Towards polymerase-mediated synthesis of artificial RNA-DNA metal base pairs

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Abstract

Metal-mediated base pairs are formed by the connection of two nucleobases via coordination to a metal cation. The resulting metal-containing duplexes have been used in a large variety of applications ranging from allosteric control of functional nucleic acids to the construction of nanowires. Recently, enzymatic approaches are being developed for the construction of metal-mediated base pairs. Here, we have studied the possibility of constructing Hg^{II}- and Ag^{I}-mediated DNA/RNA hetero base pairs using primer extension reactions. The high thermodynamic stabilities of metal base pairs can be harnessed to trigger the formation of two rU-Hg^{II}-dT base pairs. Bypass experiments demonstrate the possibility of constructing RNA containing DNA sequences which are used in multiple applications including DNAzyme selections. These findings will be useful for the enzymatic construction of xenonucleic acid (XNA) based metal pairs.

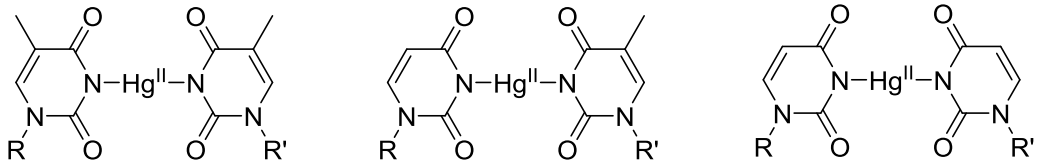
Introduction

Metal base pairs are structural analogs of canonical Watson-Crick pairs where the hydrogen pattern is substituted with the coordinative interaction of metal cations with the nucleobases.[1, 2] Metal base pairs can be constructed using the canonical nucleobases or synthetic nucleoside analogs with nucleobases that are specifically designed to serve as ligands for the specific binding of transition metal cations.[3, 4] These metal base pairs have advanced as promising candidates for a number of applications including the development of nanomolecular devices,[5] ion sensors and biosensing devices[6, 7] and metal nanowires and nanodevices[8-10] as well as for the allosteric control of functional nucleic acids.[11-14]

The formation of metal base pairs mainly occurs by annealing short synthetic oligonucleotides together with specific metal cations. While this approach has allowed the identification of metal base pairs based on natural and modified nucleotides, it is restricted in oligonucleotide size and in terms of diversity of functional groups that can be explored due to the rather harsh conditions imposed by solid-phase synthesis.[15] Alternatively, metal base pairs can be formed via enzymatic synthesis where polymerases incorporate modified or natural nucleotides into DNA in the strict presence of metal cations.[16-20]

So far, most synthetic efforts have been dedicated to the identification of modified nucleobases that can act as potent ligands for metal coordination. Surprisingly, very little attention has been devoted to combining sugar and/or backbone modifications and metal base pairs,[21-24] especially in the context of enzymatic synthesis. This might be ascribed to the fact that DNA polymerases are finely tuned biological machineries that have the capacity of strongly distinguishing dNTPs from sugar modified nucleotides including NTPs.[25, 26] The strong discrimination of NTPs (by a factor of up to 10^5) stems from a steric exclusion caused by a clash between the 2'-hydroxyl moiety of the incoming rNTP and an active site residue, usually equipped with a bulky side chain such as tryptophan or phenylalanine.[26-29] Hence, most naturally occurring DNA polymerases predominantly incorporate deoxynucleoside monophosphates (dNMPs) and depending on the polymerase, insert only one ribonucleoside monophosphate every hundred thousand correct nucleotides.[30] This rather strong discrimination can be alleviated by using naturally occurring polymerases that lack such a steric gate,[31, 32] engineered polymerases equipped with an alternate, more permissive steric gate[33, 34] or by adding Mn^{2+} cofactors which also promote the incorporation of NTPs by DNA polymerases.[26] Here, we demonstrate that the thermodynamic stabilities of metal base pairs[35, 36] can be harnessed to facilitate enzymatic RNA synthesis by DNA polymerases.

A)



B)

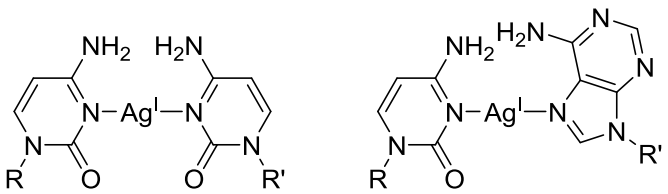


Figure 1. Chemical structures of A) Hg^{II}-mediated and B) Ag^{I}-mediated metal base pairs with canonical nucleotides.

The dT-Hg^{II}-dT undoubtedly is the most prominent and best studied metal base pair[35] and both the RNA equivalent, rU-Hg^{II}-rU,[37] and the chimeric RNA-DNA variant, rU-Hg^{II}-dT,[38, 39] have been identified (Figure 1A). The addition of mercury cations to these mismatches leads to large thermal stabilizations of duplexes (ΔT_m ranging from +6 to +10°C) driven by favorable enthalpy and entropy of formation.[40] These favorable assets have allowed the enzymatic construction of single[41] and multiple[42] dT-Hg^{II}-dT base pairs under primer extension (PEX) reaction conditions. Similarly, silver cations have been shown to stabilize duplexes containing dC-dC and dC-dA (Figure 1B) mismatches (ΔT_m = +8.3°C and +4.0°C, respectively) [43, 44] which also could be produced enzymatically.[45-47] However, the possibility of using these stable metal base pairs to form RNA-DNA heteroduplexes by enzymatic synthesis has never been investigated.

Results and discussion

In a first step towards this aim, we wanted to evaluate the possibility of forming RNA-DNA mixed metal base pairs on short synthetic oligonucleotides using UV melting experiments. To do so, we designed 4 different duplexes containing central rU-dT, rA-dA, rC-dA, and rC-dC mismatches using 13 nucleotide long sequences (Table 1).[48, 49] The thermal stability of these duplexes in the presence of Ag^{I}, Hg^{II}, and a combination of Hg^{II} and Mn^{II} was investigated by temperature-dependent UV spectroscopy (Table 1 and Supporting Information). We have also determined the T_m values of a fully matched dsDNA duplex (duplex 1) and a duplex containing a central dT-dT mismatch to compare with the stability of the duplexes containing RNA-DNA mismatches. Expectedly, this analysis revealed that Ag^{I} and Hg^{II} had little incidence on the thermal stability of a fully matched dsDNA duplex (duplex 1) and that Hg^{II} could specifically stabilize a dT-dT mismatch by formation of a metal base pair (ΔT_m = +9.8°C; dsDNA duplex 2). The insertion of an rU-dT mismatch (RNA-DNA hetereo-duplex 3) led to a further decrease in duplex stability compared to the system containing a dT-dT mismatch (ΔT_m = -3.0°C) and the presence of Hg^{II} could substantially compensate for this loss of thermal stability (ΔT_m = +8.3°C). Hence, the dT-Hg^{II}-dT and rU-Hg^{II}-dT base pairs stabilize mismatched duplexes with comparable efficiencies (ΔT_m = +9.8 and +8.3°C, respectively) in this sequence context. The introduction of rA-dA, rC-dA, and rC-dC mismatches leads to a similar destabilization as observed between duplexes 2 and 3 (ΔT_m ranging from -3.0 to -4.0°C). The addition of Ag^{I} leads to marked increases in T_m values of the systems containing rC-dA (RNA-DNA hetereo-duplex 5) and rC-dC (RNA-DNA hetereo-duplex 6) mismatches (ΔT_m = +3.8 and +4.3°C, respectively) but appears to be thermoneutral in the case of RNA-DNA hetereo-duplex 4 that contains an rA-dA mismatch (ΔT_m = 1.0°C). Hence, the gain in duplex stabilization generated by the formation of an rC-Ag^{I}-dC pair appears to be reduced compared to that of the corresponding dC-Ag^{I}-dC (Figure 1B) base pair (ΔT_m of +4.3°C and +8.3°C,[43] respectively) and compares to the stabilization imparted by an all-RNA rC-Ag^{I}-rC pair (ΔT_m = +4.0°C).[39] On the other hand, the rC-Ag^{I}-dA and dC-Ag^{I}-dA pairs (Figure 1B) display similar increases in thermal stabilities (ΔT_m of +3.8°C and +3.5°C,[50] respectively). Based on this UV-melting analysis, it appears unlikely that a silver mediated rA-Ag^{I}-dA base pair can be formed under these conditions due to the rather modest increase in T_m value observed upon addition of Ag^{I} to RNA-DNA hetereo-duplex 4.

Table 1. Oligonucleotide sequences containing mismatches and effect of Hg^{II} and Ag^{I} on duplex stability (T_m).[a]

Duplex	Sequences	Metal cation	T_m (°C)	ΔT_m (°C)[b]
Duplex 1	5'-d(GAGGGTATGAAAG) 3'-d(CTCCCATACTTTC)	-	50.6(4)	-
Duplex 1	5'-d(GAGGGTATGAAAG) 3'-d(CTCCCATACTTTC)	Ag^{I}	51.4(1)	+0.8(2)
Duplex 1	5'-d(GAGGGTATGAAAG) 3'-d(CTCCCATACTTTC)	Hg^{II}	50.3(5)	-0.3(3)
Duplex 2	5'-d(GAGGGT**T**TGAAAG) 3'-d(CTCCCA**T**ACTTTC)	-	41.8(2)	-
Duplex 2	5'-d(GAGGGT**T**TGAAAG) 3'-d(CTCCCA**T**ACTTTC)	Hg^{II}	51.6(1)	+9.8(2)

Duplex 2	5'-d(GAGGGT**T**TGAAAG) 3'-d(CTCCCA**T**ACTTTC)	Mn^{II}	41.4(2)	-0.4(2)
Duplex 2	5'-d(GAGGGT**T**TGAAAG) 3'-d(CTCCCA**T**ACTTTC)	Hg^{II}, Mn^{II}	50.8(1)	+9.0(2)
Duplex 3	5'-d(GAGGGT)**rU**d(TGAAAG) 3'-d(CTCCCA**T**ACTTTC)	-	38.8(2)	-
Duplex 3	5'-d(GAGGGT)**rU**d(TGAAAG) 3'-d(CTCCCA**T**ACTTTC)	Hg^{II}	47.1(2)	+8.3(2)
Duplex 3	5'-d(GAGGGT)**rU**d(TGAAAG) 3'-d(CTCCCA**T**ACTTTC)	Mn^{II}	39.0(1)	+0.2(2)
Duplex 3	5'-d(GAGGGT)**rU**d(TGAAAG) 3'-d(CTCCCA**T**ACTTTC)	Hg^{II}, Mn^{II}	46.4(1)	+7.6(2)
Duplex 4	5'-d(GAGGG)**rA**d(ATGAAAG) 3'-d(CTCCC**A**TACTTTC)	-	39.2(1)	-
Duplex 4	5'-d(GAGGG)**rA**d(ATGAAAG) 3'-d(CTCCC**A**TACTTTC)	Ag^{I}	40.2(4)	+1.0(2)
Duplex 5	5'-d(GAGGG)**rC**d(ATGAAAG) 3'-d(CTCCC**A**TACTTTA)	-	39.8(1)	-
Duplex 5	5'-d(GAGGG)**rC**d(ATGAAAG) 3'-d(CTCCC**A**TACTTTA)	Ag^{I}	43.6(1)	+3.8(1)
Duplex 6	5'-d(GAGGG)**rC**d(ATGAAAG) 3'-d(CTCCC**C**TACTTTA)	-	38.1(2)	-
Duplex 6	5'-d(GAGGG)**rC**d(ATGAAAG) 3'-d(CTCCC**C**TACTTTA)	Ag^{I}	42.4(4)	+4.3(3)

[a] Standard deviations are given in parenthesis. [b] ΔT_m is calculated for each duplex system as the difference in T_m between a measurement performed in the presence and one in the absence of metal cations.

Having established the possibility of constructing RNA-DNA chimeric metal base pairs with synthetic oligonucleotides, we next sought to evaluate whether this could be translated to enzymatic synthesis. To do so, we carried out primer extension (PEX) reactions with templates containing an overhang of seven consecutive dT (T1), dC (T2), dA (T3), or dG (T4) nucleotides immediately following the 3′-terminus of the FAM-labelled primer P1 (Table 2).[51] Initial PEX reactions were carried out with five different commercially available DNA polymerases (Taq, *Bst*, Vent (*exo*$^-$), *Sulfolobus* DNA Polymerase IV (Dpo4), and the Klenow fragment of DNA polymerase I *exo*$^-$ (Kf *exo*$^-$)) and in the presence or absence of either Hg^{II} or Ag^{I}. All the PEX reaction products were analyzed by 20% denaturing gel electrophoresis and visualised using fluorescence imaging.

Table 2. Primer and templates used for primer extension reactions.

Name	Sequence
P1	5‘-FAM-d(CAT GGG CGG CAT GGG)
T1	5’-d(**TTT TTT T**CC CAT GCC GCC CAT G)
T2	5’-d(**CCC CCC C**CC CAT GCC GCC CAT G)
T3	5’-d(**AAA AAA A**CC CAT GCC GCC CAT G)
T4	5’-d(**GGG GGG G**CC CAT GCC GCC CAT G)

Expectedly, all DNA polymerases investigated were capable of incorporating one (for *Bst*) or multiple deoxythymidine monophosphate (dTMP) units opposite templating dT nucleotides in the presence of Hg^{II} when the primer/template P1/T1 system and dTTP were used (Figure S1). Moreover, when PEX reactions were conducted in the presence of both rUTP and Hg^{II}, partial incorporation of a single uridine monophosphate (UMP) moiety could be observed when Kf *exo*$^-$ was used as a polymerase, albeit in modest yields (~20%). The control reaction performed in absence of the metal cation did not yield any extended primer product. When the reaction mixtures were supplemented with the Mn^{II} cofactor which is known to relax polymerase fidelity[52] and favor the formation of mercury metal mediated base pairs,[42] the yield of n+1 product formation significantly increased to ~50% (Figures 2A and S2). In order to confirm that Mn^{II} was not implicated in metal base pair formation but served to improve the substrate tolerance of the polymerase, we recorded UV-melting experiments with duplexes containing homo- and hetero-mismatches (dT-dT and rU-dT) in the presence of Mn^{II}. The addition of this metal cation had little effect on the T_m values of duplexes 2 and 3, confirming that Mn^{II} does not trigger metal base pair formation in duplex DNA. This is further confirmed by the small decrease in T_m observed when dsDNA duplex 2 and RNA-DNA hetereo-duplex 3 were supplemented both with Mn^{II} and Hg^{II} instead of Hg^{II} alone (-0.8 and -0.7°C, respectively).

Based on these observations, we next sought to fine tune the experimental conditions to improve both yields and number of incorporation events (Figure 2). When both the reaction time (from 3h to 12h; Figure 2C) and the UTP concentration (from 200 to 400 µM; Figure 2B) were increased, full conversion of the primer to the n+1 (~10%) and n+2 (~90%) products could be achieved when Hg^{II} and Mn^{II} were present (Figure 2D). In addition, no incorporation of a uridine moiety into DNA could be observed in the absence of Hg^{II}, underscoring the need for metal base-pair formation for the incorporation of this mismatched RNA nucleotide into DNA. We have also carried out PEX reactions with templates T1-T4 and the corresponding, complementary, NTPs (Figure S3) under similar experimental conditions. This analysis reveals that in PEX reactions with template T3, which contains seven dA units, two UMPs are appended on the 3’-end of primer P1 (Figure S3) with a comparable efficiency to that of the misincoporation of UMPs opposite templating dT units triggered by mercury cations shown in Figures 1C and 1D. Surprisingly, Kf *exo*$^-$ was capable of appending up to 7 rNMP units onto primer P1 when templates T1, T2, and T4 were used in PEX reactions in conjunction with the corresponding nucleoside triphosphate (Figure S3), confirming earlier findings that Pol I can incorporate matched ribonucleotides *in vitro*.[28]

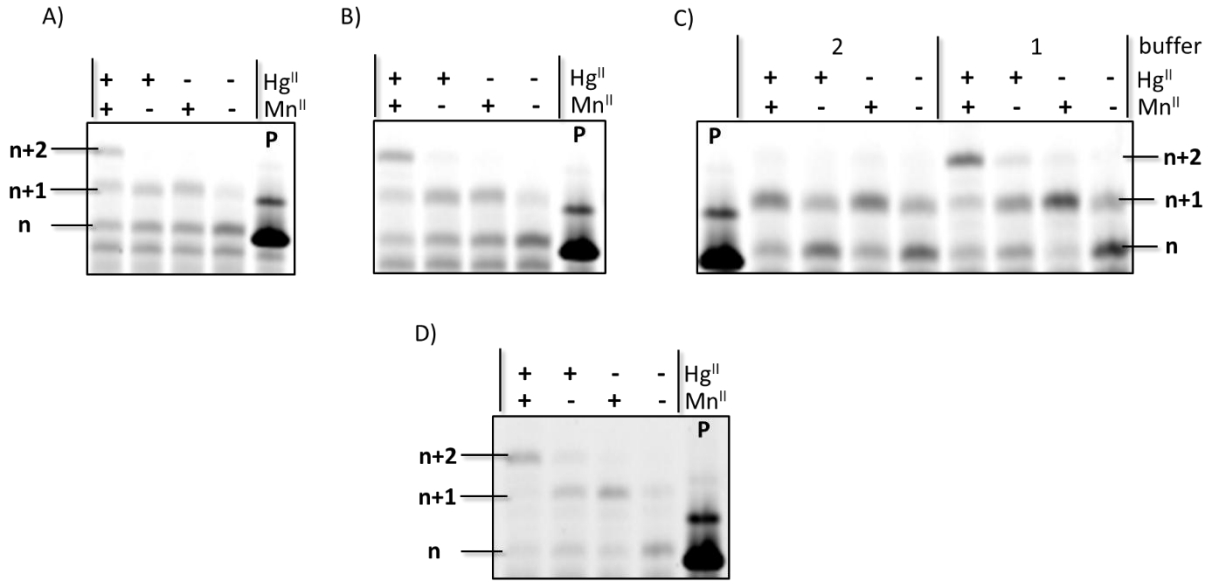


Figure 2. Gel images (PAGE 20%) of PEX reactions carried out with primer P1 and template T1 using different UTP concentrations, reaction times, and metal cations. All reactions were performed with 5U of Kf *exo*$^-$ at 37°C. A) reactions with 200 µM UTP and 3h reaction time in buffer 1; B) reactions with 400 µM UTP and 3h reaction time in buffer 1; C) reactions with 200 µM UTP and 12h reaction time; D) reactions in buffer 1 with 400 µM UTP and 12h reaction time. 5U of Kf *exo*$^-$ were used in all reactions. Buffer 1 did not contain any source of Cl^- and buffer 2 is the supplied buffer. In all cases, $Hg(ClO_4)_2$ was used as source of mercury. **P** indicates unreacted primer.

Having established conditions that enable the enzymatic formation of an rU-Hg^{II}-dT base pair, we next questioned whether this metal base pair could be bypassed once installed so that DNA synthesis could resume.[53] To do so, we carried out PEX reactions with the P1/T1 system and UTP to install rU-Hg^{II}-dT base pairs (Figure 3A). The resulting products were then incubated with dTTP and Hg^{II} (Figure S5) or dATP (Figure 2B-F) following an experimental protocol established for silver-mediated artificial base pairs (see Supporting Information).[53] Full length products (n+7) could be observed upon the addition of dATP and Kf *exo*$^-$ albeit in low yields (Figure 3B). Interestingly, when Kf *exo*$^-$ was substituted by other polymerases including *Bst*, Vent (*exo*$^-$), and Dpo4, the expected full lengths products could be formed in high yields (Figure 3C, 3E, and 3F, respectively), while reactions conducted with Therminator produced slower running products that stem from untemplated addition of dAMP residues (Figure 3D). These results suggest that the rU-Hg^{II}-dT base pairs did not induce termination of DNA synthesis and can be bypassed. On the other hand, excess Hg^{II} used for the installation of the rU-Hg^{II}-dT base pairs appears to inhibit the extension reactions with dATP and need to be removed with EDTA prior to the bypass reactions (Figure S5C and Supporting Information). Lastly, formation of dT-Hg^{II}-dT base pair after the installation of the rU-Hg^{II}-dT hetero-metal base pairs did not proceed very well and was observed in low yields only (Figure S5B).

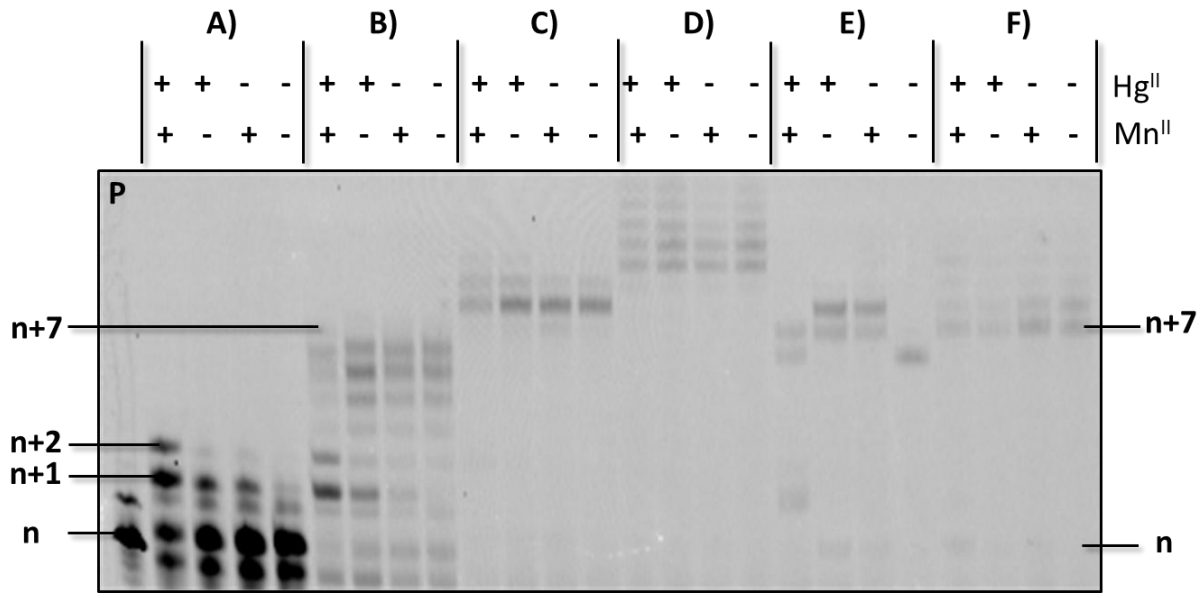


Figure 3. Gel analysis (PAGE 20%) of products from the bypass experiments carried out with the primer P1/ template T1 system. A) reactions with UTP (400 μM) and Kf *exo*$^{-}$ (5U) at 37°C; B) reaction with UTP (400 μM) and Kf *exo*$^{-}$ (5U) at 37°C for 3h followed by removal of excess Hg^{II}, addition of dATP (200 μM), and reaction for 1h; C) as in B) but with *Bst* (8 U) instead of Kf *exo*$^{-}$; D) as in B) but with Therminator (2 U) instead of Kf *exo*$^{-}$; E) as in B) but with Vent (*exo*$^{-}$) (2 U) instead of Kf *exo*$^{-}$; as in B) but with Dpo4 (2 U) instead of Kf *exo*$^{-}$. **P** indicates unreacted primer.

Having established the possibility of forming rU-Hg^{II}-dT base pairs, we next considered the possibility of using Ag^{I} to trigger the formation of DNA/RNA hetero base pairs. UV melting experiments revealed that the rC-dA and rC-dC mismatches could be stabilized by the addition of Ag^{I}, albeit to a lesser extent than rU-dT mismatches with Hg^{II}. Nonetheless, we attempted to use CTP as a substrate for polymerases to enzymatically construct rC-Ag^{I}-dA and rC-Ag^{I}-dC base pairs since similar syntheses have been reported for the corresponding DNA base pairs.[45-47] We thus carried out PEX reactions with the primer/template systems P1/T2 (Figure S4) and P1/T3 (Figure S6) in the presence of different DNA polymerases and Ag^{I}. Even by changing multiple reaction parameters (CTP and polymerase concentration, reaction times) we could not observe any difference with the control reactions carried out in absence of metal cofactor and the incorporation of the RNA nucleotide remained modest. These results suggest that the strength of the rC-Ag^{I}-dA and rC-Ag^{I}-dC base pairs might not be sufficient to coerce the introduction of mismatched RNA nucleotides into DNA duplexes under enzymatic conditions. Finally, when PEX reactions were conducted with ATP with primer P1 and template T3, no extended primer products could be observed (data not shown) which confirms the absence of enzymatic rA-Ag^{I}-dA metal base pair formation observed in UV melting experiments (RNA-DNA hetereo-duplex 4 in Table 1).

Conclusions

In conclusion, we have investigated the possibility of using the favorable thermodynamic parameters of metal base pair formation to insert RNA nucleotides into an all-DNA setting using DNA polymerases. In a first step towards this aim, we have used UV melting experiments on short synthetic duplexes to confirm the possibility of forming rU-Hg^{II}-dT base pairs. These mercury-mediated base pairs stabilized mismatched duplexes albeit to a slightly

lesser extent than the parent dT-Hg^{II}-dT base pair. A similar trend was observed with a duplex containing rC-Ag^{I}-dA base pair but not with rC-Ag^{I}-dC which is less stable than in the all-DNA case. These trends are reflected in PEX reactions using natural RNA nucleotides since efficient rU-Hg^{II}-dT base pair formation could be observed. On the other hand, the stability of silver-mediated pairs appeared to be insufficient to coerce DNA polymerases to misincorporate RNA nucleotides even in the presence of metal cations. Taken together, the favorable thermodynamic parameters of mercury-mediated base pairs can be hijacked to generate RNA-DNA oligonucleotides using polymerase-assisted synthesis. Such an approach alleviates the synthetic efforts required for the generation of such chimeric oligonucleotides which are used in numerous applications such as substrates for DNAzyme selections.[54, 55] Finally, we are currently investigating the compatibility of other sugar chemistries with the enzymatic synthesis of artificial metal base pairs in order to eventually achieving our long standing aim of creating orthogonal xenonucleic acids based on metal base pairs.[56] The use of engineered polymerases[20] with a broader substrate tolerance will certainly advance as important tools in this context since they will permit the efficient construction of additional RNA-DNA hetero-metal base pairs and expand this concept to XNAs.

Conflicts of interest

There are no conflicts to declare

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