taken with permission from Elsevier.

Based on these favorable assets combined with a relative ease of synthesis, the compatibility of the **Im**-Ag^{I}-**Im** base pair with enzymatic synthesis was investigated using four polymerases and 12 metal cations, including Ag^{I}. The incorporation of **dImTP** was favored with manganese and to a smaller extent with silver ions when the Kf (*exo*$^{-}$) was used as polymerase but natural dNTPs could also be misincorporated equally well opposite a templating **dIm** unit. In addition, once installed the **Im**-**Im** mispair could not be by-passed by polymerases and multiple incorporations were difficult to achieve.[33] These results clearly demonstrated that thermal stabilization and low structural perturbations were not the only important parameters required for the enzymatic synthesis of metal base pairs as had been observed for natural nucleotides.

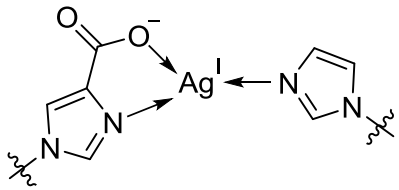
Hence, this work led to the construction of a second imidazole nucleotide which possesses a carboxylic group on the imidazole ring, which sustains the formation of [2+1] and [2+2] coordination environments (**Figure 16**).[78, 79] Enzymatic study of the incorporation of an **dIm^{C}** triphosphate with a **dIm^{C}** containing template revealed a modest formation of a metal base pair in the presence of Mn^{II}, Fe^{II}, Co^{II} or Cd^{II}, with the Taq polymerase. No multiple incorporations could be achieved and the metal base pair could not be

by-passed by polymerases. However, when the **dIm^{C}-dIm** system was used, a metal base pair in an unusual [2+1] coordination environment was formed in the strict presence of Ag^{I} cations. The base pair could be achieved with Kf (*exo*$^{-}$) polymerase with the **dIm** triphosphate and a **dIm^{C}** containing template and with the Taq polymerase for the reverse system. The incorporation of **dIm^{C}** seemed to be specific of the modification (**dIm^{C}** or **dIm**) as no n+1 products could be observed with unmodified oligonucleotide templates.[78]

The imidazole-4-carboxylate **dIm^{C}** has also been studied in thermal denaturation studies, which confirmed the formation of a stable **dIm^{C}**-Ag^{I}-**dIm^{C}** base pair (ΔT_m = +17.4°C).[79] However, this study also revealed the formation of an even more stable **dIm^{C}**-Cu^{II}-**dIm^{C}** base pair (ΔT_m = +19.9°C) which was not reflected in PEX reactions. The **dIm^{C}**-Cu^{II}-**dIm^{C}** base pair has been used for the generation of a Cu^{II}-responsive DNAzyme which showed no activity in the absence of copper(II) cations but expressed an activity in the presence of 1 equivalent of Cu^{II}.[80]



Im^{C}-Ag^{I}-**Im^{C}**



Im^{C}-Ag^{I}-**Im**

Figure 16. Putative structures of **Im^{C}**-Ag^{I}-**Im^{C}** and **Im^{C}**-Ag^{I}-**Im** base pairs.

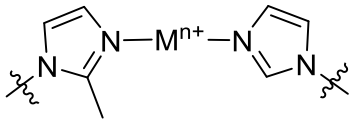
dIm^{2Me}, dIm^{4Me} and dIm^{2,4Me}

A third generation of imidazole analogs has also been investigated, which corresponds to an imidazole nucleobase bearing a methyl group on position 2 (**dIm^{2Me}**) or 4 (**dIm^{4Me}**) or on both positions of the imidazole ring (**dIm^{2,4Me}**). The thermal stability of a **dIm^{2Me}**-Ag^{I}-**dIm^{2Me}** and of a **dIm^{4Me}**-Ag^{I}-**dIm^{4Me}** base pair was studied. The presence of a methylated imidazole ring was found to have a stabilizing effect on the metal base pair compared to the parent **dIm**-Ag^{I}-**dIm** base pair ($\Delta\Delta T_m$ = +2.0°C for **dIm^{4Me}**-Ag^{I}-**dIm^{4Me}** and $\Delta\Delta T_m$ = +3.0°C for **dIm^{2Me}**-Ag^{I}-**dIm^{2Me}**). This result was explained by the presence of the methyl group which offers a better shielding of the silver cation from the solvent.[81]

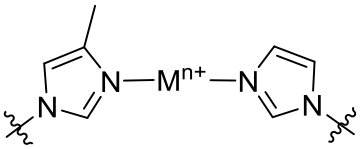
These favorable thermal denaturation studies have later led to the enzymatic investigation of **dIm^{nMe}**-M^{n+}-**dIm^{nMe}** base pairs, using 7 different polymerases and 12 metal species.[82] It was found that the homo **dIm^{nMe}**-M^{n+}-**dIm^{nMe}** base pairs led to exonucleolytic degradation of the primer with most polymerases.

Therefore, hetero base pairs were considered with **dIm^{nMe}** as incoming triphosphates and a template containing a series of three consecutive **dIm** nucleotides to reduce the possibility of steric clashes (**Figure 17**). A significant trend raised from this study, showing that 1) **dIm^{2Me}** was a poor substrate for polymerases, 2) **dIm^{2,4Me}** was a moderate candidate with the formation of a **dIm^{2,4Me}**-Ag^{I}-**dIm** base pair with 50% yield using Kf (*exo*$^{-}$) polymerase and 3) **dIm^{4Me}** was a good substrate for the enzymatic formation of metal-mediated base pairs as a **dIm^{4Me}**-Ag^{I}-**dIm** base pair could be formed in ~90% yield in the specific presence of silver ions, using Dpo4 polymerase. This trend was further confirmed by specificity experiments which showed that **dIm^{2,4Me}** triphosphate was not incorporated opposite templates containing natural nucleotides whereas **dIm^{4Me}** was partially incorporated opposite natural nucleotides. Interestingly, the trend observed in the enzymatic studies is not in accordance with the pK_a values of the methylimidazole candidates nor with the thermal denaturation experiments.

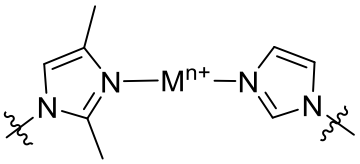
In order to complete the enzymatic study, the effect of ten underexplored metal species on the construction of a metal-mediated base pair was investigated. Ten metal species ($GaCl_3$, $IrCl_3$, $NaAuCl_4$, $PdCl_2$, $RuCl_3$, $SbCl_3$, $ScCl_3$, $SrCl_2$, VCl_3, $KCr(SO_4)_2$) were first shown to be compatible with enzymatic DNA synthesis for most polymerases under both PCR and PEX conditions, using only unmodified nucleotides and oligonucleotides. Subsequent experiments with the three hetero systems revealed that only **dIm^{2Me}** led to the formation of a promising base pair as a **dIm^{2Me}**-Cr^{III}-**dIm** base pair could be formed in ~60% yield using *Bst* polymerase and in the specific presence of chromium.[82]



Im^{2Me}-M^{n+}-**Im**



Im^{4Me}-M^{n+}-**Im**

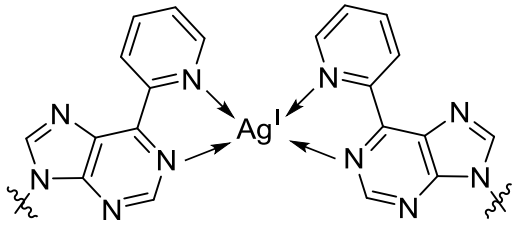


Im^{2,4Me}-M^{n+}-**Im**

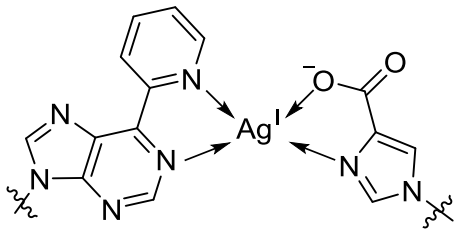
Figure 17. Putative structures of hetero metal base pairs consisting of methylated imidazole and unsubstituted imidazole nucleotides.

Pur^{P}-Ag^{I}-Pur^{P} and Im^{C}-Ag^{I}-Pur^{P} base pairs

The potential of an adenine analog bearing a pyridine moiety instead of the exocyclic amine (**Pur^{P}**) to form a homo metal base pair was investigated in 2005 by Switzer and coworkers.[83] Indeed, thermal denaturation experiments revealed that Ni^{II}, Co^{II}, Cu^{II}, Zn^{II} and Ag^{I} led to an increase of the melting temperature (ΔT_m = +18.1, +10.3, +2.9, +2.3 and +2.0°C respectively). This analysis clearly showed that the presence of Ni^{II} confers an important stability to the artificial base pair comparable to that of a C-G pair.[83] Based on this report, the enzymatic construction of **Pur^{P}**-M^{n+}-**Pur^{P}** base pair was studied, using 12 different metal cations and 7 polymerases.[34] Surprisingly, no incorporation could be detected when the PEX reactions were supplemented with Ni^{II} under any of the experimental conditions tested. On the other hand, Kf (*exo*$^{-}$) was found to effectively form the metal base pair in the specific presence of Ag^{I} (**Figure 18**). As **dIm^{C}** possesses two coordination sites and also sustained the formation of artificial base pairs in the presence of silver cations, the **dIm^{C}** triphosphate was then tested opposite a **Pur^{P}** containing template. Vent (*exo*$^{-}$) was able to form a **dIm^{C}**-Ag^{I}-**Pur^{P}** pair in high yields (**Figure 18**). The metal base pair could be by-passed using a two-polymerase strategy (formation of the base pair with Vent (*exo*$^{-}$) and further incorporation of natural nucleotides using Therminator polymerase). However, selectivity experiments revealed the incorporation of natural dNTPs opposite a templating **dPur^{P}** in the presence and absence of Ag^{I} ions. Interestingly, when the opposite system was used (**Pur^{P}** as a triphosphate, **Im^{C}** in the template), the artificial metal base pair was not specific of silver and could be formed in the absence of metal ions.[34]



Pur^{P}-Ag^{I}-**Pur^{P}**

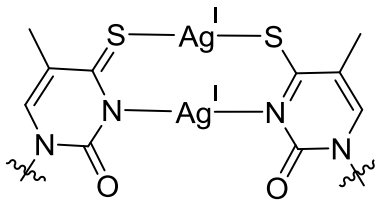


Pur^{P}-Ag^{I}-**Im^{C}**

Figure 18. Representation of **Pur^{P}**-Ag^{I}-**Pur^{P}** and **Pur^{P}**-Ag^{I}-**Im^{C}** base pairs.

Thiolated and pK_a perturbed base pairs

The 4-thiothymidine nucleotide **S4T** was investigated during thermal denaturation assays with 12 different metal cations. It was shown to form a very stable base pair in the presence of two Ag^{I} cations (ΔT_m = +23°C).[84] The formation of the **S4T**-2Ag^{I}-**S4T** base pair (**Figure 19**) was further investigated by an X-ray structural analysis which confirmed that the DNA duplex was undisturbed and adopted a B-form conformation (**Figure 20**).[85]



S4T-2Ag^{I}-**S4T**

Figure 19. Chemical structure of **S4T**-2Ag^{I}-**S4T** base pair.

Recently, the enzymatic formation of this artificial metal base pair was studied using 6 different polymerases.[29] Of these polymerases, *Bst* was able to form a metal-mediated base pair with high efficiency in the presence of different metal cations including Ag^{I}, Cd^{II}, Cu^{II} and Hg^{II}. Evidence for the formation of a **S4T**-Ag^{I}-dC base pair, the modified equivalent of a dC-Ag^{I}-dT base pair could also be obtained. The modified **S4T**-2Ag^{I}-**S4T** base pair could be by-passed by *Bst* polymerase. However, **S4T** nucleotides can also be incorporated opposite natural nucleotides especially in the case of dG and dA.[86] In this study, hetero artificial base pairs with other sulfur or fluorine containing nucleotides such as **S6G**-Hg^{II}-**S4T**, **S2C**-Hg^{II}-**S4T**, **5FU**-Ag^{I}-**S4T** and **5FU**-Hg^{II}-**S4T** could also be constructed under PEX reaction conditions.[29]

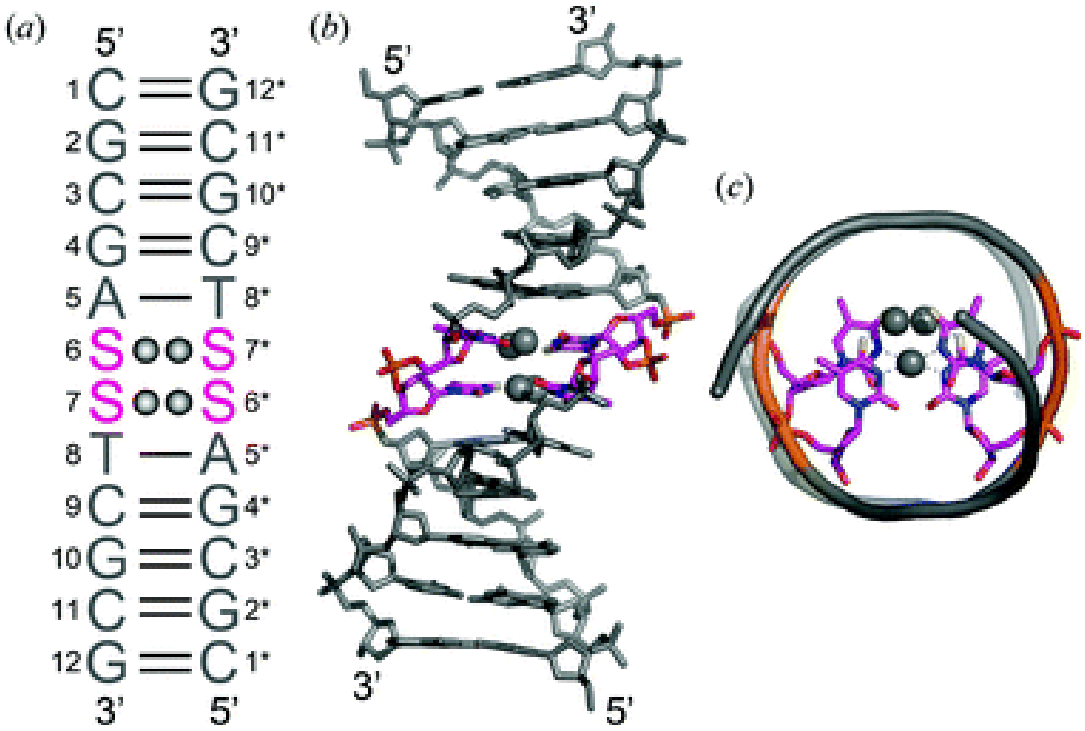


Figure 20. a) Secondary and b) crystal structure of a B-form DNA duplex containing two consecutive **S4T**-2Ag^{I}-**S4T** base pairs; c) top view of **S4T**-2Ag^{I}-**S4T** base pair. Figure taken with permission from The Royal Society of Chemistry.[85]

Artificial metal base pairs and RNA oligonucleotides

The development of artificial metal base pair has mainly focused on DNA duplexes, due to the higher stability of DNA compared to RNA. However, RNA duplexes are less prone to hydrolysis compared to single-stranded RNA and can therefore also be considered for the construction of metal base pairs.[87] Even though the RNA analog of the dT-Hg^{II}-dT base pair, U-Hg^{II}-U, has been shown to confer a substantial thermal stability to RNA duplexes (ΔT_m = +6°C),[88, 89] no enzymatic synthesis of this metal base pair has been reported yet. However, Sigel *et al.* have used T7 RNA transcription reactions to construct RNA duplexes containing up to twenty uracil-uracil mismatches in order to investigate the stabilizing effect of Hg^{II} on such systems.[89]

On the other hand, a chemoenzymatic approach has been devised by Srivatsan and co-workers for the construction of RNA-RNA and RNA-DNA artificial metal base pairs which were used as fluorescent probes for the detection of metal base pairs.[90] To do so, a fluorescent 5-methoxybenzofuran uracil triphosphate was synthesized (**Figure 21**) and incorporated into RNA transcripts using the T7 RNA polymerase. The RNA transcript containing one modification was then hybridized to: 1) its full complementary RNA and DNA oligonucleotides with a dA base opposite the modification or 2) with an rU (RNA) or a dT (DNA) base opposite the modification. An enhancement of the fluorescence was noticed when the 5-methoxybenzofuran modification was opposite an rU or a dT base, showing its ability to detect RNA-RNA or RNA-DNA mismatches. Upon the addition of Hg^{II} cations, the fluorescence intensity dropped significantly suggesting formation of the expected metal base pairs. In order to further demonstrate the formation of 5-methoybenzofuran rU-Hg^{II}-rU or 5-methoybenzofuran rU-Hg^{II}-dT base pairs, thermal denaturation experiments were conducted, which revealed a stabilization of the mispairs upon the addition of Hg^{II} ions (ΔT_m = +17.2°C). Titration experiments yielded K_d values of 104 nM and of 57 nM for the RNA-DNA duplex. These values are comparable to the dT-Hg^{II}-dT metal base pair but suggest a higher binding affinity for the RNA-DNA duplex compared the RNA-RNA duplex. This system can therefore be used to detect RNA-DNA mismatches and reveal the presence of Hg^{II} ions in the environment of the probe.[90]

The potential of modified nucleoside to serve in the metal ion-mediated base pairing between unmodified RNA oligonucleotides and short 2'-OMe-modified RNA sequences has also been investigated. Depending on the sequence composition, the presence of a bidentate 3,5-dimethylpyrazolyl-bearing purine nucleotides permitted to stabilize duplexes in the presence of Cu(II).[91]

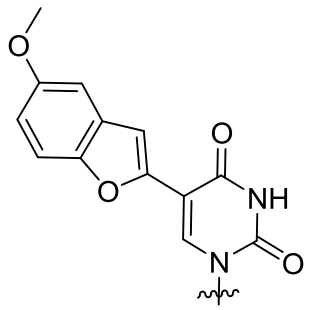


Figure 21. Chemical structure of the modified 5-methoxybenzofuran uracil.

Analytical tools

The formation of metal base pairs obtained by enzymatic strategies can be investigated via different analytical tools. A part of these methods have been initially developed to study oligonucleotides that have been synthesized through solid-phase synthesis and then adapted to enzymatic synthesis products. Another part of analytical tools is derived by the biochemical characterization of triphosphates. In this section, we will discuss the most relevant analytical methods, namely X-ray crystallization, NMR spectroscopy, mass spectrometry, UV spectroscopy and CD spectroscopy.

X-ray crystallographic analysis

The formation of metal base pairs can be investigated by the determination of crystal structures through X-ray diffraction analysis. In fact, this technique provides information not only on the exact location and configuration of the nucleobases but also on the specific sites where the metal ions are bound and the coordination geometry.[92] Before the studies on metal base pairs, the X-ray diffraction has been widely applied to the investigation of the roles played by metal ions that are bound to nucleic acids in natural structures.[93, 94]

However, application of X-ray diffraction analysis to DNA duplexes containing artificial metal base pairs has been proven to be quite challenging due to the difficulty of obtaining high-quality single crystals of the structures.[28] Therefore, there is very limited number of reported crystal structures, most of which are highlighted in the different figures of this Review article. In 2009, Schlegel *et al.* have extensively worked on the hydroxypyridone homo-base pair which is mediated by Cu^{II}. The crystal structures of GNA duplexes containing both one and two consecutive Cu^{II}-mediated hydroxypyridone metal-base pairs have been reported.[76, 95] In 2011, Kaul *et al.* have worked with the fully orthogonal and replicable **Salen** metal base pair **Salen**-Cu^{II} and have obtained its crystal structure inside a polymerase. With this crystal structure, they showed the possibility of having reversible chemistry inside the polymerase leading to the insertion of the **Salen** artificial base pair in DNA duplexes via natural polymerases.[31] In 2014, Kondo *et al.* have reported the first crystal structure of a B-DNA duplex characterized by two consecutive T-Hg^{II}-T base pairs, which was shown as a relevant bionanomaterial to be used in ion sensing and the construction of nanodevices.[45] Moreover, in 2015 Kondo *et al.* have reported the crystal structure of an RNA duplex containing the metal-base pair C-Ag^{I}-C, which is characterized by a linear coordination environment where the metal is connected to the *N*3 atom of both the pyrimidine nucleotides.[48, 96] Finally, as already mentioned in the applications section of the introduction, in 2017, Kondo *et al.* have synthesized a DNA nanowire completely composed of artificial base pairs mediated by Ag^{I} and resolved its crystal structure.[12]

The structure of the **Dipic**-Cu^{II}-**3Py** base pair was investigated in 2001 by Schultz and co-workers. They reported an X-ray crystal structure of a DNA duplex containing two **Dipic**-Cu^{II}-**3Py** base pairs (**Figure 22**). The DNA duplex was found to adopt a Z-DNA topology. Analysis of the local environment of the hetero base pair showed that the copper cation was coordinated to the artificial ligands in a Jahn-Teller distorted octahedral geometry.[67]

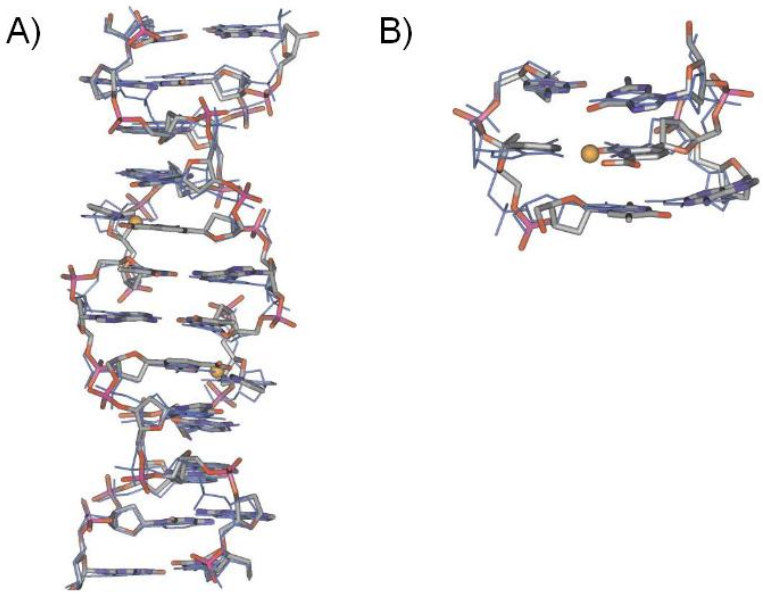


Figure 22. a) Crystal structure of a Z-DNA duplex containing two **Dipic**-Cu^{II}-**3Py** base pairs; b) local environment of **Dipic**-Cu^{II}-**3Py**. Figures taken with permission from the American Chemical Society.[67]

NMR spectroscopy

Before the introduction of metal base pairs, NMR spectroscopy has been widely applied to the investigation of the capability of RNA or DNA to bind and interact with metal ions for catalytic activity[97] or for structural purposes.[8] For example, the catalytic function of ribozymes has been frequently associated with the presence of metal ions.[98-100] However, ribozymes often depend on closed-shell metal ions which are spectroscopically silent; therefore, the study by means of NMR spectroscopy of the specific role covered by the functional metal ions has been challenging.[92]

NMR spectroscopy is used to confirm the formation and incorporation of metal-base pairs, but can be applied only if the analysed oligonucleotides contain *N*-labelled nucleotides. In fact, this label used in the context of metal base pairs often leads to significant shifts of the ^{15}N-NMR signals. Moreover, it can detect a scalar coupling between the ^{15}N atom involved in the metal-base pair and the NMR active metal nucleus showing the exact site of metalation.[28] The use of ^{14}N NMR to investigate the interaction of metal cations with nucleic acids, particularly in the context of metal base pairs, would be a highly interesting and versatile tool but is not sufficiently developed as yet .[101]

In this context, Johannsen *et al.* published in 2010 the NMR solution structure of a self-complementary DNA oligonucleotide containing three consecutive imidazole nucleotides in its centre. The structure has been reported both in the absence and presence of Ag^{I} ions. Without silver ions, the oligonucleotide was shown to have a hairpin structure whose loop was formed by the artificial nucleotides, while in the presence of Ag^{I} a canonical duplex was observed. The structure investigation has been performed through heteronuclear single quantum coherence (HSQC) spectra exploiting the presence of ^{15}N-labelled imidazole nucleosides in the studied oligonucleotides. The unstructured hairpin loop found in the absence of Ag^{I} has been suggested in the spectrum by a few cross peaks which were not well resolved. The

addition of one equivalent of Ag^{I} provoked a remarkable improvement in the spectrum together with an up-field shift of all nitrogen resonances. This observation was related to the more rigid structure of a double helix.[3]

Moreover, in 2015, Dairaku *et al.* have used ^{199}Hg NMR spectroscopy, widely applied to investigate coordination modes of metal complexes, to study the J-coupling of the highly stable T-Hg^{II}-T base pair. This analysis further confirmed that mercuriation of sp^2 nitrogen atoms occurred upon addition of Hg^{II}.[96] Subsequently, in 2016, Dairaku *et al.* reported ^{1}H, ^{15}N and ^{109}Ag NMR spectroscopy studies which allowed to unambiguously determine the chemical structure of the C-Ag^{I}-C base pair. In the same work, they also showed the 3D structure of an anti-parallel DNA duplex containing the same metal-base pair.[102]

Moreover, in 2020, Brown *et al*. have used a combination of NOESY, COSY and TOCSY NMR experiments to study the molecular interaction between Cr^{III} and DNA, which is thought to play a key role in the mutagenic and carcinogenic mechanism of action of Cr^{VI}. The study confirmed that the metal exclusively interacts with guanine *N*7 positions, excluding interactions of Cr^{III} with other nucleobases or backbone phosphates.[103]

Mass spectrometry

Mass spectrometry is an extremely relevant technique for the characterization of duplexes containing metal mediated base pairs. The most common mass spectrometry technique in the investigation of metal base pairs is electrospray ionization (ESI-MS).[104] The reason for its wide application in the study of oligonucleotides and metal base pairs is that ESI-MS is a soft ionization technique which overcomes the tendency of biological macromolecules to fragment while ionized.[105] Another relevant soft mass spectrometry type is matrix-assisted laser desorption/ionization (MALDI). MALDI forms ions with minimal fragmentation from large biomolecules through a laser energy absorbing matrix.[106] Therefore, both ESI-MS and MALDI are effective methods to obtain ions of biomolecules in the gas-phase, with the major difference that MALDI usually produces less multi-charged ions.

It is important to outline that mass spectrometry can be used only if the investigated metal base pair is kinetically and thermodynamically stable in order to clearly recognize the specific binding interactions and the formed duplexes.[28]

In 2006, Tanaka *et al.* applied electrospray ionization-time-of-flight mass spectrometry (ESI-TOF MS) to study the stable duplex structures that are formed by oligonucleotides in the presence of Cu^{II}–mediated hydroxypyridone (**H**) homo-base pairs. In this study, the capability of different metal ions to be complexed by modified DNA nucleobases was investigated.[107]

Moreover, in 2011, Megger *et al.* conducted laser-induced liquid bead ion desorption (LILBID) mass spectrometry to confirm the formation of an artificial base pair composed by 1,3-dideaza-2'-deoxyadenosine and thymidine, mediated by Ag^{I}. The LILBID technique was found to be an extremely relevant method to study biomolecules due to the low quantity of analyte needed. The characterization was completed by spectroscopic analysis.[108]

In 2018 Swasey *et al.* used ESI-MS to characterize homocytosine and homoguanine DNA strands where the interaction of the base pairs was mediated by silver ions Ag^{I}. The study was conducted by annealing dG strands or dC strands of different lengths in presence of 1 equivalent per base of $AgNO_3$ and 50 mM

NH_4OAc to provide enough ionic strength. ESI-MS showed that the main product is the duplex containing one Ag^I ion per base pair (**Figure 23**).[109]

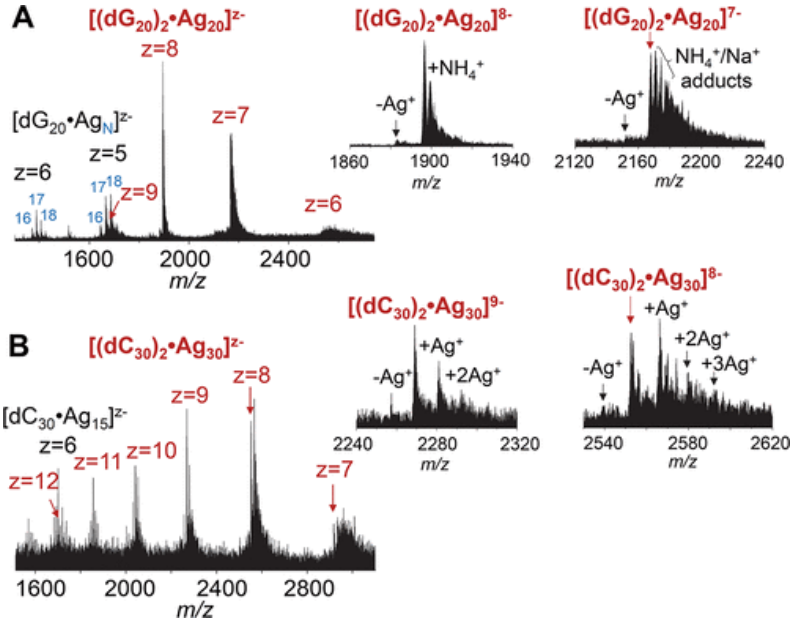


Figure 23. ESI-MS spectra of the dG-duplex $[dG_{20}]_2 \cdot Ag_{20}$ (A) and the dC-duplex $[dC_{30}]_2 \cdot Ag_{30}$. Both in the case of the homoguanine duplex and of the homocytosine duplex the major product is the duplex characterized by one Ag^I per base pair. However, while the homoguanine case is characterized by a monodispersed main product, the homocytosine case has more polydisperse of products. Figure reprinted with permission from Reference [109].

Finally, in 2019, Fujii *et al.* applied ESI-MS to study the structure of the newly synthesized metal base pair 9-TAP (N). The self-base pair 9-TAP is formed by two 1,2,9-triaza-2-oxophenoxazine whose interaction is mediated by three Ag^I. ESI-MS profiles showed clear peaks associated to a duplex containing the base pair mediated by three Ag^I (**Figure 24**). The N-3 Ag^I–N coordination was characterized by two deprotonated mass peaks, while only one less prominent deprotonated peak was observed for N-2Ag^I-N and no peak was found for N-Ag^I-N.[110]

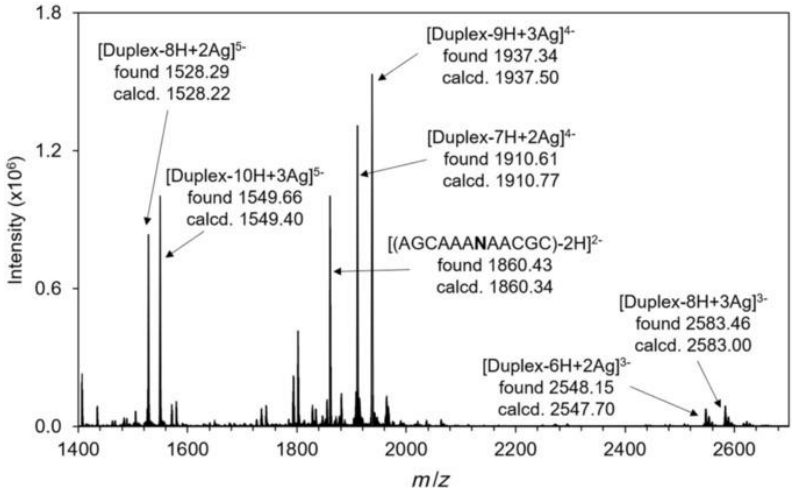


Figure 24. ESI-MS spectrum of duplex characterized by the metal self-base-pair N-3Ag^{I}-N. Figure taken with permission from Wiley Online Library from reference 110.

UV and CD spectroscopy

The formation of metal base pairs has extensively been investigated via spectroscopic techniques, such as UV and CD spectroscopy often associated with thermal duplex denaturation.

The most widely used spectroscopic method is the T_m melting experiment where the absorbance of duplexes is measured at 260 nm with varying temperatures. This technique allows to obtain the value of T_m which gives an indication of the stability of the duplex. Moreover, T_m melting experiments permit the extraction of the thermodynamic parameters ΔG, ΔH and ΔS for duplex formation.

T_m melting can be applied to investigate the nature and stability of metal base pairs while related titration experiments can be used to outline the stoichiometry and the number of metal ions that are involved in the interaction with the nucleobases of the unnatural base pairs. In UV melting and titration experiments, ideally, an increase in concentration of metal ions leads to higher melting temperatures until the saturation of the coordination sites is reached. In most of the cases where the formation of a biphasic curve is observed in the presence of substoichiometric amounts of metal ions, the lower T_m can be attributed to the dissociation of the metal-free duplex, while the higher to the dissociation of the complete molecule containing fully formed metal-base pairs.[28] The biphasic melting curve can be observed in the presence of only one metal-binding site per complex in the case of the existence of a dynamic equilibrium between the metal-free duplex and the metalated species, both characterized by their individual T_m.[28] Moreover, in the presence of duplexes with more than one metal-binding site, a biphasic melting curve can indicate a cooperative formation of the metal base pairs. Indeed, a biphasic curve in the presence of a binuclear metal base pair or of two mononuclear metal base pairs can suggest that the coordination of the first metal ion leads to the preferred binding of the second metal ion.[28]

It must be noticed that UV spectroscopy is particularly useful in the study of metal base pairs characterized by ligands with OH groups. Indeed, the UV spectra of these nucleobases show significant changes upon formation of a metal base pair due to the deprotonation of the phenolic OH groups. A relevant example is given by the hydroxypyridone nucleobase **H**, which has been reported in the previous section of this review. As discussed, **H** forms the homobase pair **H**-Cu^{II}-**H** which is a square-planar

complex. An increase in the concentration of Cu^{II} causes a decrease of the absorbance peak at 280 nm together with the appearance of a new peak at 307 nm with two isosbestic points in the range 0 to 5 for the ratio [Cu^{II}]/[duplex]. This variation indicates the level of deprotonation of the phenolic hydroxy groups upon complexation with Cu^{II} .[16, 69]

Moreover, in 2015 the same group studied the bifacial nucleobase 5-hydroxy-uracil (U^{OH}) which was expected to use two adjacent 4-carbonyl and 5-hydroxyl groups as coordination sites for the formation of a metal homo base pair. UV-based titration experiments revealed that the addition of Gd^{III} ions to a duplex containing three consecutive U^{OH} caused the appearance of a new band at 310 nm due to the deprotonation of the 5-OH groups. The results suggested the presence of one metal ion Gd^{III} per homobase pair (**Figure 25**).[111]

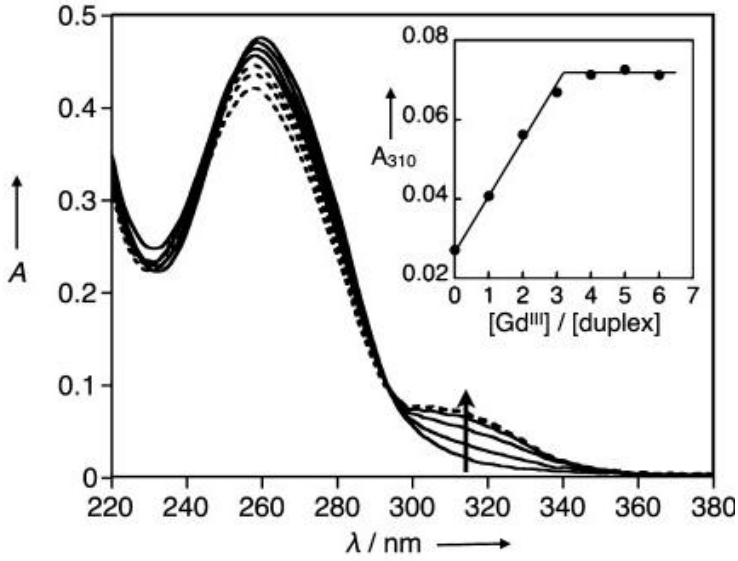


Figure 25. UV absorption spectra of a duplex containing three consecutive U^{OH} homo base pairs in presence of increasing concentrations of Gd^{III} metal ions. Figure taken with permission from Wiley Online Library from reference[111].

Another important example is the homobase pair **Salen**-Cu^{II}-**Salen**, which has been previously extensively discussed. A series of variations in the UV absorption spectrum can be observed during the formation of the metal base pair. A double strand containing two salicylic aldehydes facing each other is characterized by an absorption peak at 330 nm, caused by the aromatic chromophores, and a peak at 260 nm which is decreased when compared to the one of a natural double strand. The addition of an excess of ethylenediamine, needed for the formation of the **Salen** base pair, causes the appearance of a new band at 410 nm, which is compatible with the values of a deprotonated **Salen** ligand, and a decrease in the salicylic aldehyde peak at 330 nm. Finally, the coordination of the metal ion Cu^{II} generates a shift in the absorption band to 360 nm and the appearance of a new band at 570 nm typical for the N_2O_2-Cu chromophore.[65]

The studies performed on the **Salen** homobase pair have been followed by the synthesis and characterization of the pyrazole homobase pair (**Pz**) mediated by Cu^{II}, where the main difference with the **Salen** system bresides in the lack of the linking ethylendiamine. Carrell *et al*. have studied the stability of

duplexes containing two **Salen**-Cu^{II}-**Salen** base pairs and a **Pz**-Cu^{II}-**Pz** base pair. The spectroscopic titration performed at 360 nm plotting the absorbance in function of an increasing number of Cu^{II} equivalents proved that at first the coordination of the **Pz** homo base pair occurs, followed by the **Salen** base pair. Interestingly, when **Pz** was substituted by the variant **Pm** where the pyrazole moiety is maintained but the phenolic group is methylated, the spectroscopic titration showed the complexation only of the **Salen** homo base pairs. Therefore, these UV spectroscopic studies highlighted the fundamental role played by the deprotonation of the phenolic group of **Pz** in the formation of the metal base pair.[112]

A further confirmation of the formation of the metal base pairs can be given by CD spectroscopy. Indeed, CD spectroscopy can be used to determine the thermal and structural duplex stabilities in the presence and absence of metal ions, indicating the formation of metal base pairs.[113] CD spectroscopy can also provide structural information which is important to assess whether a metal base pair leads to structural deviations from the natural A, B, or Z-DNA structures.

A relevant example of the combined application of UV spectroscopic titration and CD studies is the work by Jash *et al.* where the Hg^{II}-binding properties of the artificial nucleobase 1*H*-imidazo[4,5-f][1,10]phenanthroline (**P**) have been investigated. Specifically, the mentioned techniques have been applied to study of the **P**-Hg^{II}-**P** and the **P**-Hg^{II}-**T** metal base pairs. The inset of **Figure 26** represents the UV spectrum of the free nucleoside P which is characterized by an absorption maxima at 249 nm and 283 nm. The progressive addition of Hg^{II} ions provoked the disappearance of the absorption peak at 249 nm accompanied by an increase in the absorption at 283 nm and the appearance of a new absorption bands at 315 nm. The changes in the spectrum suggest the formation of new species and a plot of the absorbance at 315 nm against the added equivalents of Hg^{II} ions has indicated the formation of the **P**-Hg^{II}-**P** base pairs in aqueous medium.[114] Moreover, in 2017, Jash *et al.* successfully studied the metal-base pair formed by natural cytosine and the same modified nucleotide P, whose interaction is mediated by Ag^{I} using temperature dependent UV spectroscopy and CD spectroscopy. In particular, UV spectroscopy allowed to study the stability of the unnatural base pairs while CD was applied to investigate the differences in the structures of the duplexes under different pH conditions.[115]

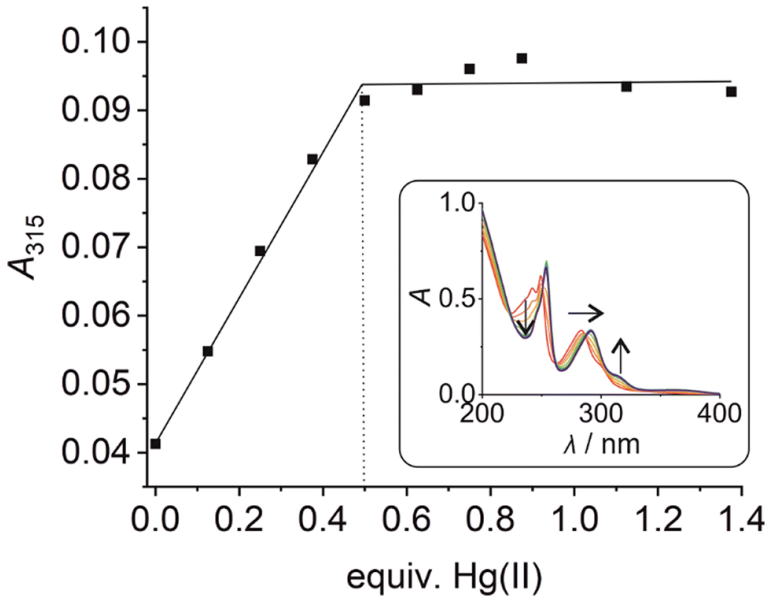


Figure 26. UV absorbance of the artificial nucleobase P at 315 nm against the equivalents of Hg^{II} added to the solution. Inset: UV-Vis spectrum of P in presence of different amounts of Hg^{II} where the changes are indicated by the arrows. Figure reproduced with permission from Springer from reference 114.

In 2012, Petrovec *et al.* applied UV and CD spectroscopy to investigate the formation and coordination of two consecutive **Im**-Ag^{I}-**Im** base pairs. The UV thermal denaturation showed that the incorporation of the imidazole homo-base pair in the absence of metal ions caused a general destabilization of the duplex. However, in the presence of Ag^{I} and of one **Im**-**Im** mispair, the T_m increases by 6°C suggesting the formation of one **Im**-Ag^{I}-**Im** base pair. Moreover, in the presence of two neighbouring **Im**-**Im** mispairs, the addition of one equivalent of Ag^{I} provoked a biphasic melting curve, suggesting that contiguous **Im**-Ag^{I}-**Im** are formed in a cooperative manner while the addition of two equivalents of Ag^{I} leaded to an increase in the T_m of 11 °C.[77]

In 2015, Yang *et al*. synthesized Pyr^{DC} nucleosides characterized by pyridyl and phenyl residues linked to position 6 of the pyrrolo[2,3-d]pyrimidine base. The described nucleobases were incorporated in oligonucleotide duplexes and used as Ag^{I} binding sites. The application of T_m studies on the duplexes showed remarkably enhanced duplex stability and selective and specific silver-ion binding.[116]

As previously mentioned, in 2019, Fujii *et al.* developed artificial nucleic acids containing 9-TAP nucleobases. Also in this case, T_m measurements were recorded in order to evaluate the ability of the nucleobase to form metal base pairs. The self-base formed by two 1,2,9-triaza-2-oxophenoxazine (N-N) was shown to be very stable with three equivalents of Ag^{I}. The presence of two inflection points in the first-derivatives of the melting curves upon addition of one or two equivalents of Ag^{I} together with the disappearance of inflection points with more than three equivalents, demonstrated that the N-N base pair is mediated by three Ag^{I}.[110]

ESR/EPR spectroscopy

Electron spin resonance (ESR) or electron paramagnetic resonance (EPR) spectroscopy is a powerful tool that has been used to confirm the structure of metal base pairs. Even if it is less popular than the other

introduced analytical tools, ESR has been widely applied in the study of the binding mode or coordination structure of metal base pairs.

In 2013 Ehrenschwender *et al.* introduced the novel metal mediated homo base pair **Hq**-Cu^{II}-**Hq** based on the hydroxyquinoline ligand inserted on a C-nucleoside. EPR was applied to study the interaction of the metal ion Cu^{II} with the nucleobase **Hq**. **Figure 27a** displays the continuous-wave X-band EPR in the case of a duplex containing one metal base pair **Hq**-Cu^{II}-**Hq**. The Figure shows the typical features of a square-planar Cu^{II} system. Moreover, **Figure 27b** shows the EPR spectrum of a duplex characterized by two consecutive metal base pairs. The spectrum shows the broad signal of the dinuclear duplex and an additional signal indicating also the presence of mononuclear Cu^{II} complex as impurity.[18]

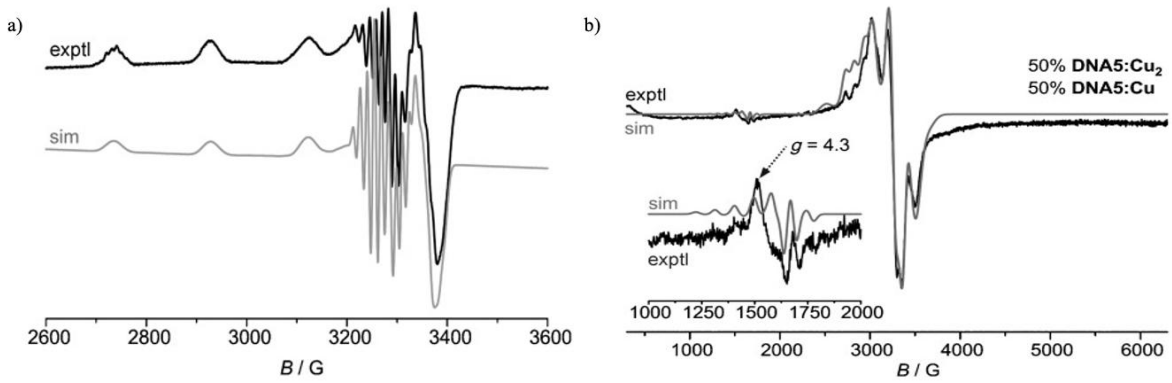


Figure 27. (a) X-band CW EPR spectrum of a duplex containing one **Hq**-Cu^{II}-**Hq** metal-base pair overlaid with the simulation (gray). (b) X-band CW EPR spectrum of a duplex containing two consecutive **Hq**-Cu^{II}-**Hq** metal base pairs overlaid with the simulation (gray). Figure taken with permission from Wiley Online Library from reference [18].

EPR spectroscopy was also used to study the widely discussed metal homo base pair **H**-Cu^{II}-**H**. The technique was applied to compare the isolated metal base pair **H**-Cu^{II}-**H** with the characteristics of the complex formed by the same metal base pair inserted in a natural duplex. EPR spectroscopy showed that both spectra are compatible with a Cu^{II} as center of a square-planar ligand field. The spectra were differentiated by a line sharpening in the duplex due the sheltering of Cu^{II} from hydrated water by an hydrophobic environment. Therefore, EPR spectroscopy confirmed the presence of a ferromagnetic Cu^{II} center within the right-handed double-strand structure of the oligonucleotide.[69]

In 2010 Clever *et al.* performed a EPR study to compare the magnetic properties of the metal base pairs **H**-Cu^{II}-**H** and **Salen**-Cu^{II}-**Salen.** Both systems are characterized by the metal ion Cu^{II} which is coordinated to the ligands in a square planar geometry. However, the EPR spectrum of a duplex containing two neighbouring **H**-Cu^{II}-**H**[16] was proven to be significantly different from the one of a duplex with two consecutive **Salen**-Cu^{II}-**Salen**[117] suggesting relevant differences in the magnetic interactions of the metal centres. The study revealed that stacking two **Salen**-Cu^{II}-**Salen** base pairs leads to antiferromagnetic coupling while ferromagnetic coupling was detected in the case of two consecutive **H**-Cu^{II}-**H** base pairs.[117]

Another relevant example of the application of EPR spectroscopy to metal base pair is represented by the work of Meggers *et al*. who have synthesized the base pair **Dipic**-Cu^{II}-**Py** composed by a pyridine-2,6-dicarboxylate nucleobase used as a planar tridentate ligand and a pyridine nucleobase as single donor ligand. EPR spectroscopy was applied to qualitatively compare the signal generated by a single Cu^{II} ion used as a control and the signal of a duplex containing the **Dipic**-**Py** base pair in presence of one equivalent of Cu^{II}. The signals confirmed a substantial difference between the two cases. EPR spectroscopy showed in the duplex case a copper complex characterized by a square-planar or square-pyramidal geometry where the metal ion is coordinated as expected to two nitrogen and two oxygen ligands thus confirming the presence of the metal base pair **Dipic**-Cu^{II}-**Py**.[39]

Conclusions

The development of metal-mediated base pairs has seen a tremendous expansion over the last decades. The majority of these studies is based on the chemical incorporation of natural or modified nucleotides by solid-phase synthesis at a specific site of DNA and to a certain extend RNA oligonucleotides. However, chemical synthesis is restricted to small oligonucleotides (i.e., < 100 nucleotides) and the harsh conditions used in automated solid-phase synthesis are not always suitable with all modified nucleotides. The formation of metal base pairs by polymerases can alleviate some of these shortcomings and constitutes an important prerequisite for their use in various applications such as selection of functional nucleic acids or formation large metal wires. This synthetic strategy is indeed not restricted in terms of oligonucleotide size and uses mild conditions compatible with the use of most modified nucleotides. The enzymatic construction of natural artificial base pairs by the addition of Hg^{II} or Ag^{I} cations has been used for the preparation of metal nanowires or as tools for the detection of mercury or silver species. These advances in the field have led to the development of more diversified systems with unnatural ligands able to coordinate other metal species, which were successfully used as metal nanowires, DNA-based logic gates, ion sensors and charge transfer devices. The characterization of such base pairs can be performed by X-ray crystallization, NMR spectroscopy, mass spectrometry, UV and CD spectroscopy. However, comparison of thermal denaturation studies and enzymatic experiments indicates that duplex stabilization and enzymatic construction of metal base pairs might not be driven by the same rules. The trends observed in enzymatic reactions differ from those obtained with oligonucleotides synthesized through solid-phase synthesis. More thorough studies will be required in order to better understand the parameters that govern the formation of metal base pairs under both conditions. Enzymatic synthesis of natural or unnatural metal base pairs, once successful, will play an important contribution to the construction of nanodevices, biomaterials, and functional nucleic acids with enhanced properties.

Conflicts of interest

There are no conflicts to declare.

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Data availability

No data has been acquired in this study.

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