Journal of Inorganic Biochemistry 191 (2019) 154–163

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

journal homepage: www.elsevier.com/locate/jinorgbio

Towards the enzymatic formation of artificial metal base pairs with a carboxy-imidazole-modified nucleotide

Pascal Röthlisberger[a], Fabienne Levi-Acobas[a], Ivo Sarac[a], Philippe Marlière[b], Piet Herdewijn[c], Marcel Hollenstein[a,*]

[a] Institut Pasteur, Department of Structural Biology and Chemistry, Laboratory for Bioorganic Chemistry of Nucleic Acids, CNRS UMR3523, 28, rue du Docteur Roux, 75724 Paris Cedex 15, France
[b] University of Paris Saclay, CNRS, iSSB, UEVE, Genopole, 5 Rue Henri Desbrueres, 91030 Evry, France
[c] KU Leuven, Rega Institute for Medical Research, Medicinal Chemistry, Herestraat, 3000 Leuven, Belgium

ARTICLE INFO

Keywords:
Metal-mediated base pairs
DNA
Nucleoside triphosphate
DNA polymerases
Enzymatic synthesis
Silver

ABSTRACT

The identification of synthetic nucleotides that sustain the formation of orthogonal, unnatural base pairs is an important goal in synthetic biology. Such artificial synthons have been used for the generation of semi-synthetic organisms as well as functional nucleic acids with enhanced binding properties. The enzymatic formation of artificial metal-base pairs is a vastly underexplored and alluring alternative to existing systems. Here, we report the synthesis and biochemical characterization of 1-(2-deoxy-β-D-ribofuranosyl) imidazole-4-carboxylate nucleoside triphosphate (**dIm^{C}TP**) which is equipped with a carboxylic acid moiety on the imidazole moiety in order to increase the coordination environment to [2 + 2] and [2 + 1]. A clear metal dependence was observed for the single incorporation of the modified nucleotide into DNA by the DNA polymerase from *Thermus aquaticus* (Taq). The presence of Ag^{I} in primer extension reactions conducted with combinations of 1-(2-deoxy-β-D-ribofuranosyl) imidazole nucleoside triphosphate (**dImTP**) and **dIm^{C}TP** supported the unusual [2 + 1] coordination pattern. The efficiency of the tailing reactions mediated by the terminal deoxynucleotidyl transferase (TdT) was markedly improved when using **dIm^{C}TP** instead of **dImTP**. Even though products with multiple modified nucleotides were not observed, the appendage of additional metal binding ligands on the imidazole nucleobase appears to be a valid approach to improve the biochemical properties of modified triphosphates in the context of an expansion of the genetic alphabet with metal base pairs.

1. Introduction

The expansion of the genetic code is a long standing aim in synthetic and molecular biology that strives to create proteins with hitherto unknown functions and three dimensional structures as well as to refine our understanding of the translational machinery [1]. Expanded genetic systems can be achieved by introducing non-canonical amino acids into proteins by suppressing the amber stop codon [2]. Another approach that is gaining significant momentum involves the construction of unnatural base pairs (UBPs) that complement the 4 natural nucleotides which form the Watson-Crick A-T and G-C pairs [3–6]. Synthetic genetic polymers equipped with UBPs have been used for the creation of semi-synthetic organisms that are capable of storing and retrieving this increased information load [7,8] and even transcribe DNA modified with UBPs to proteins containing noncanonical amino acids [9]. Expanded genetic alphabets have also been used for the in vitro isolation of potent aptamers that bind their respective targets with high affinity and selectivity [10–17]. Successful UBPs differ from natural nucleotides in terms of nucleobase composition and interact through carefully designed alternate hydrogen bond patterns [6] or via stacking or hydrophobic forces [18] or by shape complementarity [19]. However, some of these UBPs are prone to dephosphorylation [7,20] and are not uniformly retained by semi-synthetic organisms [18]. Moreover, UBP consisting of cyclic π-conjugated organic molecules can be photoactivated by near-visible light which leads to the formation of reactive oxygen species and ultimately to photodamage of DNA and cells [21,22]. Alternatively, UBPs can be constructed by identifying ligand-type nucleoside analogs capable of interacting together through coordination of a metal cation [23,24]. The resulting artificial metal base pairs are orthogonal to the canonical base pairs, present a high thermal stability, and usually cause minimal structural perturbations to DNA duplexes [25–33]. Even though artificial metal-mediated base pairs

* Corresponding author.
E-mail address: marcel.hollenstein@pasteur.fr (M. Hollenstein).

https://doi.org/10.1016/j.jinorgbio.2018.11.009
Received 31 August 2018; Received in revised form 8 November 2018; Accepted 13 November 2018
Available online 15 November 2018
0162-0134/

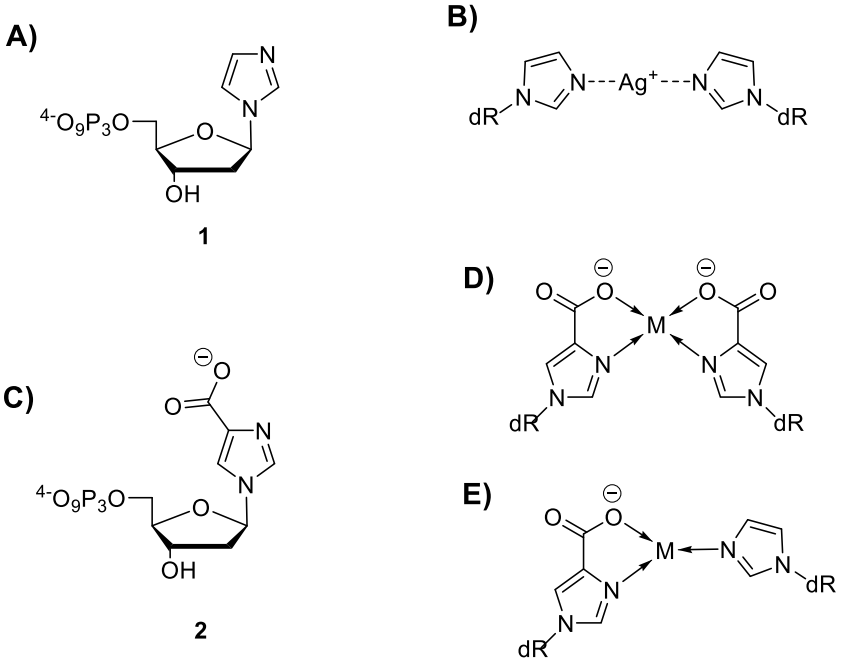


Fig. 1. Chemical structures of the modified triphosphates **dImTP 1** A) and **dIm^{C}TP 2** C) and the metal mediated **dIm**-Ag^{+}-**dIm** B), **dIm^{C}**-M^{n+}-**dIm^{C}** D), and **dIm^{C}**-M^{n+}-**dIm** E).

have been explored extensively [34], very little is known on their enzymatic construction [35–38] which is an important prerequisite for their involvement in the expansion of the genetic alphabet and ultimately in SELEX for the identification of functional nucleic acids [39,40]. In this context, we have recently questioned whether the imidazole nucleotide 1-(2-deoxy-β-D-ribofuranosyl) imidazole nucleoside triphosphate (**dImTP 1** in Fig. 1A) could be used as a substrate for DNA polymerases to construct artificial metal base pairs [41,42]. We rationalized that the imidazole nucleoside represented an ideal candidate since it is capable of forming highly stable **dIm**-Ag^{+}-**dIm** base pairs that only cause a minimal distortion of the B-DNA structure (Fig. 1B) [35,43,44]. Despite these favorable assets, metal cations with the exception of Mn^{2+} were not strictly required for single incorporation of modified nucleotides opposite templating **dIm** units, suggesting that the metal base pair could not be formed under these conditions. This biochemical characterization thus revealed that base pair stability and limited structural distortion are not the only key elements to be considered for the enzymatic construction of metal UBPs [42]. Particularly, size-complementarity, π-stacking, and interactions with residues of the active sites of polymerases are important parameters that appear to be suboptimal in the case of the **dIm** system. Here, we report the synthesis and the biochemical characterization of the 4-carboxyimidazole nucleotide 1-(2-deoxy-β-D-ribofuranosyl) imidazole-4-carboxylate nucleoside triphosphate (**dIm^{C}TP 2** in Fig. 1C). This second generation imidazole nucleotide is equipped with a carboxylic moiety that can serve as an additional metal binding ligand and increase the coordination environment from a [1 + 1] geometry in the **dIm** system to [2 + 1] or [2 + 2]. Moreover, the presence of a hydrogen bond acceptor on the imidazole moiety is expected to reduce pairing with the canonical nucleotides. To test this hypothesis, we subjected **dIm^{C}TP** to primer extension reactions with different polymerases and assessed whether metal cations could promote the formation of **dIm^{C}**-M^{n+}-**dIm^{C}** pairs.

2. Experimental

2.1. Methods

All reactions were performed under N_2 in flame-dried glassware. Anhydrous solvents for reactions were obtained from Sigma Aldrich. Flash chromatography was performed using silica gel (230–400 mesh) from Sigma Aldrich. Thin layer chromatography was carried out on precoated glass-backed plates of silica gel (0.25 mm, UV_{254}) from Macherey-Nagel. All chemicals and solvents used were purchased from Sigma-Aldrich and Alfa Aesar. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400.13 MHz for ^{1}H, 100.62 MHz for ^{13}C, and 161.62 MHz for ^{31}P) and all spectra were referenced to the signals of the corresponding solvent. Chemical shifts are given in ppm (δ scale) and coupling constants (J) in Hz. Assignation of the NMR signals was performed by using a combination of ^{1}H/^{1}H-COSY, ^{13}C-DEPT-135, and ^{13}C/^{1}H-HMBC experiments. High resolution electrospray ionization (ESI) mass spectra (MS, m/z) were recorded on a Waters Q-Tof Micro MS in the positive-ion electrospray ionization (ESI^{+}) mode. Solutions were prepared using 1:1 MeCN/H_2O containing 0.1% formic acid or MeOH/water containing 10 mM ammonium acetate in the case of sensitive compounds. HPLC purification was performed using an Äkta™ pure system (GE Healthcare) equipped with a Phenomenex Luna semi-preparative RP-HPLC column (5 µm C18 100 Å, 250 × 10.0 mm). Unmodified DNA oligonucleotides were purchased from Microsynth. DNA oligonucleotides containing **dIm^{C}** nucleotides were synthesized on an H-8 DNA synthesizer from K&A on a 1 µmol scale. Natural DNA phosphoramidites (dT, dC^{4bz}, dG^{2DMF}, dA^{6Bz}) and solid support (dA^{6Bz}-lcaa-CPG 500 Å) were all purchased from ChemGenes. The **dIm^{C}** phosphoramidite was prepared by application of a literature protocol [45]. Natural DNA phosphoramidites as well as the modified phosphoramidite were prepared as 0.07 M solutions in MeCN and were coupled using 50 s and 490 s steps, respectively. 5-(ethylthio)-1*H*-tetrazole (0.25 M in MeCN) was used as coupling agent. Capping, oxidation, and detritylation were performed using standard conditions. Cleavage from the solid support and deprotection of oligonucleotides was achieved by treatment with concentrated ammonia at room temperature for 16 h. After centrifugation, the supernatants were collected

and the resulting solutions were evaporated to dryness on a speed-vac. Crude oligonucleotides were purified by anion exchange HPLC (Dionex - DNAPac PA200). Buffer solutions of 25 mM Tris-HCl in H_2O, pH 8.0 (buffer A) and 25 mM Tris-HCl, 1.25 M NaCl in H_2O, pH 8.0 (buffer B) were used. The purified oligonucleotides were then desalted with SepPack C-18 cartridges. Oligonucleotide concentrations were quantitated by UV spectroscopy using a UV5Nano spectrophotometer (Mettler Toledo). The chemical integrity of oligonucleotides was assessed by UPLC-MS analysis: UPLC was performed on a BEH C18 column (130 Å, 1.7 µm, 2.1 mm × 50 mm) from Waters, installed on an ACQUITY UPLC H-Class System (SQ Detector 2). A Buffer containing 20 mM TEA and 400 mM HFIP in H_2O was used with a linear gradient from 18 to 31% Methanol within 5 min and a flow rate of 0.3 mL/min. All the DNA polymerases (Therminator, Vent (*exo*$^-$), Deep Vent, Bst, Taq, TdT, and Klenow fragment of DNA polymerase I *exo*$^-$ (Kf *exo*$^-$)) were purchased from New England Biolabs as well as the natural dNTPs. Acrylamide/bisacrylamide (29:1, 40%) was obtained from Fisher Scientific. Visualization of PAGE gels was performed by fluorescence imaging using a Storm 860 phosphorimager with the ImageQuant software (both from GE Healthcare). The following salts were used: $AgNO_3$, $CdCl_2$, $CuSO_4$, $CoCl_2$, $EuCl_3$, $FeSO_4$, $FeCl_3$, $HgCl_2$, $MnCl_2$, $NiSO_4$, $Pb(OAc)_2$, $ZnSO_4$.

2.2. Reaction buffers

The following 10× buffers without any Cl-source were prepared for PEX reactions in presence of Ag^I. Buffer 1 is used for Taq, buffer 2 for Kf *exo*$^-$, and buffer 3 for Bst.

Buffer 1: 100 mM Tris acetate, 500 mM $KClO_4$, 15 mM $MgSO_4$, pH 8.3 @ 25 °C; buffer 2: 500 mM $NaClO_4$, 100 mM Tris acetate, 100 mM $MgSO_4$, 100 µM DTT, pH 7.9; buffer 3: 200 mM Tris acetate, 100 mM $(NH_4)_2SO_4$, 500 mM $KClO_4$, 20 mM $MgSO_4$, 0.1% Tween® 20, pH 8.8 @ 25 °C.

2.3. Synthesis of **dIm^{C}TP 2**

2.3.1. Synthesis of ethyl 5′-O-(4,4′-dimethoxytrityl)-3′-O-acetyl-1-(2-deoxy-β-D-ribofuranosyl)-imidazole-4-carboxylate (4)

Nucleoside analog **3** [45] (220 mg, 0.38 mmol) was dissolved in dry pyridine (10 mL) at RT under N_2. To this solution, 2-(dimethylamino) pyridine (15 mg, 0.11 mmol, 0.3 eq.) was added followed by triethylamine (134 µL, 0.95 mmol, 2.5 eq.) and subsequently acetic anhydride (55 µL, 0.6 mmol, 1.5 eq.). After 4 h of stirring, the reaction mixture was quenched with $NaHCO_3$ sat. (20 mL) and extracted with DCM (3 × 30 mL). The organic layers were combined dried over $MgSO_4$ and concentrated. The crude product was purified by flash chromatography (DCM/MeOH 2% with Et_3N 1%) to yield 230 mg of **4** as a yellowish oil (quant.).

R_f (DCM/MeOH 5%) = 0.7

HR-MS $C_{34}H_{37}N_2O_8^+$ calculated: 601.2544; found: 601.2538.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.64 (dt, J = 4.3, 1.7, 1H), 7.78 (d, J = 1.4, 1H), 7.73–7.64 (m, 2H), 7.45–7.39 (m, 2H), 7.33–7.27 (m, 4H), 7.26–7.20 (m, 1H), 6.90–6.74 (m, 4H), 6.01 (dd, J = 8.5, 5.5, 1H), 5.42 (dt, J = 5.9, 1.9, 0H), 4.34 (qd, J = 7.1, 1.8, 2H), 4.23 (td, J = 3.8, 1.9, 1H), 3.62–3.18 (m, 2H), 2.76–2.45 (m, 2H), 2.11 (s, 3H), 1.33 (t, J = 7.1, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 170.1, 162.6, 158.7, 144.3, 136.5, 135.5, 135.4, 134.7, 130.0, 129.9, 129.0, 128.2, 128.1, 128.0, 127.0, 122.7, 113.3, 86.9, 86.6, 86.5, 85.7, 84.5, 75.0, 63.6, 62.6, 60.5, 55.2, 20.9, 14.4, 14.3.

2.3.2. Synthesis of ethyl 3′-O-acetyl-1-(2-deoxy-β-D-ribofuranosyl)-imidazole-4-carboxylate (5)

The starting material **4** (230 mg, 0.38 mmol) was dissolved in dry chloroform (15 mL) at RT under a N_2 atmosphere. To this solution, dichloroacetic acid (0.32 mL, 38 mmol, 10 eq.) was added and the resulting orange solution was stirred for 20 min at RT. The reaction mixture was quenched with $NaHCO_3$ sat. (10 mL), extracted with DCM (3 × 20 mL), dried over $MgSO_4$ and concentrated under reduced pressure. The crude product was purified by flash chromatography (DCM/MeOH 2–5%) to yield 90 mg (78%) of compound **5** as a white solid.

R_f (DCM/MeOH 5%) = 0.3

MS $C_{13}H_{19}N_2O_6^+$ calculated: 299.1238; found: 299.1249.

^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.87 (d, J = 1.4, 1H), 7.81 (d, J = 1.4, 1H), 6.04 (dd, J = 8.2, 5.8, 1H), 5.61–5.19 (m, 1H), 4.39 (q, J = 7.1, 2H), 4.19 (q, J = 3.0, 1H), 3.91 (s, 2H), 2.67 (ddd, J = 14.3, 8.2, 6.2, 1H), 2.55 (ddd, J = 14.0, 5.8, 2.2, 1H), 2.14 (s, 3H), 1.61 (s, 2H), 1.40 (t, J = 7.1, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ = 170.4, 136.6, 134.6, 122.8, 86.7, 85.8, 75.0, 62.6, 60.6, 39.5, 20.9, 14.4.

2.3.3. Synthesis of **dIm^{C}TP (2)**

Nucleoside **5** (40 mg, 0.13 mmol) was dissolved in dry pyridine (0.2 mL) and dry dioxane (0.4 mL) at RT under N_2. To this clear solution, 2-chloro-1,3,2-bonzodioxaphosphorin-4-one (38 mg, 0.18 mmol, 1.4 eq.) was added and the reaction mixture was stirred for 45 min. A solution of tributylammonium pyrophosphate (95 mg, 0.17 mmol, 1.3 eq.) in dry DMF (0.17 mL) and tributylamine (60 µL) was added dropwise and the reaction mixture stirred for another 45 min. The reaction mixture was then oxidized by the addition of iodine (56 mg, 0.21 mmol, 1.6 eq.) in pyridine (0.98 mL) and H_2O (0.02 mL). After 30 min of stirring, the excess of iodine was quenched with a sodium thiosulfate solution (10% w/v in water) and the resulting clear solution was concentrated under reduced pressure at 30 °C. The concentrated mixture was treated with ammonium hydroxide 30% (12 mL) for 2 h. The yellow suspension was again concentrated under reduced pressure at 30 °C. The yellow residue was dissolved in H_2O (2 mL) and precipitated by the addition of $NaClO_4$ 2% in acetone (12 mL). The crude product was purified by RP-HPLC (30% B in 20 min; Buffer A) TEAB 50 mM pH = 8; Buffer B) TEAB 50 mM pH = 8, 50% ACN) to give 3.7 mg (6%) of the pure triphosphate **2**.

ESI-MS $C_9H_{14}N_2O_{14}P_3^-$ calculated: 465.9580; found: 465.9564.

^{1}H NMR (400 MHz, D_2O) δ = 7.99 (s, 1H), 7.94 (s, 1H), 6.13 (t, J = 6.7, 1H), 4.15 (s, 1H), 4.06 (dt, J = 8.6, 5.3, 2H), 2.53 (dt, J = 13.3, 6.5, 1H), 2.48–2.37 (m, 1H).

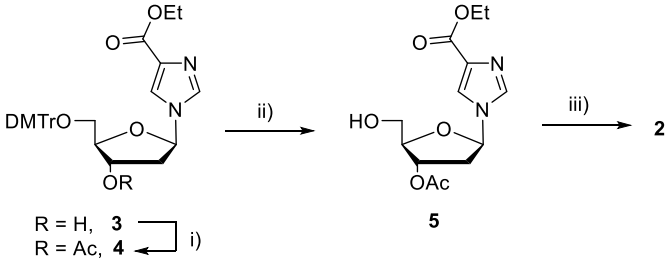
^{31}P NMR (162 MHz, D_2O) δ = −6.02 (d, J = 19.2), −9.99 (d, J = 19.6), −20.68 (t, J = 19.3).

2.4. Primer extension experiments

The 5′-FAM-labelled primer **P1** (30 pmol) was annealed to the appropriate template (45 pmol) in the presence of 10× enzyme buffer (provided by the supplier of the DNA polymerase or prepared as described in Section 2.2) by heating to 95 °C and then gradually cooling to room temperature (over 30 min). The appropriate DNA polymerase and metal cation solutions were then added to the annealed oligonucleotides mixture on ice. Finally, natural dNTPs and/or modified triphosphate 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 Storm 860 phosphorimager.

2.5. TdT assays

A solution containing 40 pmol of primer **P1** and 10 U of TdT was added to a mixture composed of **dIm^{C}TP** (100 µM final concentration), reaction buffer, 1 U of thermostable inorganic pyrophosphatase, the appropriate metal cofactor (1 mM final concentration), and H_2O (for a total reaction volume of 10 µL). The reaction mixtures were then



Scheme 1. Synthesis of **dIm^{C}TP 2**. Reagents and conditions: (i) Ac_2O, DMAP, NEt_3, pyridine, 0 °C, 4 h, 96%; (ii) DCAA, CH_2Cl_2, rt, 20 min, 78%; (iii) 1. 2-Chloro-1,3,2-benzodioxaphosphorin-4-one, pyridine, dioxane, rt, 45 min; 2. $(nBu_3NH)_2H_2P_2O_7$, DMF, nBu_3N, rt, 45 min; 3. I_2, pyridine, H_2O, rt, 30 min; 4. NH_3(aq.), rt, 1.5 h, 6% (4 steps).

incubated at 37 °C for various amounts of time and quenched by addition of 10 μL of loading buffer. The reaction products were then resolved by electrophoresis (PAGE 20%) and visualized by phosphorimager analysis.

3. Results

3.1. Synthesis of *dIm^{C}TP 2* and modified oligonucleotides

The phosphoramidite building block for the automated solid-phase synthesis of the **Im^{C}**-modified templates was obtained by application of a published protocol [45]. The synthesis of **dIm^{C}TP** is highlighted in Scheme 1 and involved phosphorylation with the Ludwig-Eckstein protocol [46] of the suitably protected precursor **5**.

All the templates containing modified nucleotides were obtained by solid-phase synthesis using standard phosphoramidite chemistry and characterized by LCMS (Table 1 and Supporting information). In order to assay the enzymatic synthesis of the metal ion mediated artificial base pair, template **T1** containing a single **dIm^{C}** nucleotide located immediately after the 3′-end of the 5′-fluorescently-labelled primer **P1** was synthesized. In addition, templates **T6** and **T7** containing 3 and 5 **dIm^{C}** nucleotide overhangs, respectively, were synthesized to assess whether multiple metal base pairs could be formed. Templates **T1** and **T6** and **T7** were designed to favor a [2 + 2] coordination geometry while templates **T8** and **T9** containing **dIm** nucleotides were used to probe [2 + 1] coordination patterns. Lastly, templates **T2–T5** contained each of the natural nucleotides instead of the modified nucleotide in **T1** and were designed to evaluate the specificity of the incorporation of **dIm^{C}TP**.

3.2. Screening of polymerases and metal cations under PEX reactions conditions

We first sought to evaluate the possibility of constructing a single **dIm^{C}**-M^{n+}-**dIm^{C}** base pair by enzymatic synthesis. Therefore, 6 different polymerases (i.e. Therminator, Vent (*exo*$^{-}$), Deep Vent, Bst, Taq, and the Klenow fragment of DNA polymerase I *exo*$^{-}$ (Kf *exo*$^{-}$); see list of Abbreviations) known to accept modified triphosphates [47,48] were evaluated for their capacity at accepting **dIm^{C}TP** as a substrate and incorporate this modification opposite a templating **dIm^{C}** nucleotide in the presence of 12 different metal cations under primer extension reaction (PEX) conditions. This initial screen revealed the complete inefficacy of both Deep Vent and Vent (*exo*$^{-}$) to accept the modified triphosphate as a substrate since no extended primer could be observed even after 5 h of incubation (Fig. S1A and B). On the other hand, the Kf *exo*$^{-}$ and Bst polymerases accepted **dIm^{C}TP** as a substrate regardless of the presence or the nature of the metal ions but with a rather modest efficiency (~60% of conversion of the primer to the n + 1 product; see Fig. S1C and D). The reactions catalyzed by Therminator led to a distribution of products indicating that this polymerase incorporated **dIm^{C}MP** units not only opposite a modified nucleotide but also indiscriminately opposite the natural nucleotides constituting the 5′-overhang of template **T1** (Fig. S1E). The addition of a **dIm^{C}** nucleotide on primer **P1** mediated by Therminator was slightly more efficient in the presence of certain metal cations such as Ag^{+}, Fe^{2+}, and Cu^{2+} but remained generally rather low yielding (< 30% of conversion of the primer). On the other hand, full conversion of the primer to the n + 1 product was observed when the Taq polymerase was used in conjunction with Mn^{2+} (Fig. 2A), as observed in the case of the parent **dImTP** but with Kf *exo*$^{-}$ as polymerase [42]. The addition of Fe^{2+}, Co^{2+}, and to a lesser extent Cd^{2+} also improved the yield of the n + 1 product compared to the control reaction that contained no additional metal cations (Fig. 2B). Therefore, a clear dependence on the nature of the metal cation on the incorporation efficiency could be observed with the Taq polymerase. In order to improve the reaction yields, we then investigated the effect of increasing metal concentrations on the outcome of the reaction.

The yield of incorporation of **dIm^{C}** nucleotides opposite a single **dIm^{C}** appears to be highly dependent on the concentrations of Co^{2+} and Mn^{2+} as shown in Fig. 3 but is rather insensitive to variations in concentrations of Ag^{+}, Fe^{2+}, and Cd^{2+} (data not shown). On the other hand, the incorporation of a modification opposite a single templating **dIm^{C}** was rather dependent on the concentration of **dIm^{C}TP** when Co^{2+} and Fe^{2+} served as cofactors (Fig. S2). Indeed, low concentrations of **dIm^{C}TP** (20 μM) led to poor incorporation efficiencies regardless of the reaction times, while higher concentrations (50–200 μM) of the modified triphosphates resulted in high incorporation yields.

Moreover, full conversion of the primer to the n + 1 product can be observed with Mn^{2+} concentrations of at least 45 μM which is comparable to what had been observed with **dImTP**. An increase in [Co^{2+}] from 15 μM to 100 μM was reflected by a ~2-fold improvement of the yield of the polymerization reaction (from 30 to 60%).

In order to address the question of whether the modified base could be bypassed by polymerases we first conducted PEX reactions with the primer **P1**/template **T1** system in the presence of 4 different metal cations (Ag^{+}, Cd^{2+}, Mn^{2+}, and Co^{2+}). The resulting products were then further extended by adding the 4 canonical dNTPs (Fig. S3). In all cases, n + 6 products were observed which correspond to full length products with a supplementary nucleotide stemming from a non-templated addition. However, the n + 1 product formed by the initial incorporation of a **dIm^{C}MP** opposite a modified nucleotide was not bypassed by the polymerases. A similar result was obtained when each canonical dNTP was added separately to the reaction mixture (Fig. S4).

3.3. Specificity of the incorporation of *dIm^{C}TP*

The presence of the cofactors Co^{2+} and Mn^{2+} enables the Taq polymerase to incorporate a single **dIm^{C}** nucleotide into DNA. In order to investigate the specificity of this incorporation, we carried out PEX reactions using the non-modified templates **T2–T5** (Table 1) that contain each one of the canonical bases at the site of the modification in **T1**. This analysis clearly revealed that **dIm^{C}** nucleotides were not incorporated opposite any of the canonical nucleotides in the presence of

Table 1
DNA primer and templates used for the primer extension reaction to assay the enzymatic synthesis of the metal base pair.

P1	5′-FAM-TAC GAC TCA CTA TAG CCT C
T1	5′-GGA G**Im^{C}**G AGG CTA TAG TGA GTC GTA
T2–T5[a]	5′-GGA GNG AGG CTA TAG TGA GTC GTA
T6	5′-GG**Im^{C} Im^{C}Im^{C}**G AGG CTA TAG TGA GTC GTA
T7	5′-**Im^{C}Im^{C}Im^{C} Im^{C}Im^{C}**G AGG CTA TAG TGA GTC GTA
T8[b]	5′-GG**Im ImIm**G AGG CTA TAG TGA GTC GTA
T9[b]	5′-**ImImIm ImIm**G AGG CTA TAG TGA GTC GTA

[a] N = A (**T2**); N = T (**T3**); N = C (**T4**); N = G (**T5**).
[b] From reference [42].

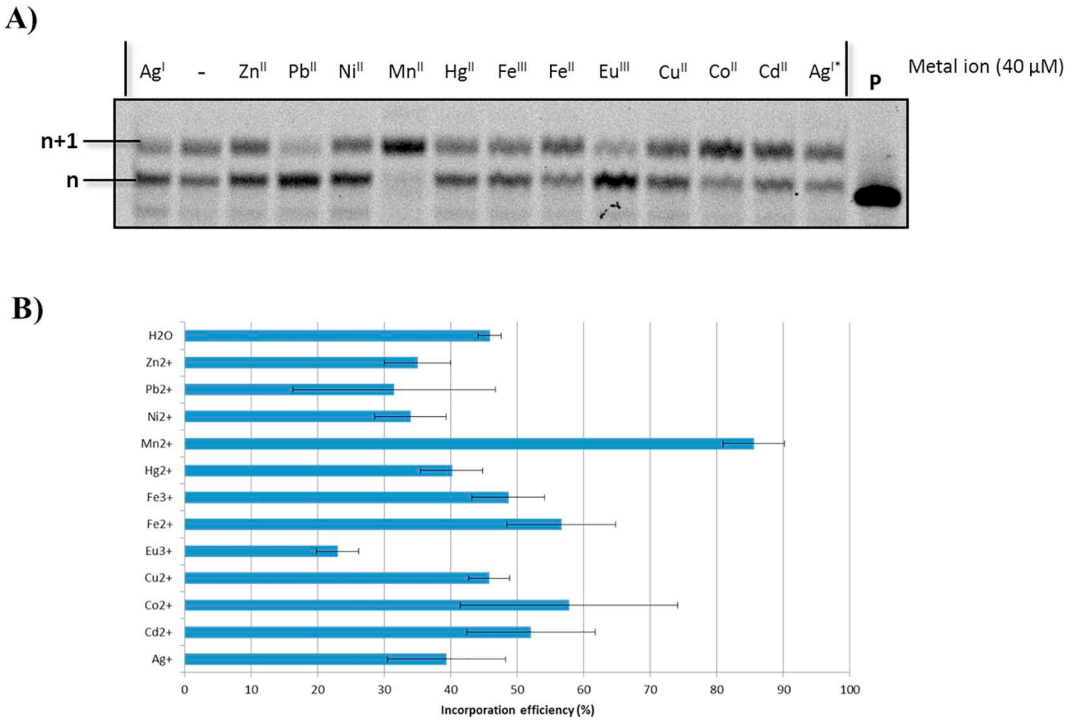


Fig. 2. A) Representative PAGE (20%) analysis of the incorporation of **dIm^{C}TP** opposite a single templating **dIm^{C}** nucleotide in the presence of various metal cations with the Taq polymerase using primer **P1** and template **T1**. All reaction mixtures contained 200 µM **dIm^{C}TP**, 1 × reaction buffer, and 5 U of Taq polymerase and were incubated at 60 °C for 5 h. **P** indicates unreacted primer. For Ag^{I} the reaction was carried out in buffer 1 while for Ag^{I*} the standard Taq reaction buffer was used. B) Quantification of the incorporation efficiency of **dIm^{C}TP** by the Taq polymerase as a function of the nature of metal cation. The results shown are the average of three independent data sets.

the preferred cofactors Co^{2+} and Mn^{2+} (Fig. 4) as well as Ag^{+} (Fig. S5). When the reactions were performed with increased concentrations of Co^{2+} and Mn^{2+} (80 µM and 50 µM, respectively), no incorporation of the modification was observed when the concentration of **dIm^{C}TP** was maintained at 20 µM (Fig. S6). On the other hand, some misincorporation (~10%) of the natural nucleotides could be observed (especially opposite dT) due to the relaxing of the substrate tolerance of the polymerase under increased metal cation concentrations. Similarly, the modified **dIm^{C}** nucleotide was moderately well incorporated opposite dT (50% in the presence of Mn^{2+} and 40% in the presence of Co^{2+}) and only marginally opposite dG (~10%) when the concentration of **dIm^{C}TP** was increased to 200 µM (Fig. S6). Since incorporation of the modification is efficient at triphosphate concentrations as low as 50 µM in the presence of 80 µM Co^{2+} (Fig. S2) we can safely conclude that the incorporation of **dIm^{C}MP** exclusively occurs when the templating nucleotide is **dIm^{C}**. Incorporation opposite a natural templating nucleotide only occurs in the presence of high metal and triphosphate concentrations. A similar observation was made previously with the parent **dImTP** analog.

3.4. PEX reactions with templates with longer modified overhangs

After identifying cofactors that sustain the polymerization reaction of **dIm^{C}TP** and demonstrating the specificity of this incorporation, we next questioned whether longer modified fragments could be obtained by PEX reactions. To do so, we carried out PEX reactions with templates **T6** and **T7** that contain longer **dIm^{C}**-modified overhangs (Fig. 5). The Taq polymerase was capable of producing substantial amounts of the n + 1 products (> 50% of conversion of the primer) in the presence of both Co^{2+} and Mn^{2+} but stalled after incorporating a single **dIm^{C}MP** since no traces of longer oligonucleotides could be detected. An increase in the concentration of the modified triphosphate or the metal cations did not lead to the incorporation of additional nucleotides (data not shown). Interestingly, when template **T6** containing three consecutive modified nucleotides was employed in PEX reactions, a significant extension of the primer could also be observed in the presence of Cd^{2+} and Hg^{2+} (Fig. 5A). All other metal cations led to yields comparable to or lower than the reaction carried out without any additional cofactor. Conducting PEX reactions with template **T7** exclusively led to the formation of the n + 1 product in the presence of Mn^{2+} (Fig. 5B). The addition of Co^{2+} and to a certain extent Cd^{2+} also increased the yields of the n + 1 product compared to the reaction carried out in absence of additional metal cations. As observed with template **T6**, no longer products were formed even at higher metal and triphosphate concentrations (data not shown). Overall, even though the outcome of the reactions is slightly and unexpectedly different when

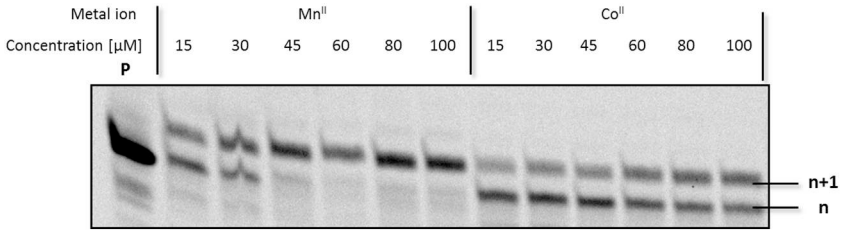


Fig. 3. Gel analysis (PAGE 20%) of the effect of the concentration of Mn^{2+} and Co^{2+} on the outcome of the PEX reactions with template **T1** and primer **P1**. The reactions were conducted in presence of 5 U of Taq polymerase at 60 °C for 4 h with 200 µM **dIm^{C}TP** and in buffer 1.

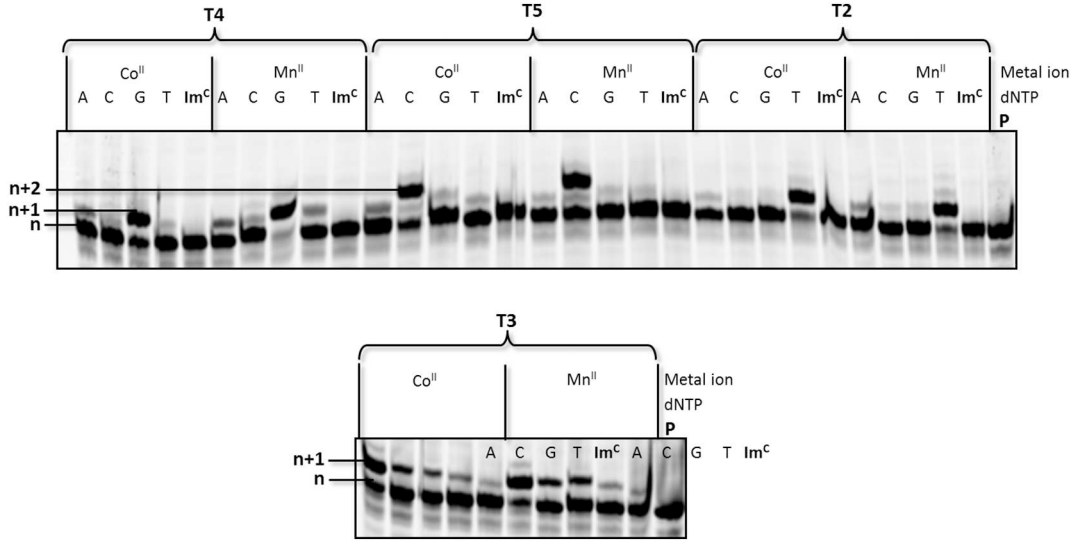


Fig. 4. Gel pictures (PAGE 20%) of the products of the PEX reactions with templates **T2–T5** and primer **P1**. The reactions were carried out using 20 μM dNTPs or **dIm^{C}TP** in the presence or absence of 15 μM Co^{2+} or Mn^{2+}. In addition, the reactions mixtures contained 0.5 U of Taq polymerase and were heated at 60 °C for 30 min in 1 × buffer 1. **P** indicates unreacted primer.

templates **T6** and **T7** are used, these experiments show that multiple incorporation events do not occur.

3.5. [2 + 1] coordination environment

The [2 + 1] coordination pattern is rather rare but has been observed in certain Fe^{II} and Fe^{III} complexes [49] as well as for Ag^{I} in the context of artificial metal base pairs [50]. This coordination mode can potentially be achieved by using the parent **dIm** nucleotide either as an incoming triphosphate on **dIm^{C}**-containing templates or vice versa. Consequently, PEX reactions were carried out first with **dImTP** together with templates **T6** and **T7**. When the Taq polymerase was used during the PEX reactions, marginal formation of the n + 1 product could be observed with template **T6** in the presence of Ag^{+}, Cd^{2+}, Co^{2+} and Mn^{2+} while no extension of the primer could be detected with template **T7** (Fig. S7). A similar trend was observed with Bst (data not shown). On the other hand, Kf *exo*$^{-}$ incorporated one **dImMP** unit opposite a templating **dIm^{C}** nucleotide only when Ag^{+} was used as a cofactor and **T6** as a template (Fig. 6A). All other metal ions did not promote the formation of the artificial base pair. Surprisingly, when template **T7** was used instead, n + 1 products could be observed with various different metal cations and in poor yields, often lower than the reaction conducted in the absence of additional cofactors (Fig. 6B). This important change in the outcome of the PEX reactions when using template **T7** instead of **T6** was rather unexpected.

When PEX reactions were carried out with the **dIm**-modified templates **T8** and **T9** in combination with **dIm^{C}TP**, only n + 1 products were formed and no bands corresponding to primers with multiple modifications could be observed regardless of the nature of the polymerase and the metal ion. When Kf *exo*$^{-}$ was used, n + 1 products were formed regardless of the nature of the metal cation (data not shown) as observed with template **T1** (Fig. S1C). However, with the Taq

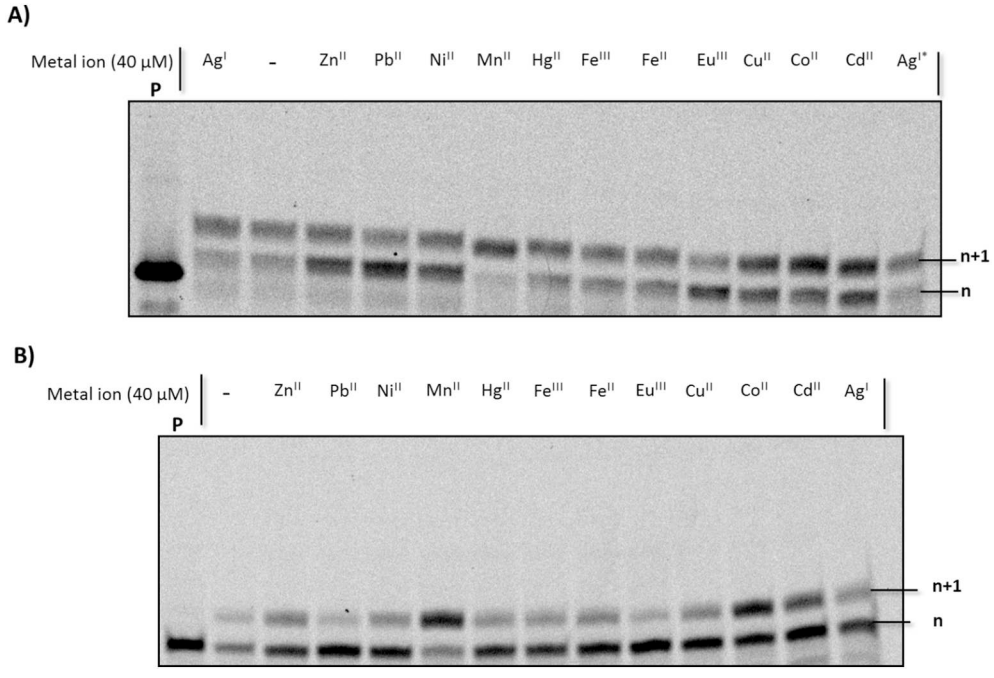
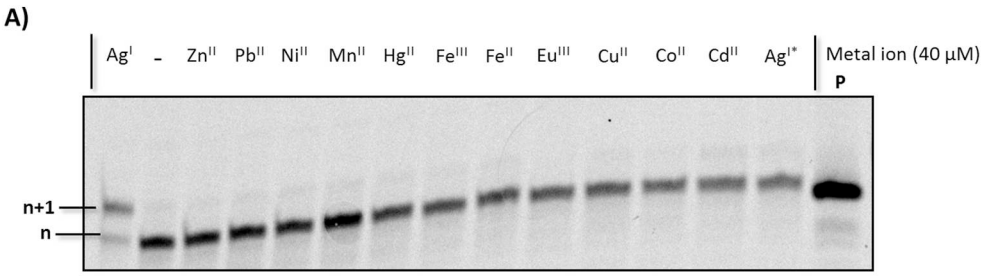


Fig. 5. Gel pictures (PAGE 20%) of the products of the PEX reactions with templates containing multiple modified nucleotide overhangs in the presence of various metal cations. A) Reactions with primer **P1** and template **T6**; B) reactions with primer **P1** and template **T7**. The reaction mixtures were incubated at 60 °C for 5 h in the presence of 0.5 U Taq polymerase, 200 μM **dIm^{C}TP**, and 1 × reaction buffer. **P** indicates unreacted primer. For Ag^{I} the reaction was carried out in 1 × buffer 1 while for Ag^{I}* the standard Taq reaction buffer was used.



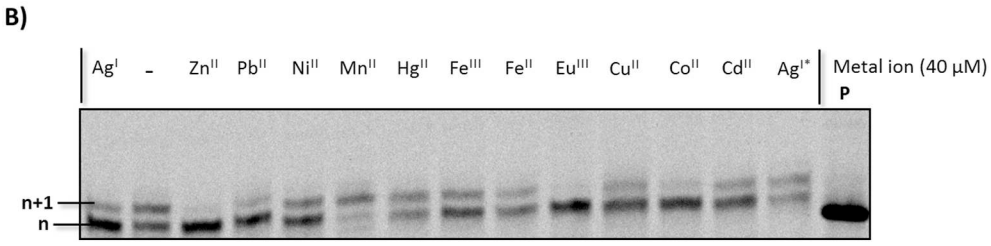
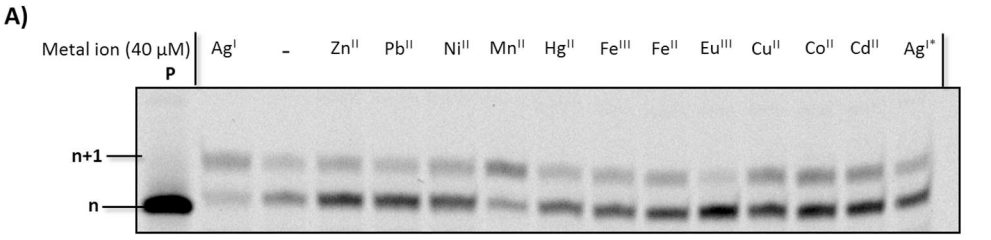


Fig. 6. Gel (PAGE 20%) analysis of the products of PEX reactions with primer **P1** and templates **T6** A) and **T7** B) with **dImTP**. All reactions were carried out in the presence of 5 U of Kf *exo*$^{-}$ and 200 μM **dImTP** at 37 °C for 5 h. **P** indicates unreacted primer. For Ag^{I} the reaction was carried out in 1 × buffer 2 while for Ag^{I*} the standard Klenow reaction buffer was used.



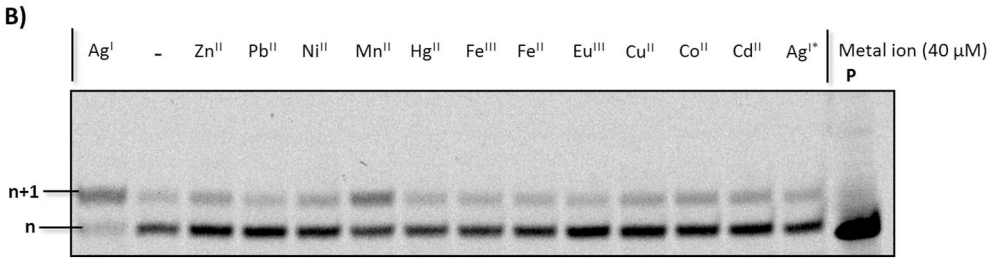


Fig. 7. Gel (PAGE 20%) analysis of the products of PEX reactions with primer **P1** and templates **T8** A) and **T9** B) with **dIm^{C}TP**. All reactions were carried out in the presence of 5 U of Taq polymerase and 200 μM **dIm^{C}TP** at 60 °C for 5 h. **P** indicates unreacted primer. For Ag^{I} the reaction was carried out in 1 × buffer 1 while for Ag^{I*} the standard Taq reaction buffer was used.

polymerase, efficient incorporation was observed with template **T8** in the presence of Ag^{+} and Mn^{2+} with appreciable yields of the n + 1 product (77% and 42%, respectively) when compared to the control reaction (only 19% of extension; Fig. 7A). Similarly, when template **T9** containing a 5 **dIm** nucleotide overhang was used in PEX reactions, the presence of Ag^{+}, Cd^{2+}, and Mn^{2+} promoted the formation of the n + 1 product with an improved efficiency compared to the PEX reaction conducted in the absence of additional metal cations (~65% compared to 28%, respectively; Fig. 7B).

3.6. TdT-mediated polymerization of *dIm^{C}TP*

The terminal deoxynucleotidyl transferase (TdT) is a Co^{2+}-dependent family X DNA polymerase which catalyzes the untemplated incorporation of nucleotides on single-stranded DNA substrates [51]. Moreover, the TdT is rather tolerant to base-modifications since nucleotides equipped with amino-acid like side chains [5,52–54], electrochemical tags [55], unnatural aromatic nucleobase analogues [56,57], and even polymerases [58] are all readily accepted as substrates. In the context of artificial metal base pairs, the TdT catalyzed the incorporation of hydroxypyridone-modified nucleotides which served for the construction of Cu^{II}-mediated metallo-DNA duplexes [37,38]. Similarly, the unmodified **dImTP** acted as an excellent substrate for the TdT which enabled the enzymatic creation of **dIm**-tags for the immobilization of DNA sequences [41]. In order to assess the impact of the presence of the carboxylic moiety on the substrate acceptance of **dIm^{C}TP** by the TdT, we conducted tailing reactions with two different metal cofactors (Mn^{2+} and Co^{2+}) and a range of reaction times (Fig. 8).

Extremely efficient TdT-mediated tailing reactions were observed when Mn^{2+} was used as cofactor since over 15 nucleotides could be incorporated in 2 h of reaction with full conversion of the primer. A further increase in the reaction time led to the formation of polydisperse, longer-sized oligonucleotides. On the other hand, when Co^{2+} was used as cofactor, 4–9 modified nucleotides were incorporated after 2 h and the formation of longer tailed sequences was not observed even after 12 h of reaction.

4. Discussion

The enzymatic construction of metal UBPs is an attractive approach to expand the genetic alphabet, yet rather strict conditions need to be fulfilled. Indeed, artificial metal UBPs need to display high thermal stabilities comparable to that of the canonical base pairs and they

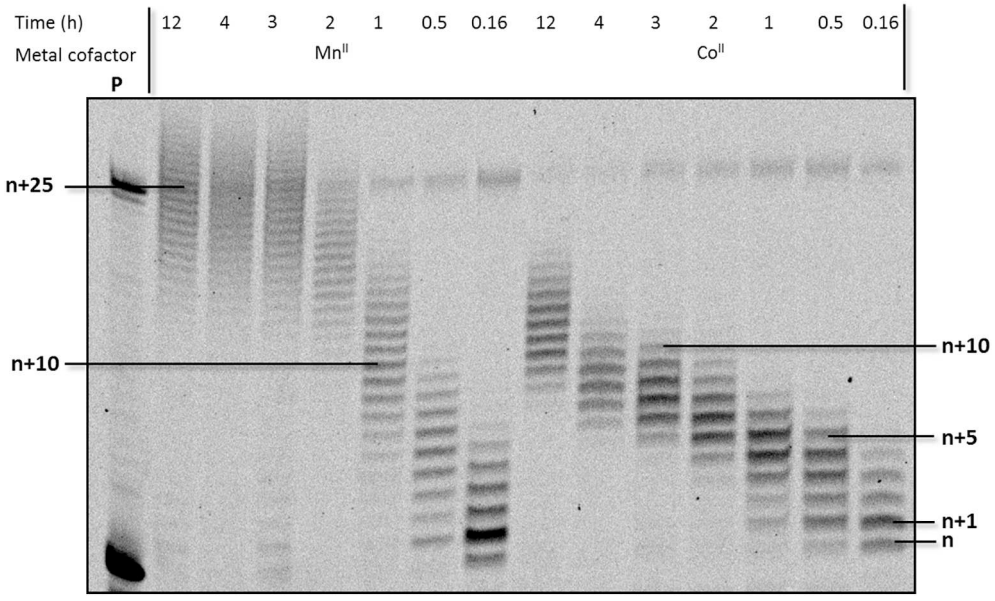


Fig. 8. Gel (PAGE 20%) image of the TdT-catalyzed extension reactions with primer **P1** and **dIm^{C}TP**.

should cause minimal distortion of B-DNA structures including primer-template duplexes while maintaining a strict orthogonality to the natural Watson-Crick system [25,35]. Also, other parameters such as size-complementarity, π-stacking, minor groove and hydrophobic interactions seem to play an important role for the enzymatic formation of these metal UBPs [42]. In the present study we strived to improve the capacity of the imidazole-modified nucleotide analog **dImTP** to form metal UBPs by including an additional ligand on the nucleobase. Appendage of this modification had various important consequences on the substrate acceptance of the resulting triphosphate **dIm^{C}TP** by DNA polymerases: 1. the modified triphosphate appears to be a rather poor substrate for Kf *exo*$^{-}$ which readily accepted **dImTP** but is rather well tolerated by the Taq polymerase instead; 2. single incorporation of **dIm^{C}MP** units clearly depended on the nature and the concentration of metal cofactors particularly when the metal ions were Mn^{2+}, Fe^{2+}, Co^{2+}, and Cd^{2+}; 3. the presence of Ag^{I} sustains the formation of a [2 + 1] coordination environment in both constellations provided that Kf *exo*$^{-}$ is the polymerase with **dImTP** as the incoming triphosphate and Taq when the triphosphate is **dIm^{C}TP**; 4. the efficiency of the TdT-mediated tailing reaction was markedly improved in presence of both cofactors compared to the homopolymerization of **dImTP**.

These features clearly demonstrate that the inclusion of an additional ligand on position 4 of the imidazole moiety improves the metal-dependence of the incorporation of the resulting triphosphate and promotes rather rare [2 + 1] coordination environment. In this context, it is not clear why the formation of the hetero base pair is favored when three (in **T6**) rather than five (in **T7**) templating **dIm^{C}** nucleotides are present.

As in the case of **dImTP**, multiple incorporation of **dIm^{C}** nucleotides into DNA was not achieved regardless of the experimental conditions that were applied. Moreover, **dIm^{C}-dIm^{C}** pairs were not bypassed by polymerases which preclude the use of this modification in SELEX experiments. Consequently, as observed for the imidazole nucleotide **dIm**, the enzymatic formation of a **dIm^{C}**-M^{n+}-**dIm^{C}** pair seems improbable. Indeed, the addition of Mn^{2+} to PEX reactions is known to relax the substrate requirements and reduce the fidelity of polymerases and enhance the incorporation of modified nucleotides into DNA including **dIm** and **dIm^{C}**. Similarly, Co^{2+} is capable of replacing Mg^{2+} of the active site and concomitantly alters the fidelity of certain polymerases including Taq [59,60].

5. Conclusions

In summary, an imidazole nucleotide equipped with an additional potential metal binding ligand was synthesized and its capacity at sustaining the enzymatic formation of metal UBPs was evaluated. A clear metal dependence was observed for the Taq-mediated single incorporation of **dIm^{C}MP** into DNA and Mn^{2+} and Co^{2+} were identified as the best metal cofactors. A [2 + 1] coordination environment was supported by the presence of Ag^{+} in primer extension reactions involving combinations of **dIm** and **dIm^{C}** nucleotides. While the **dIm^{C}** system did not support multiple incorporation events and ultimately the enzymatic formation of metal UBPs, the presence of the additional carboxylic acid moiety had an important repercussion on the biochemical properties of this nucleotide analog. Collectively, the results demonstrate that second generation imidazole nucleotides display improved substrate acceptances by DNA polymerases in the presence of specific metal cations. Other imidazole modification patterns might eventually lead to the identification of nucleotides capable of sustaining the enzymatic fabrication of artificial metal UBPs. Additionally, we are currently evaluating the possibility of using engineered polymerases to promote the enzymatic formation of imidazole-based metal UBPs [61].

Abbreviations

DMF	*N*,*N*-Dimethylformamide
dIm^{C}TP	1-(2-deoxy-β-D-ribofuranosyl) imidazole-4-carboxylate nucleoside triphosphate
dIm^{C}	1-(2-deoxy-β-D-ribofuranosyl) imidazole-4-carboxylate nucleoside
dImTP	1-(2-deoxy-β-D-ribofuranosyl) imidazole nucleoside triphosphate
dIm	1-(2-deoxy-β-D-ribofuranosyl) imidazole nucleoside
TP	Triphosphate
MP	Monophosphate
DCM	Dichloromethane
PEX	Primer extension
DMTr	4,4′-dimethoxytrityl
UBP	Unnatural base pair
DMAP	4-Dimethylaminopyridine
TEA	Triethylamine
Tris	2-Amino-2-(hydroxymethyl)propane-1,3-diol

MeCN Acetonitrile
PAGE Polyacrylamide gel electrophoresis
SELEX Systematic Evolution of Ligands by EXponential enrichment
TdT Terminal deoxynucleotidyl transferase
Vent (*exo*$^-$) Engineered form of DNA polymerase from *Thermococcus litoralis*
Therminator Engineered form of DNA polymerase from *Thermococcus* species 9°N-7.
Bst DNA polymerase from *Geobacillus stearothermophilus*
Taq DNA polymerase from *Thermus aquaticus*

Conflicts of interest

The authors declare that they have no conflict of interest.

Acknowledgements

The authors gratefully acknowledge financial support from Institut Pasteur. The start-up company DNA Script is acknowledged for help in the UPLC-MS analysis of the oligonucleotides.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jinorgbio.2018.11.009.

References

[1] J.W. Chin, Expanding and reprogramming the genetic code, Nature 550 (2017) 53–60.
[2] Y.K. Zhang, F.E. Romesberg, Semisynthetic organisms with expanded genetic codes, Biochemistry 57 (2018) 2177–2178.
[3] D.A. Malyshev, F.E. Romesberg, The expanded genetic alphabet, Angew. Chem. Int. Ed. 54 (2015) 11930–11944.
[4] A.W. Feldmann, F.E. Romesberg, Expansion of the genetic alphabet: a chemist's approach to synthetic biology, accounts, Acc. Chem. Res. 51 (2018) 394–403.
[5] K.-R. Kim, P. Röthlisberger, S.J. Kang, K. Nam, S. Lee, M. Hollenstein, D.-R. Ahn, Shaping rolling circle amplification products into DNA nanoparticles by incorporation of modified nucleotides and their application to in vitro and in vivo delivery of a photosensitizer, Molecules 23 (2018) 1833.
[6] S.A. Benner, N.B. Karalkar, S. Hoshika, R. Laos, R.W. Shaw, M. Matsuura, D. Fajardo, P. Moussatche, Alternative Watson-Crick synthetic genetic systems, Cold Spring Harb. Perspect. Biol. 8 (2016) 26.
[7] D.A. Malyshev, K. Dhami, T. Lavergne, T.J. Chen, N. Dai, J.M. Foster, I.R. Correa, F.E. Romesberg, A semi-synthetic organism with an expanded genetic alphabet, Nature 509 (2014) 385–388.
[8] Y.K. Zhang, B.M. Lamb, A.W. Feldman, A.X. Zhou, T. Lavergne, L.J. Li, F.E. Romesberg, A semisynthetic organism engineered for the stable expansion of the genetic alphabet, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) 1317–1322.
[9] Y. Zhang, J.L. Ptacin, E.C. Fischer, H.R. Aerni, C.E. Caffaro, K.S. Jose, A.W. Feldman, C.R. Turner, F.E. Romesberg, A semi-synthetic organism that stores and retrieves increased genetic information, Nature 551 (2017) 644–647.
[10] L.Q. Zhang, Z.Y. Yang, K. Sefah, K.M. Bradley, S. Hoshika, M.J. Kim, H.J. Kim, G.Z. Zhu, E. Jimenez, S. Cansiz, I.T. Teng, C. Champanhac, C. McLendon, C. Liu, W. Zhang, D.L. Gerloff, Z. Huang, W.H. Tan, S.A. Benner, Evolution of functional six-nucleotide DNA, J. Am. Chem. Soc. 137 (2015) 6734–6737.
[11] M. Kimoto, R. Yamashige, K. Matsunaga, S. Yokoyama, I. Hirao, Generation of high-affinity DNA aptamers using an expanded genetic alphabet, Nat. Biotechnol. 31 (2013) 453–457.
[12] K. Sefah, Z.Y. Yang, K.M. Bradley, S. Hoshika, E. Jimenez, L.Q. Zhang, G.Z. Zhu, S. Shanker, F.H. Yu, D. Turek, W.H. Tan, S.A. Benner, In vitro selection with artificial expanded genetic information systems, Proc. Natl. Acad. Sci. U. S. A. 111 (2014) 1449–1454.
[13] K. Matsunaga, M. Kimoto, I. Hirao, High-affinity DNA aptamer generation targeting von Willebrand factor A1-domain by genetic alphabet expansion for systematic evolution of ligands by exponential enrichment using two types of libraries composed of five different bases, J. Am. Chem. Soc. 139 (2017) 324–334.
[14] E. Biondi, J.D. Lane, D. Das, S. Dasgupta, J.A. Piccirilli, S. Hoshika, K.M. Bradley, B.A. Krantz, S.A. Benner, Laboratory evolution of artificially expanded DNA gives redesignable aptamers that target the toxic form of anthrax protective antigen, Nucleic Acids Res. 44 (2016) 9565–9577.
[15] M.R. Dunn, R.M. Jimenez, J.C. Chaput, Analysis of aptamer discovery and technology, Nat. Rev. Chem. 1 (2017) 16.
[16] P. Röthlisberger, C. Gasse, M. Hollenstein, Nucleic acid aptamers: emerging applications in medical imaging, nanotechnology, neurosciences, and drug delivery, Int. J. Mol. Sci. 18 (2017) 39.
[17] P. Röthlisberger, M. Hollenstein, Aptamer chemistry, Adv. Drug Deliv. Rev. 134 (2018) 3–21.
[18] M.P. Ledbetter, R.J. Karadeema, F.E. Romesberg, Reprograming the replisome of a semisynthetic organism for the expansion of the genetic alphabet, J. Am. Chem. Soc. 140 (2018) 758–765.
[19] K. Betz, M. Kimoto, K. Diederichs, I. Hirao, A. Marx, Structural basis for expansion of the genetic alphabet with an artificial nucleobase pair, Angew. Chem. Int. Ed. 56 (2017) 12000–12003.
[20] A.W. Feldman, V.T. Dien, F.E. Romesberg, Chemical stabilization of unnatural nucleotide triphosphates for the in vivo expansion of the genetic alphabet, J. Am. Chem. Soc. 139 (2017) 2464–2467.
[21] Q. Wang, X.Y. Xie, J. Han, G.L. Cui, QM and QM/MM studies on excited-state relaxation mechanisms of unnatural bases in vacuo and base pairs in DNA, J. Phys. Chem. B 121 (2017) 10467–10478.
[22] M. Pollum, B. Ashwood, S. Jockusch, M. Lam, C.E. Crespo-Hernandez, Unintended consequences of expanding the genetic alphabet, J. Am. Chem. Soc. 138 (2016) 11457–11460.
[23] B. Jash, J. Müller, Metal-mediated base pairs: from characterization to application, Chem. Eur. J. 23 (2017) 17166–17178.
[24] Y. Takezawa, J. Müller, M. Shionoya, Artificial DNA base pairing mediated by diverse metal ions, Chem. Lett. 46 (2017) 622–633.
[25] S. Johannsen, N. Megger, D. Bohme, R.K.O. Sigel, J. Muller, Solution structure of a DNA double helix with consecutive metal-mediated base pairs, Nat. Chem. 2 (2010) 229–234.
[26] C. Switzer, S. Sinha, P.H. Kim, B.D. Heuberger, A purine-like nickel(II) base pair for DNA, Angew. Chem. Int. Ed. 44 (2005) 1529–1532.
[27] I. Sinha, C.F. Guerra, J. Muller, A highly stabilizing Silver(I)-mediated base pair in parallel-stranded DNA, Angew. Chem. Int. Ed. 54 (2015) 3603–3606.
[28] S.K. Jana, X.R. Guo, H. Mei, F. Seela, Robust silver-mediated imidazolo-dC base pairs in metal DNA: dinuclear silver bridges with exceptional stability in double helices with parallel and antiparallel strand orientation, Chem. Commun. 51 (2015) 17301–17304.
[29] X.R. Guo, P. Leonard, S.A. Ingale, F. Seela, Gemcitabine, pyrrologemcitabine, and 2′-fluoro-2′-deoxycytidines: synthesis, physical properties, and impact of sugar fluorination on silver ion mediated base pairing, Chem. Eur. J. 23 (2017) 17740–17754.
[30] H. Mei, I. Rohl, F. Seela, Ag^+-mediated DNA base pairing: extraordinarily stable pyrrolo-dC-pyrrolo-dC pairs binding two silver ions, J. Organomet. Chem. 78 (2013) 9457–9463.
[31] B. Jash, P. Scharf, N. Sandmann, C.F. Guerra, D.A. Megger, J. Muller, A metal-mediated base pair that discriminates between the canonical pyrimidine nucleobases, Chem. Sci. 8 (2017) 1337–1343.
[32] H. Weizman, Y. Tor, 2,2′-Bipyridine ligandoside: a novel building block for modifying DNA with intra-duplex metal complexes, J. Am. Chem. Soc. 123 (2001) 3375–3376.
[33] E. Meggers, P.L. Holland, W.B. Tolman, F.E. Romesberg, P.G. Schultz, A novel copper-mediated DNA base pair, J. Am. Chem. Soc. 122 (2000) 10714–10715.
[34] G.H. Clever, C. Kaul, T. Carell, DNA-metal base pairs, Angew. Chem. Int. Ed. 46 (2007) 6226–6236.
[35] C. Kaul, M. Müller, M. Wagner, S. Schneider, T. Carell, Reversible bond formation enables the replication and amplification of a crosslinking salen complex as an orthogonal base pair, Nat. Chem. 3 (2011) 794–800.
[36] E.K. Kim, C. Switzer, Polymerase recognition of a Watson-Crick-like metal-mediated base pair: purine-2,6-dicarboxylate center dot copper(II) center dot pyridine, ChemBioChem 14 (2013) 2403–2407.
[37] T. Kobayashi, Y. Takezawa, A. Sakamoto, M. Shionoya, Enzymatic synthesis of ligand-bearing DNAs for metal-mediated base pairing utilising a template-independent polymerase, Chem. Commun. 52 (2016) 3762–3765.
[38] Y. Takezawa, T. Kobayashi, M. Shionoya, The effects of magnesium ions on the enzymatic synthesis of ligand-bearing artificial DNA by template-independent polymerase, Int. J. Mol. Sci. 17 (2016) 10.
[39] D.M. Perrin, T. Garestier, C. Helene, Bridging the gap between proteins and nucleic acids: a metal-independent RNaseA mimic with two protein-like functionalities, J. Am. Chem. Soc. 123 (2001) 1556–1563.
[40] M. Hollenstein, C.J. Hipolito, C.H. Lam, D.M. Perrin, A self-cleaving DNA enzyme modified with amines, guanidines and imidazoles operates independently of divalent metal cations (M-2), Nucleic Acids Res. 37 (2009) 1638–1649.
[41] P. Röthlisberger, F. Levi-Acobas, I. Sarac, B. Baron, P. England, P. Marliere, P. Herdewijn, M. Hollenstein, Facile immobilization of DNA using an enzymatic histag mimic, Chem. Commun. 53 (2017) 13031–13034.
[42] P. Röthlisberger, F. Levi-Acobas, I. Sarac, P. Marliere, P. Herdewijn, M. Hollenstein, On the enzymatic incorporation of an imidazole nucleotide into DNA, Org. Biomol. Chem. 15 (2017) 4449–4455.
[43] S. Kumbhar, S. Johannsen, R.K.O. Sigel, M.P. Waller, J. Müller, A QM/MM refinement of an experimental DNA structure with metal-mediated base pairs, J. Inorg. Biochem. 127 (2013) 203–210.
[44] K. Schweizer, J.C. Leon, B.J. Ravoo, J. Müller, Thermodynamics of the formation of Ag(I)-mediated azole base pairs in DNA duplexes, J. Inorg. Biochem. 160 (2016) 256–263.
[45] S. Pochet, L. Dugue, Imidazole-4-carboxamide and 1,2,4-triazole-3-carboxamide deoxynucleotides as simplified DNA building blocks with ambiguous pairing capacity, Nucleosides Nucleotides 17 (1998) 2003–2009.
[46] J. Ludwig, F. Eckstein, Rapid and efficient synthesis of nucleoside 5′-O-(1-thiotriphosphates), 5′-triphosphates and 2′,3′-cyclophosphorothioates using 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one, J. Organomet. Chem. 54 (1989) 631–635.
[47] M. Hocek, Synthesis of base-modified 2′-deoxyribonucleoside triphosphates and

their use in enzymatic synthesis of modified DNA for applications in bioanalysis and chemical biology, J. Organomet. Chem. 79 (2014) 9914–9921.
[48] J. Aschenbrenner, A. Marx, DNA polymerases and biotechnological applications, Curr. Opin. Biotechnol. 48 (2017) 187–195.
[49] P.L. Holland, Electronic structure and reactivity of three-coordinate iron complexes, Acc. Chem. Res. 41 (2008) 905–914.
[50] T. Richters, J. Müller, A metal-mediated base pair with a 2 + 1 coordination environment, Eur. J. Inorg. Chem. 2014 (2014) 437–441.
[51] E.A. Motea, A.J. Berdis, Terminal deoxynucleotidyl transferase: the story of a misguided DNA polymerase, Biochim. Biophys. Acta, Proteins Proteomics 1804 (2010) 1151–1166.
[52] M. Takahara, R. Wakabayashi, K. Minamihata, M. Goto, N. Kamiya, Primary amine-clustered DNA aptamer for DNA-protein conjugation catalyzed by microbial transglutaminase, Bioconjug. Chem. 28 (2017) 2954–2961.
[53] M. Hollenstein, Deoxynucleoside triphosphates bearing histamine, carboxylic acid, and hydroxyl residues - synthesis and biochemical characterization, Org. Biomol. Chem. 11 (2013) 5162–5172.
[54] R. Gu, T. Oweida, Y.G. Yingling, A. Chilkoti, S. Zauscher, Enzymatic synthesis of nucleobase-modified single-stranded DNA offers tunable resistance to nuclease degradation, Biomacromolecules 19 (2018) 3525–3535.
[55] P. Horakova, H. Macickova-Cahova, H. Pivonkova, J. Spacek, L. Havran, M. Hocek, M. Fojta, Tail-labelling of DNA probes using modified deoxynucleotide triphosphates and terminal deoxynucleotidyl transferase. Application in electrochemical DNA hybridization and protein-DNA binding assays, Org. Biomol. Chem. 9 (2011) 1366–1371.
[56] M. Hollenstein, F. Wojciechowski, C.J. Leumann, Polymerase incorporation of pyrene-nucleoside triphosphates, Bioorg. Med. Chem. Lett. 22 (2012) 4428–4430.
[57] Y.J. Cho, E.T. Kool, Enzymatic synthesis of fluorescent oligomers assembled on a DNA backbone, ChemBioChem 7 (2006) 669–672.
[58] S. Palluk, D.H. Arlow, T. de Rond, S. Barthel, J.S. Kang, R. Bector, H.M. Baghdassarian, A.N. Truong, P.W. Kim, A.K. Singh, N.J. Hillson, J.D. Keasling, De novo DNA synthesis using polymerase-nucleotide conjugates, Nat. Biotechnol. 36 (2018) 645–650.
[59] A.K. Vashishtha, J.M. Wang, W.H. Konigsberg, Different divalent cations alter the kinetics and fidelity of DNA polymerases, J. Biol. Chem. 291 (2016) 20869–20875.
[60] G. Stengel, B.W. Purse, R.D. Kuchta, Effect of transition metal ions on the fluorescence and Taq-catalyzed polymerase chain reaction of tricyclic cytidine analogs, Anal. Biochem. 416 (2011) 53–60.
[61] V.B. Pinheiro, A.I. Taylor, C. Cozens, M. Abramov, M. Renders, S. Zhang, J.C. Chaput, J. Wengel, S.Y. Peak-Chew, S.H. McLaughlin, P. Herdewijn, P. Holliger, Synthetic genetic polymers capable of heredity and evolution, Science 336 (2012) 341–344.