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On the enzymatic formation of metal base pairs with thiolated and pK_a-perturbed nucleotides

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Abstract: The formation of artificial metal base pairs is an alluring and versatile method for the functionalization of nucleic acids. Access to DNA functionalized with metal base pairs is granted mainly by solid-phase synthesis. An alternative, yet underexplored method, envisions the installation of metal base pairs via the polymerization of modified nucleoside triphosphates. Herein, we have explored the possibility of using thiolated and pK_a-perturbed nucleotides for the enzymatic construction of artificial metal base pairs. The thiolated nucleotides **S2C**, **S6G**, and **S4T** as well as the fluorinated analog **5FU** are readily incorporated opposite a templating **S4T** nucleotide through the guidance of metal cations. Multiple incorporation of the modified nucleotides along with polymerase bypass of the unnatural base pairs are also possible under certain conditions. The thiolated nucleotides **S4T**, **S4T**, **S2C**, and **S6G** were also shown to be compatible with the synthesis of modified, high molecular weight ssDNA products through TdT-mediated tailing reactions. Thus, sulfur-substitution and pK_a perturbation represent alternative strategies for the design of modified nucleotides compatible with the enzymatic construction of metal base pairs.

Introduction

The role of DNA is often considered to be restricted to the carrier of genetic information in all forms of life. This biopolymer can be viewed as a supramolecular right-handed duplex maintained together by the canonical Watson-Crick base pairs AT and CG. However, chemical modulation of any of the constituting elements has propelled DNA into applications that markedly deviate from this rather simplistic view.[1] For instance, blending chemically modified nucleoside triphosphates (dN*TPs) [2] with Darwinian *in vitro* evolution methods have enabled the isolation of M^{2+}-independent ribonucleases[3] and aptamers with improved binding affinity and nuclease resistance.[4] Moving a step further, morphing of DNA into artificial genetic polymers can be achieved by integrating synthetic elements into the nucleosidic scaffold that substantially deviate from the natural building blocks.[4e, 5] Particularly, the replacement of the nucleobase by aromatic entities has led to the creation of DNA alphabets that have been augmented by one or even two additional unnatural base pairs (UBPs).[5e, 6] These nucleobase surrogates can interact through hydrophobic and packing forces,[7] size complementarity,[8] or via rearranged hydrogen bond patterns.[9] Such an expansion of the genetic alphabet has crystallized in the creation of semi-synthetic organisms capable of storing and self-replicating modified DNA[10] and even of its transcription into modified proteins.[11] Alternatively, aptamers containing five or six-nucleotides could be evolved and shown to display improved properties compared to systems restricted to canonical nucleic acids.[12]

Canonical nucleobases can also be replaced by artificial metal base pairs.[13] The presence of a metal ion in the artificial base pair confers unique properties to DNA such as electron conductivity and transportation in nanowires,[14] robust and enhanced stability compared to natural nucleic acids,[15] and a chiral environment suitable for catalysis.[16] So far, access to metal-DNA is mainly granted by automated solid-phase synthesis. The enzymatic construction of metal UBPs represents an alluring alternative to solid phase synthesis which would allow the crafting of long DNA metal nanowires and the construction of functional nucleic acids with an expanded genetic alphabet. So far, only few reports have tried to address the prerequisites for the polymerization of dN*TPs for the construction of metal UBPs.[17] Here, we have investigated the possibility of using thiolated and pK_a-perturbed purine and pyrimidine nucleotide analogs to build metal UBPs enzymatically (Figure 1). Previously, nucleotides containing carbonyl to thiocarbonyl substitutions have been employed to investigate the effects controlling the incorporation of triphosphates into DNA by polymerases.[18] These analogs are also known to form very stable Ag^+-mediated DNA metal base pairs[19] and thus were hypothesized to be ideal candidates for the enzymatic formation of metal UBPs. We clearly demonstrate that **S2C**, **S6G**, and **S4T** are incorporated opposite templating **S4T** nucleotides only in the presence of specific metal cations such as Hg^{2+} and Ag^+, while **S2T** seems to be rather reluctant in participating in the enzymatic construction of metal UBPs, both as a templating and an incoming nucleotide. The pK_a-perturbed analog **5FU**[20] is also capable of sustaining the formation of

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5FU-Ag^+-**S4T** and **5FU**-Hg^{2+}-**S4T** metal base pairs during enzymatic synthesis. Lastly, we have explored the possibility of using these modified nucleotides in TdT-mediated tailing reactions which might grant access to long, dsDNA systems maintained together by metal base pairs.

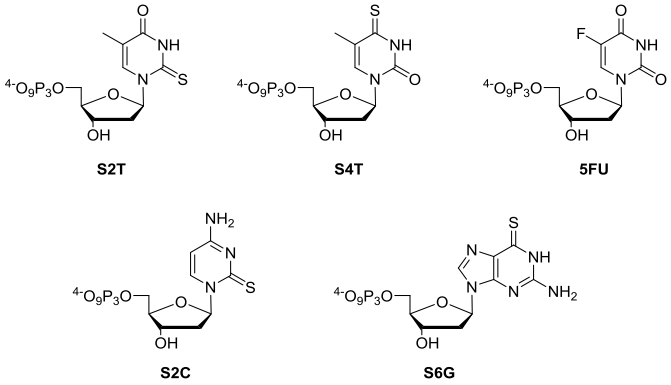

Figure 1. Chemical structures of the modified nucleotides that were used in this study. The following code was used: 2-Thiothymidine-5'-Triphosphate (**S2T**), 4-Thiothymidine-5'-Triphosphate (**S4T**), 5-fluoro-2'-deoxyuridine (**5FU**), 2-Thio-2'-deoxycytidine (**S2C**), and 6-Thio-2'-deoxyguanosine (**S6G**). TP will be used as an abbreviation for triphosphate.

Results

Single incorporation of thiolated pyrimidine nucleotides

DNA duplexes containing **S4T** nucleotides are strongly stabilized by the presence of Ag^+ (ΔT_m = 23°C)[19a] and the formation of **S4T**-2Ag^+-**S4T** metal base pairs was recently confirmed by X-ray structural analysis.[19b] In order to evaluate whether **S4T** could be used for the enzymatic construction of metal base pairs we carried out primer extension (PEX) reactions. The template used for these PEX reactions contained a single **S4T** modification (**T1** in Table 1) immediately downstream of the FAM-labelled primer **P1**. Various polymerases (Therminator, Vent (*exo*$^-$), *Bst*, *Sulfolobus* DNA Polymerase IV (Dpo4), the Klenow fragment of DNA polymerase I *exo*$^-$ (Kf *exo*$^-$), and Taq) were asked to incorporate an **S4T**–nucleotide opposite the **S4T** modification in the presence of one of 12 different metal cations. This initial screen revealed that some polymerases (i.e. Therminator, Vent (*exo*$^-$), and Taq) led to incorporation of the modified nucleotide regardless on the presence and the nature of the metal cation (data not shown). On the other hand, the efficiency of the *Bst*-mediated incorporation of an **S4T**–nucleotide was much improved in the presence of specific metal cations such as Ag^+, Cd^{2+}, Hg^{2+}, and Cu^{2+} compared to the control reaction performed in the absence of additional cofactors (Figure 1A). Interestingly, when PEX reactions were performed in the presence of 30 µM and 60 µM Ag^+, the primer was fully converted to the n+2 product which contains the known **S4T**-2Ag^+-**S4T**[19b] metal base pair and an additional **S4T**-Ag^+-dC pair (Figure 1B). The formation of a **S4T**-Ag^+-dC pair was also confirmed by PEX reactions with templates **T2**-**T5** (*vide infra*). The formation of this base pair is expected since the enzymatic construction of a dC-Ag^+-dT base pair has recently been reported.[21] Higher concentrations of Ag^+ (> 60 µM) abolished the formation of the metal base pairs, while an increase in the concentrations of the metal cations Cd^{2+} and Cu^{2+} had little incidence on the incorporation efficiency of the **S4T** nucleotide (Figure 1B). We then evaluated the incorporation efficiency of natural dNTPs opposite a templating **S4T** unit in the presence and absence of metal ions. Expectedly, the *Bst* polymerase efficiently incorporated dA nucleotides opposite the modification independently of the presence or the nature of the metal cation (Figure S1 in the Supporting Information). When the **S4T**–triphosphate was replaced by its natural counterpart dTTP, misincorporation opposite **S4T** was only observed in the presence of Hg^{2+} (with ~50% of conversion of the primer) presumably through the formation of a dT-Hg^{2+}-**S4T** base pair.[22] No other metal cation promoted the incorporation of a dTMP opposite the modified nucleotide, suggesting that both thiol moieties are required for the formation of the artificial metal base pair (Figure S1). When dGTP was used as the incoming triphosphate, full conversion to the n+3 product corresponding to one dG-**S4T** pair[18] followed by two dG-dC base pairs was observed in the presence of Hg^{2+} and to a lower extent with Ag^+. The formation of the n+3 product was also observed in the absence of metal cations, albeit with much reduced yields (Figure S1). Misincorporation of dG opposite an **S4T** nucleotide has been reported previously and was ascribed to the presence of a favorable enethiol tautomer. Lastly, little (< 20% conversion of the primer in the presence of Ag^+) or no incorporation was observed when dCTP was used instead of the modified **S4T**–triphosphate.

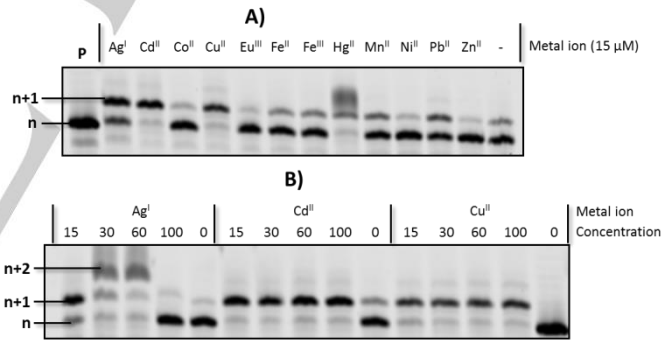

Figure 1. Gel image (PAGE 20%) of the PEX reactions with template **T1** and primer **P1** and triphosphate **S4T** and with A) 0.5U *Bst* and various metal ions. B) Analysis of the PEX reactions with varying concentrations of Ag^+, Cd^{2+}, and Cu^{2+}. All reactions were carried out with template **T1**, primer **P1**, 20 µM of the triphosphate **S4T**, and 0.5U of *Bst* at 60°C for 10 min.

When Kf *exo*$^-$ served as polymerase, the **S4T**–nucleotide was incorporated opposite a templating **S4T** analog only when Ag^+ was present albeit in rather modest yields (Figure 2). Increasing the reaction time or the concentration of Ag^+ only marginally improved the yield of formation of the n+1 product (Figure S2). On the other hand, when PEX reactions were carried out with natural dNTPs, the expected n+1 product was observed with dATP but the presence of Ag^+ negatively impacted on the efficiency of the formation of the **S4T**-dA base pair. No misincorporation events were observed with the other natural triphosphates (Figure S3).

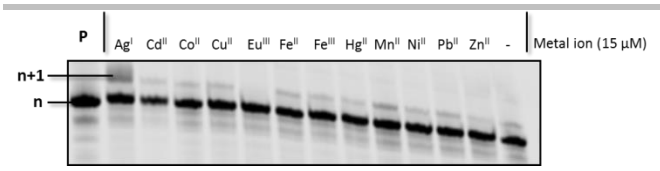

Figure 2. Gel image (PAGE 20%) of the PEX reactions with template **T1** and primer **P1** and triphosphate **S4T**. The reactions were carried out with 0.5U Kf *exo*$^{-}$ for 15 min with 20 µM final concentration of the modified triphosphate. **P** represents unreacted primer.

Table 1. Primers and templates used for primer extension reactions

Name	Sequence
P1	5'-FAM- TAC GAC TCA CTA TAG CCT C -3'
T1	5'- GAC C**X**G AGG CTA TAG TGA GTC GTA -3'[a]
T2-T5	5'- GGA G**N**G AGG CTA TAG TGA GTC GTA -3'[b]
T6	5'- GAC C**Y**G AGG CTA TAG TGA GTC GTA -3'[a]
T7	5'- GC**X XX**G AGG CTA TAG TGA GTC GTA -3'

[a] **X** correspond to **S4T** nucleotides and **Y** to **S2T** nucleotides. [b] **N** = C (**T2**); **N** = G (**T3**); **N** = A (**T4**); **N** = T (**T5**).

Having established conditions for the enzymatic formation of **S4T**-M^{n+}-**S4T** metal base pairs, we next questioned the possibility of incorporating the **2ST** isomer opposite **S4T**. An initial screen revealed that most polymerases incorporated the modified analog irrespectively of the nature of metal cation (data not shown). However, with Kf *exo*$^{-}$ enzymatic synthesis was best when Ni^{2+} was used as prosthetic factor since an appreciable yield (~50%) of the n+1 was formed while all other metal cations did not promote the incorporation of the modified nucleotide (Figure 3A). However, a complete conversion of the primer into the n+1 product could never be achieved even when the concentration of the metal cation and the reaction time were increased (Figure S4). These results can be rationalized by the fact that the 2-thio group points into the minor grove[23] and is not readily available for the formation of metal base pairs. We also carried out PEX reactions with the **S2T** nucleotide serving at the same time as the incoming and the templating nucleotide. To do so, we carried out PEX reactions with template **T6** which contains a single **S2T** nucleotide. Under these conditions, certain polymerases such as Kf *exo*$^{-}$ did not recognize the **S2T** nucleotide as a substrate (data not shown), while other polymerases including Vent (*exo*$^{-}$), Dpo4, and *Bst* extended the primer with one modified nucleotide regardless of the presence and the nature of the metal ion (Figure 3B). On the other hand, nearly full conversion of the primer to the n+2 product could be observed when the Taq polymerase was used in conjunction with Ag^{+} as cofactor (Figure 3B). This corresponds to the formation of a single **S2T**-Ag^{+}-**S2T** pair followed by a **S2T**-Ag^{+}-dC pair which had been observed in the case of the **4S** isomer.

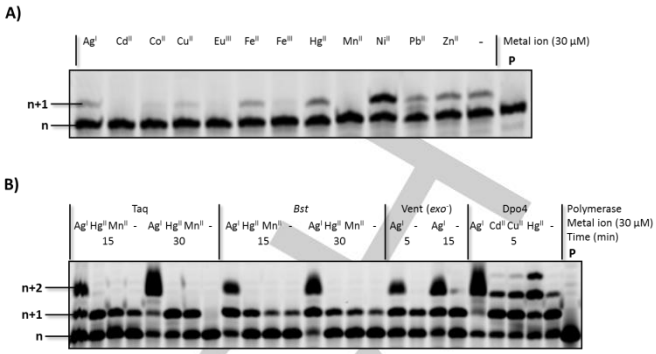

Figure 3. A) gel image (PAGE 20%) of the PEX reactions of the incorporation of the nucleotide **S2T** opposite a templating **S4T** nucleotide by the Kf *exo*$^{-}$ in presence of different metal cations. All the reactions were incubated at 37°C for 20 min in the presence of 0.5U of Kf *exo*$^{-}$ and 20 µM of the modified triphosphate. B) Gel (PAGE 20%) analysis of the incorporation of nucleotide **S2T** opposite a templating **S2T** moiety. All reactions were carried out in the presence of 0.05U of the polymerases and 20 µM of the **S2T**–triphosphate. **P** represents unreacted primer.

We next investigated the possibility of forming **S2C**-**S4T** pyrimidine mispairs via the guidance of metal cations. PEX reactions with template **T1** and primer **P1** and various polymerases revealed that Kf *exo*$^{-}$ not only incorporated the **S2C**-nucleotide opposite **S4T** but also opposite the two dC nucleotides located immediately next to the site of the modification and upstream on the template strand (Figure S5). These incorporation events only occurred when Ag^{+} was included in the reaction mixtures. However, the yields for the formation of **S2C**-Ag^{+}-**S4T** and **S2C**-Ag^{+}-dC base pairs remained modest even when the conditions (i.e. reaction time, concentration of **S2C** and Ag^{+}) of the reactions were varied (Figure S5). On the other hand, the Taq polymerase efficiently promoted the formation of Hg^{2+}-mediated metal base pairs (Figure 4A). At high polymerase concentrations however, the incorporation of the modified nucleotide became independent on the presence and nature of the metal cofactor (Figure 4B). Interestingly, Taq also promoted the formation of **S2C**-Ag^{+}-**S4T** and **S2C**-Ag^{+}-dC base pairs with an efficiency superior to that of Kf *exo*$^{-}$ since the primer was completely converted to the n+2 product. Other metal cations did not promote the formation of a **S2C**-M^{n+}-**S4T** pair (Figure 4C).

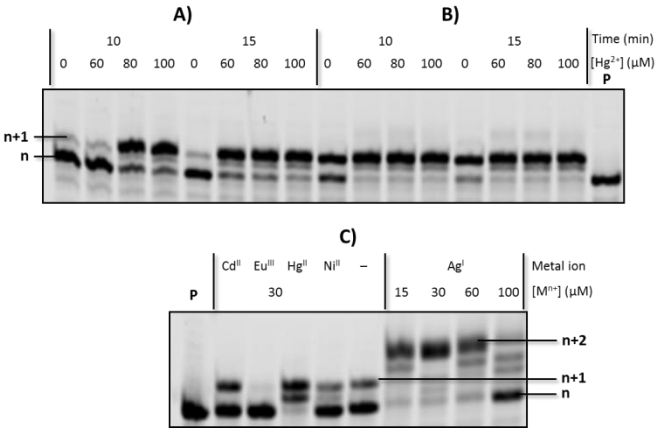

Figure 4. Gel (PAGE 20%) analysis of the incorporation of the nucleotide **S2C** opposite a templating **S4T** modification by the Taq polymerase in the presence of Hg^{2+}. Reaction mixtures containing primer **P1**, template **T1**, 200 μM of **S2C**-TP and A) 0.05 U of Taq or B) 0.5 U of Taq. All reactions were incubated at 60°C for given amounts of time. C) Reaction mixtures contained 0.5U Taq and 20 μM **S2C**-TP and were incubated at 60°C for 30 min **P** represents unreacted primer.

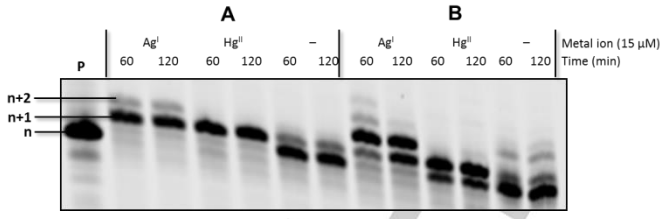

Figure 5. Gel image (PAGE 20%) of the PEX reactions of the incorporation of the nucleotide **5FU** opposite a templating **S4T** nucleotide (on template **T1**) in the presence of Ag^{+} and Hg^{2+} with A) Taq and B) Vent (*exo*$^{-}$) DNA polymerases. All the reactions were incubated at 60°C for given amounts of time in the presence of 0.05U of the respective polymerases and 200 μM of the modified triphosphate. **P** represents unreacted primer.

Single incorporation of a pK_a purine analog and a thiolated pyrimidine nucleotide

The last modifications that were considered as potential candidates for the enzymatic formation of metal base pairs were **5FU** and **S6G** (Figure 1). The pK_a-perturbed **5FU** is known to form stable Ag^{+}-mediated base pairs,[24] especially at high pH, and to form Hg^{2+}-**5FU** bonds under acidic conditions albeit of lower strength than the parent canonical thymidine.[20] On the other hand, the RNA- and DNA-targeting cytotoxin 6-thioguanine[25] is capable of forming stable Cd^{2+}-mediated base pairs.[26] Also, **S6G**-TP appears to be a good substrate for DNA polymerases and has been used to generate long, fully modified oligonucleotides that are not accessible by chemical methods.[27] Our polymerase screen revealed that both the Vent (*exo*$^{-}$) and the Taq polymerases enabled the formation of **5FU**-M^{n+}-**S4T** base pairs. In the case of the Taq polymerase, both Hg^{2+} and Ag^{+} appeared to be excellent cofactors for the formation of metal base pairs since the n+1 product formed exclusively (Figure 5A). Only little incorporation was observed in the absence of metal cofactor even after 120 min of reaction. However, both the reduction of the reaction time and an increase of the concentration of the polymerase were detrimental to the outcome of the PEX reactions (Figure S6). When Vent (*exo*$^{-}$) was used as polymerase, the PEX reactions conducted in the presence of Ag^{+} mainly led to the formation of the n+2 product which involved the formation of the **5FU**-Ag^{+}-**S4T** base pair as well as the expected **5FU**-Ag^{+}-dC mispair (Figure 5B). On the other hand, when Hg^{2+} was used as cofactor, only the n+1 product was obtained albeit in slightly lower yields than with the Taq polymerase.

PEX reactions with the **S6G**-triphosphate showed that when the Y-family polymerase *Sulfolobus* DNA Polymerase IV (Dpo4) was used with Cu^{2+} or Hg^{2+} high incorporation efficiencies of the modified nucleotide could be achieved (Figure 6). With Hg^{2+}, complete conversion to the n+2 (corresponding to a **S6G**-Hg^{2+}-**S4T** pair followed by a single **S6G**-dC pair) was observed. On the other hand, the n+3 product (containing a second **S6G**-dC pair) was not observed even when the reaction time and the concentration of the metal cation were increased (Figure S7B) presumably due to the formation of less stable C=S H–NH– interaction.[23] When Cu^{2+} was used as cofactor, a distribution of the n+1, n+2, and n+3 products was observed (Figure 6) and full and exclusive conversion to the n+3 product could be obtained by increasing the reaction time and the concentration of the metal cation (Figure S7A). Other metal cations did not sustain metal base formation with this system since yields comparable to that of the expected metal-independent **S6G**-**S4T** mispair were observed (data not shown).

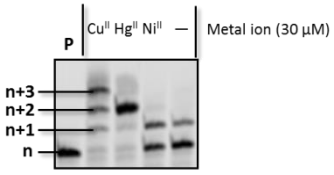

Figure 6. Gel image (PAGE 20%) of PEX experiments with the **S6G**-triphosphate and Dpo4. Reaction mixtures containing template **T1**, primer **P1**, 20 μM of the modified triphosphate, and 0.5 U of polymerase were incubated at 55°C for 20 min. **P** represents unreacted primer.

Formation of metal base pairs with natural nucleotides

Having established conditions for the enzymatic formation of artificial metal base pairs using thiol- and fluorine-modified nucleotides, we next questioned whether these analogs could also engage in metal-mediated incorporation events opposite natural nucleotides. Therefore, PEX reactions were carried out under the experimental conditions that led to the formation of the artificial metal base pairs and with templates **T2**-**T5** (Table 1) that contain one of the natural nucleotides immediately after the 3'-end of primer **P1**. This analysis showed that the **5FU**-modified nucleotide is incorporated in low yields opposite a templating cytosine nucleotide in the presence of Ag^{+} by the Vent (*exo*$^{-}$) polymerase while some misincorporation was observed opposite dG but only in the absence of the metal cation (Figure 7). Under

these conditions, formation of the known dA-Ag^+-dC[28] and to a lesser extent dT-Ag^+-dC[21a] pairs were also observed but only when dA and dT served as incoming triphosphates. In the presence of Hg^{2+} and Vent (*exo*$^-$), only the known dT-Hg^{2+}-dT base pair [21a] and the unusual dG-Hg^{2+}-dT formed and the modified nucleotide showed a lower propensity at forming a **5FU**-Hg^{2+}-dT pair compared to the unmodified counterpart (Figure S8A). Moreover, when the polymerase was changed to Taq, **5FU** was only incorporated opposite dA regardless of the nature of the cofactor (Figure S8B). Increasing the reaction time to 1h led to the formation of dA-Ag^+-dC, dT-Ag^+-dC, and **5FU**-Ag^+-dC[29] base pairs albeit in moderate yields (Figure S8C).

When the **S4T**-triphosphate was assayed with the *Bst* polymerase and Ag^+ as a cofactor, some incorporation of the modified nucleotide was observed opposite dC and dG (Figure S9A). However, the yields of n+1 product formation were similar to those obtained with reactions carried out in the absence of metal cations (Figure S9B). On the other hand, when Cu^{2+} was used as metal cation, more mispairing was observed (Figures S10 and S9B). Particularly, the **S4T**-nucleotide misincorporated well opposite natural dT and dC nucleotides (full conversion of the primer) and correct pairing with dA was not impaired by the presence of the metal cofactor. Also, these experimental conditions favored mispairing of the natural nucleotides since the efficient formation of dG-dT, dA-dA, and to a certain extent the previously reported dC-dC[30] mispairs were observed (Figure S10).

In the case of **S2C**, the presence of the metal cofactor Ag^+ mainly reduced the formation of the **S2C**-dG base pair (compare Figures S11A and S11B). In addition, the yield of misincorporation opposite natural nucleotides was low and did not depend on the presence of the metal cation. Lastly, Dpo4 favored the incorporation of the **S6G**-nucleotide opposite a templating cytidine but in the presence of Hg^{2+} the formation of the **S6G**-dC pair was severely impaired. The opposite was observed with a templating dT nucleotide since the **S6G**-dT pair formed in the presence but not in the absence of the metal cofactor, suggesting the formation of a **S6G**-Hg^{2+}-dT pair (Figure S12). The formation of the unusual dG-Hg^{2+}-dT was also observed but to a lesser extent than with the Vent (*exo*$^-$) DNA polymerase (*vide supra*).

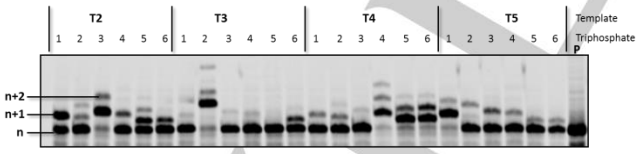

Figure 7. Gel image (PAGE 20%) of PEX reactions with the **5FU**-triphosphate and natural triphosphates (20 µM final concentration) and templates **T2**-**T5** with 0.05U Vent (*exo*$^-$) and 15 µM Ag^+. All reactions were incubated at 60°C for 60 min. Lanes 1: dATP; lanes 2: dCTP; lanes 3: dGTP; lanes 4: dTTP; lanes 5: **5FU**-triphosphate with Ag^+; lanes 6: **5FU**-triphosphate without Ag^+. **P** represents unreacted primer.

Multiple incorporation of modified nucleotides

We then wanted to capitalize on the conditions that sustained metal-dependent single incorporation events of the different modified nucleotides to incorporate more than one nucleotide analog. Therefore, we performed PEX reactions with template **T7** that contains three consecutive **S4T** nucleotides immediately downstream of primer **P1** using the conditions established for single incorporation of modified nucleotides. Surprisingly, very little extended products formed and with the exception of **S6G**, no multiple incorporation events could be observed (Figure S13); this suggests that these conditions were not suitable for the enzymatic incorporation of modified nucleotides with template **T7**. We therefore performed a screen with a limited subset of polymerases (i.e. Taq, Bst, Vent (*exo*$^-$), Kf *exo*$^-$, and Dpo4) and metal ions (Ag^+, Cd^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, and Pd^{2+}) along with the modified triphosphates of **S4T**, **5FU**, **S2C**, and **S6G** to identify conditions supporting multiple incorporation events (Figure S14). This analysis showed that multiple incorporation of the modified nucleotide could be achieved with **S4T** and **S6G** and to some extent with **S2C**. Under optimized conditions, up to three modified **S6G** nucleotides were incorporated by the *Bst* and Taq polymerases in the presence of Hg^{2+} (Figure 8). However, the misincorporation of a single **S6G** nucleotide by the Bst polymerase was quite efficient in the absence of the metal cation but no further elongation after this mispair was observed. Similarly, the incorporation of two **S4T** analogs was promoted by the combined use of Vent (*exo*$^-$) and Cd^{2+} as cofactor. An extension of the primer by two modified nucleotides was also observed when the **S2C**-triphosphate was used in conjunction with Dpo4 and Hg^{2+}, albeit in moderate yields.

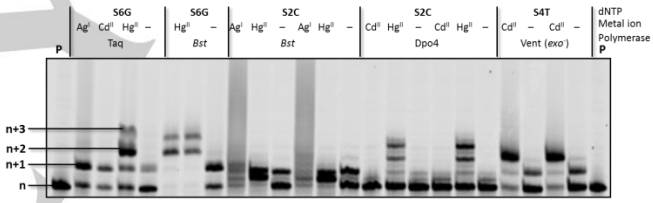

Figure 8. Gel image (PAGE 20%) of PEX reactions with primer **P1** and template **T7** and triphosphates **S4T**, **S6G**, and **S2C**.Conditions used: for reactions with **S6G**: 0.05U polymerase, [dN*TP] = 20 µM, [M^{n+}] = 30 µM; 60°C, 15min or 30 and 60 min with *Bst*; for reactions with **S2C**: 0.05U *Bst*, [dN*TP] = 200 µM, [M^{n+}] = 100 µM; 60°C, 120 min (left lanes) and 180 min (right lanes) or 0.01U Dpo4, [dN*TP] = 200 µM, [M^{n+}] = 30 µM; 60°C, 5 min (left lanes) and 10 min (right lanes); for reactions with **S4T**: 0.1U Vent (*exo*$^-$), [dN*TP] = 20 µM, [Cd^{2+}] = 100 µM; 60°C, 120 min (left lanes) and 180 min (right lanes). **P** represents unreacted primer.

Bypass of the metal base pairs by the polymerases

An important prerequisite of potent UBPs is that they sustain enzymatic synthesis beyond the site of the artificial base pair without causing polymerases to halt.[31] In order to probe whether single, metal-mediated artificial base pairs perturbed further enzymatic DNA synthesis, we first installed the mispairs and then carried out PEX reactions with the natural dNTPs (Figure 9). The presence of **5FU**-Ag^+-**S4T**, **5FU**-Hg^{2+}-**S4T** and **5FU**-Ag^+-dC pairs could be partially bypassed by the Vent (*exo*$^-$) and Taq polymerases since formation of the n+5 product (and n+6 which corresponds to non-templated addition of a natural, presumably dA, nucleotide[32]) was observed in all circumstances with only little truncated products. On the other hand, the incorporation of **S2C** and **S6G** precluded further elongation of the primer by natural nucleotides. The bypass efficiency of an **S4T**-M^{n+}-**S4T** pair strongly depended on the nature of the metal

cation. For instance, with Cu^{2+} and Cd^{2+}, very little conversion to the fully extended primer could be observed, while the presence of Ag^{+} did not have a negative influence on further enzymatic synthesis with natural dNTPs.

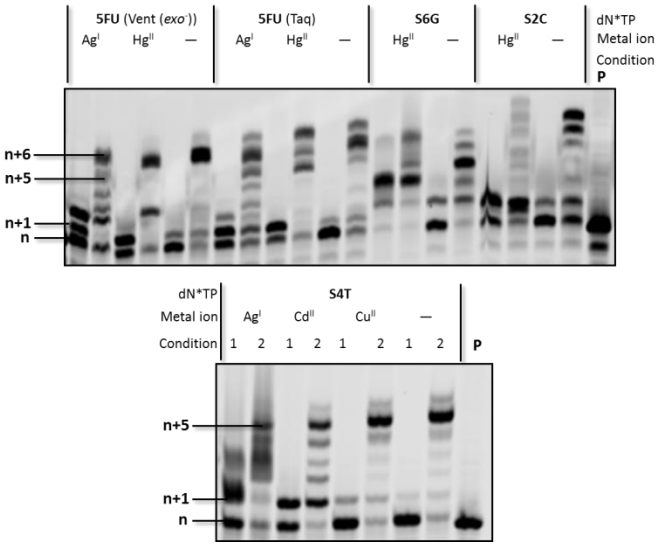

Figure 9. Gel analysis (PAGE 20%) of the PEX experiments for the evaluation of complete enzymatic synthesis beyond the metal UBPs. In all cases the concentration of the metal cations was 15 µM except for **S2C** (60 µM) and Ag^{+} with **S4T** (30 µM). Reaction times with the modified triphosphates were 60 min for **5FU**, 15 min for **S2C**, and 10 min for **S4T** and **S6G**. Reaction mixtures containing primer **P1**, template **T1**, dN*TPs (20 µM final for **S6G** and **S4T**, 200 µM for **5FU** and **S2C**) and 0.05U of the polymerases (*Bst* for **S4T**, Taq and Vent (*exo*$^{-}$) for **5FU**, Dpo4 for **S6G**, and Taq for **S2C**) were incubated at the appropriate temperature and reaction times. Natural dNTPs were then added (20 µM final, 60 min reaction). Lanes 1: PEX reactions only with the modified triphosphates; lanes 2: PEX reactions with first the modified triphosphates and then with the dNTPs. **P** represents unreacted primer.

Incorporation by the Terminal deoxynucleotidyl Transferase

The Terminal deoxynucleotidyl Transferase (TdT) is a member of the X Family of DNA polymerases and catalyzes the untemplated addition of nucleotides at the 3'-end of ssDNA primers.[33] This polymerase is rather tolerant to modifications on nucleotides and hence has been involved in the enzymatic construction of metal base pairs.[17d, f] We thus wanted to assess whether bulkier thiol groups and pK_a-perturbations on the nucleobase had an incidence on the substrate acceptance of the nucleotides by the TdT. In order to address this question, we carried out tailing reactions with primer **P1** and the modified triphosphates in the presence of two TdT cofactors (Co^{2+} and Mn^{2+}) and compared the outcome of the reactions with those of the corresponding unmodified nucleotides. With the exception of **5FU**, all tailing reactions were more efficient with Co^{2+} as cofactor than with Mn^{2+}; a trend which is also observed with the canonical nucleotides under these experimental conditions (Figures S15-17). Moreover, all modified nucleotides with the exception of **S2T** (Figure 10) were not as well accepted by the polymerase as their corresponding unmodified counterparts (Figures S15-17) since larger product dispersities and lower size averages were both observed. These observations suggest that even though the thiolated nucleobases might have a strong stacking capacity with the 3'-terminal nucleotide,[18] the binding affinity (K_d) of the nucleotides to the polymerase might be reduced which negatively impacts on their incorporation efficiency.[34] On the other hand, a significant increase in molecular weight of the modified products could be obtained when the feed ratio of monomers (i.e. modified triphosphates) to initiator (i.e. primer) was increased (Figures S18 and S19).[35] Indeed, when the ration monomer vs. initiator was increased to 1000, over 50 **S6G** nucleotides could be appended at the 3'-end of primer **P1** (Figures S18B and S19). Lastly, in order to exclude the possibility of formation of self-assembled higher-order structures especially in the presence of Co^{2+} and **S6G**,[36] the TdT-tailing reactions were performed in the presence of three different cofactors (i.e. Co^{2+}, Mn^{2+}, and Mg^{2+}). All the tailing reactions led to the formation of similar product distributions regardless of the nature of the cofactor (Figure S19), thus confirming that the products stem from polymerization of the nucleotide rather than from self-assembly.

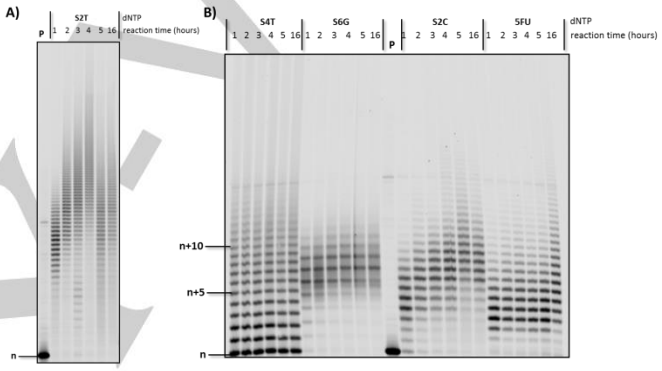

Figure 10. Gel image (PAGE 20%) of the TdT-mediated tailing reactions with A) **S2T**-TP and B) **S4T**-TP, **S6G**-TP, **S2C**-TP, and **5FU**-TP with primer **P1**. All reactions were incubated at 37°C for given amounts of time in the presence of 20 pmol P1, 1U/µL polymerase, 0.25 mM Co^{2+} (for **S2T**-TP, **S4T**-TP, **S6G**-TP,and **S2C**-TP) or 1 mM Mn^{2+} (for **5FU**-TP) and 200 µM of the modified triphosphate. **P** indicates unreacted primer.

Discussion

The thio-isomers **S4T**, **S2C**, and **S6G** together with the pK_a-perturbed **5FU** nucleotide sustain the enzymatic construction of metal base pairs under specific conditions. Indeed, the known capacity of **S4T** and **5FU** to form stable Ag^{+}-mediated base pairs could also be observed under PEX reaction conditions. In addition to this known interaction, the enzymatic incorporation of **S4T** and **5FU** could also be guided by the presence of thiophilic metal cations including Hg^{2+}, Cu^{2+}, and Cd^{2+}.[20] While the metal base pair formation of **S4T** and **5FU** nucleotides has been well documented, little is known on the **S2C** and **S6G** analogs. Under PEX reaction conditions, we found that Hg^{2+} triggered the incorporation of these modified nucleotides opposite a templating **S4T** nucleotide, suggesting the formation of **S6G**-Hg^{2+}-**S4T** and **S2C**-Hg^{2+}-**S4T** pairs.[37] We also observed the formation of an **S2C**-Ag^{+}-**S4T** pair, the modified version of the known dC-Ag^{+}-dT pair.[29] On the other hand, the thiol group of the **S2T** analog is not involved in Watson Crick interactions with functional groups on dA and points into the minor grove which probably accounts for the rather poor capacity of this modified

nucleotide to form metal base pairs other than with itself, both as an incoming triphosphate and a templating unit.

The replacement of functional groups of nucleobases by thiocarbonyl units has been suggested as an efficient method to provide orthogonality to xeno nucleic acids since Watson-Crick base pairing with canonical nucleic acids is disrupted.[38] Here, we observe that the combination of thiolated nucleotides and metal cations ensures the formation of stable self-pairs that can even be formed under enzymatic polymerization conditions. This important prerequisite with view on a potential expansion of the genetic alphabet with metal base pairs is further accompanied by the fact that a **S4T**-Ag^+-**S4T** pair could be by-passed by polymerases without disrupting enzymatic synthesis. In addition, up to two consecutive **S4T**-Ag^+-**S4T** pairs could be built under PEX reaction conditions. However, unlike other systems such as the 2-methyl-6-thiopurine base, thiolated nucleotides such as **S4T** are not completely orthogonal since they are still incorporated opposite canonical nucleotides (dA and dG).

Lastly, the presence of the larger sulfur atom and the electronegative fluorine has a negative impact on the substrate acceptance of the modified triphosphates by the TdT when compared to the natural counterparts. However, conditions could be met where the primer was elongated by >20 **S2C** modified nucleotides and >50 of the **S6G**, **S2T,** and **S4T** analogs (Figure S18). Hence, the combination of TdT-mediated tailing reactions with these modified nucleotides could potentially be used for the construction long, dsDNA constructs via the interaction with metal cations such as Hg^{2+}, Cd^{2+}, or Ag^+. A similar approach has already been reported with a modified hydroxypyridone nucleotide.[17f]

Conclusions

The enzymatic formation of artificial metal base pairs was investigated with nucleotides containing thiolated or pK_a-perturbed nucleobases. In some instances, we enzymatically constructed metal base pairs that have previously been observed in short DNA duplexes obtained by solid-phase synthesis (i.e. **S4T**-Ag^+-**S4T**, **S2T**-Ag^+-**S2T**, and **5FU**-Ag^+-dC).[24] The formation of new metal pairs could also be generated under certain PEX reaction conditions (such as **S6G**-Hg^{2+}-**S4T** and **S2C**-Hg^{2+}-**S4T**). In addition, we could demonstrate that some of these metal base pairs presented other important features such as multiple incorporation and artificial base pair by-pass which are important in the context of an expansion of the genetic code. However, incorporation of these modified nucleotides opposite natural templating nucleotides, either as Watson-Crick pairs or as mispairs, precludes their immediate use in SELEX. Nonetheless, thiolated or pK_a-perturbed nucleotides represent alluring candidates for an expansion of the genetic alphabet and other analogs are currently explored. Lastly, the presence of these modifications on the different nucleobases only led to a slight decrease in the efficiency of the TdT-mediated tailing reactions. We are currently evaluating the possibility of combining TdT-catalyzed polymerization reactions and metal cations to construct large, metal-containing dsDNA constructs.

In conclusion, we clearly demonstrate that nucleotides equipped with thiol and fluorine moieties can be used for the enzymatic construction of artificial metal base pairs. Hence, longer sequences are accessible than by conventional solid-phase synthesis which might permit the creation of novel metal sensors,[39] long, metallated DNA nanowires,[14a] multistep replicating systems,[21a, 40] or novel immobilization methods for nucleic acids.[41]

Experimental Section

General protocol for PEX reactions

The 5'-FAM-labelled primer **P1** (10 pmol) was annealed to the appropriate template (15 pmol) in the presence of 10× enzyme buffer (provided by the supplier of the DNA polymerase or prepared as described in the supporting information) by heating to 95 °C and then gradually cooling to room temperature (over 45 min). The appropriate DNA polymerase and metal cation solutions were then added to the annealed oligonucleotides mixture at 4 °C. Finally, natural dNTPs and/or modified triphosphates were added for a total reaction volume of 10 µL. Following incubation at the optimal temperature for the enzyme, the reactions were quenched by adding stop solution (10 µL; formamide (70%), ethylenediaminetetraacetic acid (EDTA; 50 mM), bromophenol (0.1%), xylene cyanol (0.1%)). The reaction mixtures were subjected to gel electrophoresis in denaturing polyacrylamide gel (20%) containing trisborate-EDTA (TBE) 1× buffer (pH 8) and urea (7 M). Visualization was performed by fluorescence imaging using a Typhoon Trio phosphorimager.

General protocol for the TdT-mediated polymerization reactions

A solution containing 20 or 2 pmol primer **P1**, 1U/µL of TdT, dNTPs (100 µM for natural triphosphates or 200 µM for modified nucleotides), 1x reaction buffer, and H_2O (for a total reaction volume of 10 µL) was incubated at 37°C for 5 hours. The reaction mixtures were then purified by Nucleospin columns and quenched by the addition of an equal volume of loading buffer. The reaction products were then resolved by electrophoresis (PAGE 20%) and visualized by phosphorimager analysis.

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Keywords: metal base pairs • polymerases • thiolated nucleotides • DNA synthesis • expanded genetic alphabet

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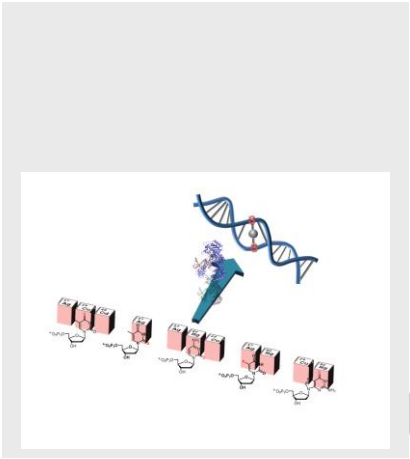
FULL PAPER

We report the usefulness of thiol-modified and pK_a-perturbed nucleotides for the enzymatic construction of artificial metal base pairs. In addition, the presence of single and multiple metal base pairs did not impair enzymatic synthesis with natural nucleotides. These artificial nucleotides are also substrates for the TdT polymerase. Functionalization of nucleotides with thiol and fluorine moieties appears to be favourable for the formation of artificial metal base pairs using polymerases.



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Page No. – Page No.

On the enzymatic formation of metal base pairs with thiolated and pK_a-perturbed nucleotides