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Summary of the Ph.D. Thesis

Syntéza vybraných funkcionalizovaných nukleosidů a nukleosid trifosfátů a jejich inkorporace do DNA

Synthesis of functionalized nucleosides and nucleotides and their incorporation into DNA

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Abstract

This PhD thesis describes the synthesis of modified DNA containing electrochemical active labels such as anthraquinone, benzofurazane, azide and nitrophenyltriazole with the application in bioanalysis. The methodology of the construction of base-modified triphosphates based on Suzuki-Miyaura coupling of halogenated deoxynucleoside triphosphates with arylboronic acids, Sonogashira coupling of dNTPs with terminal acetylenes or triphosphorylation of modified nucleosides was developed. Enzymatic incorporation of functionalized dNTPs into DNA by primer extension experiment and the electrochemical properties of DNA bearing redox labels were studied by cyclic and square-wave voltammetry. At first anthraquinone was tested as an electrochemical label. Modified 2'-deoxynucleoside triphosphates bearing anthraquinone attached through an acetylene or propargylcarbamoyl linker at 5-position of 2'-deoxycytidine 5'-triphosphate and at the 7-position of 7-deaza-2'-deoxyadenosine-5'-triphosphate were prepared by Sonogashira cross-coupling. Enzymatic incorporation of the anthraquinone-labeled dNTPs into DNA by primer extension has been developed. The electrochemical properties of the anthraquinone-labeled nucleosides, dNTPs and DNA were studied by cyclic voltammetry which shows a reversible couple of peaks around -0.4 V. The combination of two reducible labels, anthraquinone and nitrophenyl group, was tested by voltammetry. One unresolved broad peak was observed. Then benzofurazane as a new redox label has been attached to nucleosides and dNTPs either directly or through the acetylene linker. The benzofurazane-modified dNTPs were incorporated into DNA by primer extension. The voltammetric properties of the benzofurazane-modified nucleosides, nucleotides and DNA were studied by using cyclic voltammetry which produced intense cathodic peaks in the region -0.70 and -0.85 V. By combination of benzofurazane, nitrophenyl and aminophenyl labels we have successfully developed a three-potential coding of DNA bases. The combination of benzofurazane and nitrophenyl reducible labels has proved to be excellent for ratiometric analysis of nucleotide sequences and is suitable for bioanalytical applications. New redox labeling of DNA by azido group which can be chemically transformed to nitrophenyltriazole or silenced to phenyltriazole was developed. This tranformation was utilized in the electrochemical detection of DNA-protein interactions (p53 protein) since only those azidophenyl groups in the parts of the DNA which was not shielded by the bound p53 protein were transformed to nitrophenyltriazoles whereas those covered by the protein were not.

Abstrakt

Disertační práce popisuje syntézu modifikované DNA nesoucí elektrochemické aktivní značky (antrachinon, benzofurazan, azidoskupinu a nitrofenyltriazol) pro aplikaci v bioanalýze. Byla vyvinuta metoda přípravy modifikovaných trifosfátů, která je založena na vodné Suzukiho cross-couplingové reakci halogenovaných deoxynukleosid trifosfátů s arylboronovými kyselinami, na Sonogashirově cross-couplingové reakci jodovaných deoxynukleosid trifosfátů s terminálními alkyny, nebo na fosforylaci již modifikovaných nukleosidů. Enzymatická inkorporace funcionalizovaných nukleosid trifosfátů do DNA byla testována prostřednictvím primer extension experimentu a elektrochemické vlastnosti DNA nesoucí redoxní značky byly studovány pomocí cyklické a "square wave" voltametrie. Jako první byl jako elektrochemická značka testován antrachinon. Modifikované 2'-deoxynukleosid trifosfáty nesoucí antrachinon připojený přes acetylenový nebo přes propargylkarbamoylový linker v pozici 5 2'-deoxycytidin 5'-trifosfátu a v pozici 7 7-deaza-2'-deoxyadenosin-5'-trifosfátu byly připraveny pomoci Sonogashirova cross-couplingu. Deoxynukleosid trifosfáty značené antrachinonem byly inkorporovány do DNA prostřednictvím primer extension experimentu. Elektrochemické vlastnosti antrachinonem značených nukleosidů, nukleotidů a DNA byly studovány pomocí cyklické voltametrie, při které byla pozorována reverzibilní dvojice píků při -0.4 V. Kombinace dvou redukovatelných značek, antrachinonu a nitrofenylu byla testována pomocí voltametrie a poskytla jeden nerozlišený široký signál. Benzofurazan jako nová elektrochemická značka byl připojen k deoxynukleosidům a deoxynukleosid trifosfátům přímo, nebo přes acetylenový linker. Benzofurazanem značené nukleotidy byly inkorporovány do DNA prostřednictvím primer extension experimentu. Elektrochemické vlastnosti nukleosidů, nukleotidů a DNA značených benzofurazanem byly studovány pomocí cyklické voltametrie, která zobrazuje intenzívní katodický signál v oblasti -0.7 a -0.85 V. Kombinací benzofurazanu s nitrofenylem a aminofenylem jsme úspěšně vyvinuli trojpotenciálové kódování DNA bází. Kombinace benzofurazanu s nitrofenylem je vynikající pro poměrové analýzy nukleotidových sekvencí a je vhodná pro bioanalytické aplikace. Redoxní značení DNA azido skupinou, která může být chemicky transformována na nitrofenyltriazol nebo na fenyltriazol bylo využito pro elektrochemickou detekci interakce DNA-protein (protein p53). Pouze azidofenylové skupiny v úsecích DNA, které nejsou zakryty proteinem p53, byly transformovány na nitrofenyltriazoly.

List of abbreviations

AQ	antraquinone
CuAAC	copper catalysed alkyne-azide cycloaddition
CV	cyclic voltammetry
dATP	2'-deoxyadenosine triphosphate
dCTP	2'-deoxycytidine triphosphate
dGTP	2'-deoxyguanosine triphosphate
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside triphosphate
dppf	1,1'-bis(diphenylphosphino)ferrocene
dsDNA	double-stranded DNA
dTTP	2'-deoxythymidine triphosphate
equiv.	equivalents
Fc	ferrocene
HMDE	hanging mercury drop electrode
*i*Pr	isopropyl
ON	oligonucleotide
PAGE	polyacrylamide gel electrophoresis
PEX	primer extension experiment
PGE	pyrolytic graphite electrode
Ph	phenyl
RP-HPLC	reverse phase high performance liquid chromatography
SWV	square wave voltammetry
TBTA	tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine
TEAB	triethylammonium bicarbonate
TPPTS	tris(3-sulfophenyl)phosphine trisodium salt

Contents

1 Introduction

DNA is important in living cells because it is a carrier of the genetic information.[1] Base-modified oligonucleotides are synthesized either chemically by the phosphoramidite method on solid support[2] or by enzymatic synthesis of modified 2′-deoxynucleoside triphosphates. In the 1950's E. Paleček discovered the electrochemical activity od DNA[3]. The electrochemical properties of DNA were studied by square-wave voltammetry at HMDE by Paleček.[4] For the study of the reduction of nucleic acids the mercury electrode is preferable whereas solid electrodes such as carbon electrode are suitable for the oxidation study of nucleic acids.[5] Electrochemical detection of redox-labeled DNA[6] is a less expensive alternative to fluorescence techniques for DNA sequencing and diagnostics. Our research is focused on seeking redox labels or their combinations to develop a generally applicable and orthogonal multicolor coding scheme for electrochemical DNA labeling, which could be applied in detection of multiple variants of mutations of certain important genes. Redox labeling by polymerase incorporation of base-modified deoxynucleoside triphosphates bearing a number of redox labels based on ferrocene,[7] amino- and nitrobenzene,[8] Ru- and $Os(bpy)_3$ complexes,[9] tetrathiafulvalene,[10] sulfides,[11] hydrazones[12] have been developed. Although the proof of concept for multicolor redox coding was succesful, the first-generation labels were not practical and there is still a need to develop other redox labels in order to provide a set of four labels which can be readily incorporated into DNA and be independently readable in the presence of all the other labels.

2 Specific aims of the thesis

1. Synthesis of anthraquinone-modified nuleosides, nucleotides and DNA for electrochemical detection.
2. Synthesis of benzofurazane-modified nuleosides, nucleotides and DNA and their electrochemical study with combination of nitrophenyl and aminophenyl labels.
3. Synthesis of azidophenyl-modified nuleosides, nucleotides and DNA and their transformation to nitrophenyltriazole for electrochemical detection of DNA-protein interactions.

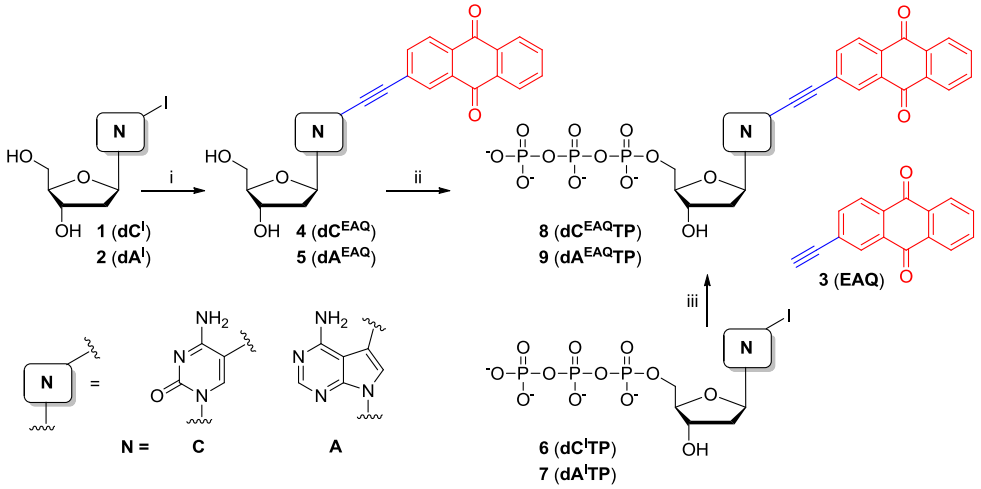
3 Results and discussion

3.1 Anthraquinone-labeled nucleosides and dNTPs. Synthesis, enzymatic incorporation and electrochemical detection

3.1.1 Synthesis of anthraquinone-modified nucleosides and dNTPs

3.1.1.1 Synthesis of EAQ-modified nucleosides and dNTPs

The Sonogashira cross-coupling reactions of iodinated nucleosides **1** (**dC^{I}**) and **2** (**dA^{I}**) with 2-ethynylanthraquinone **3** (**EAQ**) in DMF in the presence of [Pd(PPh$_3$)$_2$Cl$_2$], CuI and (iPr)$_2$EtN proceeded very smoothly (Scheme 1) to give the corresponding **EAQ**-nucleoside conjugates **4** (**dC^{EAQ}**) and **5** (**dA^{EAQ}**) in good yields of 79 % (Table 1, entries 1, 2). Attempted cross-couplings under aqueous conditions of nucleosides **4** (**dC^{EAQ}**) and **5** (**dA^{EAQ}**) with **3 (EAQ)** did not proceed (Table 1, entries 3, 4).



Scheme 1. Synthesis of **EAQ**-modified nucleosides **4** (**dC^{EAQ}**), **5** (**dA^{EAQ}**) and nucleotides **8** (**dC^{EAQ}TP**), **9** (**dA^{EAQ}TP**): i) **3** (**EAQ**), [Pd(PPh$_3$)$_2$Cl$_2$], CuI, (iPr)$_2$EtN, DMF, 1h, 75 °C; ii) 1. PO(OMe)$_3$, POCl$_3$, 0 ºC; 2. (NHBu$_3$)$_2$H$_2$P$_2$O$_7$, Bu$_3$N, DMF, 0 ºC; 3. TEAB; iii) **3** (**EAQ**), [Pd(PPh$_3$)$_2$Cl$_2$],CuI, (iPr)$_2$EtN, DMF:H$_2$O (4:1), 1 h, 75 °C.

Attempted cross-couplings of **3** (**EAQ**) with halogenated triphosphates **6** (**dC^{I}TP**) and **7** (**dA^{I}TP**) under the same aqueous conditions did not proceed either (Table 1, entries 5-7). Therefore I have also tried the cross-coupling of halogenated triphosphates **6** (**dC^{I}TP**) and **7** (**dA^{I}TP**) with 2-ethynylanthraquinone **3** (**EAQ**) in DMF:H$_2$O (4:1) and the reaction proceeded to give the desired **8** (**dC^{EAQ}TP**) and **9** (**dA^{EAQ}TP**) in moderate yields of ca 30 % (Table 1, entries 8, 9). Triphosphorylation of modified nucleosides **4** (**dC^{EAQ}**) and **5** (**dA^{EAQ}**) with POCl$_3$ in PO(OMe)$_3$ followed by the addition of (NHBu$_3$)$_2$H$_2$P$_2$O$_7$, Bu$_3$N and treatment

with TEAB (Scheme 1) gave the desired **8** (**dC^{EAQ}TP**) and **9** (**dA^{EAQ}TP**) in good yields (65-68 %)(Table 1, entries 10, 11) after isolation by RP-HPLC.

Table 1. Synthesis of **EAQ**-modified nucleosides **4** (**dC^{EAQ}**), **5** (**dA^{EAQ}**) and nucleotides **8** (**dC^{EAQ}TP**), **9** (**dA^{EAQ}TP**).

Entry	Starting compound	Reagent	Catalyst	Additives	Solvent	Product	Yield (%)[a]
1	**2** (**dA^{I}**)	**3**	$[Pd(PPh_3)Cl_2]$	CuI, $(iPr)_2NEt$	DMF	**5** (**dA^{EAQ}**)	79
2	**1** (**dC^{I}**)	**3**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	DMF	**4** (**dC^{EAQ}**)	79
3	**2** (**dA^{I}**)	**3**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**5** (**dA^{EAQ}**)	-
4	**1** (**dC^{I}**)	**3**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**4** (**dC^{EAQ}**)	-
5	**7** (**dA^{I}TP**)	**3**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**9** (**dA^{EAQ}TP**)	-
6	**6** (**dC^{I}TP**)	**3**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**8** (**dC^{EAQ}TP**)	-
7	**7** (**dA^{I}TP**)	**3**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**9** (**dA^{EAQ}TP**)	-
8	**7** (**dA^{I}TP**)	**3**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	$DMF:H_2O$ (4:1)	**9** (**dA^{EAQ}TP**)	30
9	**6** (**dC^{I}TP**)	**3**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	$DMF:H_2O$ (4:1)	**8** (**dC^{EAQ}TP**)	31
10	**5** (**dA^{EAQ}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3.TEAB				**9** (**dA^{EAQ}TP**)	65
11	**4** (**dC^{EAQ}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3.TEAB				**8** (**dC^{EAQ}TP**)	68

[a] isolated yield

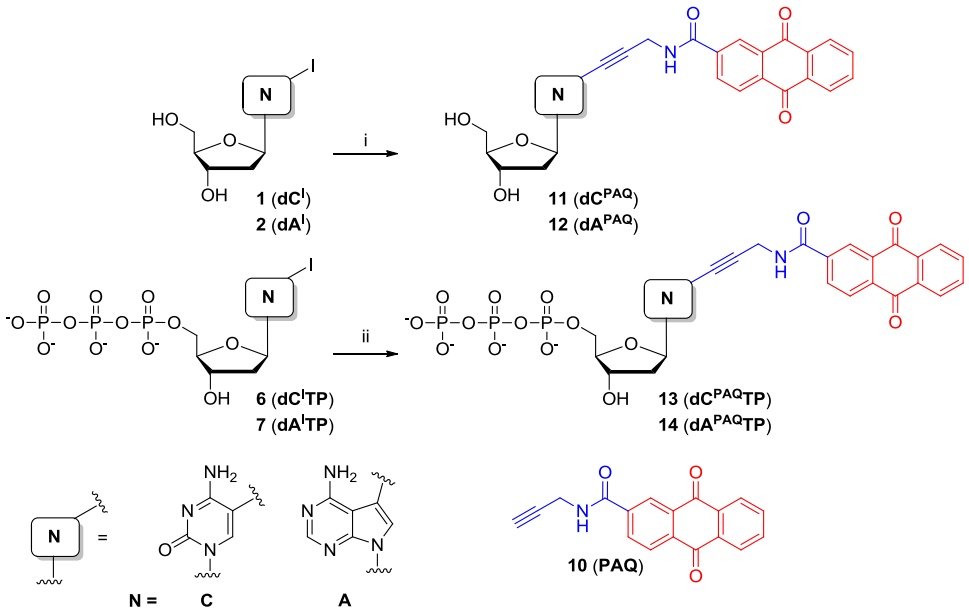
3.1.1.2 Synthesis of PAQ-modified nucleosides and dNTPs

Nucleosides **1** (**dC^{I}**) and **2** (**dA^{I}**) easily reacted with 2-(2-propynylcarbamoyl)anthraquinone **10** (**PAQ**) in DMF in presence of $[Pd(PPh_3)_2Cl_2]$, CuI and $(iPr)_2EtN$ to give the desired derivatives **11** (**dC^{PAQ}**) and **12** (**dA^{PAQ}**) in good yields of 80-83 % (Scheme 2) (Table 2, entries 1, 2), whereas the reaction under aqueous conditions did not proceed (Table 2, entries 3,4). Modified nucleotides **13** (**dC^{PAQ}TP**) and **14** (**dA^{PAQ}TP**) were prepared under the aqueous Sonogashira reaction of halogenated triphosphates **6** (**dC^{I}TP**) and **7** (**dA^{I}TP**) with **10** (**PAQ**) in presence of $Pd(OAc)_2$, TPPTS, CuI and $(iPr)_2EtN$ in $CH_3CN:H_2O$ (1:2) in excellent yields of ca 80 % (Table 2, entries 5, 6).

Table 2. Synthesis of **PAQ**-modified nucleosides **11** (**dC^{PAQ}**), **12** (**dA^{PAQ}**) and nucleotides **13** (**dC^{PAQ}TP**), **14** (**dA^{PAQ}TP**).

Entry	Starting compound	Reagent	Catalyst	Additives	Solvent	Product	Yield (%)
1	**2** (**dA^{I}**)	**10**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	DMF	**12** (**dA^{PAQ}**)	80
2	**1** (**dC^{I}**)	**10**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	DMF	**11** (**dC^{PAQ}**)	83
3	**2** (**dA^{I}**)	**10**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**12** (**dA^{PAQ}**)	-
4	**1** (**dC^{I}**)	**10**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**11** (**dC^{PAQ}**)	-
5	**7** (**dA^{I}TP**)	**10**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**14** (**dA^{PAQ}TP**)	80
6	**6** (**dC^{I}TP**)	**10**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**13** (**dC^{PAQ}TP**)	79

[a] isolated yield



Scheme 2. Synthesis of **PAQ**-modified nucleosides **11** (**dC^{PAQ}**), **12** (**dA^{PAQ}**) and nucleotides **13** (**dC^{PAQ}TP**), **14** (**dA^{PAQ}TP**): i) **10** (**PAQ**), [Pd(PPh$_3$)$_2$Cl$_2$], CuI, (iPr)$_2$EtN, DMF, 1h, 75 °C; ii) **10** (**PAQ**), Pd(OAc)$_2$, TPPTS, CuI, (iPr)$_2$EtN, CH$_3$CN:H$_2$O (2:1), 1h, 75 °C.

3.1.2 Enzymatic incorporation of anthraquinone-modified dNTPs into DNA

Multiple incorporations of anthraquinone-modified nucleotides into the random sequence containing all four bases (temp^{rnd16}) were tested (Table 3) using DNA polymerases such as KOD XL, Vent (*exo*-), PWO and Deep Vent (*exo*-). Both flexible propargylamide linked dNTPs **13** (**dC^{PAQ}TP**) and **14** (**dA^{PAQ}TP**) (Figure 1, lanes 6, 8) gave the fully extended ON whereas the rigid **dN^{EAQ}TP**s gave either a shorter ON in case of **9** (**dA^{EAQ}TP**) (Figure 1, lane 5) or a full-length but slightly impure product in case of **8** (**dC^{EAQ}TP**) (Figure 1, lane 7). I tried to increase the concentration of the modified triphosphates **dN^{XAQ}TP**s 10 times relatively to the natural dNTPs in order to increase the efficacy of the incorporation of the modified **dN^{XAQ}TP**s by KOD XL polymerase (Figure 1, lanes 9-12). Increased concentration of **8** (**dC^{EAQ}TP**), **9** (**dA^{EAQ}TP**), **13** (**dC^{PAQ}TP**) and **14** (**dA^{PAQ}TP**) resulted in an inhibition of the PEX reaction.

Table 3. Primer and template used for PEX experiments.

	Sequences
Prim^{rnd}	5′-CATGGGCGGCATGGG-3′
Temp^{rnd16}	5′-**CTAGCATGAGCTCAGT***CCCATGCCGCCCATG*-3′

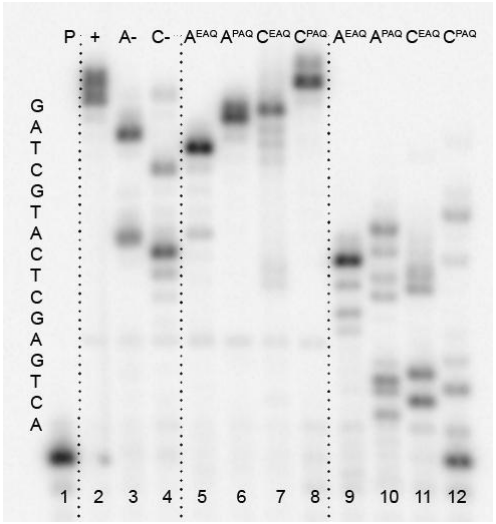


Figure 1. PEX incorporations into 31-nt DNA using anthraquinone-modified dNTPs **8** (**dC^{EAQ}TP**), **9** (**dA^{EAQ}TP**), **13** (**dC^{PAQ}TP**) and **14** (**dA^{PAQ}TP**), template temp^{rnd16}, prim^{rnd} and KOD XL polymerase. P: primer; +: natural dNTPs; A-: dCTP, dGTP, dTTP; C-: dATP, dGTP, dTTP; A^{EAQ}: **dA^{EAQ}TP**, dCTP, dGTP, dTTP; A^{PAQ}: **dA^{PAQ}TP**, dCTP, dGTP, dTTP; C^{EAQ}: dATP, **dC^{EAQ}TP**, dGTP, dTTP; C^{PAQ}: dATP, **dC^{PAQ}TP**, dGTP, dTTP.

3.1.3 Electrochemical studies of anthraquinone-modified nucleosides, dNTPs and DNA

Electrochemical properties of the anthraquinone-modified nucleosides, dNTPs and ONs were studied by cyclic and/or square-wave voltammetry at hanging mercury drop electrode (HMDE) and pyrolytic graphite electrode (PGE). The voltammetry of anthraquinone/anthrahydroquinone moiety in building blocks, nucleosides, dNTPs and ONs shows a distinct reversible couple of peaks around -0.4 V. We tested the combination of two reducible labels in DNA- **AQ** labels and previously reported nitrophenyl (**PhNO$_2$**)[13] (Scheme 3). We studied how easily can we distinguish between them. For this experiment we prepared *pex*^{rnd16}(**C**^{X}) and *pex*^{rnd16}(**A**^{X}) products in which **15** (**dA^{PhNO2}TP**) and **14** (**dA^{PAQ}TP**) were combined at various ratios (in Figure 2a shown for **A**^{X}). Reduction of the nucleobases occurs at potentials around -1.5 V (C, A) or more negative at -1.8 V (G) and there is no interference between signals resulting from nucleobase reduction and signals due to the external reducible tags such as nitrophenyl and AQ. Reduction peaks of anthraquinone label at -0.41 V and nitrophenyl at -0.49 V could be distinguish independently. In case both labels are in the same ON chain, their reduction signals overlapped with small difference in their peak potentials (~80 mV). It is difficult to detect peak AQ^{red} if one **PAQ** per three **PhNO$_2$** (or vice versa) were incorporated. CV can distinguish between these two labels since the reduction of **AQ** is

reversible whereas reduction of **PhNO$_2$** is irreversible. The **PhNO$_2$** labels could be observed indirectly during the anodic voltage scan using a signal at -0.01 V corresponding to reversible oxidation of hydroxylamine (the product of four-electron reduction of the nitro group) to nitroso group (Figure 2a).

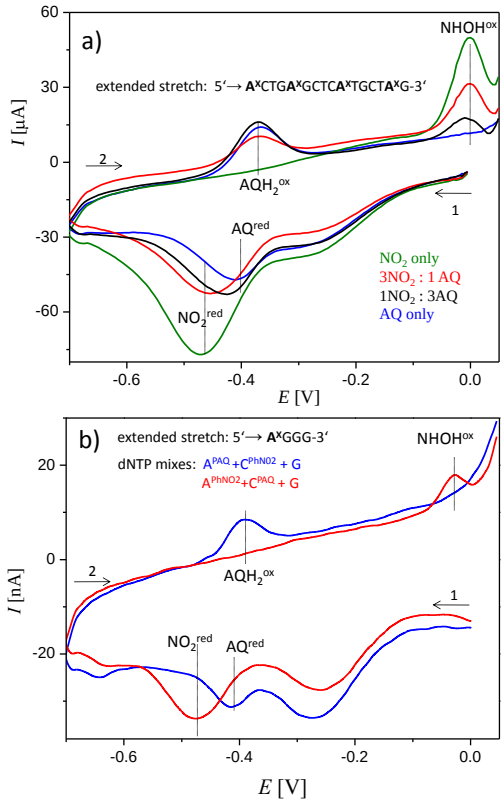
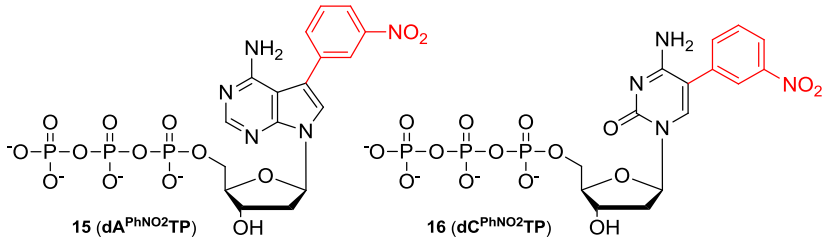


Figure 2. a) CV responses of *pex*^{rnd16} (**A^X**) products with incorporated **A^{PAQ}** and **A^{PhNO2}** conjugates at different ratios (given in the panel); b) CV responses obtained for sequence-specific incorporation of a single **A^X** bearing either **PAQ** or **PhNO$_2$** label. PEX reactions were performed with temp^A template and **dN$^{(X)}$TP** mixes given in the panel. CVs were measured with initial potential 0.0 V, switching potential -0.7 V and final potential +0.05 V.



Scheme 3. Structures of modified dNTPs used for study.

In Figure 2b we tested the possibility of electrochemical monitoring of the sequence-specific incorporation of a single nucleotide labeled with either **PAQ** or **PhNO$_2$** tag. We performed PEX reaction with the temp^A template and mixtures containing either **14** (**dA^{PAQ}TP**) + **16** (**dC^{PhNO2}TP**) + dGTP, or **15** (**dA^{PhNO2}TP**) + **13** (**dC^{PAQ}TP**) + dGTP. CV

responses resulting from these experiments showed perfectly specific incorporation of labeled $\mathbf{A^X}$ against thymine residue in the template strand: in case the reaction mixture contained **14** (**dA$^{\mathbf{PAQ}}$TP**), only peaks AQ^{red} and AQH_2^{ox} were detected and no presence of peak NO_2^{red} and $NHOH^{ox}$ what signified no misincorporation of the labeled cytosine **16** (**dC$^{\mathbf{PhNO2}}$TP**). The same results are shown in case for the reaction mixture contained **15** (**dA$^{\mathbf{PhNO2}}$TP**) + **13** (**dC$^{\mathbf{PAQ}}$TP**) + dGTP (Figure 2b).

3.2 Benzofurazane-labeled nucleosides and dNTPs. Synthesis, enzymatic incorporation and electrochemical detection

3.2.1 Synthesis of benzofurazane-modified nucleosides and dNTPs

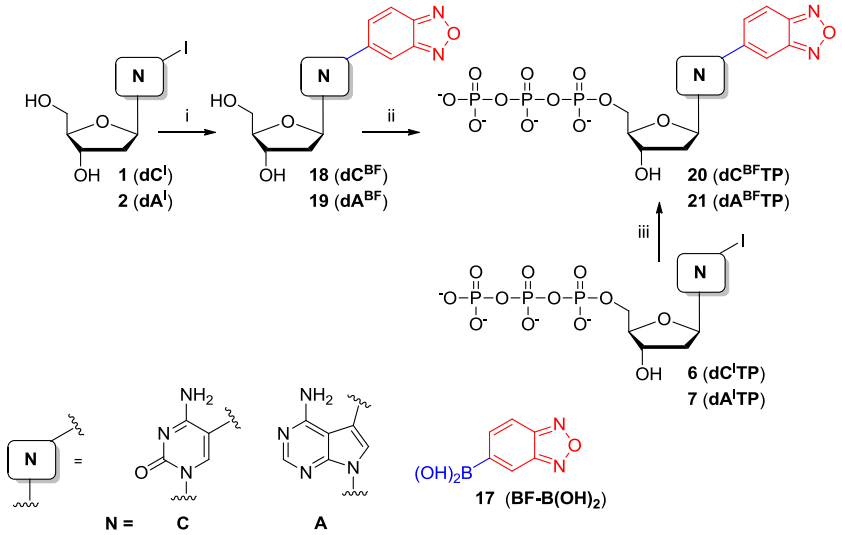
Directly linked benzofurazane modified derivatives of nucleosides **18** (**dC$^{\mathbf{BF}}$**) and **19** (**dA$^{\mathbf{BF}}$**) were prepared by Suzuki-Miyaura cross-coupling[14] of unprotected halogenated nucleosides 5-iodocytidine **1** (**dC$^{\mathbf{I}}$**) and 7-deaza-7-iodoadenosine **2** (**dA$^{\mathbf{I}}$**) (Scheme 4) with benzo[c][1,2,5]oxadiazole-5-boronic acid (**17**) in the presence of $Pd(OAc)_2$, TPPTS, Cs_2CO_3 in CH_3CN:H_2O (1:2) at 75 ºC for 1 h in good yields of 69-74 % (Table 4, entries 1, 2).

Table 4. Preparation of benzofurazane-modified nucleosides and nucleotides.

Entry	Starting compound	Reagent	Catalyst	Additives	Solvent	Product	Yield (%)[a]
1	**2** (**dA$^{\mathbf{I}}$**)	**17**	$Pd(OAc)_2$, TPPTS	Cs_2CO_3	CH_3CN:H_2O (1:2)	**19** (**dA$^{\mathbf{BF}}$**)	74
2	**1** (**dC$^{\mathbf{I}}$**)	**17**	$Pd(OAc)_2$, TPPTS	Cs_2CO_3	CH_3CN:H_2O (1:2)	**18** (**dC$^{\mathbf{BF}}$**)	69
3	**7** (**dA$^{\mathbf{I}}$TP**)	**17**	$Pd(OAc)_2$, TPPTS	Cs_2CO_3	CH_3CN:H_2O (1:2)	**21** (**dA$^{\mathbf{BF}}$TP**)	22
4	**6** (**dC$^{\mathbf{I}}$TP**)	**17**	$Pd(OAc)_2$, TPPTS	Cs_2CO_3	CH_3CN:H_2O (1:2)	**20** (**dC$^{\mathbf{BF}}$TP**)	10
5	**19** (**dA$^{\mathbf{BF}}$**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3.TEAB				**21** (**dA$^{\mathbf{BF}}$TP**)	70
6	**18** (**dC$^{\mathbf{BF}}$**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3.TEAB				**20** (**dC$^{\mathbf{BF}}$TP**)	24

[a] isolated yield

Under the same aqueous conditions of the aqueous Suzuki-Miyaura cross-coupling of halogenated **dNTPs 6** (**dC$^{\mathbf{I}}$TP**) and **7** (**dA$^{\mathbf{I}}$TP**) I observed the desired benzofurazane-modified **dNTPs 20** (**dC$^{\mathbf{BF}}$TP**) and **21** (**dA$^{\mathbf{BF}}$TP**) in moderate yields (10-22 %, Table 4, entries 3, 4). I have applied the triphosphorylation of the corresponding nucleosides **18** (**dC$^{\mathbf{BF}}$**) and **19** (**dA$^{\mathbf{BF}}$**) (Scheme 4) and I observed the desired triphosphates **20** (**dC$^{\mathbf{BF}}$TP**) and **21** (**dA$^{\mathbf{BF}}$TP**) (Table 4, entries 5, 6) in 24 or 70 % yield after isolation by RP HPLC.



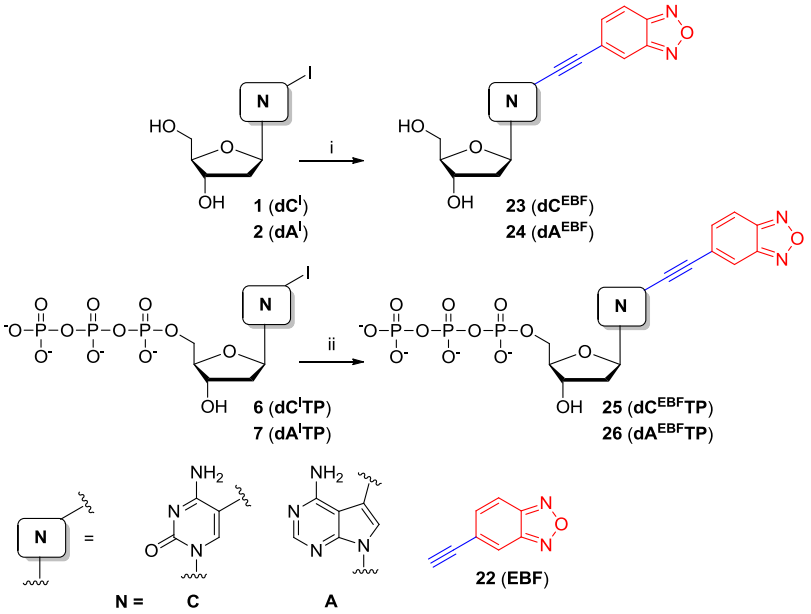
Scheme 4. Reagents and conditions: i), iii) **17** [BF-B(OH)$_2$], Pd(OAc)$_2$, TPPTS, Cs_2CO_3, CH_3CN:H_2O (1:2), 1h, 75 ºC; ii) 1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3.TEAB.

For attachment of benzofurazane-moiety linked through an acetylene tether I used the Sonogashira cross-coupling reactions[14a,15] of 5-ethynyl-benzo[c][1,2,5]oxadiazole[16] **22** with unprotected halogenated nucleosides. The reactions of 5-ethynyl-benzo[c][1,2,5]oxadiazole **22** with 5-iodocytidine **1** (**dC^I**) and 7-deaza-7-iodoadenosine **2** (**dA^I**) were performed in the presence of [Pd(PPh$_3$)$_2$Cl$_2$], (iPr)$_2$EtN, CuI in DMF at 75 ºC for 1 h to give the desired nucleosides **23** (**dC^{EBF}**) and **24** (**dA^{EBF}**) in good yields (60-70 %; Scheme 5, Table 5, entries 1, 2). The same reactions under aqueous conditions in the presence of Pd(OAc)$_2$, TPPTS, CuI and (iPr)$_2$EtN in CH_3CN:H_2O (1:2) proceeded less efficiently to give **23** (**dC^{EBF}**) and **24** (**dA^{EBF}**) in moderate yields (28-45 %; Table 5, entries 3, 4).

Table 5. Preparation of BF-modified nucleos(t)ides.

Entry	Starting compound	Reagent	Catalyst	Additives	Solvent	Product	Yield (%)[a]
1	**2** (**dA^I**)	**22**	[Pd(PPh$_3$)$_2$Cl$_2$]	CuI, (iPr)$_2$EtN	DMF	**24** (**dA^{EBF}**)	70
2	**1** (**dC^I**)	**22**	[Pd(PPh$_3$)$_2$Cl$_2$]	CuI, (iPr)$_2$EtN	DMF	**23** (**dC^{EBF}**)	60
3	**2** (**dA^I**)	**22**	Pd(OAc)$_2$, TPPTS	CuI, (iPr)$_2$EtN	CH_3CN:H_2O (1:2)	**24** (**dA^{EBF}**)	28
4	**1** (**dC^I**)	**22**	Pd(OAc)$_2$, TPPTS	CuI, (iPr)$_2$EtN	CH_3CN:H_2O (1:2)	**23** (**dC^{EBF}**)	45
5	**7** (**dA^{I}TP**)	**22**	Pd(OAc)$_2$, TPPTS	CuI, (iPr)$_2$EtN	CH_3CN:H_2O (1:2)	**26** (**dA^{EBF}TP**)	54
6	**6** (**dC^{I}TP**)	**22**	Pd(OAc)$_2$, TPPTS	CuI, (iPr)$_2$EtN	CH_3CN:H_2O (1:2)	**25** (**dC^{EBF}TP**)	52

[a] isolated yield



Scheme 5. Reagents and conditions: i) **22** (BF-C≡CH), $[Pd(PPh_3)_2Cl_2]$, $(iPr)_2EtN$, CuI, DMF, 1h, 75 ºC; ii) **22**, $Pd(OAc)_2$, TPPTS, $(iPr)_2EtN$, CuI, $CH_3CN:H_2O$ (1:2), 1h, 75 ºC.

For the synthesis of modified nucleotides **25** (**dC^{EBF}TP**) and **26** (**dA^{EBF}TP**) I applied the analogous aqueous Sonogashira cross-coupling. The reactions of **6** (**dC^{I}TP**) and **7** (**dA^{I}TP**) with 5-ethynyl-benzo[c][1,2,5]oxadiazole **22** (Scheme 5) in the presence of $Pd(OAc)_2$, TPPTS, CuI and $(iPr)_2EtN$ in $CH_3CN:H_2O$ (1:2) gave the desired **25** (**dC^{EBF}TP**) and **26** (**dA^{EBF}TP**) in good 52-54 % yields (Table 5, entries 5, 6).

3.2.2 Enzymatic incorporation of benzofurazane-modified dNTPs into DNA

3.2.2.1 PEX of benzofurazane-modified dNTPs

Incorporations of all four modified nucleotides **20** (**dC^{BF}TP**), **21** (**dA^{BF}TP**), **25** (**dC^{EBF}TP**) and **26** (**dA^{EBF}TP**) into random sequences were tested using a temp^{rnd16} template in the presence of several DNA polymerases: KOD XL, PWO, Vent (*exo*-), Deep Vent, Deep Vent (*exo*-) was studied. Three of the modified nucleotides **20** (**dC^{BF}TP**), **25** (**dC^{EBF}TP**) and **26** (**dA^{EBF}TP**) were succesfully incorporated into DNA providing full lenth products in PAGE analysis, whereas **26** (**dA^{EBF}TP**) gave an ON product that was apparently shorter on PAGE (Figure 3).

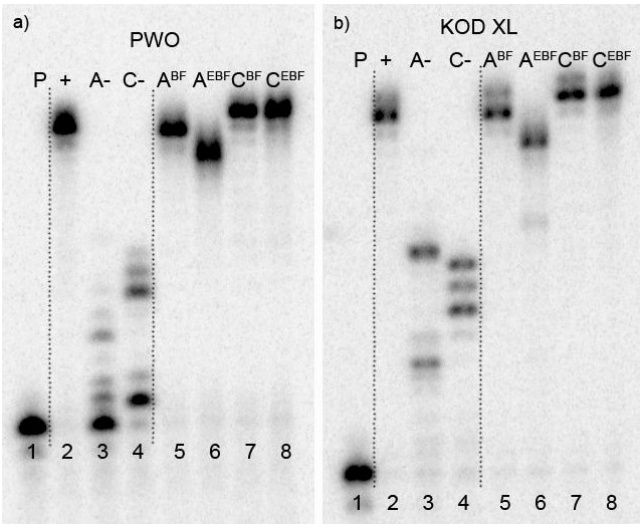


Figure 3. PEX incorporations into 31-nt DNA using benzofurazane-modified dNTPs **20** (**dC^{BF}TP**), **21** (**dA^{BF}TP**), **25** (**dC^{EBF}TP**) and **26** (**dA^{EBF}TP**), temp^{rnd16} template and DNA polymerase: a) PWO DNA polymerase; b) KOD XL DNA polymerase. P: primer; +: natural dNTPs; A-: dCTP, dGTP, dTTP; C-: dATP, dGTP, dTTP; A^{BF}: **dA^{BF}TP**, dCTP, dGTP, dTTP; A^{EBF}: **dA^{EBF}TP**, dCTP, dGTP, dTTP; C^{BF}: dATP, **dC^{BF}TP**, dGTP, dTTP; C^{EBF}: dATP, **dC^{EBF}TP**, dGTP, dTTP.

3.2.2.2 Multipotential coding of DNA

The synthesis of ONs with three different redox labels **20** (**dC^{BF}TP**), **15** (**dA^{NO2}TP**) and **27** (**dT^{NH2}TP**) (Scheme 6) by PEX was tested using template temp^{rnd16}. The results from Figure 4 indicated that the combination of **20** (**dC^{BF}TP**), **15** (**dA^{NO2}TP**) and **27** (**dT^{NH2}TP**) resulted in good incorporation of all modified ONs in the PEX experiments and the desired full-length ONs were obtained.

5'-CATGGGCGGCATGGGA^{NO2}C^{BF}T^{NH2}GA^{NO2}GC^{BF}T^{NH2}C^{BF}A^{NO2}T^{NH2}GC^{BF}T^{NH2}A^{NO2}G-3'

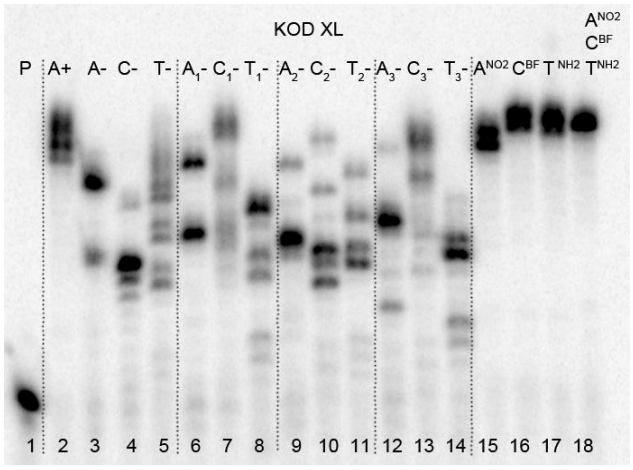
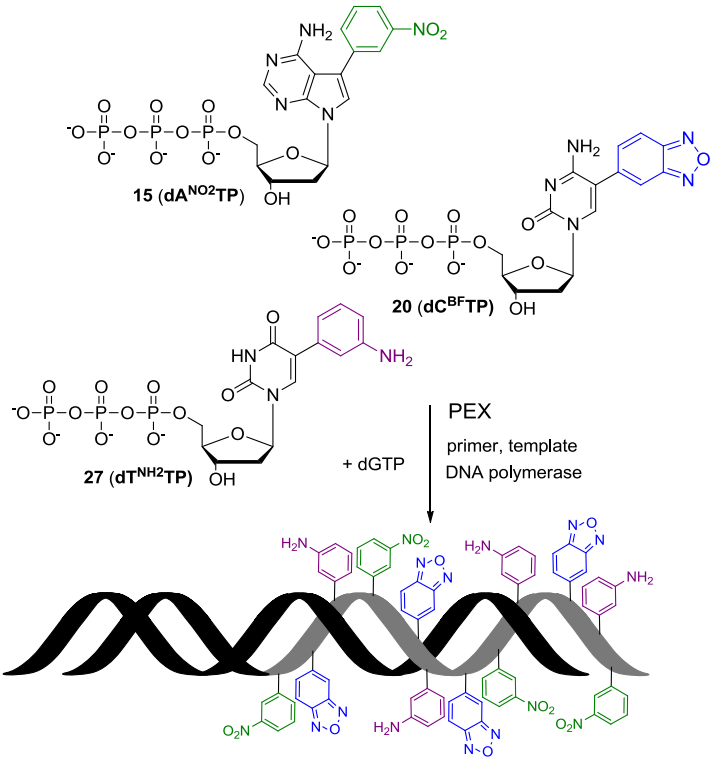


Figure 4. PEX incorporation of **15** (**dA^{NO2}TP**), **20** (**dC^{BF}TP**) and **27** (**dT^{NH2}TP**) in one ON using temp^{rnd16} template to form 31-nt DNA products. P: primer; +: natural dNTPs; A-: dCTP, dGTP, dTTP; C-: dATP, dGTP,

dTTP; T-: dATP, dCTP, dGTP; A_1-: **dC^{BF}TP**, dGTP, dTTP; C_1-: dATP, dGTP, **dT^{NH2}TP**; T_1-: **dA^{NO2}TP**, dCTP, dGTP; A_2-: dCTP, dGTP, **dT^{NH2}TP**; C_2-: **dA^{NO2}TP**, dGTP, dTTP; T_2-: dATP, **dC^{BF}TP**, dGTP; A_3-: **dC^{BF}TP**, dGTP, **dT^{NH2}TP**; C_3-: **dA^{NO2}TP**, dGTP, **dT^{NH2}TP**; T_3-: **dA^{NO2}TP**, **dC^{BF}TP**, dGTP; A^{NO2}: **dA^{NO2}TP**, dCTP, dGTP, dTTP; C^{BF}: dATP, **dC^{BF}TP**, dGTP, dTTP; dT^{NH2}: dATP, dCTP, dGTP, **dT^{NH2}TP**; A^{NO2}+C^{BF}+T^{NH2}: **dA^{NO2}TP**, **dC^{BF}TP**, dGTP, **dT^{NH2}TP**.



Scheme 6. Structures of modified dNTPs **20** (**dC^{BF}TP**), **15** (**dA^{NO2}TP**) and **27** (**dT^{NH2}TP**) and PEX for multipotential coding of DNA.

Table 6. ONs used for multipotential coding of DNA.

ON^{3A2C1T}	5'-CATGGGCGGCATGGG**A^{NO2}**G**C^{BF}**G**A^{NO2}**G**C^{BF}**G**T^{NH2}**G**A^{NO2}**GG-3'
ON^{3A1C3T}	5'-CATGGGCGGCATGGG**T^{NH2}**G**A^{NO2}**G**T^{NH2}**G**A^{NO2}**G**T^{NH2}**G**A^{NO2}**G**C^{BF}**G-3'
ON^{1A3C2T}	5'-CATGGGCGGCATGGG**T^{NH2}**GG**C^{BF}**G**T^{NH2}**GG**C^{BF}**G**A^{NO2}**G**C^{BF}**G-3'
ON^{1A2C3T}	5'-CATGGGCGGCATGGG**T^{NH2}**GG**C^{BF}**G**T^{NH2}**GG**C^{BF}**T^{NH2}**G**A^{NO2}**G-3'
ON^{2A1C3T}	5'-CATGGGCGGCATGGG**T^{NH2}**G**A^{NO2}**GG**T^{NH2}**G**C^{BF}**G**T^{NH2}**G**A^{NO2}**GG-3'
ON^{2A3C1T}	5'-CATGGGCGGCATGGG**A^{NO2}**G**C^{BF}**G**T^{NH2}**GG**C^{BF}**G**A^{NO2}**G**C^{BF}**-3'
ON^{3A3C3T}	5'-CATGGGCGGCATGGG**T^{NH2}**G**A^{NO2}**C^{BF}**G**T^{NH2}**A^{NO2}**G**C^{BF}**T^{NH2}**G**A^{NO2}**C^{BF}**G-3'
ON^{3A0C3T}	5'-CATGGGCGGCATGGG**T^{NH2}**G**A^{NO2}**GG**T^{NH2}**G**A^{NO2}**G**T^{NH2}**G**A^{NO2}**GG-3'
ON^{3A3C0T}	5'-CATGGGCGGCATGGG**A^{NO2}**G**C^{BF}**GG**A^{NO2}**G**C^{BF}**G**A^{NO2}**G**C^{BF}**-3'
ON^{0A3C3T}	5'-CATGGGCGGCATGGG**T^{NH2}**GG**C^{BF}**G**T^{NH2}**G**C^{BF}**G**T^{NH2}**GG**C^{BF}**-3'

After the successful incorporation of three different redox labels **20** (**dC^{BF}TP**), **15** (**dA^{NO2}TP**) and **27** (**dT^{NH2}TP**) into DNA, I designed 10 templates for synthesis of ON probes containing different combinations of three redox labels in different sequences (Table 6). These labelled ON probes (Table 6) were then used for the electrochemical characterization.

3.2.3 Electrochemical studies of benzofurazane-modified nucleosides, dNTPs and DNA

The voltammetric properties of benzofurazane-modified nucleosides, nucleoside triphosphates and DNA were studied using cyclic voltammetry at a hanging mercury drop electrode or a basal-plane pyrolytic graphite electrode. My proposed mechanism of the electrochemical reduction of benzofurazane is irreversible reduction process and involves six electrons and six protons to reduce two C=N double bonds in the furazane ring and releases of a water molecule, giving rise to a diaminobenzene derivative. The benzofurazane conjugates **18** (**dC^{BF}**), **19** (**dA^{BF}**), **23** (**dC^{EBF}**), **24** (**dA^{EBF}**), **20** (**dC^{BF}TP**), **21** (**dA^{BF}TP**), **25** (**dC^{EBF}TP**) and **26** (**dA^{EBF}TP**) produced intense cathodic peaks (denoted as BF^{red}) at the HMDE in the region between -0.70 and -0.85 V, in addition to signals known to correspond to reduction of cytosine or adenine at potentials more negative than -1.2 V[6]. All PEX products containing **A^{BF}**, **A^{EBF}**, **C^{BF}** or **C^{EBF}** produced well developed, symmetrical, and irreversible cathodic peaks at around -0.8 V which could be assigned to reduction of the BF moieties.

3.2.4 Multipotential redox coding of DNA (in collaboration with Doc. Fojta research group)

We focused our attention on multipotential DNA coding by combining benzofurazane with previously developed irreversibly reducible nitrophenyl ($PhNO_2$) tag with multiple electrons and an irreversibly oxidizable aminophenyl ($PhNH_2$) label.[13] At first we tested if the incorporation of two reducible tags **20** (**dC^{BF}TP**) and **15** (**dA^{NO2}TP**) into one PEX product would allow their independent detection without significant mutual interference.

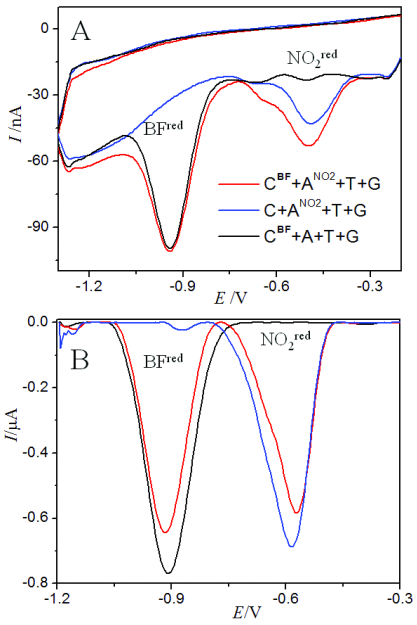


Figure 5. CV responses at HMDE (A) and SWV responses at PGE (B) obtained for PEX product synthesized with $temp^{3A3C3T}$ template and combinations of BF and/or $PhNO_2$ **$dN^{X}TP$** with unmodified dNTPs, as indicated by nucleobase symbols in legend (valid for both panels).

Figure 5a shows the voltammetric responses of PEX products obtained with the $temp^{3A3C3T}$ template and dNTP mixes containing either **20** (**$dC^{BF}TP$**), **15** (**$dA^{NO2}TP$**) or the combination of both incorporated in one reaction. Signals in voltammograms of PEX products containing three BF and/or three $PhNO_2$ labels at the HMDE reflected the composition of the PEX products (Figure 5). The relative intensities of the signals corresponded to the number of electrons expected to be involved in the given reduction process per label ($6e^-$ for benzofurazane and $4e^-$ for $PhNO_2$) and no overlapping of signals was observed. Figure 5b shows analogous results which were obtained from the same PEX products measured at the PGE. ON probes which were designed for incorporating the three labeled nucleotides at different quantities and ratios were used for the electrochemical characterization. Figure 6A shows intensities of peak BF^{red} and peak NO_2^{red} obtained for the individual PEX products. The intensities of the reducible label signals varied consistently with the variation in the number of respective conjugates incorporated as dictated by the template nucleotide sequence. In case complementary base was missing in the template, a negligible signal corresponding to the given tag was observed (probably due to a small level of misincorporation).

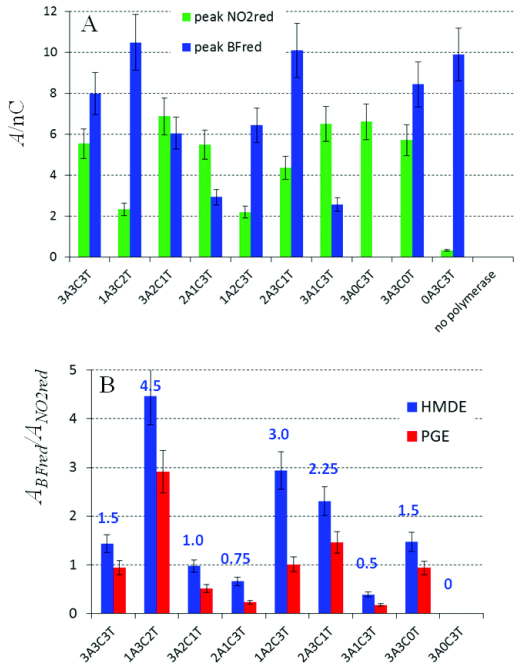


Figure 6. a) Areas of AdTS CV peaks BF^{red}, NO_2^{red} and G obtained at the HMDE for PEX products synthesized with $temp^{xAyCzT}$ templates (numbers of A, C and T residues in the synthesized sequences are indicated in the graph) and **20** (**$dC^{BF}TP$**) + **15** (**$dA^{NO2}TP$**) + **27** (**$dT^{NH2}TP$**) + dGTP mix; b) Ratios of areas of peaks BF^{red}/NO_2^{red} obtained for the same PEX products. Blue numbers indicate expected values calculated from number of labels per ON and number of electrons consumed per label.

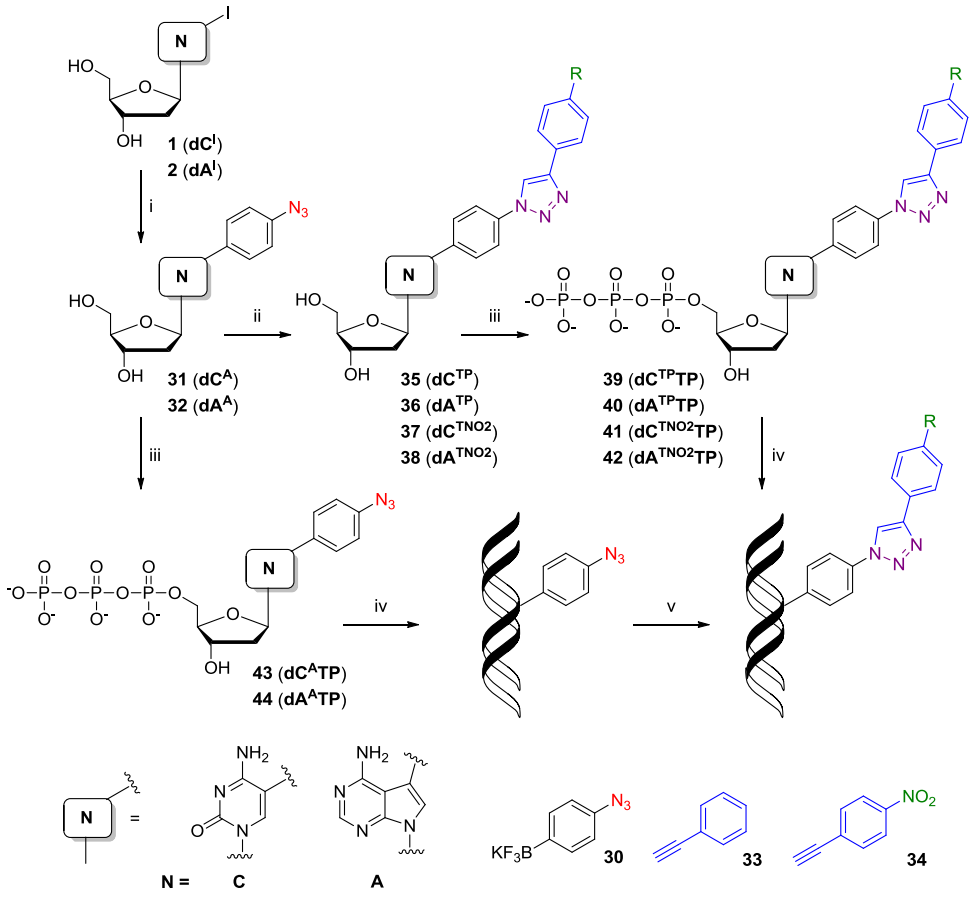
Absolute signal intensities (Figure 6) obtained from the PEX products containing identical number of labeled nucleotides (e.g., three C^{BF}s) varied to a small extent with samples with an identical number of label, which can be explained in terms of natural variations in the yields of individual ONs after the isolation procedure. The method how to eliminate the effect of DNA concentration variations is the calculation of the ratio between the intensities of signals yielded by two independently detectable labels. Figure 6b shows ratios of peak areas BF^{red}/NO_2^{red} which are plotted. Blue numbers in Figure 6b indicate expected values calculated from number of labels per ON and number of electrons consumed per label.

3.3 Azidophenyl as a click-transformable redox label of DNA suitable for electrochemical detection of DNA-protein interactions

3.3.1 Synthesis of modified nucleosides and dNTPs bearing azido and (nitrophenyl)triazole group

The syntheses of the azidophenyl-modified nucleosides were based on the Suzuki-Miyaura cross-coupling reaction 5-iodocytidine **1** (**dC^I**) and 7-deaza-7-iodoadenosine **2** (**dA^I**)

with 4-azidophenyltrifluoroborate[17] **30** in the presence of [$PdCl_2$(dppf)] and Cs_2CO_3 in MeOH. The reactions provided the desired modified nucleosides **31** (**dC^{A}**) and **32** (**dA^{A}**) in good yields of 58-63 % (Scheme 7, Table 7, entries 1, 2). The Huisgen-Sharpless CuAAC reaction[18] between the azidophenyl-modified nucleosides **31** (**dC^{A}**) and **32** (**dA^{A}**) and an alkyne **33** (phenylacetylene) or **34** (1-ethynyl-4-nitrobenzene) gave 1,4-disubstituted 1,2,3-triazoles **35** (**dC^{TP}**), **36** (**dA^{TP}**), **37** (**dC^{TNO2}**) and **38** (**dA^{TNO2}**) in good yields of 40-94 % (Scheme 7, Table 7, entries 5-8). For the preparation of **43** (**dC^{A}TPs**) and **44** (**dA^{A}TPs**) I have applied a triphosphorylation of the corresponding nucleosides **dN^{A}**s and gave the desired **43** (**dC^{A}TPs**) and **44** (**dA^{A}TPs**) (Table 7, entries 3, 4) in 21 and 34 % yield after isolation by RP HPLC. Triazole-modified triphosphates **39** (**dC^{TP}TP**), **40** (**dA^{TP}TP**), **41** (**dC^{TNO2}TP**), **42** (**dC^{TNO2}TP**) were prepared by the same way by triphosphorylation of modified nucleosides **35** (**dC^{TP}**), **36** (**dA^{TP}**), **37** (**dC^{TNO2}**) and **38** (**dA^{TNO2}**) (Scheme 7, Table 7, entries 9-12) in 13-52 % yield.



Scheme 7. Synthesis of modified nucleosides: i) Suzuki-Miyaura cross-coupling: **30** (4-azidophenyltrifluoroborate), $PdCl_2$(dppf), Cs_2CO_3, MeOH, 2 h, 80 ºC; ii) CuAAC: **33** (phenylacetylene) / **34** (1-ethynyl-4-nitrobenzene), sodium ascorbate, $CuSO_4.5H_2O$, *t*BuOH:H_2O (1:1), 12 h, rt; iii) Triphosphorylation of modified nucleosides: 1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB; iv) PEX

experiment; v) Azide-alkyne Huisgen cycloaddition: **33** / **34**, sodium ascorbate, CuBr, TBTA ligand, *t*BuOH:DMSO (1:3), 2 h, 37 ºC.

Table 7. Synthesis of modified nucleosides and nucleotides.

Entry	Starting compound	Reagent	Additives	Solvent	Product	Reaction time	Yield (%)[a]
1	**2** (**dA^{I}**)	**30**	$PdCl_2$(dppf), Cs_2CO_3	MeOH	**32** (**dA^{A}**)	2 h, 80 ºC	58
2	**1** (**dC^{I}**)	**30**	$PdCl_2$(dppf), Cs_2CO_3	MeOH	**31** (**dC^{A}**)	2 h, 80 ºC	63
3	**32** (**dA^{A}**)	1. $PO(OMe)_3$, $POCl_3$, 0ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**43** (**dA^{A}TP**)	6 h, 0 ºC	34
4	**31** (**dC^{A}**)	1. $PO(OMe)_3$, $POCl_3$, 0ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**44** (**dC^{A}TP**)	6 h, 0 ºC	21
5	**32** (**dA^{A}**)	**33**	sodium ascorbate, $CuSO_4.5H_2O$	*t*BuOH:H_2O (1:1)	**36** (**dA^{TP}**)	12 h, rt	72
6	**31** (**dC^{A}**)	**33**	sodium ascorbate, $CuSO_4.5H_2O$	*t*BuOH:H_2O (1:1)	**35** (**dC^{TP}**)	12 h, rt	40
7	**32** (**dA^{A}**)	**34**	sodium ascorbate, $CuSO_4.5H_2O$	*t*BuOH:H_2O (1:1)	**38** (**dA^{TNO2}**)	12 h, rt	94
8	**31** (**dC^{A}**)	**34**	sodium ascorbate, $CuSO_4.5H_2O$	*t*BuOH:H_2O (1:1)	**37** (**dC^{TNO2}**)	12 h, rt	62
9	**36** (**dA^{TP}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**40** (**dA^{TP}TP**)	18 h, 0 ºC	13
10	**35** (**dC^{TP}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**39** (**dC^{TP}TP**)	18 h, 0 ºC	52
11	**38** (**dA^{TNO2}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**42** (**dA^{TNO2}TP**)	18 h, 0 ºC	18
12	**37** (**dC^{TNO2}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**41** (**dC^{TNO2}TP**)	18 h, 0 ºC	18

[a] isolated yields

3.3.2 Electrochemical studies of modified dNTPs

Electrochemical properties of all six modified dNTPs **44** (**dA^{A}TP**), **43** (**dC^{A}TP**), **40** (**dA^{TP}TP**), **39** (**dC^{TP}TP**), **42** (**dA^{TNO2}TP**) and **41** (**dC^{TNO2}TP**) were studied by cyclic voltammetry at the hanging mercury drop electrode. The azidophenyl modified nucleotides **44** (**dA^{A}TP**) and **43** (**dC^{A}TP**) exerted a strong reduction peak at -0.9 V (peak N_3^{red}), whereas the phenyltriazole derivatives **40** (**dA^{TP}TP**) and **39** (**dC^{TP}TP**) did not give any redox signal of the label. On the other hand, the nitrophenyltriazole derivatives **42** (**dA^{TNO2}TP**) and **41** (**dC^{TNO2}TP**) gave a strong reduction peak at -0.4 V (peak NO_2^{red}). The azidophenyl derivatives can be easily transformed to both types of triazoles by CuAAC reactions with alkynes. The click reaction with phenylacetylene can be utilized for silencing of the redox signal of the azido group whereas the click reaction with nitrophenylacetylene can be used for conversion of one redox label (azido) into another one (nitro) exerting a different redox potential.

3.3.3 Enzymatic synthesis of modified DNA

The next aim was the polymerase-catalyzed synthesis of DNA bearing azidophenyl labels and the study of their conversion to (nitro)phenyltriazole groups by CuAAC of the azidophenyl modified DNA with acetylenes (**33** phenylacetylene or **34** 1-ethynyl-4-nitrobenzene) in presence of CuBr, TBTA ligand and sodium ascorbate in aqueous *t*BuOH:DMSO (1:3) at 37 ºC for 2 hours. Figure 7 shows PEX products after Cu(I)-catalyzed CuAAC reaction with no apparent degradation of DNA. In comparison, the direct incorporation of triazole-modified nucleotides, using **40** (**dA^{TP}TP**), **39** (**dC^{TP}TP**), **42** (**dA^{TNO2}TP**) and **41** (**dC^{TNO2}TP**) as substrates into DNA were also attempted (not shown).

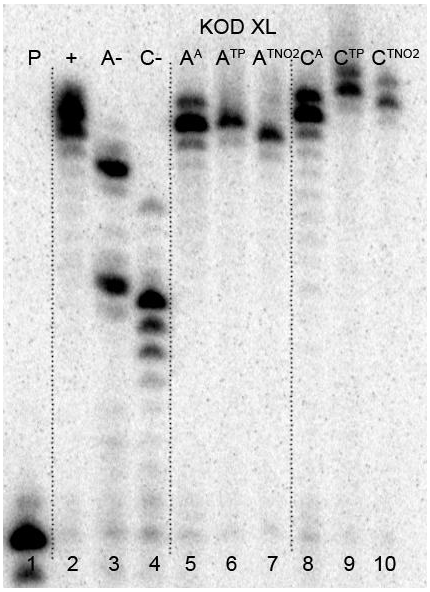


Figure 7. PEX incorporations into 31-nt DNA using **44** (**dA^{A}TP**) and **43** (**dC^{A}TP**), template temp^{rnd16} and KOD XL polymerase followed by click reaction with 1-ethynyl-4-nitrobenzene **34** and phenylacetylene **33**.

3.3.4 Electrochemical studies of the modified DNA

The voltammetric properties of modified DNA (PEX products) containing **N^{A}** , **N^{TP}** and **N^{TNO2}** were studied by using cyclic voltammetry at the HMDE. Figure 8 shows cyclic voltammograms of PEX products prepared with temp^{rnd16} template containing azidophenyl-modified nucleobase (**A^{A}** or **C^{A}**). The irreversible cathodic peak at around -0.9 V corresponds to the reduction of azido group (peak N_3^{red}, red curves). Negative control experiment of PEX reactions with no polymerase added to the mixture detected no signal of azido group what exclude the presence of unremoved **dN^{A}TPs** in the mixture. PEX products containing phenyltriazole group **A^{TP}** and **C^{TP}** which were prepared by click reaction of **N^{A}**-modified DNA with **33** (phenylacetylene) gave no redox signal of label (blue curves). On the other

hand, the CuAAC click reaction of $\mathbf{N^A}$-modified DNA with **34** (1-ethynyl-4-nitrobenzene) gives DNA products containing nitrophenyltriazole group $\mathbf{A^{TNO2}}$ and $\mathbf{C^{TNO2}}$ which exert the irreversible cathodic peak at around -0.4 V due to the reduction of nitro group (peak NO_2^{red}, green curves).

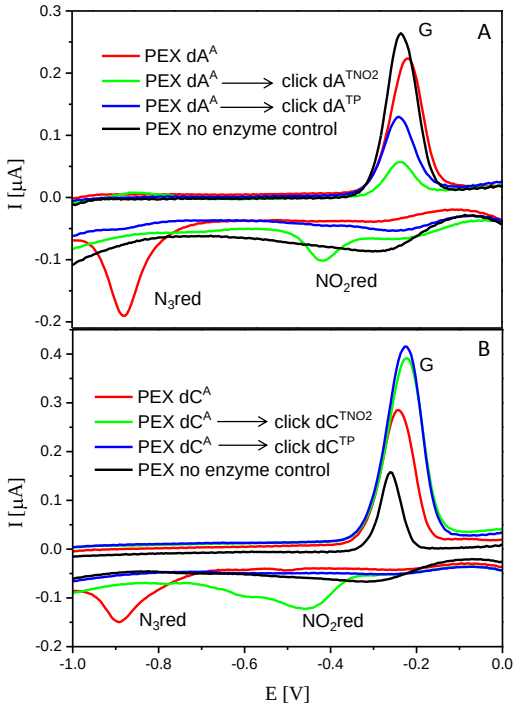
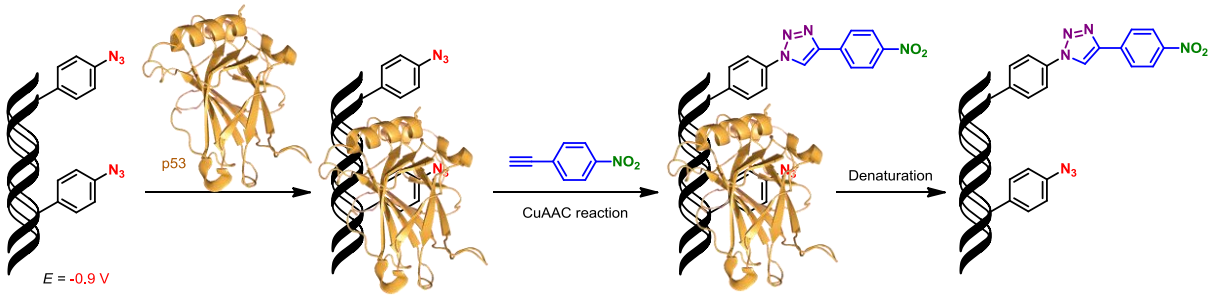


Figure 8. Detail of CV responses at HMDE of PEX products synthesized with $temp^{rnd16}$ template and dNTP mixes containing **dN^ATP** conjugate (as specified in legend) complemented with three respective unmodified dNTPs and PEX products after click reaction with **33** (phenylacetylene) / **34** (1-ethynyl-4-nitrobenzene).

3.3.5 Application of the click transformations of the redox labels in detection of DNA-protein interactions

The transformation of azido redox label to nitrophenyltriazole could be applied as a new method for detection of DNA-protein interaction (Scheme 8). I supposed that if I incorporate several azidophenyl-modifications into DNA probe, incubate the probe with protein and perform the CuAAC click reaction with nitrophenylacetylene, only the freely accessible azido-groups (not shielded by the protein) should be converted to nitrophenyltriazoles and the ratio of azido/nitro redox signals should indicate whether the protein was bound.



Scheme 8. The principle of electrochemical detection of protein-DNA interaction.

I have chosen tumor-suppressor protein p53[19,20] (p53CD_GST) as a biologically relevant example of a sequence-specific[21] binder to DNA. I designed two different sequences of 50-bp DNA (templates temp^{p53_1a2G} and temp^{p53_2CON4}) in which 6 azido-groups are inside and 6 azido-groups are outside of sequence specifically recognized by p53.

Table 8. Sequences of ONs[a]

Sequences	
ON $^{p53\_2CON4}$	5'-GAATTCGATATCAAG***A*****G*****A*****C*****A***TGTCT***A*****G*****A*****C*****A***TGTCT**A**TT**A**TGG**A**T**AA**GGT**A**-3'
ON $^{p53\_1a2G}$	5'-GAATTCGATATCAAG***A*****G*****A*****C*****A***TGCCT***A*****G*****A*****C*****A***TGTTT**A**TT**A**TCC**A**T**AA**CCT**A**-3'

[a] p53 recognition sequence are in italics and nucleotides bearing modification are in bold and underlined

After all tested experiments of stability of DNA-protein complexes (DNA_p53CD_GST complex), I proceeded to the experiments with electrochemical detection of DNA-protein interactions. Cyclic voltammetry of the **A**$^{\mathbf{A}}$-linked DNA (50-bp, **ON**$^{p53\_1a2G}$) containing 12 azidophenyl groups shows the characteristic peak of N_3 reduction at -0.9 V (Figure 9, red curve). This **A**$^{\mathbf{A}}$-linked DNA was then reacted with **34** (4-nitrophenylacetylene), CuBr, TBTA ligand, sodium ascorbate at 20 ºC for 1 h under the previously optimized conditions which are suitable for survival of DNA-protein complexes (Figure 9). The voltammetric analysis shows full conversion of all azido-groups to nitrophenyltriazoles which was confirmed by the disappearance of the signal at -0.9 V and appearance of the new signal at -0.4 V due to reduction of NO_2 group (Figure 9, green curve) In next experiment **A**$^{\mathbf{A}}$-linked DNA was incubated with 1.2 equiv. of p53CD_GST (for 45 min on ice) to form the DNA_p53CD_GST complex and then treated with **34** (4-nitrophenylacetylene) under the above mentioned conditions, followed by denaturation. CV of the product (Figure 9, violet curve) revealed a ca. 50 % decrease in intensity of the peak N_3^{red} of reduction of azido-group and an increase of the peak NO_2^{red} corresponding to the reduction of the nitro group (to ca. 50 % intensity compared to the reaction in the absence of p53). This confirms that only those azido-groups which are not shielded by the protein binding can

undergo the click tranformation to nitrophenyltriazole whereas the N_3 groups covered by the protein remain intact. As a control, I carried out the CuAAC reaction of $\mathbf{A^A}$-linked DNA in presence of bovine serum albumine (BSA), which does not bind DNA, to give the same results as the experiment in the absence of any protein (Figure 9, black curve, all azido groups were converted to nitrophenyltriazoles).

ON$^{p53\_1a2G}$: 5'-GAATTCGATATCAAG*AGACATGCCTAGACATGTTT*ATTATCCATAACCTA-3'

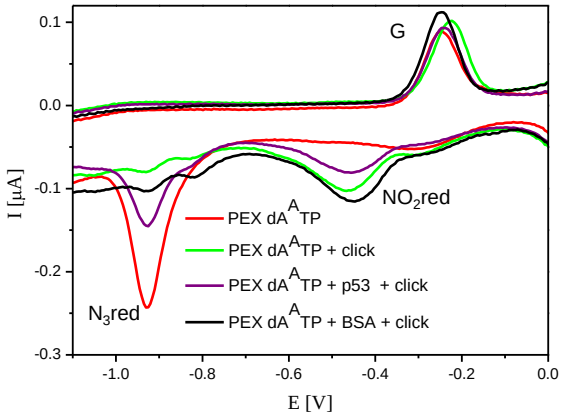


Figure 9. Detail of CV responses at HMDE of PEX products synthesized with temp^{p53_1a2G} template and **44** (**dA^{A}TP**) conjugate complemented with three respective unmodified dNTPs (red curve) and PEX products after click reaction with **34** (4-nitrophenylacetylene) (green curve), DNA-p53 complex after click reaction followed by denaturation (violet curve), the control with BSA (black curve).

4 Conclusion

Anthraquinone-modified nucleosides and nucleotides linked via conjugate acetylene or non-conjugate propargylcarbamoyl linker were prepared and anthraquinone-modified nucleotides were tested as substrates for DNA polymerases. In multiple incorporations, the more flexible **dN^{PAQ}TP**s were better substrates than the rigid **dN^{EAQ}TP**s. Under 10-fold higher concentration of **dN^{XAQ}TP**s, inhibition of the polymerase was observed. Electrochemical studies of the anthraquinone-modified nucleosides, nucleotides and DNA by voltammetry showed peaks of reversible reduction of the anthraquinone moiety at -0.4 V. The combination of anthraquinone modification with previously reported nitrophenyl label gave one unresolved broad reduction peak. However cyclic voltammetry can distinguish between these two labels since the reduction of NO_2 is irreversible and reduction of anthraquinone is reversible. The independent detection of the nitrophenyl in the presence of anthraquinone is possible through oxidation of hydroxylamine, product of the nitrophenyl reduction. Since the **dC^{PAQ}TP** and **dA^{PAQ}TP** linked via propargylcarbamoyl group are good substrates for

polymerase incorporation, the antraquionone can be used to complete the palette of redox labels for multicolor DNA coding in combination with some of the previously reported or future novel redox-labels.

In the second part of the project, benzofurazane attached to nucleosides and dNTPs directly or through acetylene linker were prepared. Benzofurazane-labeled nucleotides were successfully tested as substrates for DNA polymerases exhibiting facile and precise incorporation of the benzofurazane-labeled nucleotides into different DNA sequences by primer extension. Electrochemical reduction of the furazane ring consumes 6 e^- and gives rise to the intense cathodic signal measurable at both mercury and carbon electrodes, occuring at a potential -0.8 V not overlapping with potentials of reduction of natural nucleobases. DNA labeling with combinations of reducible labels benzofurazane, nitrophenyl and the oxidizable label aminophenyl showed no significant interference between benzofurazane and nitrophenyl reductions and no effect of the aminophenyl on signals of any of the reducible tags. ON probes which were designed for incorporating the three labeled nucleotides **$dA^{NO2}TP$, $dC^{BF}TP$** and **$dT^{NH2}TP$** at different quantities and ratios were used for the electrochemical characterization. Quantities of benzofurazane and nitrophenyl labels incorporated into a nucleotide sequence could be determined independently and relative intensities of their signals exhibited excellent correlation with number of complementary bases in the template, making them applicable for ratiometric analysis of nucleotide sequences (such as electrochemical detection of mutations in a DNA stretch based on a change in ratio of two nucleobases encoded by two different redox labels). Aminophenyl has appeared to be suitable for qualitative but not (semi)quantitative ratiometric electrochemical probing of nucleotide sequences.

In the third part of the project, azidophenyl-modified nucleosides and dNTPs were prepared and the polymerase incorporation of azidophenyl-modified nucleotides to DNA by primer extension using azidophenyl-modified nucleotides as substrates was developed. Nucleotides and DNA bearing the azidophenyl modifications exert the strong reduction peak around -0.9 V in voltammetry. The azido-group was transformed to phenyltriazole or nitrophenyltriazole by the CuAAC click reaction. The phenyltriazole-modified dNTPs do not give any reduction signal in voltammetry whereas nitrophenyltriazoles gave the strong reduction signal at -0.4 V. The azidophenyl-modified nucleotides are useful redox labels for DNA which can be either silenced or transformed to the different redox label with distinctly

different redox potential. This transformation was utilized in detection of DNA-protein interactions. Azidophenyl-labeled DNA which was incubated with the protein, binding to a part of its sequence, followed by the CuAAC reaction with 4-nitrophenylacetylene, the free azidophenyl-groups not covered by the protein reacted with 4-nitrophenylacetylene whereas the azides in close contact with the protein remained intact. The electrochemical readout is used for the analysis of the outcome and one can not only distinguish whether or not the protein formed a stable complex with the DNA but also directly deduce the length of the sequence in contact with the particular protein (from the ratio of transformed versus not transformed labels resulting in changes in intensity of the corresponding redox peaks in voltammetry).

5 Úvod

DNA je nositeľkou genetickej informácie živých organizmov.[1] Modifikované oligonukleotidy sa pripravujú buď chemicky fosforamiditovou syntézou na pevnej fáze alebo enzymatickou syntézou modifikovaných 2′-deoxynukleozid trifofátov. V roku 1950 E. Paleček objavil elektrochemické vlastnosti DNA[2], ktoré boli študované pomocou "square-wave" voltametrie na ortuťovej elektróde.[3] Pre štúdium redukčných vlastností nukleových kyselín sa používa ortuťová elektróda, zatiaľ čo pevné elektródy ako napr. uhlíková elektróda sa používa pre štúdium oxidačných vlastností nukleových kyselín.[4] Elektrochemická detekcia redoxne značenej DNA[5] je cenovo dostupnou alternatívou fluorescenčným technikám pre DNA sekvenovanie a diagnostiku. Pracujeme na vývoji nových redoxne-aktívnych značkách a na plne ortogonálnej sade redoxných značiek, ktoré môžu byť použité pre redoxné kódovanie DNA na detekciu mutácií určitých dôležitých génov. Redoxné značenie pomocou polymerázovej inkorporácie modifikovaných deoxynukleozid trifosfátov nesúcich redoxné značky ako napr. ferocén,[6] amino- a nitrobenzén,[7] Ru a Os komplexy,[8] tetratiafulvalén,[9] sulfidy,[10] hydrazóny[11] bolo študované. Hoci už bola pripravená prvá generácia redoxných značiek, je potrebné hľadať ďalšie pre plný set štyroch značiek, ktoré budú inkorporované do DNA a budú elektrochemicky detekovateľné bez prekryvu.

6 Cieľe práce

4. Syntéza antrachinónom modifikovaných nuleozidov, nukleotidov a DNA pre electrochemickú detekciu.
5. Syntéza benzofurazánom modifikovaných nuleozidov, nukleotidov a DNA a štúdium ich elektrochemických vlastností v kombinácii s nitrofenylovou a aminofenylovou značkou.
6. Syntéza azidofenylom modifikovaných nuleozidov, nukleotidov a DNA a ich premena na nitrofenyltriazol pre elektrochemickú detekciu interakcie DNA-proteín.

7 Výsledky a diskusia

7.1 Antrachinónom-modifikované nukleozidy a nukleotidy. Syntéza, enzymatická inkorporácia a elektrochemická detekcia

7.1.1 Syntéza antrachinónom-modifikovaných nukleozidov a nukleotidov

7.1.1.1 Syntéza EAQ-modifikovaných nukleozidov a nukleotidov

Sonogashirová reakcia jodovaných nukleozidov **1** (**dC^{I}**) a **2** (**dA^{I}**) s 2-etynylantrachinónom **3** (**EAQ**) v DMF v prítomnosti [$Pd(PPh_3)_2Cl_2$], CuI a $(iPr)_2EtN$ (Schéma 1) poskytla **EAQ**-nukleozidové konjugáty **4** (**dC^{EAQ}**) a **5** (**dA^{EAQ}**) vo výťažku 79 % (Tabuľka 1, riadok 1, 2). Vodné cross-couplingové reakcie týchto nukleozidov **4** (**dC^{EAQ}**) a **5** (**dA^{EAQ}**) s **3 (EAQ)** neprebiehali (Tabuľka 1, riadok 3, 4).

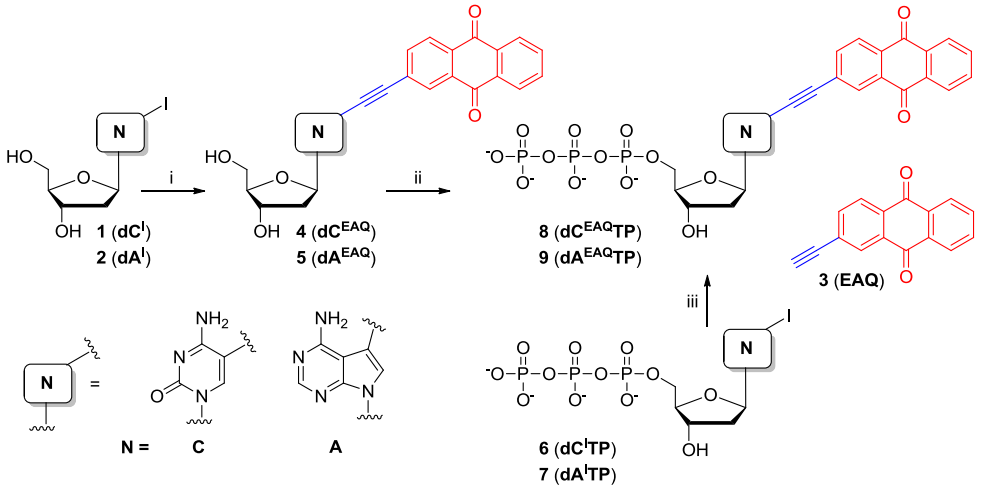


Schéma 1. Syntéza **EAQ**-modifikovaných nukleozidov **4** (**dC^{EAQ}**), **5** (**dA^{EAQ}**) a nukleotidov **8** (**dC^{EAQ}TP**), **9** (**dA^{EAQ}TP**): i) **3** (**EAQ**), $[Pd(PPh_3)_2Cl_2]$, CuI, $(iPr)_2EtN$, DMF, 1h, 75 °C; ii) 1. $PO(OMe)_3$, $POCl_3$, 0 °C; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 °C; 3. TEAB; iii) **3** (**EAQ**), $[Pd(PPh_3)_2Cl_2]$,CuI, $(iPr)_2EtN$, DMF:H_2O (4:1), 1 h, 75 °C.

Cross-couplingové reakcie **3** (**EAQ**) s halogenovanými trifosfátmi **6** (**dC^{I}TP**) a **7** (**dA^{I}TP**) za rovnakých podmienok neprebiehali (Table 1, riadok 5-7). Preto sme skúšali cross-couplingové reakcie halogenovaných trifosfátov **6** (**dC^{I}TP**) a **7** (**dA^{I}TP**) s 2-etynylantrachinónom **3** (**EAQ**) v DMF:H_2O (4:1) a získali sme **8** (**dC^{EAQ}TP**) a **9** (**dA^{EAQ}TP**) vo výťažku 30 % (Tabuľka 1, riadok 8, 9). Trifosforyláciou modifikovaných nukleozidov **4** (**dC^{EAQ}**) a **5** (**dA^{EAQ}**) s $POCl_3$ v $PO(OMe)_3$ a následnom pridaní $(NHBu_3)_2H_2P_2O_7$, Bu_3N a spracovaním s TEAB-om (Schéma 1) sme získali **8** (**dC^{EAQ}TP**) a **9** (**dA^{EAQ}TP**) v dobrom výťažku (65-68 %)(Tabuľka 1, riadok 10, 11) po separácií na RP-HPLC.

Tabuľka 1. Syntéza **EAQ**-modifikovaných nukleozidov **4** (**dC^{EAQ}**), **5** (**dA^{EAQ}**) a nukleotidov **8** (**dC^{EAQ}TP**), **9** (**dA^{EAQ}TP**).

Poradie	Východisková látka	Reagent	Katalyzátor	Aditíva	Rozpúšťadlo	Produkt	Výťažok (%)[a]
1	**2** (**dA^{I}**)	**3**	$[Pd(PPh_3)Cl_2]$	CuI, $(iPr)_2NEt$	DMF	**5** (**dA^{EAQ}**)	79
2	**1** (**dC^{I}**)	**3**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	DMF	**4** (**dC^{EAQ}**)	79
3	**2** (**dA^{I}**)	**3**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**5** (**dA^{EAQ}**)	-
4	**1** (**dC^{I}**)	**3**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**4** (**dC^{EAQ}**)	-
5	**7** (**dA^{I}TP**)	**3**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**9** (**dA^{EAQ}TP**)	-
6	**6** (**dC^{I}TP**)	**3**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**8** (**dC^{EAQ}TP**)	-
7	**7** (**dA^{I}TP**)	**3**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**9** (**dA^{EAQ}TP**)	-
8	**7** (**dA^{I}TP**)	**3**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	DMF:H_2O (4:1)	**9** (**dA^{EAQ}TP**)	30
9	**6** (**dC^{I}TP**)	**3**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	DMF:H_2O (4:1)	**8** (**dC^{EAQ}TP**)	31
10	**5** (**dA^{EAQ}**)	1. $PO(OMe)_3$, $POCl_3$, 0 °C; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 °C; 3.TEAB				**9** (**dA^{EAQ}TP**)	65
11	**4** (**dC^{EAQ}**)	1. $PO(OMe)_3$, $POCl_3$, 0 °C; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 °C; 3.TEAB				**8** (**dC^{EAQ}TP**)	68

[a] izolovaný výťažok

7.1.1.2 Syntéza PAQ-modifikovaných nukleozidov a nukleotidov

Nukleozidy **1** (**dC^{I}**) a **2** (**dA^{I}**) reagujú s 2-(2-propynylkarbamoyl)antrachinónom **10** (**PAQ**) v DMF v prítomnosti $[Pd(PPh_3)_2Cl_2]$, CuI a $(iPr)_2EtN$ a poskytli **11** (**dC^{PAQ}**) a **12** (**dA^{PAQ}**) vo výťažku 80-83 % (Schéma 2, Tabuľka 2, riadok 1, 2), zatiaľ čo za vodných podmienok reakcia neprebieha (Tabuľka 2, riadok 3, 4). Modifikované nukleotidy **13** (**dC^{PAQ}TP**) a **14** (**dA^{PAQ}TP**) boli pripravené pomocou vodnej Sonogashirovej reakcie halogenovaných trifosfátov **6** (**dC^{I}TP**) a **7** (**dA^{I}TP**) s **10** (**PAQ**) v prítomnosti $Pd(OAc)_2$, TPPTS, CuI a $(iPr)_2EtN$ v $CH_3CN:H_2O$ (1:2) vo výťažku 80 % (Tabuľka 2, riadok 5, 6).

Tabuľka 2. Syntéza **PAQ**-modifikovaných nukleozidov **11** (**dC^{PAQ}**), **12** (**dA^{PAQ}**) a nukleotidov **13** (**dC^{PAQ}TP**), **14** (**dA^{PAQ}TP**).

Poradie	Východisková látka	Reagent	Katalyzátor	Aditíva	Rozpúšťadlo	Produkt	Výťažok (%)
1	**2** (**dA^{I}**)	**10**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	DMF	**12** (**dA^{PAQ}**)	80
2	**1** (**dC^{I}**)	**10**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2NEt$	DMF	**11** (**dC^{PAQ}**)	83
3	**2** (**dA^{I}**)	**10**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**12** (**dA^{PAQ}**)	-
4	**1** (**dC^{I}**)	**10**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**11** (**dC^{PAQ}**)	-
5	**7** (**dA^{I}TP**)	**10**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**14** (**dA^{PAQ}TP**)	80
6	**6** (**dC^{I}TP**)	**10**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2NEt$	$CH_3CN:H_2O$ (1:2)	**13** (**dC^{PAQ}TP**)	79

[a] izolovaný výťažok

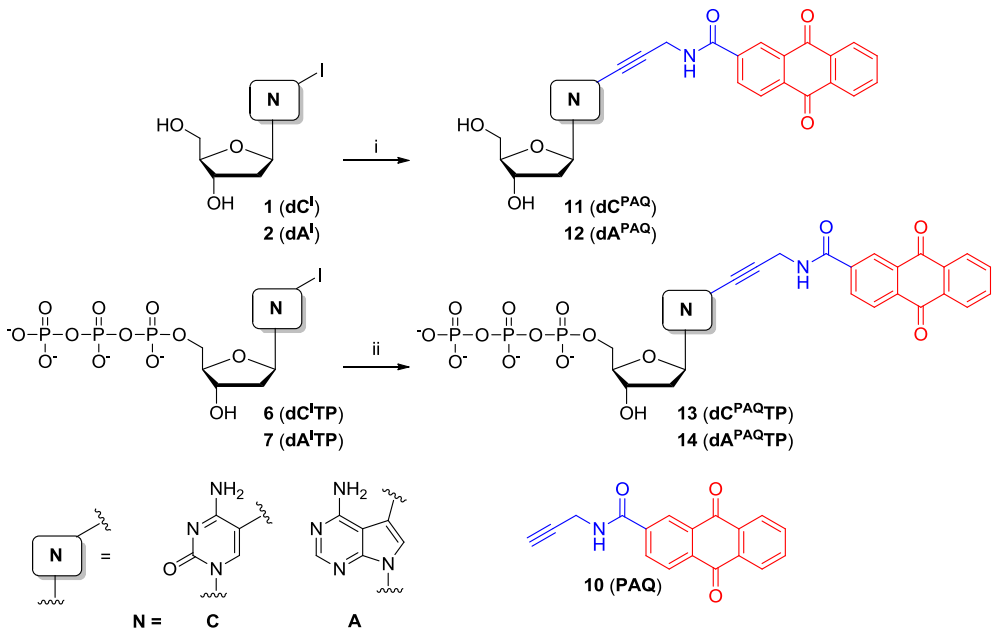


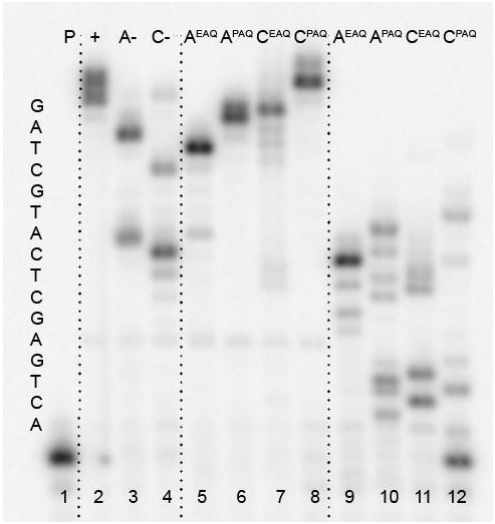
Schéma 2. Syntéza **PAQ**-modifikovaných nukleozidov **11** (**dC^{PAQ}**), **12** (**dA^{PAQ}**) a nukleotidov **13** (**dC^{PAQ}TP**), **14** (**dA^{PAQ}TP**): i) **10** (**PAQ**), $[Pd(PPh_3)_2Cl_2]$, CuI, $(iPr)_2EtN$, DMF, 1h, 75 °C; ii) **10** (**PAQ**), $Pd(OAc)_2$, TPPTS, CuI, $(iPr)_2EtN$, CH_3CN:H_2O (2:1), 1h, 75 °C.

7.1.2 Enzymatická inkorporácia antrachinónom-modifikovaných nukleotidov do DNA

Inkorporácia antrachinónom-modifikovaných nukleotidov do DNA (temp^{rnd16}, Tabuľka 3) bola testovaná pomocou DNA polymeráz KOD XL, Vent (*exo*-), PWO a Deep Vent (*exo*-). Flexibilné propargylamidom viazané nukleotidy **13** (**dC^{PAQ}TP**) a **14** (**dA^{PAQ}TP**) (Obrázok 1, pás 6, 8) poskytli plne dosyntetizovaný oligonukleotid, zatiaľ čo rigidné **dN^{EAQ}TP** poskytli buď kratší ON v prípade **9** (**dA^{EAQ}TP**) (Obrázok 1, pás 5) alebo dosyntetizovaný, ale znečistený produkt v prípade **8** (**dC^{EAQ}TP**) (Obrázok 1, pás 7). Zvýšili sme koncentráciu modifikovaných trifosfátov **dN^{XAQ}TP** desaťnásobne v porovnaní s prirodzenými trifosfátmi kvôli zvýšeniu efektivity inkorporácie s KOD XL DNA polymerázou (Obrázok 1, pás 9-12). Inhibícia PEX reakcie pomocou **8** (**dC^{EAQ}TP**), **9** (**dA^{EAQ}TP**), **13** (**dC^{PAQ}TP**) a **14** (**dA^{PAQ}TP**) bola pozorovaná.

Tabuľka 3. Primer a templát pre PEX experimenty.

Sekvencie	
Prim^{rnd}	5´-CATGGGCGGCATGGG-3´
Temp^{rnd16}	5´-**CTAGCATGAGCTCAGT***CCCATGCCGCCCATG*-3´

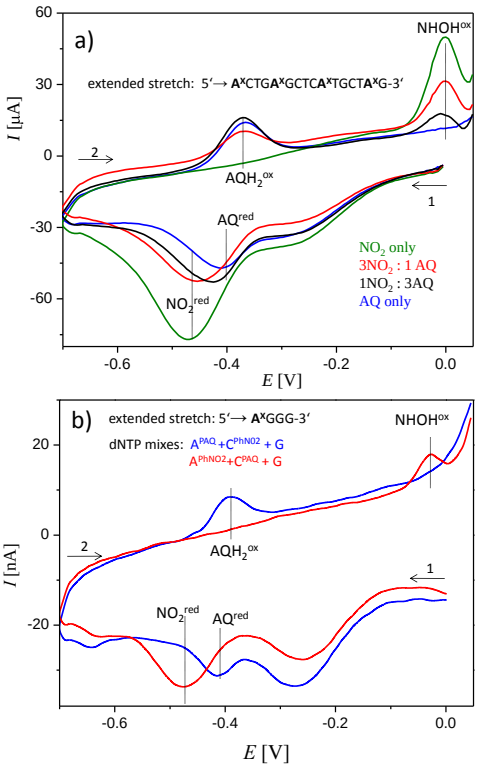


Obrázok 1. PEX inkorporácie do DNA (31 báz) pomocou antrachinónom-modifikovaných nukleotidov **8** (**dC^{EAQ}TP**), **9** (**dA^{EAQ}TP**), **13** (**dC^{PAQ}TP**) a **14** (**dA^{PAQ}TP**), templátu temp^{rnd16}, prim^{rnd} a KOD XL polymerázy. P: primer; +: prirodzené dNTPs; A-: dCTP, dGTP, dTTP; C-: dATP, dGTP, dTTP; A^{EAQ}: **dA^{EAQ}TP**, dCTP, dGTP, dTTP; A^{PAQ}: **dA^{PAQ}TP**, dCTP, dGTP, dTTP; C^{EAQ}: dATP, **dC^{EAQ}TP**, dGTP, dTTP; C^{PAQ}: dATP, **dC^{PAQ}TP**, dGTP, dTTP.

7.1.3 Elektrochemické štúdie antrachinónom-modifikovaných nukleozidov, nukleotidov a DNA

Elektrochemické vlastnosti antrachinónom-značených nukleozidov, nukleotidov a DNA boli študované pomocou cyklickej a "square-wave" voltametrie na ortuťovej a uhlíkovej elektróde. Antrachinónová skupina v nukleozidoch, nukleotidoch a DNA pomocou voltametrie poskytuje nevratnú dvojicu signálov (antrachinón/antrahydrochinón) pri -0.4 V. Skúšali sme kombináciu dvoch redukovateľných značiek v jednej DNA- **AQ** značku a nitrofenyl (**PhNO$_2$**)[11] (Schéma 3). Študovali sme ako ľahko je možné rozpoznať medzi týmito značkami. Pre tento experiment sme pripravili *pex*^{rnd16}(**C**^{X}) a *pex*^{rnd16}(**A**^{X}) produkty, v ktorých **15** (**dA^{PhNO2}TP**) a **14** (**dA^{PAQ}TP**) boli kombinované v rôzných pomeroch (Obrázok 2a pre **A**^{X}). Redukcie nukleobáz sú pozorované v oblasti okolo -1.5 V (C, A) a pri -1.8 V (G) a nie je pozorovaný žiaden prekryv signálov nukleobáz s externými značkami nitrofenylom a AQ. Redukčné signály antrachinónu sa vyskytujú pri potenciáli -0.41 V a u nitrofenylu pri -0.49 V. V prípade, že oba značky sa nachádzajú v tom istom ON, ich redukčné signály sa prekrývajú s malým rozdielom v potenciáli (~80 mV). Je obtiažné detekovať signál AQ^{red}, ak je inkorporovaných pomerovo 1:3 (**PAQ**:**PhNO$_2$**) modifikácií a opačne. CV rozpozná tieto značky, pretože redukcia **AQ** je vratná, kým redukcia **PhNO$_2$** je nevratná. Značka **PhNO$_2$** môže byť detekovaná tiež nepriamo cez anodický signál pri -0.01 V, ktorý odpovedá vratnej

oxidácie hydroxylamínu (produkt 4e$^-$ redukcie nitro-skupiny) na nitrozo-skupinu (Obrázok 2a).



Obrázok 2. a) CV záznamy *pex*^{rnd16} (**A^X**) produktov s inkorporovanými **A^{PAQ}** a **A^{PhNO2}** konjugátmi v rôznych pomeroch; b) CV získané pre sekvenčne špecifickú inkorporáciu **A^X** nesúca buď **PAQ** alebo **PhNO$_2$**. PEX reakcie boli uskutočnené s temp^A templátom a **dN$^{(X)}$TP** zmesou. CV boli merané s počiatočným potenciálom 0.0 V, "switching potenciál" -0.7 V a konečným potenciálom +0.05 V.

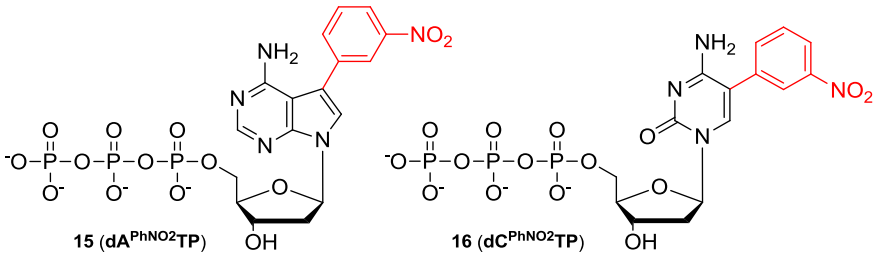


Schéma 3. Štruktúra modifikovaných trifosfátov.

Obrázok 2b zobrazuje možnosť elektrochemického monitorovania sekvenčne špecifickej inkorporácie značeným nukleotidom buď pomocou **PAQ** alebo **PhNO$_2$** značky. Uskutočnili sme PEX reakcie s temp^A templátom a zmesou obsahujúcou buď **14** (**dA^{PAQ}TP**) + **16** (**dC^{PhNO2}TP**) + dGTP alebo **15** (**dA^{PhNO2}TP**) + **13** (**dC^{PAQ}TP**) + dGTP. CV zobrazuje špecifickú inkorporáciu značeného **A^X** oproti tymínu v templáte. V prípade, že reakčná zmes obsahuje iba **14** (**dA^{PAQ}TP**), tak iba signály AQ^{red} a AQH$_2$^{ox} boli pozorované a žiaden signál

NO_2^{red} a $NHOH^{ox}$ nebol pozorovaný. Z toho vyplýva, že nenastala chybná inkorporácia značeného cytozínu **16** (**dC^{PhNO2}TP**). Rovnaký výsledok bol získaný v prípade, ak reakčná zmes obsahovala **15** (**dA^{PhNO2}TP**) + **13** (**dC^{PAQ}TP**) + dGTP (Obrázok 2b).

7.2 Benzofurazánom-modifikované nukleozidy a nukleotidy. Syntéza, enzymatická inkorporácia a elektrochemická detekcia.

7.2.1 Syntéza benzofurazánom-modifikovaných nukleozidov a nukleotidov

Benzofurazánom modifikované nukleozidy **18** (**dC^{BF}**) a **19** (**dA^{BF}**) boli pripravené Suzuki-Miyaurovou cross-couplingovou reakciou[14a,15] nechránených halogenovaných nukleozidov 5-jodocytidínu **1** (**dC^{I}**) a 7-deaza-7-jodoadenozínu **2** (**dA^{I}**) (Schéma 4) s benzo[c][1,2,5]oxadiazol-5-boronovou kyselinou[16] **17** v prítomnosti $Pd(OAc)_2$, TPPTS, Cs_2CO_3 v $CH_3CN:H_2O$ (1:2) pri 75 ºC počas 1 h vo výťažku 69-74 % (Tabuľka 4, riadok 1, 2).

Tabuľka 4. Príprava benzofurazánom modifikovaných nukleozidov a nukleotidov.

Poradie	Východisková látka	Reagent	Katalyzátor	Aditíva	Rozpúšťadlo	Produkt	Výťažok (%)[a]
1	**2** (**dA^{I}**)	**17**	$Pd(OAc)_2$, TPPTS	Cs_2CO_3	$CH_3CN:H_2O$ (1:2)	**19** (**dA^{BF}**)	74
2	**1** (**dC^{I}**)	**17**	$Pd(OAc)_2$, TPPTS	Cs_2CO_3	$CH_3CN:H_2O$ (1:2)	**18** (**dC^{BF}**)	69
3	**7** (**dA^{I}TP**)	**17**	$Pd(OAc)_2$, TPPTS	Cs_2CO_3	$CH_3CN:H_2O$ (1:2)	**21** (**dA^{BF}TP**)	22
4	**6** (**dC^{I}TP**)	**17**	$Pd(OAc)_2$, TPPTS	Cs_2CO_3	$CH_3CN:H_2O$ (1:2)	**20** (**dC^{BF}TP**)	10
5	**19** (**dA^{BF}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3.TEAB				**21** (**dA^{BF}TP**)	70
6	**18** (**dC^{BF}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3.TEAB				**20** (**dC^{BF}TP**)	24

[a] izolovaný výťažok

Za rovnakých podmienok vodnej Suzuki-Miyaurovej cross-couplingovej reakcie halogénovaných trifosfátov **6** (**dC^{I}TP**) a **7** (**dA^{I}TP**) sme získali benzofurazánom modifikované trifosfáty **20** (**dC^{BF}TP**) a **21** (**dA^{BF}TP**) vo výťažku (10-22 %, Tabuľka 4, riadok 3, 4). Aplikovali sme aj fosforyláciu modifikovaných nukleozidov **18** (**dC^{BF}**) a **19** (**dA^{BF}**) (Scheme 4) a získali sme modifikované trifosfáty **20** (**dC^{BF}TP**) a **21** (**dA^{BF}TP**) (Tabuľka 4, riadok 5, 6) vo výťažku 24 alebo 70 % po izolácií na RP HPLC.

Benzofurázanová skupina môže byť tiež pripojená k nechráneným halogénovaným nukleozidom cez acetylénový mostík. Pomocou Sonogashirovej cross-couplingovej reakcie[14a,] 5-iodocytidínu **1** (**dC^{I}**) a 7-deaza-7-jodoadenozínu **2** (**dA^{I}**) s 5-etynyl-

benzo[c][1,2,5]oxadiazolom **22** pomocou $[Pd(PPh_3)_2Cl_2]$, $(iPr)_2EtN$, CuI v DMF pri 75 ºC počas 1 h boli pripravené modifikované nukleotidy **23** (**dC^{EBF}**) a **24** (**dA^{EBF}**) vo výťažku (60-70 %; Schéma 5, Tabuľka 5, riadok 1, 2). Za použitia vodných podmienok v prítomnosti $Pd(OAc)_2$, TPPTS, CuI a $(iPr)_2EtN$ v $CH_3CN:H_2O$ (1:2) reakcia prebiehala s nižšou efektivitou a poskytla **23** (**dC^{EBF}**) a **24** (**dA^{EBF}**) vo výťažku (28-45 %; Tabuľka 5, riadok 3, 4).

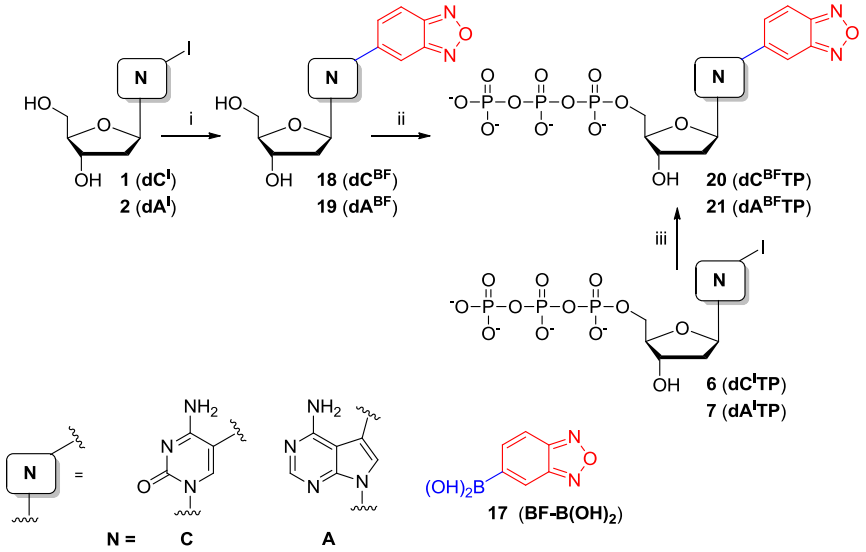


Schéma 4. Reakčné podmienky: i), iii) 17 [BF-B(OH)$_2$], $Pd(OAc)_2$, TPPTS, Cs_2CO_3, $CH_3CN:H_2O$ (1:2), 1h, 75 ºC; ii) 1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3.TEAB.

Pre syntézu modifikovaných nukleotidov **25** (**dC^{EBF}TP**) a **26** (**dA^{EBF}TP**) sme aplikovali analogickú Sonogashirovú cross-couplingovú reakciu. Reakcie **6** (**dC^{I}TP**) a **7** (**dA^{I}TP**) s 5-ethynyl-benzo[c][1,2,5]oxadiazolom[21] **22** (Schéma 5) v prítomnosti $Pd(OAc)_2$, TPPTS, CuI a $(iPr)_2EtN$ v $CH_3CN:H_2O$ (1:2) poskytli **25** (**dC^{EBF}TP**) a **26** (**dA^{EBF}TP**) vo výťažku 52-54 % (Tabuľka 5, riadok 5, 6).

Tabuľka 5. Príprava benzofurazánom-modifikovaných nukleozidov a nukleotidov.

Poradie	Východisková látka	Reagent	Katalyzátor	Aditíva	Rozpúšťadlo	Produkt	Výťažok (%)[a]
1	**2** (**dA^{I}**)	**22**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2EtN$	DMF	**24** (**dA^{EBF}**)	70
2	**1** (**dC^{I}**)	**22**	$[Pd(PPh_3)_2Cl_2]$	CuI, $(iPr)_2EtN$	DMF	**23** (**dC^{EBF}**)	60
3	**2** (**dA^{I}**)	**22**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2EtN$	$CH_3CN:H_2O$ (1:2)	**24** (**dA^{EBF}**)	28
4	**1** (**dC^{I}**)	**22**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2EtN$	$CH_3CN:H_2O$ (1:2)	**23** (**dC^{EBF}**)	45
5	**7** (**dA^{I}TP**)	**22**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2EtN$	$CH_3CN:H_2O$ (1:2)	**26** (**dA^{EBF}TP**)	54
6	**6** (**dC^{I}TP**)	**22**	$Pd(OAc)_2$, TPPTS	CuI, $(iPr)_2EtN$	$CH_3CN:H_2O$ (1:2)	**25** (**dC^{EBF}TP**)	52

[a] izolovaný výťažok

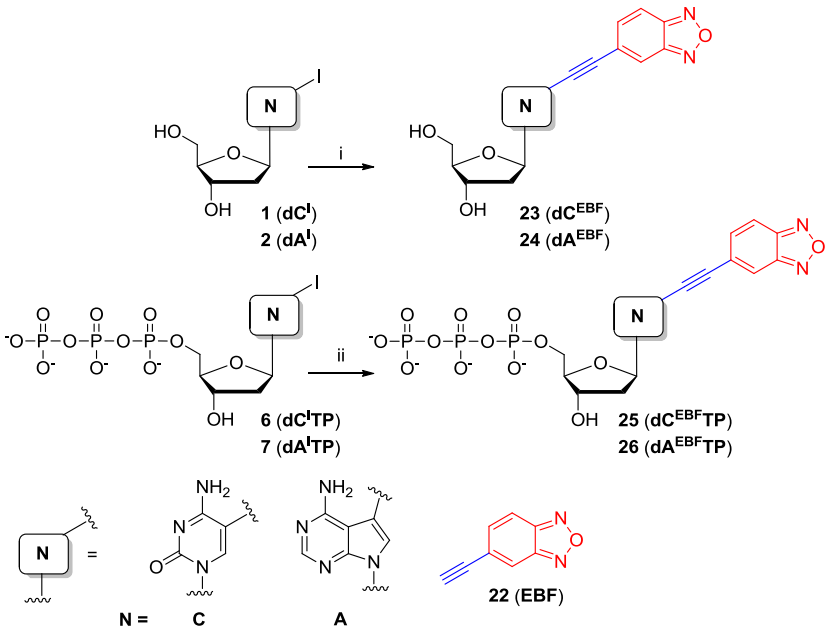
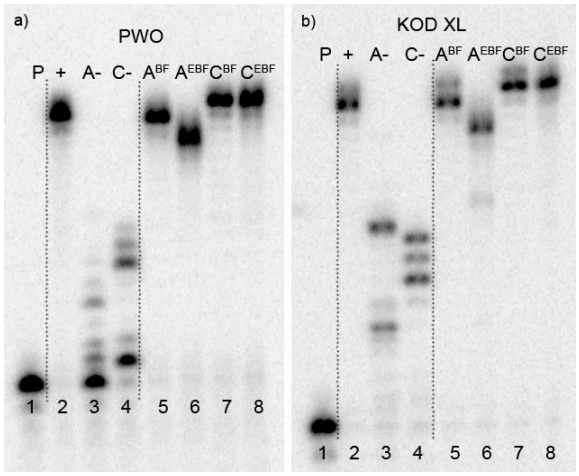


Schéma 5. Reakčné podmienky: i) **22** (BF-C≡CH), $[Pd(PPh_3)_2Cl_2]$, $(iPr)_2EtN$, CuI, DMF, 1h, 75 ºC; ii) **22**, $Pd(OAc)_2$, TPPTS, $(iPr)_2EtN$, CuI, $CH_3CN:H_2O$ (1:2), 1h, 75 ºC.

7.2.2 Enzymatická inkorporácia benzofurazánom-modifikovaných nukleotidov do DNA

7.2.2.1 PEX experiment benzofurazánových nukleotidov

Inkorporácie všetkých štyroch modifikovaných nukleotidov **20** (**dC^{BF}TP**), **21** (**dA^{BF}TP**), **25** (**dC^{EBF}TP**) a **26** (**dA^{EBF}TP**) pomocou temp^{rnd16} templátu v prítomnosti niekoľkých DNA polymeráz: KOD XL, PWO, Vent (*exo*-), Deep Vent, Deep Vent (*exo*-) boli študované. Modifikované nukleotidy **20** (**dC^{BF}TP**), **25** (**dC^{EBF}TP**) a **26** (**dA^{EBF}TP**) boli úspešne inkorporované do DNA s poskytnutím plne dosyntetizovaných produktov na PAGE, zatiaľ čo **26** (**dA^{EBF}TP**) poskytol kratší ON produkt (Obrázok 3).

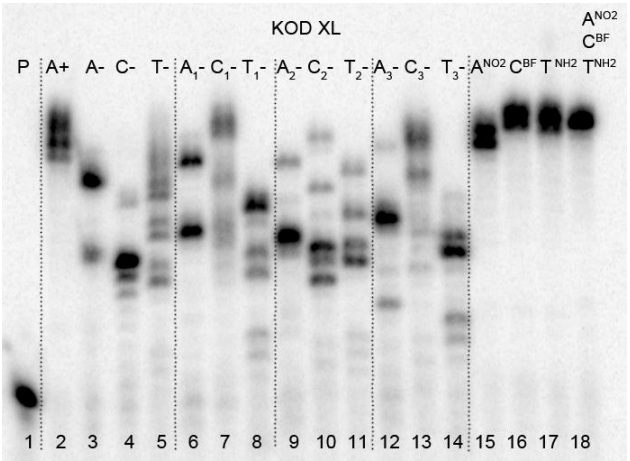


Obrázok 3. PEX inkorporácie do DNA (31 báz) použitím benzofurazánom-modifikovaných nukleotidov **20** (**dC^{BF}TP**), **21** (**dA^{BF}TP**), **25** (**dC^{EBF}TP**) a **26** (**dA^{EBF}TP**), temp^{rnd16} templátu a DNA polymerázy: a) PWO DNA polymeráza; b) KOD XL DNA polymeráza. P: primer; +: prirodzené dNTPs; A-: dCTP, dGTP, dTTP; C-: dATP, dGTP, dTTP; A^{BF}: **dA^{BF}TP**, dCTP, dGTP, dTTP; A^{EBF}: **dA^{EBF}TP**, dCTP, dGTP, dTTP; C^{BF}: dATP, **dC^{BF}TP**, dGTP, dTTP; C^{EBF}: dATP, **dC^{EBF}TP**, dGTP, dTTP.

7.2.2.2 Multipotenciálové kódovanie DNA

Syntéza oligonukleotidov s troma rôznymi redoxnými značkami **20** (**dC^{BF}TP**), **15** (**dA^{NO2}TP**) a **27** (**dT^{NH2}TP**) (Schéma 6) pomocou PEX experimentu bola testovaná pomocou templátu temp^{rnd16}. Výsledky z Obrázok 4 zobrazujú, že kombinácia značiek **20** (**dC^{BF}TP**), **15** (**dA^{NO2}TP**) a **27** (**dT^{NH2}TP**) je vhodná pre inkorporáciu do DNA v PEX experimentoch a boli získané plne dosyntetizované oligonukleotidy.

5'-CATGGGCGGCATGGGA^{NO2}C^{BF}T^{NH2}GA^{NO2}GC^{BF}T^{NH2}C^{BF}A^{NO2}T^{NH2}GC^{BF}T^{NH2}A^{NO2}G-3'



Obrázok 4. PEX inkorporácie **15** (**dA^{NO2}TP**), **20** (**dC^{BF}TP**) a **27** (**dT^{NH2}TP**) v jednom ON použitím temp^{rnd16} templátu pre formovanie DNA (31 báz) produktov. P: primer; +: prirodzené dNTPs; A-: dCTP, dGTP, dTTP; C-: dATP, dGTP, dTTP; T-: dATP, dCTP, dGTP; A$_1$-: **dC^{BF}TP**, dGTP, dTTP; C$_1$-: dATP, dGTP, **dT^{NH2}TP**; T$_1$-: **dA^{NO2}TP**, dCTP, dGTP; A$_2$-: dCTP, dGTP, **dT^{NH2}TP**; C$_2$-: **dA^{NO2}TP**, dGTP, dTTP; T$_2$-: dATP, **dC^{BF}TP**, dGTP;

A_3-: **dC^{BF}TP**, dGTP, **dT^{NH2}TP**; C_3-: **dA^{NO2}TP**, dGTP, **dT^{NH2}TP**; T_3-: **dA^{NO2}TP**, **dC^{BF}TP**, dGTP; A^{NO2}: **dA^{NO2}TP**, dCTP, dGTP, dTTP; C^{BF}: dATP, **dC^{BF}TP**, dGTP, dTTP; dT^{NH2}: dATP, dCTP, dGTP, **dT^{NH2}TP**; A^{NO2}+C^{BF}+T^{NH2}: **dA^{NO2}TP**, **dC^{BF}TP**, dGTP, **dT^{NH2}TP**.

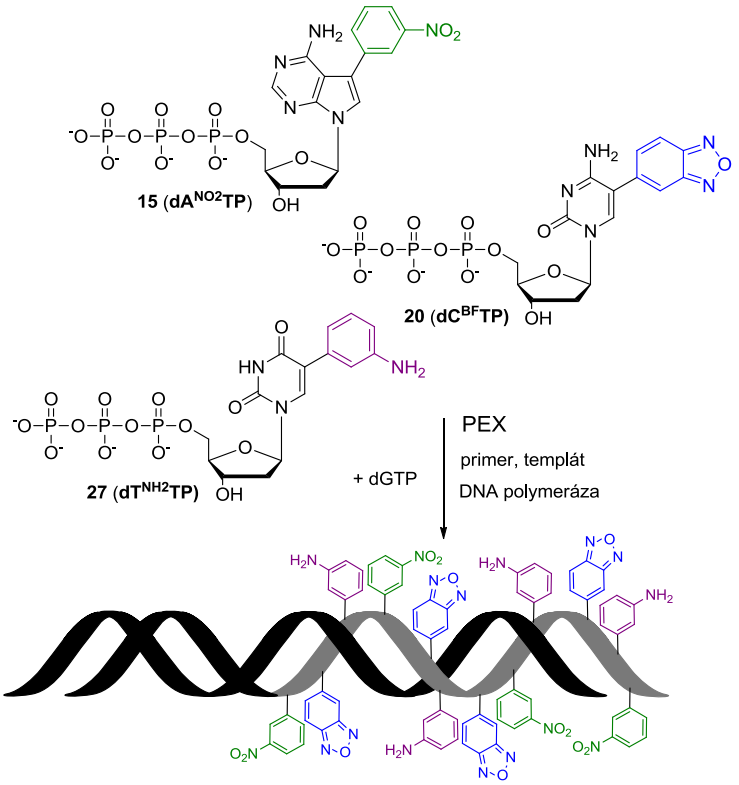


Schéma 6. Štruktúra modifikovaných nukleotidov **20** (**dC^{BF}TP**), **15** (**dA^{NO2}TP**) a **27** (**dT^{NH2}TP**) a PEX experiment pre multipotenciálové kódovanie DNA.

Tabuľka 6. ON pre multipotenciálové kódovanie DNA.

ON^{3A2C1T}	5'-CATGGGCGGCATGGGGA^{NO2}GC^{BF}GA^{NO2}GC^{BF}GT^{NH2}GA^{NO2}GG-3'
ON^{3A1C3T}	5'-CATGGGCGGCATGGGT^{NH2}GA^{NO2}GT^{NH2}GA^{NO2}GT^{NH2}GA^{NO2}GC^{BF}G-3'
ON^{1A3C2T}	5'-CATGGGCGGCATGGGT^{NH2}GGC^{BF}GT^{NH2}GGC^{BF}GA^{NO2}GC^{BF}G-3'
ON^{1A2C3T}	5'-CATGGGCGGCATGGGT^{NH2}GGC^{BF}GT^{NH2}GGC^{BF}T^{NH2}GA^{NO2}G-3'
ON^{2A1C3T}	5'-CATGGGCGGCATGGGT^{NH2}GA^{NO2}GGT^{NH2}GC^{BF}GT^{NH2}GA^{NO2}GG-3'
ON^{2A3C1T}	5'-CATGGGCGGCATGGGGA^{NO2}GC^{BF}GT^{NH2}GGC^{BF}GA^{NO2}GC^{BF}-3'
ON^{3A3C3T}	5'-CATGGGCGGCATGGGT^{NH2}GA^{NO2}C^{BF}GT^{NH2}A^{NO2}GC^{BF}T^{NH2}GA^{NO2}C^{BF}G-3'
ON^{3A0C3T}	5'-CATGGGCGGCATGGGT^{NH2}GA^{NO2}GGT^{NH2}GA^{NO2}GT^{NH2}GA^{NO2}GG-3'
ON^{3A3C0T}	5'-CATGGGCGGCATGGGGA^{NO2}GC^{BF}GGA^{NO2}GC^{BF}GA^{NO2}GC^{BF}-3'
ON^{0A3C3T}	5'-CATGGGCGGCATGGGT^{NH2}GGC^{BF}GT^{NH2}GC^{BF}GT^{NH2}GGC^{BF}-3'

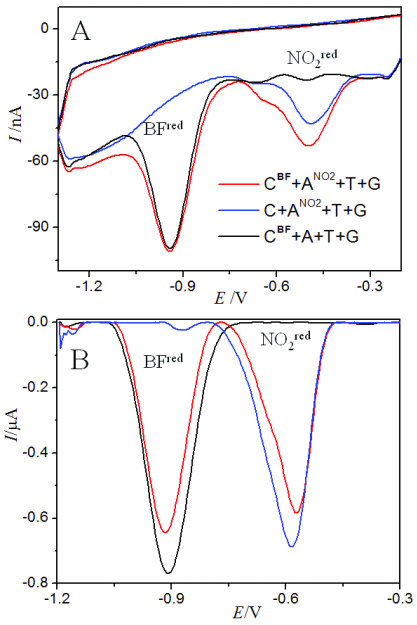
Po úspešných inkorporáciach troch rôznych redoxných značiek **20** (**dC^{BF}TP**), **15** (**dA^{NO2}TP**) a **27** (**dT^{NH2}TP**) do DNA sme navrhli 10 templátov pre syntézu ON obsahujúcich rozdielné kombinácie týchto redoxných značiek v rôznych sekvenciách (Tabuľka 6). Tieto modifikované ON (Tabuľka 6) boli použité pre electrochemické štúdie.

7.2.3 Electrochemické štúdie benzofurazánom-modifikovaných nukleozidov, nukleotidov a DNA

Elektrochemické vlastnosti benzofurazánom-modifikovaných nukleozidov, nukleotidov a DNA boli študované pomocou cyklickej voltametrie na ortuťovej alebo uhlíkovej elektróde. Predpokladaný mechanizmus elektrochemickej nevratnej redukcie benzofurazánu zahŕňa šesť elektrónov a šesť protónov a dochádza k redukcii dvoch C=N dvojitých väzieb a k uvoľneniu molekuly vody. Benzofurazánové konjugáty **18** (**dC^{BF}**), **19** (**dA^{BF}**), **23** (**dC^{EBF}**), **24** (**dA^{EBF}**), **20** (**dC^{BF}TP**), **21** (**dA^{BF}TP**), **25** (**dC^{EBF}TP**) a **26** (**dA^{EBF}TP**) produkujú na ortuťovej elektróde intenzívny katodický signál (prislúchajúc k BF^{red}) v rozmedzí -0.70 a -0.85 V a signály prislúchajúce redukciám cytozínu alebo adenínu pri potenciáli -1.2 V[6]. Všetky PEX produkty obsahujúce **A^{BF}**, **A^{EBF}**, **C^{BF}** alebo **C^{EBF}** poskytujú nevratný symetrický katodický signál pri potenciáli -0.8 V, ktorý je priradený k redukcii BF skupiny.

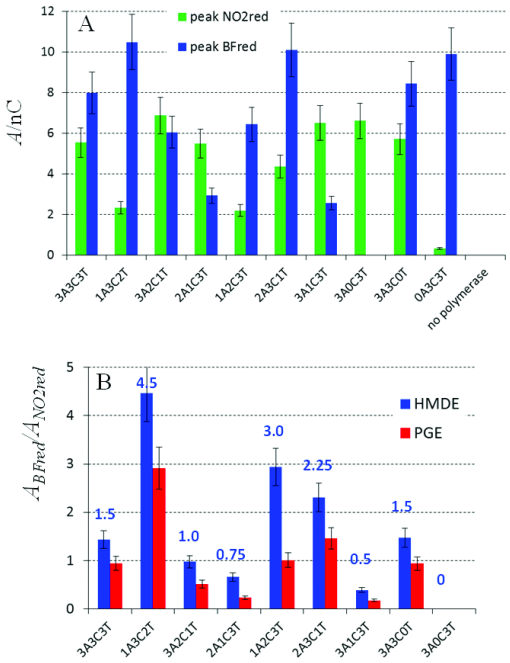
7.2.4 Multipotenciálové redoxné kódovanie DNA (v spolupráci so skupinou Doc. Fojty)

Našu pozornosť sme zamerali na multipotenciálové DNA kódovanie kombináciou benzofurazánu s redukovateľnou nitrofenylovou ($PhNO_2$) a oxidovateľnou aminofenylovou ($PhNH_2$) značkou.[13] Najprv sme testovali inkorporácie dvoch redukovateľných značiek **20** (**dC^{BF}TP**) a **15** (**dA^{NO2}TP**) do jedného vlákna DNA, čo by nám malo umožniť nezávislú detekciu modifikácií bez značného vzájomného prekryvu.



Obrázok 5. CV záznamy na ortuťovej elektróde (A) a SWV na uhlíkovej elektróde (B) získané z PEX produktov syntetizované použitím temp^{3A3C3T} templátov a kombináciou BF a/alebo $PhNO_2$ **dN^{X}TP** s nemodifikovanými dNTPs, ako je znázornéné symbolmi in popise platné pre oba obrázky.

Obrázok 5a zobrazuje voltamogram PEX produktov získaných z temp^{3A3C3T} templátov a dNTP zmesi obsahujúcej buď **20** (**dC^{BF}TP**), **15** (**dA^{NO2}TP**) alebo kombináciu oboch inkorporovaných v jednej reakcií. Signály vo voltamogramoch PEX produktov obsahujúcich tri BF a/alebo tri $PhNO_2$ značky na ortuťovej elektróde zobrazujú zloženie PEX produktov (Obrázok 5). Intenzity signálov odpovedajú počtu očakávaných elektrónov, ktoré by sa mali podieľať na redukčnom procese každej značky ($6e^-$ pre benzofurazán a $4e^-$ pre $PhNO_2$) a zároveň žiaden prekryv signálov nebol pozorovaný. Obrázok 5b zobrazuje analogické výsledky získané z tých istých PEX produktov meraných na uhlíkovej elektróde. Oligonukleotidy, ktoré boli navrhnuté pre inkorporácie troch značených nukleotidov v rôznych pomeroch boli použité pre elektrochemické štúdie (Tabuľka 6). Obrázok 6A zobrazuje intenzity signálov BF^{red} a NO_2^{red} získaných pre jednotlivé PEX produkty. Intenzity signálov redukovateľných značiek sa líšia so zmenou počtu inkorporovaných modifikácií v DNA v jednotlivých sekvenciách.



Obrázok 6. a) CV signály BF^{red}, NO_2^{red} a G získané na ortuťovej elektróde z PEX produktov syntetizovaných s $temp^{xAyCzT}$ templátmi (počet A, C a T v syntetizovaných sekvenciách sú znázornené v grafe) a **20** (**$dC^{BF}TP$**) + **15** (**$dA^{NO2}TP$**) + **27** (**$dT^{NH2}TP$**) + dGTP zmes; b) Pomery signálov BF^{red}/NO_2^{red} získané z tých istých PEX produktov. Modré hodnoty znázorňujú očakávané hodnoty vyrátané z pomeru počtu značiek v ON a počtu elektrónov spotrebovaných modifikáciou počas redoxného procesu.

Intenzity signálov (Obrázok 6) získané z PEX produktov obsahujúcich identický počet značených nukleotidov (napr. tri C^{BF}) sa líšia v malej miere v porovnaní so vzorkami obsahujúcich rovnaký počet značiek. Výsledkom môže byť rozdielný výťažok jednotlivých ON po izolácií. Spôsob ako eliminovať tento efekt rozdielných koncentrácií DNA je určenie pomeru intenzít signálov dvoch nezávisle detekovateľných značiek. Figure 6b zobrazuje pomery signálov BF^{red}/NO_2^{red}, pričom modré hodnoty v Obrázok 6b zobrazujú očakávané hodnoty pomerov počtu značiek v ON a počtu elektrónov spotrebovaných počas redoxného procesu značkou.

7.3 Azidofenylová akupina ako transformovateľná redoxná značka vhodná pre elektrochemickú detekciu interakcií DNA-proteín

7.3.1 Syntéza modifikovaných nukleozidov a nukleotidov nesúcich azido- a (nitrofenyl)triazolovú skupinu

Syntéza azidofenyl-modifikovaných nukleozidov je založená na Suzuki-Miyaurovej cross-couplingovej reakcií 5-jodocytidínu **1** (**dC^I**) a 7-deaza-7-jodoadenozínu **2** (**dA^I**) s 4-

azidofenyltrifluoroborátom[17] **30** v prítomnosti **[**$PdCl_2$(dppf)] a Cs_2CO_3 v MeOH poskytla modifikované nukleozidy **31** (**dC^{A}**) a **32** (**dA^{A}**) vo výťažku 58-63 % (Schéma 7, Tabuľka 7, riadok 1, 2). Huisgen-Sharplessová reakcia[18] medzi azidofenylom-modifikovanými nukleozidmi **31** (**dC^{A}**) a **32** (**dA^{A}**) a alkínmi **33** (fenylacetylén) alebo **34** (1-etynyl-4-nitrobenzén) poskytli 1,4-disubstituované 1,2,3-triazoly **35** (**dC^{TP}**), **36** (**dA^{TP}**), **37** (**dC^{TNO2}**) a **38** (**dA^{TNO2}**) vo výťažku 40-94 % (Schéma 7, Tabuľka 7, riadok 5-8). Pre prípravu **43** (**dC^{A}TPs**) a **44** (**dA^{A}TPs**) sme aplikovali trifosforyláciu modifikovaných nukleozidov **dN^{A}** a získali sme **43** (**dC^{A}TP**) a **44** (**dA^{A}TP**) (Schéma 7, Tabuľka 7, riadok 3, 4) v 21 a 34 % výťažku po izolácii na RP HPLC. Triazolom-modifikované trifosfáty **39** (**dC^{TP}TP**), **40** (**dA^{TP}TP**), **41** (**dC^{TNO2}TP**), **42** (**dC^{TNO2}TP**) boli pripravené trifosforyláciou modifikovaných nukleozidov **35** (**dC^{TP}**), **36** (**dA^{TP}**), **37** (**dC^{TNO2}**) a **38** (**dA^{TNO2}**) (Schéma 7, Tabuľka 7, riadok 9-12) vo výťažku 13-52 %.

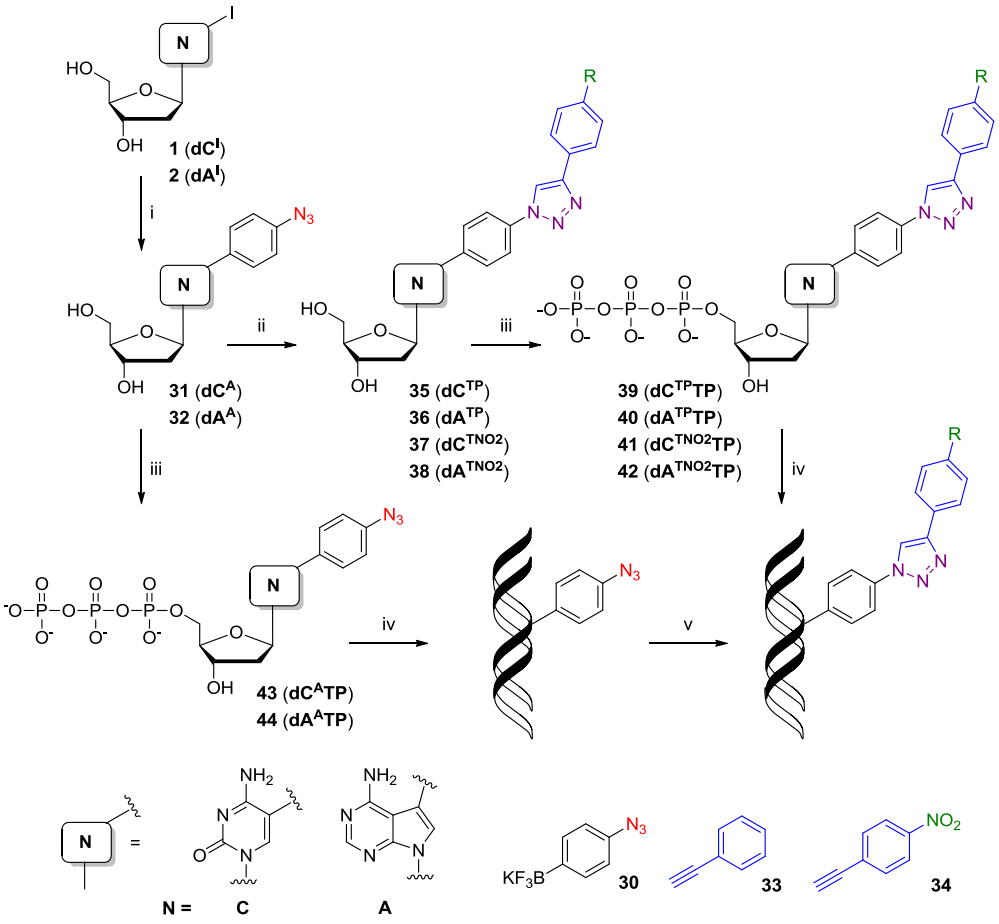


Schéma 7. Syntéza modifikovaných nukleozidov: i) Suzuki-Miyaurová cross-couplingová reakcia: **30** (4-azidofenyltrifluoroborát), $PdCl_2$(dppf), Cs_2CO_3, MeOH, 2 h, 80 ºC; ii) CuAAC: **33** (fenylacetylén) / **34** (1-etynyl-4-nitrobenzén), askorbát sodný, $CuSO_4.5H_2O$, *t*BuOH:H_2O (1:1), 12 h, rt; iii) Trifosforylácia modifikovaných nukleozidov: 1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB; iv) PEX experiment; v) Azid-alkínová cykloadícia: **33** / **34**, askorbát sodný, CuBr, TBTA ligand, *t*BuOH:DMSO (1:3), 2 h, 37 ºC.

Tabuľka 7. Syntéza modifikovaných nukleozidov a nukleotidov.

Poradie	Východisková látka	Reagent	Aditíva	Rozpúšťadlo	Produkt	Reakčný čas	Výťažok (%)[a]
1	**2** (**dA^{I}**)	**30**	$PdCl_2$(dppf), Cs_2CO_3	MeOH	**32** (**dA^{A}**)	2 h, 80 ºC	58
2	**1** (**dC^{I}**)	**30**	$PdCl_2$(dppf), Cs_2CO_3	MeOH	**31** (**dC^{A}**)	2 h, 80 ºC	63
3	**32** (**dA^{A}**)	1. $PO(OMe)_3$, $POCl_3$, 0ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**43** (**dA^{A}TP**)	6 h, 0 ºC	34
4	**31** (**dC^{A}**)	1. $PO(OMe)_3$, $POCl_3$, 0ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**44** (**dC^{A}TP**)	6 h, 0 ºC	21
5	**32** (**dA^{A}**)	**33**	askorbát sodný, $CuSO_4.5H_2O$	*t*BuOH:H_2O (1:1)	**36** (**dA^{TP}**)	12 h, rt	72
6	**31** (**dC^{A}**)	**33**	askorbát sodný, $CuSO_4.5H_2O$	*t*BuOH:H_2O (1:1)	**35** (**dC^{TP}**)	12 h, rt	40
7	**32** (**dA^{A}**)	**34**	askorbát sodný, $CuSO_4.5H_2O$	*t*BuOH:H_2O (1:1)	**38** (**dA^{TNO2}**)	12 h, rt	94
8	**31** (**dC^{A}**)	**34**	askorbát sodný, $CuSO_4.5H_2O$	*t*BuOH:H_2O (1:1)	**37** (**dC^{TNO2}**)	12 h, rt	62
9	**36** (**dA^{TP}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**40** (**dA^{TP}TP**)	18 h, 0 ºC	13
10	**35** (**dC^{TP}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**39** (**dC^{TP}TP**)	18 h, 0 ºC	52
11	**38** (**dA^{TNO2}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**42** (**dA^{TNO2}TP**)	18 h, 0 ºC	18
12	**37** (**dC^{TNO2}**)	1. $PO(OMe)_3$, $POCl_3$, 0 ºC; 2. $(NHBu_3)_2H_2P_2O_7$, Bu_3N, DMF, 0 ºC; 3. TEAB			**41** (**dC^{TNO2}TP**)	18 h, 0 ºC	18

[a] izolovaný výťažok

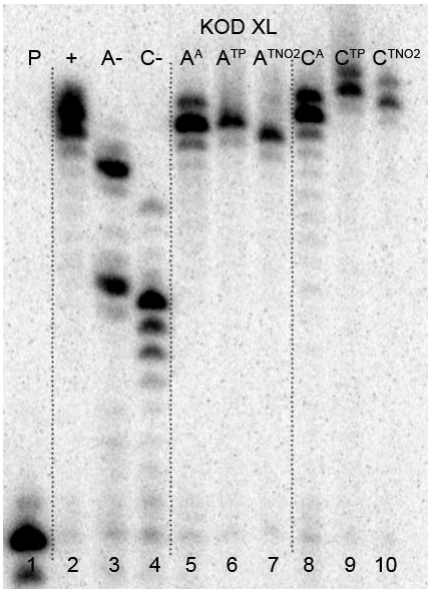
7.3.2 Electrochemické štúdie modifikovaných trifosfátov

Elektrochemické vlastnosti modifikovaných trifosfátov **44** (**dA^{A}TP**), **43** (**dC^{A}TP**), **40** (**dA^{TP}TP**), **39** (**dC^{TP}TP**), **42** (**dA^{TNO2}TP**) a **41** (**dC^{TNO2}TP**) boli študované pomocou cyklickej voltametrie na ortuťovej elektróde. Azidofenylom-modifikované nukleotidy **44** (**dA^{A}TP**) a **43** (**dC^{A}TP**) poskytujú signál pri potenciáli -0.9 V (signál N_3^{red}), zatiaľ čo fenyltriazolové deriváty **40** (**dA^{TP}TP**) a **39** (**dC^{TP}TP**) neposkytli žiaden redoxný signál. Na druhej strane nitrofenyltriazolové deriváty **42** (**dA^{TNO2}TP**) a **41** (**dC^{TNO2}TP**) poskytli redukčný signál pri potenciáli -0.4 V (peak NO_2^{red}). Azidofenylové deriváty môžu byť ľahko transformovateľné na oba typy triazolov pomocou CuAAC reakcie s alkínmi. CuAAC reakcie s fenylacetylénom môžu byť použité pre stratu redoxných vlastností, alebo reakciou s nitrofenylacetylénom pre zmenu jednej redoxnej značky na inú so zmenou redoxného potenciálu.

7.3.3 Enzymatická syntéza modifikovanej DNA

Ďalším cieľom bola polymerázou katalyzovaná syntéza DNA nesúca azidofenylové značky a štúdium ich premeny na (nitrofenyl)triazolové skupiny pomocou CuAAC reakcie azidofenylom-modifikovanej DNA s acetylénami (**33** fenylacetylén alebo **34** 1-etynyl-4-

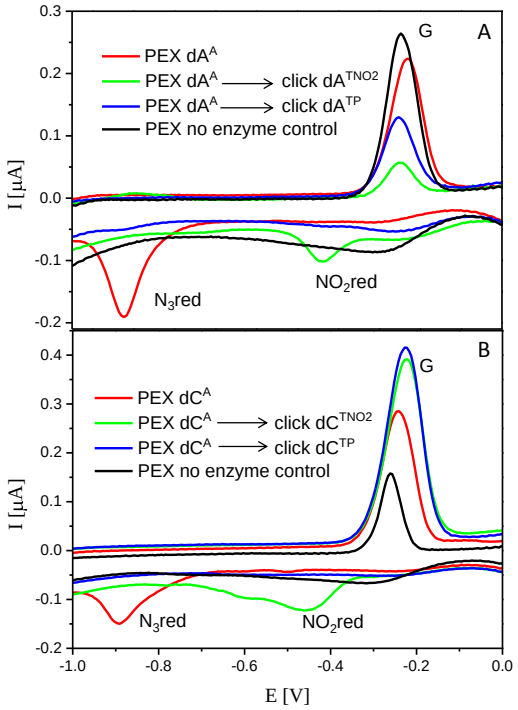
nitrobenzén) za prítomnosti CuBr, TBTA ligandu a askorbátu sodného v *t*BuOH:DMSO (1:3) pri 37 ºC počas 2 hodín. Obrázok 7 zobrazuje PEX produkty po Cu(I)-katalyzovanej CuAAC reakcie bez degradácie DNA. Pre porovnanie, aj priama inkorporácia triazolom-modifikovaných nukleotidov pomocou **40** (**dA^{TP}TP**), **39** (**dC^{TP}TP**), **42** (**dA^{TNO2}TP**) a **41** (**dC^{TNO2}TP**) ako substrátov do DNA bola testovaná (výsledok nie je zobrazený).



Obrázok 7. PEX inkorporácie do DNA (31 báz) pomocou **44** (**dA^{A}TP**) a **43** (**dC^{A}TP**), templátu temp^{rnd16} a KOD XL polymerázy a nasledovnou CuAAC reakciou s 1-etynyl-4-nitrobenzénom **34** a fenylacetylénom **33**.

7.3.4 Electrochemické štúdie modifikovanej DNA

Voltametrické vlastnosti modifikovanej DNA (PEX produkty) obsahujúcej **N^{A}** , **N^{TP}** a **N^{TNO2}** boli študované pomocou cyklickej voltametrie na ortuťovej elektróde. Obrázok 8 zobrazuje cyklický voltamogram PEX produktov pripravených použitím templátu temp^{rnd16} obsahujúcich azidofenylom-modifikované nukleobázy (**A^{A}** alebo **C^{A}**). Nevratný katodický signál pri potenciáli -0.9 V odpovedá redukcii azido skupiny (signál N_3^{red}, červená krivka). Kontrolný experiment PEX reakcie bez použitia polymerázy v zmesi detekuje neprítomnosť signálu azido-skupiny, čím sa vylučuje prítomnosť **dN^{A}TPs** v zmesi. PEX produkty obsahujúce fenyltriazolovú skupinu **A^{TP}** a **C^{TP}** , ktoré boli pripravené pomocou CuAAC reakcie **N^{A}**-modifikovanej DNA s **33** (fenylacetylén) a neposkytli žiaden redoxný signál (modrá krivka). Na druhej strane CuAAC reakcia **N^{A}**-modifikovanej DNA s **34** (1-etynyl-4-nitrobenzénom) poskytla DNA produkty obsahujúce nitrofenyltriazolové skupiny **A^{TNO2}** a **C^{TNO2}**, ktoré poskytli nevratný katodický signál pri potenciáli -0.4 V, čo odpovedá redukcii nitro-skupiny (signál NO_2^{red}, zelená krivka).



Obrázok 8. Detail CV PEX produktov syntetizovaných s temp^{rnd16} templátom a dNTP zmesou obsahujúcou **dN^{A}TP** konjugáty a PEX produkty po CuAAC rekcii s **33** (fenylacetylénom) / **34** (1-etynyl-4-nitrobenzénom) merané na ortuťovej elektróde.

7.3.5 Transformácie redoxných značiek pomocou CuAAC reakcie s aplikáciou v detekcii interakcií DNA-proteín

Premena azidovej redoxnej značky na nitrofenyltriazolovú môže byť použitá ako nová metóda pre detekciu interakcií DNA-proteín (Schéma 8). Predpokladali sme, že ak inkorporujeme niekoľko azidofenylových modifikácií do DNA, následne DNA inkubujeme s proteínom a uskutočníme CuAAC reakciu s nitrofenylacetylénom, tak iba voľne dostupné azido-skupiny (neprikryté proteínom) môžu byť transformované na nitrofenyltriazoly a pomer azido/nitro redoxných signálov indikuje, či proteín bol naviazaný na DNA alebo nie.

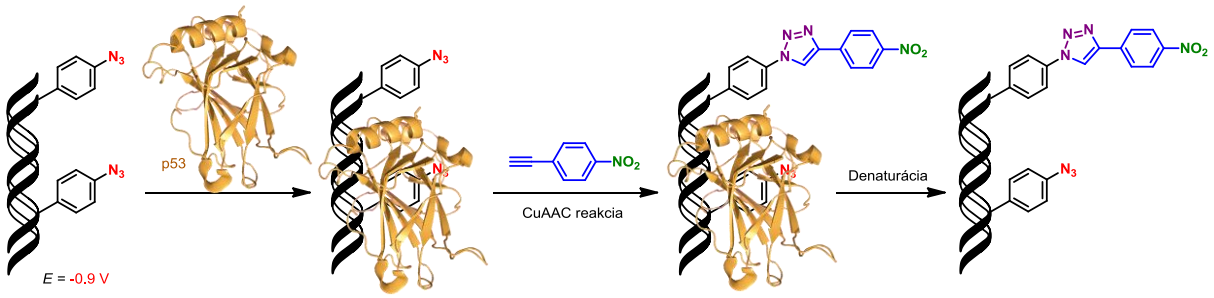


Schéma 8. Princíp elektrochemickej detekcie interakcií DNA-proteín.

Zvolili sme tumor supresorový proteín[19,20] p53 (p53CD_GST) pre jeho sekvenčne-špecifickú[21] väzbu k DNA. Navrhli sme dve rôzne sekvencie 50-bázovej DNA (templáty $temp^{p53\_1a2G}$ a $temp^{p53\_2CON4}$, Tabuľka 8), v ktorej 6 azido-skupín je vo vnútri a 6 azido-skupín je mimo dosah sekvencie špecificky rozpoznávanou proteínom p53.

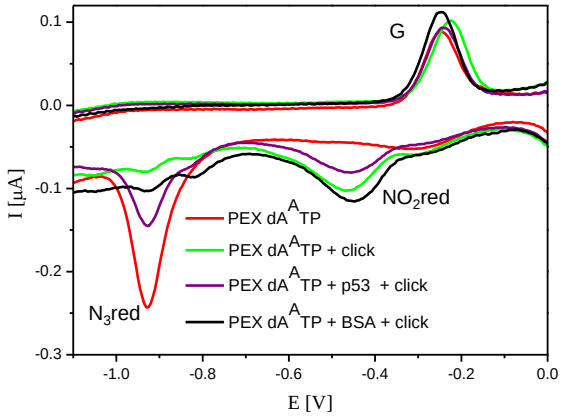
Tabuľka 8. Sekvencie ON[a]

Sekvencie	
ON $^{p53\_2CON4}$	5'-GAATTCGATATCAAG$A^AGA^ACA^ATGTCTA^AGA^ACA^ATGTCTA^ATTA^ATGGA^ATA^AA^AGGTA^A$-3'
ON $^{p53\_1a2G}$	5'-GAATTCGATATCAAG$A^AGA^ACA^ATGCCTA^AGA^ACA^ATGTTTA^ATTA^ATCCA^ATA^AA^ACCTA^A$-3'

[a] p53 rozpoznávacia sekvencia je označená kurzívou a nukleotidy nesúce modifikácie sú znázornené tučne a sú podčiarknuté.

Po všetkých experimentoch testovania stability komplexu DNA-proteín (DNA_p53CD_GST komplex) sme uskutočnili experimenty s electrochemickou detekciou interakcií DNA-proteín. Cyklická voltametria $\mathbf{A}^{\mathbf{A}}$-viazanej DNA (50-báz, **ON**$^{p53\_1a2G}$) obsahujúca 12 azidofenylových skupín zobrazuje signál N_3 redukcie pri potenciáli -0.9 V (Obrázok 9, červená krivka). $\mathbf{A}^{\mathbf{A}}$-viazaná DNA reagovala s **34** (4-nitrofenylacetylénom), CuBr, TBTA ligandom, askorbátom sodným pri 20 ºC počas 1 h za optimalizovaných podmienok, ktoré sú vhodné bez porušenia komplexu DNA-proteín. Voltametrická analýza ukazuje plnú premenu všetkých azido-skupín na nitrofenyltriazoly, čo potvrdzuje zánik signálu pri potenciáli -0.9 V a vznik nového signálu pri -0.4 V, čo odpovedá redukcii NO_2 skupiny (Obrázok 9, zelená krivka). V ďalšom experimente $\mathbf{A}^{\mathbf{A}}$-viazaná DNA bola inkubovaná s 1.2 ekvivalentom p53CD_GST (počas 45 min. na ľade) pre vznik komplexu DNA_p53CD_GST. Komplex DNA-proteín reagoval s **34** (4-nitrofenylacetylénom) za vyššie spomínaných podmienok (CuAAC reakcia) s následnou denaturáciou. CV tohto produktu (Obrázok 9, fialová krivka) ukázala 50 % úbytok intenzity signálu N_3^{red} redukcie azido-skupín a nárast signálu NO_2^{red} (50 % rozdiel intenzity signálu v porovnaní s produktom bez prítomnosti proteínu p53). To potrvdzuje, že iba voľné azido-skupiny (nezakryté proteínom) podliehajú transformácií na nitrofenyltriazoly pomocou CuAAC reakcie, zatiaľ čo N_3 skupiny prikryté proteínom sa nemenia. Ako kontrolu sme ešte uskutočnili reakciu $\mathbf{A}^{\mathbf{A}}$-viazanej DNA v prítomnosti BSA proteínu, ktorý sa neviaže na DNA s rovnakým výsledkom ako u experimentu bez prítomnosti proteínu (Obrázok 9, čierná krivka, všetky azido-skupiny sú premenené na nitrofenyltriazoly).

ON$^{p53\_1a2G}$: 5'-GAATTCGATATCAAG$A^AGA^ACA^ATGCCTA^AGA^ACA^ATGTTTA^ATTA^ATCCA^ATA^AA^ACCTA^A$-3'



Obrázok 9. Detail CV PEX produktov syntetizovaných s temp^{p53_1a2G} templátom a **44** (**dA^{A}TP**) konjugátom a PEX produkty po CuAAC reakcií s **34** (4-nitrofenylacetylénom) (zelená krivka), DNA-p53 komplex po CuAAC reakcií nasledovaná denaturáciou (fialová krivka), kontrola s BSA (čierna krivka) na ortuťovej elektróde.

8 Záver

Antrachinónom-modifikované nukleozidy a nukleotidy viazané cez konjugovaný acetylénový linker alebo cez nekonjugovaný propargylkarbamoylový linker boli pripravené a testované ako substráty pre DNA polymerázy. V enzymatických inkorporáciach bol flexibilnejší **dN^{PAQ}TP** lepší substrát než rigidný **dN^{EAQ}TP**. 10-násobne vyššia koncentrácia **dN^{XAQ}TP** spôsobuje inhibíciu polymerázy. Elektrochemické štúdie antrachinónom-modifikovaných nukleozidov, nukleotidov a DNA pomocou voltametrie zobrazuje signály vratnej redukcie antrachinónovej skupiny pri potenciáli -0.4 V. Kombinácia antrachinónovej modifikácie s nitrofenylovou skupinou poskytla jeden nerozlíšený signál. Avšak cyklická voltametria môže rozlíšiť medzi týmito značkami, pretože redukcia NO_2 je nevratná a redukcia antrachinónu je vratná. Nezávislá detekcia nitro-skupiny v prítomnosti antrachinónu je možná pomocou oxidácie hydroxylamínu, ktorý je produktom nitrofenylovej redukcie. Keďže **dC^{PAQ}TP** a **dA^{PAQ}TP** viazané cez propargylkarbamoylový mostík sú vhodnými substrátmi pre polymerázovú inkorporáciu, antrachinón môže byť použitý pre multipotenciálové DNA kódovanie v kombinácií s inými značkami.

Vdruhej časti projektu bol benzofurazán pripojený k nukleozidom a nukleotidom priamo alebo cez acetylénový mostík. Benzofurazánom modifikované nukleotidy boli testované ako substráty pre DNA polymerázy a boli inkorporované do rozdielnych sekvencií pomocou PEX experimentu. Elektrochemická redukcia furazánového kruhu spotrebuváva $6e^-$ a poskytuje intenzivný katodický signál pri potenciáli -0.8 V merateľný na ortuťovej a

uhlíkovej elektróde bez prekryvu s potenciálmi prirodzených báz. DNA značenie redukovateľnými značkami benzofurazánom, nitrofenylom a oxidovateľnou značkou aminofenylom neukazuje žiaden prekryv medzi týmito značkami. Oligonukleotidy, ktoré boli navrhnuté pre inkorporáciu troch rôzne značených nukleotidov **dA^{NO2}TP, dC^{BF}TP** a **dT^{NH2}TP** v rôznych pomeroch boli použité pre elektrochemické štúdia. Počet benzofurázanových a nitrofenylových značiek inkorporovaných do rôznych sekvencií je možné určiť nezávisle na sebe. Intenzity signálov jednotlivých modifikácii odpovedajú počtu modifikácii v sekvencii, čo by mohlo byť využité v "ratiometric analysis of nucleotide sequences" (napr. elektrochemická detekcia mutácií v DNA založená na zmene v pomere dvoch nukleobáz značených dvoma rozdielnými redoxnými značkami). Aminofenyl sa javil ako vhodná značka vhodná pre kvalitatívnu analýzu, ale nie pre (semi)kvantívne pomerové elektrochemické analýzy nukleotidovej sekvencie.

V tretej časti projektu boli pripravené azidofenylom-modifikované nukleozidy a nukleotidy a polymerázová inkorporácia azidofenylom-modifikovaných nukleotidov do DNA pomocou PEX experimentu použitím azidofenylom-modifikovaných nukleotidov ako substrátov bola študovaná. Nukleotidy a DNA nesúca azidofenylovú skupinu poskytujú pomocou voltametrie signál pri potenciáli -0.9 V. Azido-skupina bola tranformovaná na nitrofenyltriazol pomocou CuAAC reakcie. Fenyltriazolom modifikované nukleotidy neposkytujú žiaden redoxný signál, zatiaľ čo nitrofenyltriazoly poskytujú redukčný signál pri potenciále -0.4 V. Azidofenylom-modifikované nukleotidy sú užitočné redoxné značky pre DNA, ktoré môžu byť použité pre stratu redoxných vlastností, alebo pre zmenu jednej redoxnej značky na inú so zmenou redoxného potenciálu. Táto transformácia bola použitá v detekcií interakcií DNA-proteín. Prípravou azidofenylom-modifikovanej DNA, inkubáciou DNA s proteínom a následnou CuAAC reakciou s nitrofenylacetylénom, iba voľne dostupné azido-skupiny (neprikryté proteínom) môžu byť transformované na nitrofenyltriazoly a azidofenylové-skupiny prikryté proteínom zastávajú nezmenené. Elektrochemické merania sú použité pre analýzu výsledku a je možné určiť, či bol vytvorený komplex DNA-proteín alebo nie na základe pomeru intenzít signálov transformovaných a netransformovaných značiek.

9 Zoznam publikácií – Selected publications

1 J. Balintová, R. Pohl, P. Horáková, P. Vidláková, L. Havran, M. Fojta and M. Hocek: "Anthraquinone as a Redox Label for DNA: Synthesis, Enzymatic Incorporation, and Electrochemistry of Anthraquinone-Modified Nucleosides, Nucleotides, and DNA" *Chem. Eur. J.* **2011**, *17*, 14063-14073.

2 J. Balintová, M. Plucnara, P. Vidláková, R. Pohl, L. Havran, M. Fojta and M. Hocek: "Benzofurazane as a New Redox Label for Electrochemical Detection of DNA: Towards Multipotential Redox Coding of DNA Bases" *Chem. Eur. J.* **2013**, *19*, 12720-12731.

3 A. Simonova, J. Balintová, Radek Pohl, L. Havran, Miroslav Fojta and Michal Hocek: "Methoxyphenol and Dihydrobenzofuran as Oxidizable Labels for Electrochemical Detection of DNA" *ChemPlusChem* **2014**, in press, doi: 10.1002/cplu.201402194.

4 J. Balintová, J. Špaček, R. Pohl, M. Brázdová, L. Havran, M. Fojta and M. Hocek: "Azidophenyl as a click-transformable redox label of DNA suitable for electrochemical detection of DNA-protein interactions" *Chem. Sci.* **2014**, in press, doi: 10.1039/C4SC01906G.

10 Konferenčné príspevky- Conference contributions

1. "Evolving DNA Polymerases: Chemistry meets Biology" Meeting, Monte Verita, Switzerland 10/2010
2. XVth Symposium on Chemistry of Nucleic Acid Component, Český Krumlov, Czech Republic 06/2011(poster)
3. 17th European Symposium On Organic Chemistry, Crete, Greece 07/2011 (poster)
4. Challenges in Organic Chemistry and Chemical Biology, Edinburgh, UK 06/2012 (poster)
5. 4th EuCheMS Chemistry Congress. Prague, Czech Republic 09/2012 (poster)
6. Heterocycles in Bio-organic Chemistry, Riga, Latvia 05/2013 (poster)
7. SYLICA Workshop on chemical modification and redox labeling of biopolymers for biosensing, Brno, Czech republic 09/2013 (poster)
8. 48th Advances in Organic, Bioorganic and Pharmaceutical Chemistry, Špindlerův Mlýn, Czech Republic, 11/2013 (lecture)
9. XVIth Symposium on Chemistry of Nucleic Acid Components, Český Krumlov, Czech Republic 06/2014 (lecture, poster)
10. 49th Advances in Organic, Bioorganic and Pharmaceutical Chemistry, Lázně Bělohrad, Czech Republic, 11/2014 (lecture)

11 Curriculum vitae

2009- **PhD study – Organic chemistry**

Faculty of Science, Charles University in Prague

Institute of Organic Chemistry and Biochemistry AS CR, Prague

Ph. D. thesis: *Synthesis of functionalized nucleosides and nucleotides and their incorporation into DNA*

Supervisor: *Prof. Michal Hocek, Ph.D., DSc.*

2004 - 2009 **undergraduate study – Organic chemistry**

Faculty of Science, P.J.Šafárik University, Department of organic chemistry, Košice, Slovak Republic, MS degree

Diploma work: *Study stereoselectivity of Claisen rearrangement induced by microwave irradiation of reaction center on C-3 of compound derived from D-xylose*

Supervisor: *Prof. Jozef Gonda, DrSc.*

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