Cross-coupling reactions of nucleoside triphosphates followed by polymerase incorporation. Construction and applications of base-functionalized nucleic acids

Michal Hocek*[a] and Miroslav Fojta[b]

Received 3rd March 2008, Accepted 19th March 2008
First published as an Advance Article on the web 2nd April 2008
DOI: 10.1039/b803664k

Construction of functionalized nucleic acids (DNA or RNA) *via* polymerase incorporation of modified nucleoside triphosphates is reviewed and selected applications of the modified nucleic acids are highlighted. The classical multistep approach for the synthesis of modified NTPs by triphosphorylation of modified nucleosides is compared to the novel approach consisting of direct aqueous cross-coupling reactions of unprotected halogenated nucleoside triphosphates. The combination of cross-coupling of NTPs with polymerase incorporation gives an efficient and straightforward two-step synthesis of modified nucleic acids. Primer extension using biotinylated templates followed by separation using streptavidine-coated magnetic beads and DNA duplex denaturation is used for preparation of modified single stranded oligonucleotides. Examples of using this approach for electrochemical DNA labelling and bioanalytical applications are given.

Introduction

Functional nucleic acids (such as DNA aptamers,[1,2] and DNAzymes[1,3]) have attracted growing interest due to potential applications in chemical biology, bioanalysis or nanotechnology and material science.[4] To expand the scope of these applications, the introduction of a variety of functional groups to DNA and RNA, especially to the nucleobase, is highly desirable.

A classical approach to modified nucleic acids is the solid-phase oligonucleotide (ON) synthesis using functionalized nucleoside phosphoramidites[5] or by post-synthetic oligonucleotide modification.[6,7] The oligonucleotide synthesis is often problematic due to incompatibility of the additional functional groups with the phosphoramidite methodology (acidic detritylation, coupling, capping, oxidation and final acyl protecting groups cleavage by ammonia), necessity of using additional protecting groups, and, sometimes, low yields of the coupling step. On the other hand, post-synthetic modification of ONs requires selective and mild reactions and in most cases has been used only for further functional group transformations of already modified ONs.

Apart from chemical synthesis, modified nucleic acids can be prepared enzymatically by incorporation of modified nucleoside triphosphates (NTPs) by DNA or RNA polymerases (Scheme 1).[8] This account reviews enzymatic incorporation of modified NTPs to nucleic acids as an alternative strategy for the construction of functionalized nucleic acids and compares classical multistep approaches to the synthesis of modified NTPs with novel direct cross-coupling reactions. The most important example of polymerase

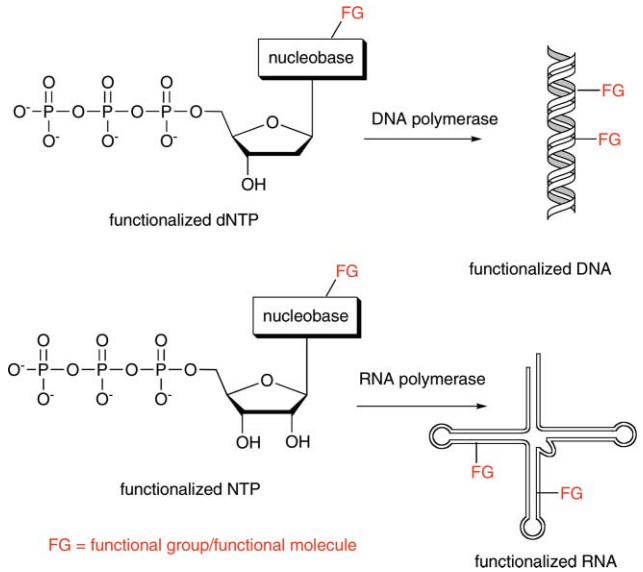
[a]*Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Gilead & IOCB Research Center, Flemingovo nam. 2, CZ-16610 Prague 6, Czech Republic. E-mail: hocek@uochb.cas.cz; Fax: +420 220183559; Tel: +420 220183324*
[b]*Institute of Biophysics, Academy of Sciences of the Czech Republic, Kralovopolska 135, CZ-612 65 Brno, Czech Republic*

Michal Hocek

Michal Hocek was educated at the ICT Prague and got his PhD at IOCB ASCR in 1996. After a postdoc at UCL in Belgium, he returned to IOCB and became a group leader in 2003. In 2006 he got his habilitation at the ICT Prague and defended his DSc degree. His research interests include synthetic, bioorganic and medicinal chemistry of nucleosides, nucleotides and nucleic acids.

Miroslav Fojta

Miroslav Fojta was educated at the Charles University in Prague and in 1995 he earned his PhD at IBP ASCR where he started his independent research and became the head of department in 2005. In the same year he finished his habilitation at the Masaryk University Brno. His research interests include electrochemistry of nucleic acids and its application in bioanalysis and DNA–protein interactions.



Scheme 1 General scheme of polymerase construction of functionalized DNA and RNA.

incorporation of modified NTPs is DNA sequencing using chain termination by fluorescently labeled dideoxynucleoside triphosphates (ddNTPs).[9] As it is beyond the scope of this account, it will not be discussed here and we will only focus on non-terminating incorporations of base-modified 2′-deoxyribonucleoside triphosphates (dNTPs) to DNA and ribonucleoside triphosphates (NTPs) to RNA.

Polymerase construction of modified nucleic acids and syntheses of dNTPs and NTPs

Polymerase incorporations of base-modified dNTPs to DNA

The very first polymerase incorporation of base-functionalized dNTPs was published in 1981 by Langer *et al.*[10] who prepared dUTP biotinylated in position 5 and found that it is a substrate for several DNA polymerases. Other biotinylated DNA syntheses by polymerases followed[11,12] soon thereafter. Latham *et al.*[13] prepared 5-(pent-1-yn-1-yl)-dUTP and performed polymerase incorporation to generate a library of DNA sequences in order to develop a thrombin aptamer. Later on, Sakthivel and Barbas[14] prepared a series of amides of 5-(3-aminoprop-1-en-1-yl)-dUTPs bearing diverse functional groups (imidazole, pyridine, carboxylate *etc.*) and performed PCR incorporation using Taq polymerase. Most of the modified dUTPs were good substrates and formed full-length modified DNA strands. A library of modified DNA was generated in order to select catalytically active DNAs.

Following these pioneering works, from 2001 other groups have entered the field and used polymerase incorporations of modified dNTPs to construct functional DNA molecules. Williams *et al*.[15] have further contributed to the enhancement of the catalytic repertoire of DNA by synthesis of dUTPs functionalized with imidazole connected *via* an amide bond to 3-aminopropargyl, -propenyl or -propyl linkers attached at position 5. 7-Deazaadenine was used[16] as an adenine surrogate in the construction of the corresponding dATP analogues bearing the same functionality. Brackmann and Lobermann[17] have used FluoroRed (TMR) labeled dUTP and rhodamine110-labeled dCTP for high-density fluorescent labeling of DNA by primer extension using Klenow exo$^-$ polymerase. Sawai *et al.*[18] prepared a series of 5-functionalized dUTPs bearing diverse amino, imidazole, carboxylate and biotin functions and studied PCR incorporations by KOD Dash, Taq, Tth and Vent polymerases to find that KOD Dash was the most efficient. Held and Benner[19] have prepared several protected (in the form of *t*-BuSS-function) thiol-containing dUTPs and performed PCR using 8 different polymerases to find that Pwo and Pfu polymerases were the most efficient to amplify DNA sequences with this modification. The Tor group has reported[20] incorporation of dUTP containing Ru or Os complexes of bipyridine ligands.

Famulok *et al.*[21,22] were the first to prepare a complete set of four modified dNTPs (5-substituted dUTPs and dCTPs and 7-substituted 7-deaza-dATPs and 7-deaza-dGTPs, as well as 8-substituted dATPs and dGTPs) and study their PCR incorporation using a series of polymerases. He found[22] that 8-substituted dATPs and dGTPs are not good substrates for polymerases, while the 7-substituted 7-deazapurine nucleoside triphosphates are efficiently incorporated. He has also found[22] that B-family polymerases, in particular Pwo and Vent (exo$^-$), are more efficient in PCR incorporation of the modified dNTPs than A-family polymerases (*i.e.* Taq).

Burgess *et al.*[23] prepared TAMRA-labeled dUTPs where the fluorescent label was attached *via* a conjugated acetylene linker to position 5 of uracil. The nucleotides were then incorporated by TaqFS polymerase by primer extension to yield fluorescent DNA conjugates. More recently, Sawai *et al.* have constructed modified DNA bearing amino and cyano groups, guanidine,[24] amino acids,[25] and acridone,[26] and have used some of them for further post-synthetic modifications.[27] Ferrocene-modified DNAs are attractive for electrochemical detection and, therefore, several types of ferrocene-modified dNTPs were prepared,[28] incorporated and used for bioanalytical applications. Ebara *et al.*[29] have recently reported saccharide-modified DNA by PCR incorporation of maltose- and lactose-containing dUTPs. Finally, the Carell group has reported on synthesis and PCR incorporation of alkyne-modified dNTPs and the post-synthetic modifications of the alkynylated DNA *via* Huisgen–Sharpless click reaction with azides. This approach has been used for attachment of sugars,[30] for DNA metallization,[31] and for assembly of gold nanoparticles.[32]

From the reported studies, no definitive conclusions about a universally applicable DNA polymerase can be made. Sawai *et al.* have recently reviewed[33] the efficiency of diverse DNA polymerases for incorporation of 5-substituted pyrimidine dNTPs to confirm that B-family polymerases (KOD Dash, Pwo and Vent) are generally more efficient than A-family polymerases. However, for each novel dNTP, screening of several polymerases is necessary in order to find an efficient one for that particular nucleotide.

Polymerase incorporations of base-modified NTPs to RNA

Due to higher structural diversity and the multiple biological roles of RNA, it is even more interesting to construct modified RNA. Base-functionalized NTPs have also been used for polymerase incorporation to RNA but have received considerably less

attention due to difficult handling of modified RNA as compared to modified DNA. Biotinylated UTP was the first example of a modified NTP incorporated[10] by T7 RNA polymerase. Eaton *et al.* have developed the synthesis of several 5-substituted UTPs and T7 polymerase-catalyzed incorporation to RNA[34] and used this approach for *in vitro* selection of RNA amide synthases.[35] Later on, the Eaton group published the construction of modified RNA bearing amino and heteroaryl groups linked *via* 5-carboxamide to U using T7-catalyzed incorporation of modified UTPs.[36] McLaughlin *et al*. made 5-aminoalkyl- and 5-sulfanylalkyl-modified UTPs, studied their incorporation by T7 polymerase and used this approach for selection of RNA aptamers.[37] Very recently, Srivatsan and Tor reported the synthesis of fluorescent 5-furylpyrimidine NTPs and their incorporation into RNA by T7 polymerase.[38] 5′-End fluorescein or biotin labelling was achieved by *in vitro* transcription with N^6-modified AMP.[39]

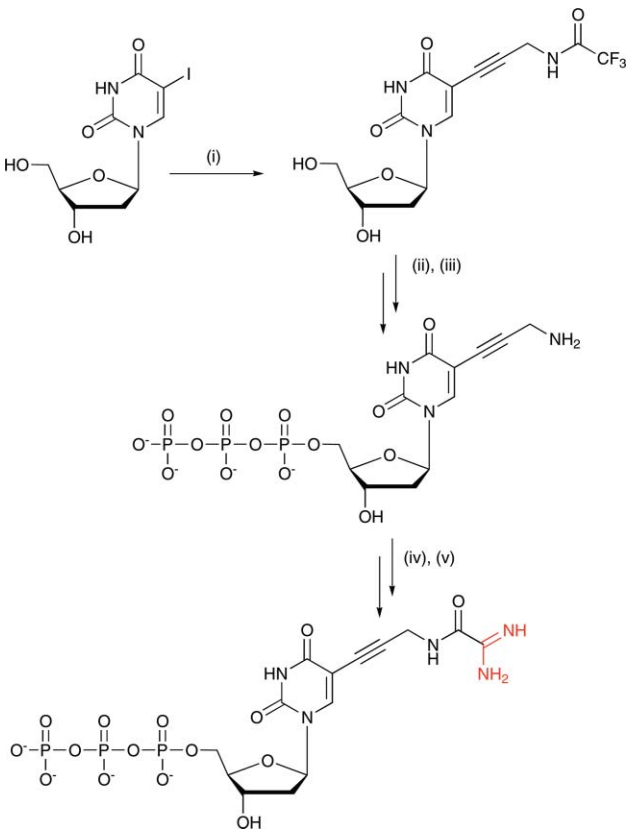
Hirao has developed two new base pairs as an extension of the genetic alphabet and has used the specific transcription of these novel base pairs for single point modification of RNA.[40] Thus iodopyridone was introduced as a photocross-linking component into RNA by T7 transcription[41] *via* a base-pair with 6-thienyl-2-aminopurine. Fluorescent labelling (FAM, TAMRA, Dansyl) of RNA was achieved by incorporation[42] of modified pyridone NTP *via* base-pairing with 6-thienyl- or 6-thiazolyl-2-aminopurine. This study also led to the development of a fluorescent aptamer for theophilin.[42] Biotinylated RNA was constructed by incorporation of biotin-modified pyridone NTP *via* a base-pair with 6-thienyl- or 6-thiazolyl-2-aminopurine[43] or by incorporation of biotin-modified pyrrole-1-carbaldehyde NTP *via* a base-pair with 6-thienyl-1-deazapurine.[44] This unique approach is apparently the best solution for incorporation of single modification into a specific position in RNA (but should be also applicable to DNA).

As a rule, all the incorporations of functionalized NTPs into RNA were catalyzed by T7 RNA polymerase and the DNA templates contained a promotor sequence.

Classical multistep syntheses of base-modified NTPs and dNTPs

The dNTP and NTP building blocks are usually prepared[10–22,24–44] by troublesome and laborious triphosphorylation of the corresponding modified nucleosides, where the functional groups usually have to be protected and deprotected. The most common approach[14–17,21,22] consists in multistep synthesis of aminopropargyl-, aminopropenyl- or aminopropyl-substituted dNTPs or NTPs *via* Sonogashira cross-coupling of halogenated nucleosides with CF_3CO-protected propargylamine, followed by triphosphorylation and deprotection (for a typical example, see Scheme 2). The desired functional molecule is then attached to the amino group *via* amide bond formation. The example[22] shown in Scheme 2 needs 5 steps and 5 separations (of which 4 separations are of rather labile triphosphates) to get the desired amidine-modified dUTP in 8% overall yield. In other cases, the total yields are higher but still the reaction sequence is rather laborious.

Another approach to 5-modification of pyrimidine dNTPs consisted in generation[10,29] of a 5-chloromercury derivative of nucleoside or dNTP followed by coupling with functionalized alkene. This approach is however hardly acceptable due to the toxicity of organomercury compounds where the residual Hg



Scheme 2 Typical multistep synthesis of modified dUTP. *Reagents and conditions*: (i) $CF_3CONHCH_2C{\equiv}CH$, CuI, $Pd(PPh_3)_4$, Et_3N, DMF (84%); (ii) 1. $POCl_3$, proton sponge, $PO(OMe)_3$; 2. $(Bu_3NH)_2H_2P_2O_7$, Bu_3N, DMF; 3. 1 M TEAB; (iii) aq. NH_3 (48% over two steps); (iv) *N*,*N*′-di-Boc-*N*″-Ms-guanidine, Et_3N, dioxane, H_2O (72%); (v) CF_3COOH (27%). In total 5 steps, 5 purifications, overall yield 8%. Example taken from ref. 22*b*.

may influence the biological activity of the prepared nucleotides and nucleic acids. Sawai *et al.* usually used[18,24–27] attachment of functionality *via* an amide bond to 5-(carboxymethyl)pyrimidine but, again, the synthesis of the modified dNTPs requires laborious multistep sequences with rather low total yields. Therefore, there was a great need for a simple and straightforward methodology for introduction of functional groups directly into dNTPs.

Aqueous cross-coupling reactions for synthesis of base-modified dNTPs/NTPs

Cross-coupling reactions are the most efficient methodology for C–C bond formation and are also commonly used for base-modification of pyrimidine and purine nucleosides.[45] In the past, the reactions were usually performed on protected nucleosides in organic solvents and the desired free nucleosides had to be prepared by deprotection of intermediates. Recently, with the discovery of water soluble catalytic systems, aqueous-phase cross-coupling reactions have been developed[46] and Shaughnessy *et al.*[47] were the first who applied them for arylation of unprotected halopurine nucleosides by Suzuki–Miyaura reaction with arylboronic acids in the presence of tris(3-sulfonylphenyl)phosphine (TPPTS), $Pd(OAc)_2$ and inorganic base (*i.e.* Na_2CO_3) in a mixture of water–acetonitrile. Burgess *et al.* have developed[23] the first Sonogashira

cross-coupling reactions of 5-iodo-dUTP with terminal acetylenes derived from fluorescent dyes which was the very first cross-coupling reaction on dNTPs.

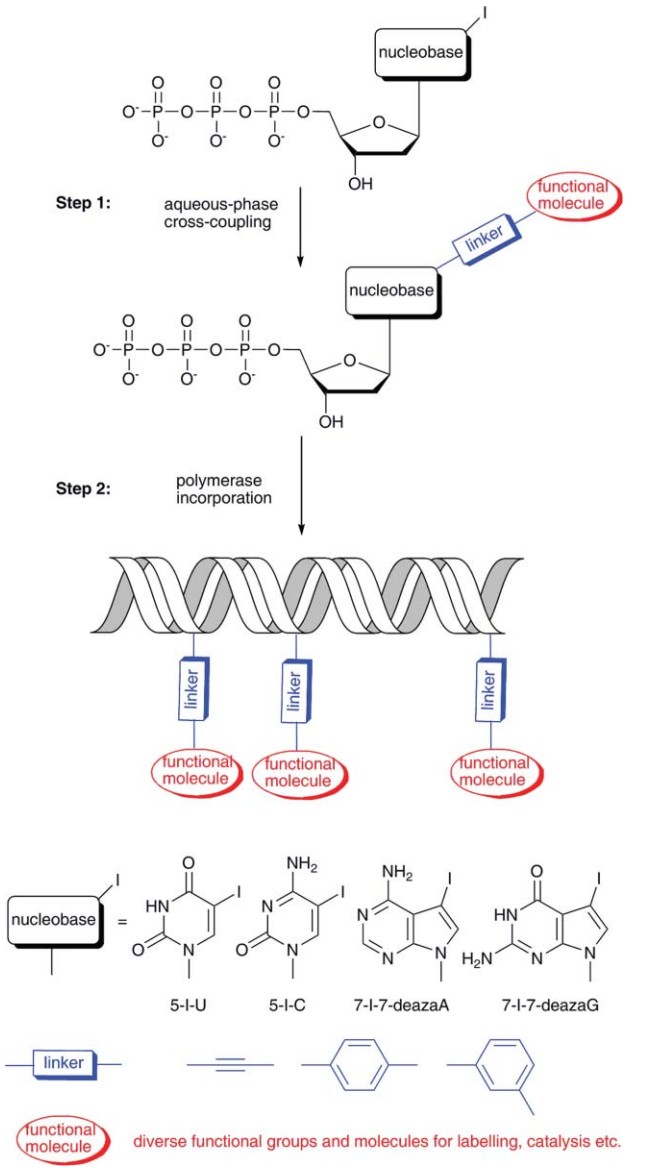
We have applied analogous aqueous-phase Suzuki–Miyaura methodology for the arylation of unprotected halopurine bases[48] and for arylation of 8-bromoadenosines with boronophenylalanine[49] and for attachment of bipyridine ligands and Ru-complexes to purine bases[50] and nucleosides.[51] Later on, we further optimized the conditions in order to apply the reactions for modification of nucleotides.[52] Nucleotides (and in particular NTPs) are rather labile compounds that easily undergo hydrolysis in aqueous solutions. We found that when using stronger base (Cs_2CO_3) and short reaction times (<1 h), the nucleotides are reasonably stable in undergoing the cross-coupling. Thus we have developed a single step arylation of 8-bromoadenosine monophosphates, NTPs and dNTPs in acceptable *ca*. 50% yields after separation by reversed phase chromatography on C18. It was the first example of the Suzuki reaction on NTPs. Wagner *et al.* have also independently reported on the arylation of cyclic 8-bromoinosine 5′-diphosphate ribose[53] and 8-bromoguanine NTPs[54] and GDP-sugars[55] under analogous conditions.

Combination of aqueous cross-coupling reactions of dNTPs with polymerase incorporations

Having an efficient single-step access to modified nucleoside triphosphates by the aqueous-phase Suzuki or Sonogashira couplings, an obvious application would be the use of these NTPs and dNTPs for polymerase incorporations to nucleic acids. Such an approach represents a very straightforward two-step methodology for the construction of modified DNA or RNA bearing diverse useful functionalities (Scheme 3). The corresponding halogenated dNTPs are accessible by chemical triphosphorylation[56] of halogenated nucleosides (5-I-dU and 5-I-dC are commercially available, 7-I-7-deaza-dA[57] and 7-I-7-deaza-dG[58] nucleosides must be prepared by multistep procedures).

The first example of the two-step construction of functionalized DNA was the paper by Burgess *et al.* in 2003[23] reporting on the first Sonogashira coupling of 5-I-dUTP with fluorescein-linked terminal acetylenes and their incorporation into DNA. However, no other example was published until we developed the complementary Suzuki–Miyaura arylation.[52] Certainly, we became very interested in utilizing this approach for modification of DNA.

Having access to purine dNTPs bearing a phenylalanine moiety in position 8 we wanted to test their polymerase incorporation. However, from previous studies[22] it was clear that 8-substituted purine dNTPs are very poor substrates for DNA polymerases probably due to sterical hindrance causing the *syn*-conformation of the nucleobase. As 7-substituted 7-deazapurine dNTPs were good substrates for some DNA polymerases, we have extended the series of phenylalanine bearing dNTPs. In order to study the influence of the linker and of the nucleobase, we have prepared[59] another four types of conjugates. The aqueous Sonogashira cross-coupling reactions of 5-I-dUTP or 7-I-7-deaza-dATP with 4-ethynylphenylalanine in the presence of TPPTS, $Pd(OAc)_2$, Et_3N and CuI in H_2O–acetonitrile at 60 °C gave the corresponding acetylene linked dNTPs (**dU^{CCPhe}TP** or **dA^{CCPhe}TP**) in good yields of 66–67% (Scheme 4). The aqueous Suzuki–Miyaura reaction

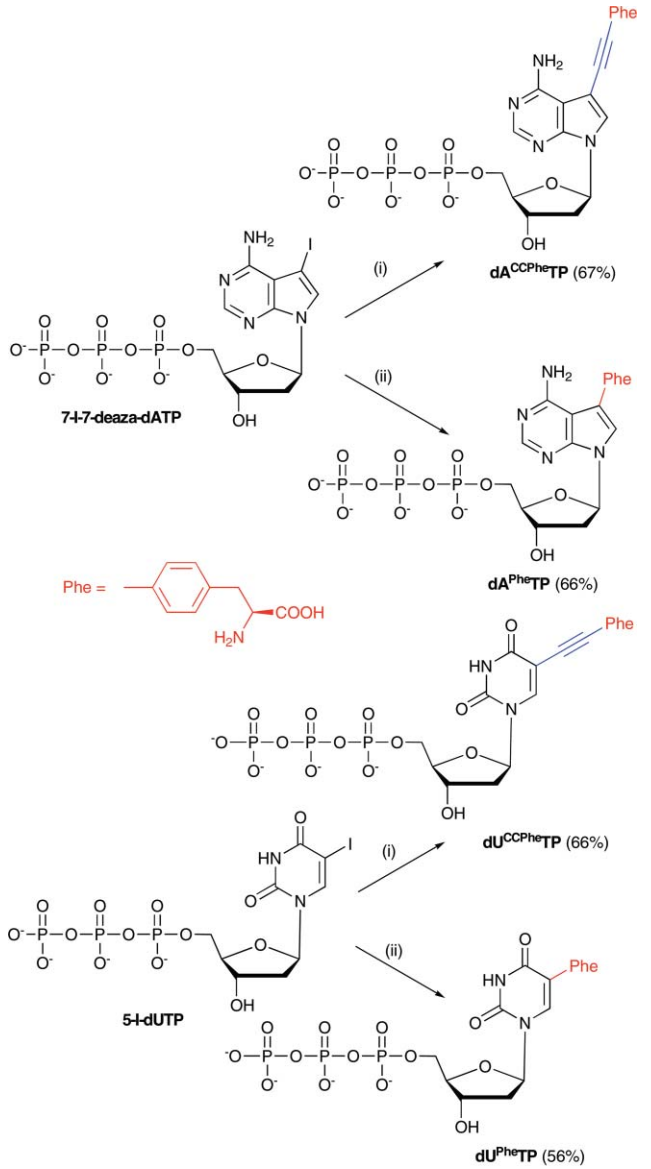


Scheme 3 General scheme for a two-step construction of functionalized DNA.

with 4-boronophenylalanine in the presence of the same catalyst and Cs_2CO_3 at 110 °C gave the aryl-linked dNTPs (**dU^{Phe}TP** and **dA^{Phe}TP**).

In order to develop electrochemical labelling of DNA, we have envisaged ferrocene (Fc) linked *via* a conjugated acetylene linker as a suitable marker for detection *via* reversible electrochemical oxidation. The conjugated linker should transfer electronic effects from nucleobase to the label and thus respond to hybridization by changing the redox potential of the label. The aqueous Sonogashira reaction of 5-I-dUTP and 7-I-7-deaza-dATP with ethynylferrocene under the above mentioned conditions gave[60] the corresponding Fc-linked dNTPs (**dU^{CCFc}TP** and **dA^{CCFc}TP**) in 42–48% yields (Scheme 5).

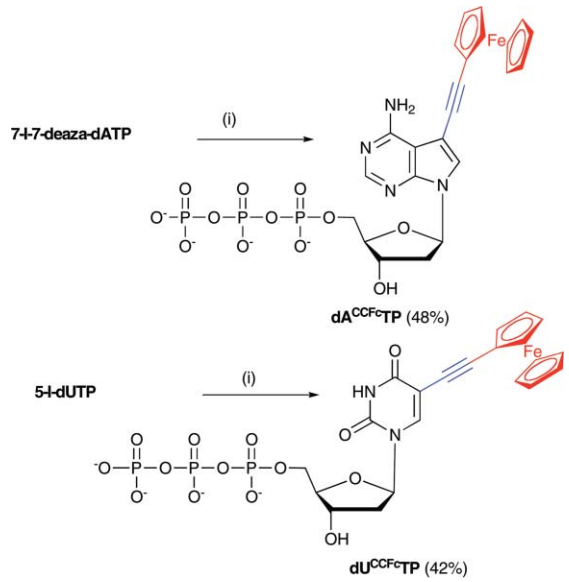
Finally, amino- and nitrophenyl groups were designed as new redox labels for DNA. Especially the nitro group appears promising for sensitive detection due to a high number of electrons (4 or 6) collected per NO_2 group reduction. The corresponding modified



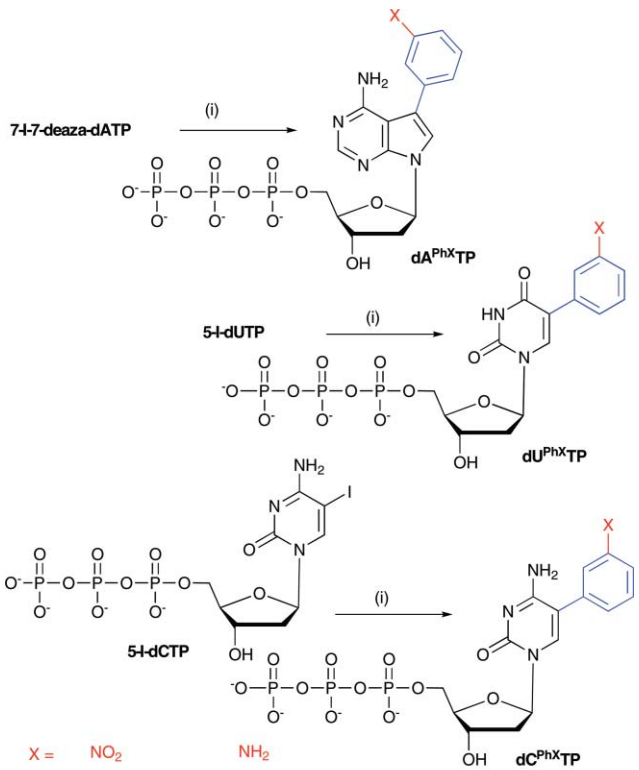
Scheme 4 Synthesis of phenylalanine-linked dNTPs. (i) 4-Ethynylphenylalanine, $Pd(OAc)_2$, TPPTS, CuI, Et_3N, acetonitrile–H_2O 1 : 2, 60–70 °C, 45–60 min; (ii) 4-boronophenylalanine, Cs_2CO_3, $Pd(OAc)_2$, TPPTS, H_2O–CH_3CN 2 : 1, 110–120 °C, 30 min; yields in parentheses for each compound.

dNTPs were prepared[61] by the aqueous Suzuki–Miyaura cross-coupling of 7-I-7-deaza-dATP, 5-I-dUTP and here also 5-I-dCTP with 3-nitrophenyl- or 3-aminophenylboronic acid (Scheme 6). The more reactive electron rich aminophenylboronic acid gave the desired products (**dA^{PhNH2}TP**, **dU^{PhNH2}TP** and **dC^{PhNH2}TP**) in better yields (40–43%) than less reactive nitrophenylboronic acid (**dA^{PhNO2}TP**, **dU^{PhNO2}TP** and **dC^{PhNO2}TP**, 26–28%).

In all cases, the functionalized dNTP products were isolated by RP HPLC or RP flash chromatography (depending on the scale of the reaction). As the procedure requires just a single chemical step and single separation, it is the most efficient method for the preparation of aryl- or alkynyl-modified dNTPs suitable for the preparation of sufficient amounts of dNTPs necessary for full characterization and for many polymerase incorporation



Scheme 5 Synthesis of ferrocene-linked dNTPs. (i) Fc-acetylene, $Pd(OAc)_2$, TPPTS, CuI, Et_3N, H_2O–CH_3CN 2 : 1, 60–70 °C, 45–60 min; yields are in parentheses for each compound.



Scheme 6 Synthesis of nitro and aminophenyl-linked dNTPs. (i) 3-NO_2-PhB$(OH)_2$ or 3-NH_2-PhB$(OH)_2$, Cs_2CO_3, $Pd(OAc)_2$, TPPTS, H_2O–CH_3CN 2 : 1, 110–120 °C, 30 min; yields in parentheses for each compound.

experiments (*vide infra*). The modified dNTPs are stored as lyophilizates at −20 °C and are sufficiently stable for several months. On the other hand, their aqueous solutions should be

Table 1 Incorporation of modified dNTPs by DNA polymerases

dNTP	Polymerase				Ref.
	Klenow	DyNAzyme	Vent (exo⁻)	Pwo	
dA^{CCPhe}TP	n.t.	n.t.	+	+++	59
dU^{CCPhe}TP	n.t.	n.t.	+	+++	59
dA^{Phe}TP	n.t.	n.t.	+	++	59
dU^{Phe}TP	n.t.	n.t.	+	++	59
dA^{CCFe}TP	+++	++	n.t.	n.t.	60
dU^{CCFe}TP	+++	++	n.t.	n.t.	60
dA^{PhNH2}TP	+++	+++	n.t.	n.t.	61
dU^{PhNH2}TP	+++	+++	n.t.	n.t.	61
dC^{PhNH2}TP	+++	+++	n.t.	n.t.	61
dA^{PhNO2}TP	+++	+++	n.t.	n.t.	61
dU^{PhNO2}TP	+++	+++	n.t.	n.t.	61
dC^{PhNO2}TP	+++	+++	n.t.	n.t.	61

+ single incorporation to a small extent, ++ good incorporation in some sequences, +++ excellent incorporation even for difficult sequences, n.t. not tested

used as soon as possible and we observed partial decomposition even when stored in the freezer after several months.

All three types of functionalized dNTPs were tested as substrates for DNA polymerases and incorporated into DNA either by primer extenstion (PEX) or by PCR (see Table 1). Amino acid modified 8-substituted dATPs were not incorporated by any polymerase. However, the corresponding amino acid derivatives of 5-substituted dUTP (**dU^{CCPhe}TP** and **dU^{Phe}TP**), as well as 7-substituted 7-deaza-dATP (**dA^{CCPhe}TP** and **dA^{Phe}TP**)[59] were all excellent substrates for thermostable Pwo polymerase and were efficiently incorporated both by PEX and by PCR. Also combinations of modified dATP and modified dUTP were efficiently incorporated by Pwo using both methods. From other tested polymerases, Vent (exo⁻) gave some incorporation to a small extent and could not have been used for PCR, while Taq polymerase did not catalyze any incorporation of those dNTPs.

The Fc-labeled dNTPs were incorporated[60] by PEX using Klenow (exo⁻) polymerase at 37 °C or using thermostable DyNAzyme at 60 °C (Fig. 1). The use of Klenow polymerase gave efficient incorporation even of two labels next to each other but, on the other hand, also gave higher frequency of erroneous nucleotide

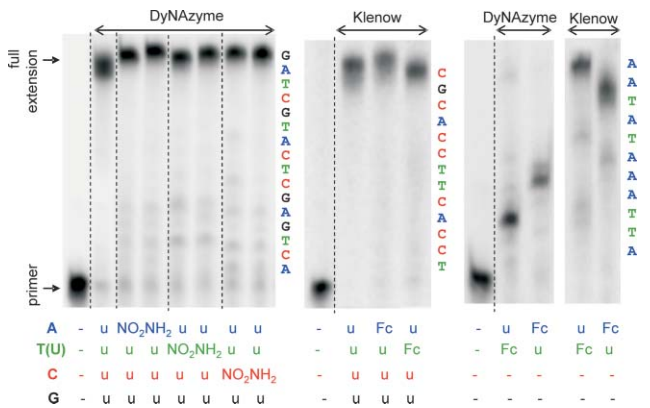


Fig. 1 Analysis of products of the PEX incorporation of nitrophenyl (NO_2), aminophenyl (NH_2) or ferrocene (Fc) conjugates of A, U or C nucleotides. Composition of the dNTP mixes and nucleotide labeling are indicated at the bottom, the DNA polymerases used at the top; u = unmodified.

incorporation. DyNAzyme gave more precise incorporation but a tendency for early termination at sites of clustered conjugate incorporation was observed (Fig. 1). Therefore the Klenow enzyme appears to be more convenient for incorporation of large number of the Fc-tags into the synthesized DNA strand (*e.g.*, tail-labelling of signaling hybridization probes), while the DyNAzyme is suitable for more precise incorporation of one or several Fc-labels (*e.g.*, DNA minisequencing).

PEX incorporation of amino- and nitrophenyl-dNTPs was also studied[61] using Klenow (exo⁻) at 37 °C or DyNAzyme at 60 °C. Both enzymes worked efficiently to synthesize all types of sequences even with multiple incorporation of modified dNTPs.

Electrochemical detection and bioanalytical applications

Ferrocene and amino- or nitrophenyl groups were designed and incorporated as redox labels for electrochemical detection. Due to electronic coupling *via* the conjugate ethynyl or phenylene bridge, redox potentials of the Fc, NH_2 and NO_2 labels depend on the nucleobase type. Moreover, the peak potentials respond to incorporation into ON by remarkable potential shifts, while differences between individual nucleobases labeled with the same marker are retained (see Fig. 2 for examples). Thus, the multipotential electrochemical DNA sensing (*vide infra*) can be achieved not only *via* combination of different DNA tags, but also *via* exploiting distinct redox properties of the same label bound to different nucleobases. On the other hand, the presented cross-coupling dNTP strategy paves the way towards preparation of a broad spectrum of electroactive DNA markers. These markers need not necessarily give reversible redox electrochemistry (that is essential for some specific applications, such as regenerable electrochemical molecular beacons[62] or some electrocatalytic systems[63]). It has been demonstrated[61] that a combination of irreversibly reducible NO_2 and irreversibly oxidizable NH_2 tags offers their easy detection and perfect discrimination within one ON chain.

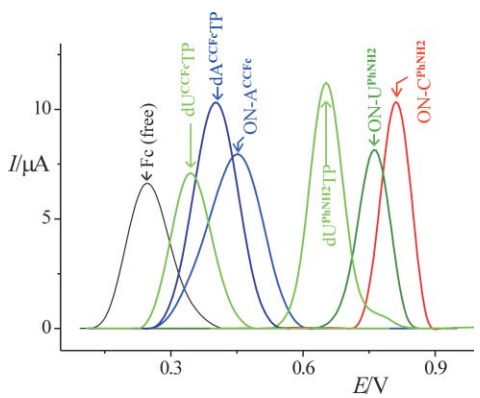
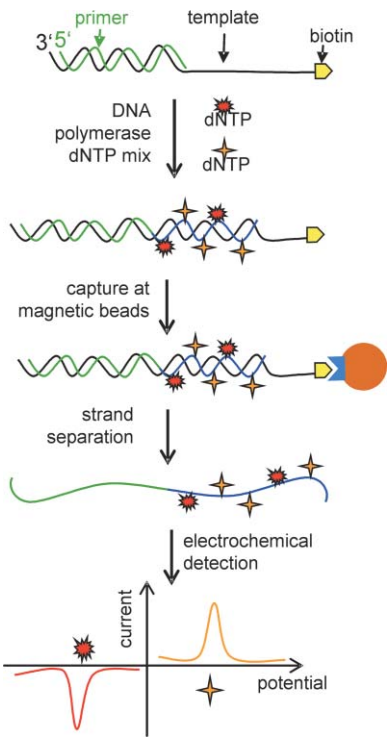


Fig. 2 Examples of voltammetric signals of ferrocene (Fc) or 3-aminophenyl tags coupled to nucleobases within dNTPs or incorporated in oligonucleotides (ON).

Review of broad applications of fluorescent dye-labeled dNTPs (or 2′,3′-dideoxy-NTPs) in DNA sequencing, arrayed primer extension (APEX)[64] genotyping or gene expression monitoring, is out of the scope of this article. Electrochemical techniques in connection with the PEX incorporation approaches have been

applied mainly with the ferrocene markers or biotin tags to which another label (such as an enzyme), was subsequently attached. Different ferrocene derivatives were developed and used[28] for electrochemical multipotential DNA coding (in some applications three ferrocenes were complemented with anthraquinone coding for the fourth base). Willner's laboratory introduced multiple ferrocene redox tags by PEX into a replica of a single-stranded circular viral DNA using a primer tethered to a gold electrode.[63] The same group proposed, using biotin-labeled dNTPs, sensors for the detection of telomerase activity[65] or a quartz crystal microbalance-based technique for single nucleotide polymorphism (SNP) detection.[66] Biotin tags introduced into DNA by PEX, followed by enzyme-amplified electrochemical detection, have recently been utilized by Horakova-Brazdilova *et al.*[67] to monitor gene expression.

Electrochemical techniques combined with magnetic separation ("double-surface" electrochemical techniques) have recently been applied in the sensing of DNA hybridization, DNA–protein interactions, immunoassays *etc*.[68] We used an analogous protocol (Scheme 7) in connection with PEX incorporation of nucleotides labeled with ferrocene,[60] nitrophenyl or aminophenyl[61] electroactive markers. This technique proved to be suitable for monitoring incorporation of different nucleotide conjugates (bearing electrochemically distinguishable labels), for testing the efficiency of the PEX reaction (as an alternative to the PAGE assay), as well as for estimating the number of labeled nucleotides introduced per ON molecule. The magnetic separation procedure can be used for preparation of signaling probes for sandwich hybridization assays,[7] labeled on demand with multiple electrochemical tags (to achieve signal amplification) chosen with respect to properties of the target DNA and the optimum detection technique. In addition, we have demonstrated that the same approach is applicable in the analysis of nucleotide sequences (*e.g.*, the SNP detection).[60,61]



Scheme 7 PEX construction of labeled ONs on 5′-biotinylated ON templates, followed by separation of the synthesized strand using streptavidin-coated magnetic beads and electrochemical detection of the incorporated labels.

When the position of the SNP (point mutation) of interest within the target nucleotide sequence is known, it is relatively easy to probe the site using PEX. A model experiment is shown in Fig. 3a. A mix of **dA^{PhNH2}TP**, **dC^{PhNO2}TP** and dGTP was used here to discriminate between nucleotides complementary to the labeled ones (T or G, respectively) in the first position of the template. The resulting electrochemical signals excellently matched the given SNP variants.[61]

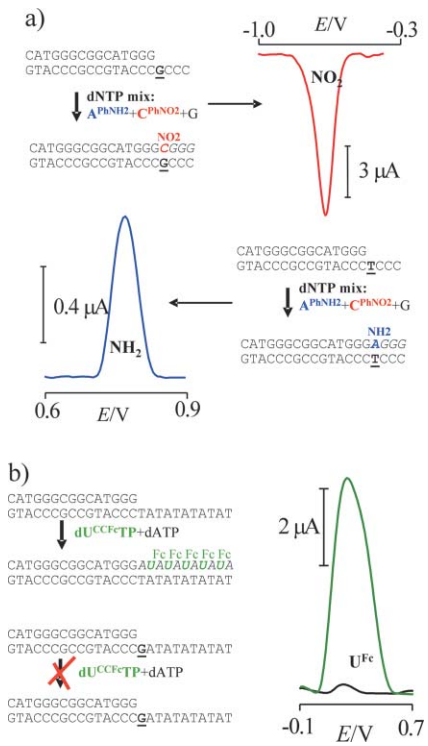


Fig. 3 Examples of using electrochemically labeled nucleotides in analysis of nucleotide sequences. a) Probing of a single nucleotide polymorphism using a mix of nitro- and amino-labeled nucleotides. The resulting electrochemical signal (reduction of nitro group or oxidation of amino group) reveals which nucleotide was incorporated *i.e.*, which nucleotide was present in the probed position of the template. b) Mismatch detection by early terminated PEX. Incorporation of multiple Fc tags in the synthesized ON stretch results in considerable signal amplification (*green curve*). When a nucleotide, the complementary dNTP counterpart to which is missing in the PEX mixture, is present at the 3′-end of the template, the PEX cannot proceed, resulting in absence of the intense signal (*black curve*).

Another possibility of SNP detection, well-suited primarily for sensing mutations within or 3′- (in the template strand) to repetitive DNA stretches, is an approach relying on stopping the DNA strand elongation at a mismatch site.[60] As shown in Fig. 3b, synthesis of the $(UA)_n$ stretch in the presence of **dU^{CCFc}TP** + dATP resulted in an intense U^{Fc} signal due to incorporation of multiple Fc tags. When, however, the first T in the template stretch was replaced by G, the resulting signal was negligible because the primer elongation could not proceed in the absence of dCTP. An analogous principle can be used for mapping homonucleotide blocks (based on the low feasibility of clustered incorporation of some nucleotide conjugates,[60,61] see Fig. 1).

Conclusions and future outlook

This review demonstrates that the polymerase incorporation of modified dNTPs and NTPs is a very simple and powerful methodology for construction of functionalized nucleic acids. In particular, the combination of cross-coupling reactions of halogenated nucleoside triphoshates with polymerase incorporation is a very efficient and straightforward two-step access to this attractive class of biomolecules. Primer extension is suitable for construction of shorter DNA molecules bearing one or several modifications in one strand, while PCR can be used for construction of longer DNA duplexes with high-density modifications in both strands. A number of DNA polymerases of the B-family can be used for these incorporations and some thermostable polymerases (DyNAzyme, Pwo or Vent) could be advantageously used not only for PCR but also for PEX. While in PEX, most 5-substituted pyrimidine and 7-substituted 7-deazapurine dNTPs are very efficiently incorporated, many of them are not sufficiently efficient in PCR.[69] Apparently, there is a great potential for *in vitro* evolution of novel polymerases[70] more efficient in PCR incorporations of modified dNTPs.

We have shown some preliminary bioanalytical applications of electrochemically labeled DNA. Our future efforts will focus on detection of changes in redox potentials due to hybridization of single strand ONs to DNA duplexes in order to develop sensors for DNA hybridization and detection of base pair mismatches. Other bioanalytical applications can involve, besides the above-mentioned sandwich hybridization assays with PEX-labeled signaling probes, mapping the abundance of particular nucleotides in specific DNA regions or detecting repetitive sequence expansions.[71] Our preliminary data suggest that using nitrophenyl-labeled ON substrates, DNA–protein interactions can easily be monitored using the "double-surface" electrochemical assay.[72]

Functionalized DNAs are useful in material and nanotechnology applications. Some of these applications have been reviewed here[30–32,63–66] and many more will definitely follow in the near future. Use of DNA as a chiral auxiliary for catalysis of organic reactions has been studied[73,74] using intercalating ligands. Polymerase incorporation of ligand-containing dNTPs should lead to DNA molecules capable of complexation of transition metals and thus catalyze some reactions. Synthesis of functionalized RNA using this approach has received much less attention and apparently a huge potential of development of new functionalized RNA aptamers, ribozymes, riboswitches or siRNAs is just awaiting its exploration.

Acknowledgements

This work is a part of the research projects Z4 055 0506 and Z5 004 0507. It was supported by the Grant Agency of the Czech Republic (203/05/0043, 203/07/1195), by the Ministry of Education (LC512, LC06035) and by Gilead Sciences, Inc. (Foster City, CA, U. S. A.).

Notes and references

1 Reviews: (*a*) R. R. Breaker, *Curr. Opin. Chem. Biol.*, 1997, **1**, 26–31; (*b*) M. Famulok, G. Mayer and M. Blind, *Acc. Chem. Res.*, 2000, **33**, 591–599; (*c*) M. Famulok, *Curr. Opin. Mol. Ther.*, 2005, **7**, 137–143; (*d*) M. Famulok, J. S. Hartig and G. Mayer, *Chem. Rev.*, 2007, **107**, 3715–3743.

2 Recent examples: (*a*) J. W. Liu and Y. Lu, *J. Am. Chem. Soc.*, 2007, **129**, 8634–8643; (*b*) J. Liu and Y. Lu, *Nat. Protocols*, 2006, **1**, 246–252; (*c*) H. Urata, K. Nomura, S.-I. Wada and M. Akagi, *Biochem. Biophys. Res. Commun.*, 2007, **360**, 459–463; (*d*) R. Nutiu and Y. Li, *Angew. Chem., Int. Ed.*, 2005, **44**, 1061–1065.

3 Recent reviews: (*a*) Y. Isaka, *Curr. Opin. Mol. Ther.*, 2007, **9**, 132–136; (*b*) A. Peracchi, *ChemBioChem*, 2005, **6**, 1316–1322.

4 (*a*) A. Condon, *Nat. Rev. Genet.*, 2006, **7**, 565–575; (*b*) F. E. Alemdaroglu and A. Herrmann, *Org. Biomol. Chem.*, 2007, **5**, 1311–1320; (*c*) U. Feldkamp and C. M. Niemeyer, *Angew. Chem., Int. Ed.*, 2006, **45**, 1856–1876.

5 Recent examples: (*a*) P. Kočalka, N. K. Andersen, F. Jensen and P. Nielsen, *ChemBioChem*, 2007, **8**, 2106–2116; (*b*) N. J. Greco and Y. Tor, *Tetrahedron*, 2007, **63**, 3515–3527; (*c*) R. H. E. Hudson and A. Ghorbani-Choghamarani, *Org. Biomol. Chem.*, 2007, **5**, 1845–1848; (*d*) Q. Xiao, R. T. Ranasinghe, A. M. P. Tang and T. Brown, *Tetrahedron*, 2007, **63**, 3483–3490; (*e*) E. Mayer-Enthart and H.-A. Wagenknecht, *Angew. Chem., Int. Ed.*, 2006, **45**, 3372–3375; (*f*) E. Rozners, R. Smicius and C. Uchiyama, *Chem. Commun.*, 2005, 5778–5780; (*g*) H. Hashimoto, M. G. Nelson and C. Schwitzer, *J. Am. Chem. Soc.*, 1993, **115**, 7128–7134; (*h*) H. Rosemeyer, N. Ramzaeva, E.-M. Becker, E. Feiling and F. Seela, *Bioconjugate Chem.*, 2002, **13**, 1274–1285; (*i*) J. A. Brazier, T. Shibata, J. Townsley, B. F. Taylor, E. Frary, N. H. Williams and D. M. Williams, *Nucleic Acids Res.*, 2005, **33**, 1362–1371.

6 (*a*) J. Gierlich, G. A. Burley, P. M. E. Gramlich, D. M. Hammond and T. Carell, *Org. Lett.*, 2006, **8**, 3639–3642; (*b*) D. M. Hammond, A. Manetto, J. Gierlich, V. A. Azov, P. M. E. Gramlich, G. A. Burley, M. Maul and T. Carell, *Angew. Chem., Int. Ed.*, 2007, **46**, 4184–4187.

7 M. Fojta, P. Kostecka, M. Trefulka, L. Havran and E. Palecek, *Anal. Chem.*, 2007, **79**, 1022–1029.

8 For a comprehensive review (book chapter), see: G. H. McGall, Nucleoside triphosphate analogs for nonradioactive labeling of nucleic acids, in *Nucleoside Triphosphates and Their Analogs*, ed. M. Vaghefi, CRC Press, Taylor Francis Group, Boca Raton,USA, 2005, pp. 269–327.

9 (*a*) J. M. Prober, G. L. Trainor, R. J. Dam, F. W. Hobbs, C. W. Robertson, R. J. Zagursky, A. J. Cocuzza, M. A. Jensen and K. Baumeister, *Science*, 1987, **238**, 336–341; (*b*) L. G. Lee, C. R. Connell, S. L. Woo, R. D. Cheng, B. F. McArdle, C. W. Fuller, N. D. Halloran and R. K. Wilson, *Nucleic Acids Res.*, 1992, **20**, 2471–2483; (*c*) B. B. Rosenblum, L. G. Lee, S. L. Spurgeon, S. H. Khan, S. M. Menchen, C. R. Heiner and S. M. Chen, *Nucleic Acids Res.*, 1997, **25**, 4500–4504; (*d*) L. G. Lee, S. L. Spurgeon, C. R. Heiner, S. C. Benson, B. B. Rosenblum, S. M. Menchen, R. J. Graham, A. Constantinescu, K. G. Upadhya and J. M. Cassel, *Nucleic Acids Res.*, 1997, **25**, 2816–2822; (*e*) C. R. Heiner, K. L. Hunkailler, S. M. Chen, J. I. Glass and E. Y. Chen, *Genome Res.*, 1998, **8**, 557–561; (*f*) J. W. Brandis, *Nucleic Acids Res.*, 1999, **27**, 1912–1918.

10 P. R. Langer, A. A. Waldrop and D. C. Ward, *Proc. Natl. Acad. Sci. U. S. A.*, 1981, **78**, 6633–6637.

11 (*a*) P. S. Nelson, C. Bahl and I. Gobbons, *Nucleosides Nucleotides*, 1986, **5**, 233–241; (*b*) G. Gebeyehu, P. Y. Rao, P. Soon Chan, D. A. Simms and L. Klevan, *Nucleic Acids Res.*, 1987, **15**, 4513–4534.

12 C. R. Petrie, A. D. Adams, M. Stamm, J. Van Ness, S. M. Watanabe and R. B. Meyer jr., *Biocojugate Chem.*, 1991, **2**, 441–446.

13 J. A. Latham, R. Johnson and J. J. Toole, *Nucleic Acids Res.*, 1994, **22**, 2817–2822.

14 K. Sakthivel and C. F. Barbas III, *Angew. Chem., Int. Ed.*, 1998, **37**, 2872–2875.

15 S. E. Lee, A. Sidorov, T. Gourlain, N. Mignet, S. J. Thorpe, J. A. Brazier, M. J. Dickman, D. P. Hornby, J. A. Grasby and D. M. Williams, *Nucleic Acids Res.*, 2001, **29**, 1565–1573.

16 T. Gourlain, A. Sidorov, N. Mignet, S. J. Thorpe, S. E. Lee, J. A. Grasby and D. M. Williams, *Nucleic Acids Res.*, 2001, **29**, 1898–1905.

17 S. Brackmann and S. Lobermann, *Angew. Chem., Int. Ed.*, 2001, **40**, 1427–1429.

18 H. Sawai, A. N. Ozaki, F. Satoh, T. Ohbayashi, M. M. Masud and H. Ozaki, *Chem. Commun.*, 2001, 2601–2605.

19 H. A. Held and S. A. Benner, *Nucleic Acids Res.*, 2002, **30**, 3857–3869.

20 H. Weizman and Y. Tor, *J. Am. Chem. Soc.*, 2002, **124**, 1568–1569.

21 O. Thum, S. Jäger and M. Famulok, *Angew. Chem., Int. Ed.*, 2001, **40**, 3990–3993.

22 (*a*) S. Jäger and M. Famulok, *Angew. Chem., Int. Ed.*, 2004, **43**, 3337–3340; (*b*) S. Jäger, G. Rasched, H. Kornreich-Leshem, M. Engeser, O. Thum and M. Famulok, *J. Am. Chem. Soc.*, 2005, **127**, 15071–15082.
23 L. H. Thoresen, G.-S. Jiao, W. C. Haaland, M. L. Metzker and K. Burgess, *Chem.–Eur. J.*, 2003, **9**, 4603–4610.
24 M. Kuwahara, J. Nagashima, M. Hasegawa, T. Tamura, R. Kitagata, K. Hanawa, S. Hososhima, T. Kasamatsu, H. Ozaki and H. Sawai, *Nucleic Acids Res.*, 2006, **34**, 5383–5394.
25 M. Kuwahara, K. Hanawa, K. Ohsawa, R. Kitagata, H. Ozaki and H. Sawai, *Bioorg. Med. Chem.*, 2006, **14**, 2518–2526.
26 A. Shoji, T. Hasegawa, M. Kuwahara, H. Ozaki and H. Sawai, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 776–779.
27 M. M. Masud, A. Ozaki-Nakamura, M. Kuwahara, H. Ozaki and H. Sawai, *ChemBioChem*, 2003, **4**, 584–588.
28 (*a*) D. A. Di Giusto, W. A. Wlassoff, S. Geisebrecht, J. J. Gooding and G. C. King, *J. Am. Chem. Soc.*, 2004, **126**, 4120–4121; (*b*) D. A. Di Giusto, W. A. Wlassoff, S. Geisebrecht, J. J. Gooding and G. C. King, *Angew. Chem., Int. Ed.*, 2004, **43**, 2809–2812; (*c*) W. A. Wlassoff and G. C. King, *Nucleic Acids Res.*, 2002, **30**, e58; (*d*) S. S. W. Yeung, T. M. H. Lee and I.-M. Hsing, *J. Am. Chem. Soc.*, 2006, **128**, 13374–13375.
29 M. Matsui, Y. Nishiyama, S. Ueji and Y. Ebara, *Bioorg. Med. Chem. Lett.*, 2007, **17**, 456–460.
30 J. Gierlich, K. Gutsmiedl, P. M. E. Gramlich, A. Schmidt, G. A. Burley and T. Carell, *Chem.–Eur. J.*, 2007, **13**, 9486–9494.
31 (*a*) G. A. Burley, J. Gierlich, M. R. Mofid, H. Nir, S. Tal, Y. Eichen and T. Carell, *J. Am. Chem. Soc.*, 2006, **128**, 1398–1399; (*b*) M. Fischler, U. Simon, H. Nir, Y. Eichen, G. A. Burley, J. Gierlich, P. M. E. Gramlich and T. Carell, *Small*, 2007, **3**, 1049–1055.
32 M. Fischler, A. Sologubenko, J. Mayer, G. Clever, G. Burley, J. Gierlich, T. Carell and U. Simon, *Chem. Commun.*, 2008, 169–171.
33 (*a*) H. Sawai, J. Nagashima, M. Kuwahara, R. Kitagata, T. Tamura and I. Matsui, *Chem. Biodiversity*, 2007, **4**, 1979–1995; (*b*) H. Sawai, A. Ozaki-Nakamura, M. Mine and H. Ozaki, *Bioconjugate Chem.*, 2002, **13**, 309–316.
34 T. W. Dewey, A. A. Mundt, G. J. Crouch, M. C. Zyznewski and B. E. Eaton, *J. Am. Chem. Soc.*, 1995, **117**, 8474–8475.
35 T. W. Wiegand, R. C. Janssen and B. E. Eaton, *Chem. Biol.*, 1997, **4**, 675–683.
36 J. D. Vaught, T. Dewey and B. E. Eaton, *J. Am. Chem. Soc.*, 2004, **126**, 11231–11237.
37 (*a*) N. K. Vaish, A. W. Fraley, J. W. Shostak and L. W. McLaughlin, *Nucleic Acids Res.*, 2000, **28**, 3316–3322; (*b*) N. K. Vaish, R. Larralde, A. W. Fraley, J. W. Shostak and L. W. McLaughlin, *Biochemistry*, 2003, **42**, 8842–8851.
38 S. G. Srivatsan and Y. Tor, *J. Am. Chem. Soc.*, 2007, **129**, 2044–2053.
39 F. Huang, G. Wang, T. Coleman and N. Li, *RNA*, 2003, **9**, 1562–1570.
40 I. Hirao, *Biotechniques*, 2006, **40**, 711–717.
41 M. Kimoto, M. Endo, T. Mitsui, T. Okuni, I. Hirao and S. Yokoyama, *Chem. Biol.*, 2004, **11**, 47–55.
42 R. Kawai, M. Kimoto, S. Ikeda, T. Mitsui, M. Endo, S. Yokoyama and I. Hirao, *J. Am. Chem. Soc.*, 2005, **127**, 17286–17295.
43 K. Moriyama, M. Kimoto, T. Mitsui, S. Yokoyama and I. Hirao, *Nucleic Acids Res.*, 2005, **33**, e129.
44 I. Hirao, M. Kimoto, T. Mitsui, T. Fujiwara, R. Kawai, A. Sato, Y. Harada and S. Yokoyama, *Nat. Methods*, 2006, **3**, 729–735.
45 Reviews on cross-coupling reactions of purines and nucleosides: (*a*) M. Hocek, *Eur. J. Org. Chem.*, 2003, 245–254; (*b*) L. A. Agrofoglio, I. Gillaizeau and Y. Saito, *Chem. Rev.*, 2003, **103**, 1875–1916; (*c*) M. K. Lakshman, *J. Organomet. Chem.*, 2002, **653**, 234–251.
46 J. P. Genet and M. Savignac, *J. Organomet. Chem.*, 1999, **576**, 305–317.
47 (*a*) E. C. Western, J. R. Daft, E. M. Johnson II, P. M. Garnnett and K. H. Shaughnessy, *J. Org. Chem.*, 2003, **68**, 6767–6774; (*b*) E. C. Western and K. H. Shaughnessy, *J. Org. Chem.*, 2005, **70**, 6378–6388; (*c*) recent comprehensive review: K. H. Shaughnessy, *Eur. J. Org. Chem.*, 2006, 1827–1835.
48 P. Čapek, M. Vrábel, Z. Hasník, R. Pohl and M. Hocek, *Synthesis*, 2006, 3515–3526.
49 P. Čapek and M. Hocek, *Synlett*, 2005, 3005–3007.
50 M. Vrábel, M. Hocek, L. Havran, M. Fojta, I. Votruba, B. Klepetářová, R. Pohl, L. Rulíšek, L. Zendlová, P. Hobza, I. Shih, E. Mabery and R. Mackman, *Eur. J. Inorg. Chem.*, 2007, 1752–1769.
51 M. Vrábel, R. Pohl, B. Klepetářová, I. Votruba and M. Hocek, *Org. Biomol. Chem.*, 2007, **5**, 2849–2857.
52 P. Čapek, R. Pohl and M. Hocek, *Org. Biomol. Chem.*, 2006, **4**, 2278–2284.
53 G. K. Wagner, A. H. Guse and B. V. L. Potter, *J. Org. Chem.*, 2005, **70**, 4810–4819.
54 A. Collier and G. Wagner, *Org. Biomol. Chem.*, 2006, **4**, 4526–4532.
55 A. Collier and G. K. Wagner, *Chem. Commun.*, 2008, 178–180.
56 T. Kovacs and L. Ötvös, *Tetrahedron Lett.*, 1988, **29**, 4525–4528.
57 F. Seela, M. Zulauf, H. Rosemeyer and H. Reuter, *J. Chem. Soc., Perkin Trans. 2*, 1996, 2373–2376.
58 (*a*) N. Ramzaeva and F. Seela, *Helv. Chim. Acta*, 1995, **78**, 1083–1090; (*b*) F. Seela and X. Peng, *Synthesis*, 2004, 1203–1210.
59 P. Čapek, H. Cahová, R. Pohl, M. Hocek, C. Gloeckner and A. Marx, *Chem.–Eur. J.*, 2007, **13**, 6196–6203.
60 P. Brázdilová, M. Vrábel, R. Pohl, H. Pivoňková, L. Havran, M. Hocek and M. Fojta, *Chem.–Eur. J.*, 2007, **13**, 9527–9533.
61 H. Cahová, L. Havran, P. Brázdilová, H. Pivoňková, R. Pohl, M. Fojta and M. Hocek, *Angew. Chem., Int. Ed.*, 2008, **47**, 2059–2062.
62 (*a*) C. Fan, K. W. Plaxco and A. J. Heeger, *Proc. Natl. Acad. Sci. U. S. A.*, 2003, **100**, 9134–9137; (*b*) A. Anne, A. Bouchardon and J. Moiroux, *J. Am. Chem. Soc.*, 2003, **125**, 1112–1113; (*c*) E. Palecek, *Trends Biotechnol.*, 2004, **22**, 55–58.
63 F. Patolsky, Y. Weizmann and I. Willner, *J. Am. Chem. Soc.*, 2002, **124**, 770–772.
64 J. M. Shumaker, J. J. Tollet, K. J. Filbin, M. P. Montague-Smith and M. C. Pirrung, *Bioorg. Med. Chem.*, 2001, **9**, 2269–2278.
65 (*a*) Y. Weizmann, F. Patolsky, E. Katz and I. Willner, *ChemBioChem*, 2004, **5**, 943–948; (*b*) Y. Weizmann, F. Patolsky, O. Lioubashevski and I. Willner, *J. Am. Chem. Soc.*, 2004, **126**, 1073–1080.
66 I. Willner, F. Patolsky, Y. Weizmann and B. Willner, *Talanta*, 2002, **56**, 847–856.
67 P. Horakova-Brazdilova, M. Fojtova, K. Vytras and M. Fojta, *Sensors*, 2008, **8**, 193–210.
68 For review see: E. Palecek and M. Fojta, *Talanta*, 2007, **74**, 276–290.
69 For example, all the Fc-modified dNTPs and nitrophenyl-dNTPs were not efficiently incorporated by PCR; M. Hocek, H. Cahová, M. Vrábel, unpublished results.
70 Recent examples of evolution of DNA polymerases: (*a*) A. M. Leconte, L. Chen and F. E. Romesberg, *J. Am. Chem. Soc.*, 2005, **127**, 12470–12471; (*b*) C. Gloeckner, K. B. M. Sauter and A. Marx, *Angew. Chem., Int. Ed.*, 2007, **46**, 3115–3117; (*c*) M. Strerath, C. Gloeckner, D. Liu, A. Schnur and A. Marx, *ChemBioChem*, 2007, **8**, 395–401.
71 (*a*) H. L. Paulson and K. H. Fischbeck, *Annu. Rev. Neurosci.*, 1996, **19**, 79–107; (*b*) M. Fojta, L. Havran, M. Vojtíšková and E. Paleček, *J. Am. Chem. Soc.*, 2004, **126**, 6532–6533.
72 M. Fojta, K. Němcová, L. Havran, H. Cahová, M. Hocek, unpublished results.
73 (*a*) B. Seelig, S. Keiper, F. Stuhlmann and A. Jäschke, *Angew. Chem., Int. Ed.*, 2000, **39**, 4576–4579; (*b*) F. Stuhlmann and A. Jäschke, *J. Am. Chem. Soc.*, 2002, **124**, 3238–3244; (*c*) M. Caprioara, R. Fiammengo, M. Engeser and A. Jäschke, *Chem.–Eur. J.*, 2007, **13**, 2089–2095.
74 (*a*) A. J. Boersma, B. L. Feringa and G. Roelfes, *Org. Lett.*, 2007, **9**, 3647–3650; (*b*) G. Roelfes and B. L. Feringa, *Angew. Chem., Int. Ed.*, 2005, **44**, 3230–3232; (*c*) G. Roelfes, A. J. Boersma and B. L. Feringa, *Chem. Commun.*, 2006, 635–637.