DNA–Protein cross-linking

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Vinylsulfonamide and Acrylamide Modification of DNA for Cross-linking with Proteins**

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Bioconjugation of nucleic acids with peptides, proteins, and other biomolecules is of paramount importance in medicinal chemistry and chemical biology.[1] In most cases, the biomolecule is linked to a sugar or phosphate at either 3′- or 5′-end of an oligonucleotide (ON).[2] However, a connection through a nucleobase offers a good programmability of the position(s) and number of modifications. Introduction of some reactive groups, that is, alkyne, alkene, azide, diene, or aldehyde groups or a cysteine residue, was achieved either by chemical phosphoramidite synthesis or by enzymatic polymerase synthesis. The modified DNAs were used for [2+3]-dipolar cycloadditions,[3,4] Staudinger ligation,[5] Diels–Alder reactions,[6] hydrazone formation,[7] reductive aminations,[8] or native peptide ligations.[9] However, none of these reactive groups allow bioorthogonal and specific cross-linking with a natural amino acid residue in a peptide or protein. Bioconjugation of native proteins with other molecules has also been extensively studied,[10] and the SH group of cysteine was shown to be the most easily targetable, as it readily undergoes Michael additions to maleimide[11] (or other Michael acceptors, MA). Herein we present the synthesis of nucleotides ON and DNA bearing reactive acrylamide or vinylsulfonamide groups and their covalent cross-linking reactions with cysteine-containing peptides and DNA binding domain of protein p53 (Figure 1).

Our strategy for the synthesis of reactive ON or DNA probes exploits the polymerase incorporation[12] of base-modified deoxyribonucleoside triphosphates (dNTPs) prepared by aqueous cross-coupling reactions of halogenated dNTPs.[13] Therefore the first goal was the synthesis of dNTPs bearing some suitable MA groups. Owing to instability of maleimide function under cross-coupling conditions, we turned our attention to acrylamide and vinylsulfonamide moieties tethered to the nucleobase through propargylic linker. The whole chemistry was developed to modify cytosine nucleotides at the easily accessible position 5, which points to the major groove of DNA. Thus the *N*-propargylacrylamide and *N*-propargyl-vinylsulfonamide were prepared and tested in aqueous Sonogashira cross-coupling reactions with **dC^{I}MP** and **dC^{I}TP** (Scheme 1). The reactions in the presence of $Pd(OAc)_2$ and triphenylphosphine-3,3′,3″-trisulfonate (TPPTS) proceeded smoothly to give the desired MA-modified nucleotides **dC^{AA}MP** (46%), **dC^{AA}TP** (22%), **dC^{VS}MP** (19%), and **dC^{VS}TP** (23% yield).

Then the modified **dC^{X}MP**s were used as model compounds (Scheme 2) for testing of the Michael addition of cysteine (Cys) and glutathione (GSH). The reactions were performed with 1.1-fold excess of thiols overnight in pH 8.3 buffer. In all cases, quantitative conversions to the desired conjugates **dC^{AA}MP_Cys**, **dC^{AA}MP_GSH**, **dC^{VS}MP_Cys**, and **dC^{VS}MP_GSH** were observed. The products were isolated by

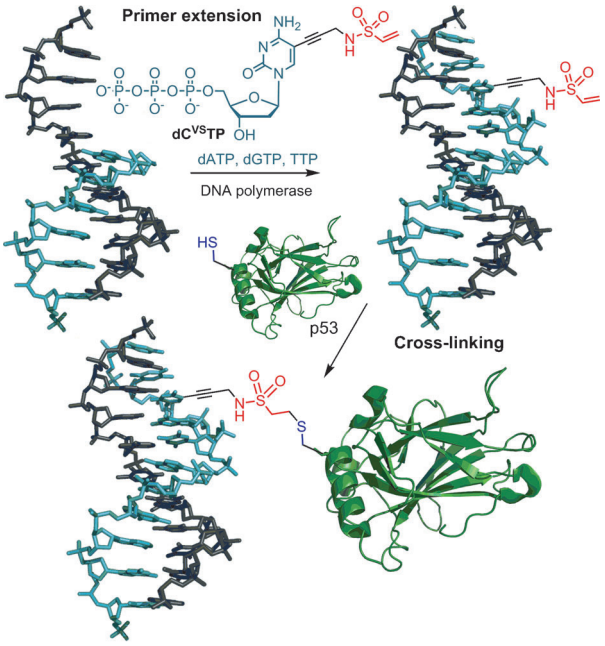

Figure 1. Synthesis of vinylsulfonamide modified DNA (**DNA^{VS}**) by PEX and its cross-linking with p53 (PDB 3EXJ[18b]).

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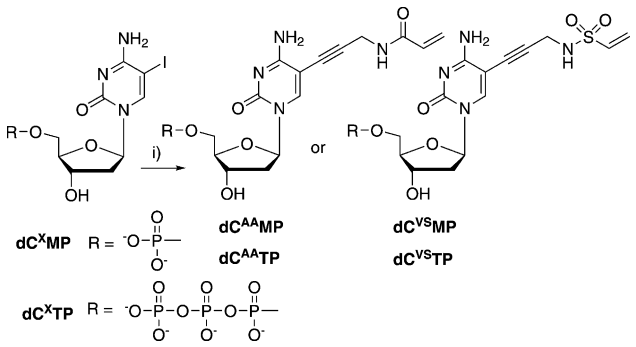
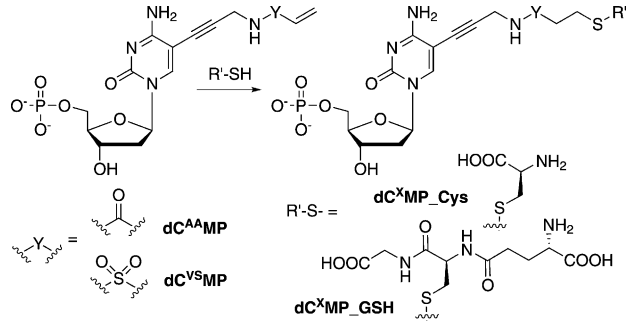


Scheme 1. Synthesis of modified **dN^{X}MP**s and **dN^{X}TP**s. Conditions: i) *N*-propargyl acrylamide or *N*-propargyl vinylsulfonamide (2 equiv), CuI (10 mol%), $Pd(OAc)_2$ (5 mol%), TPPTS (10 mol%).

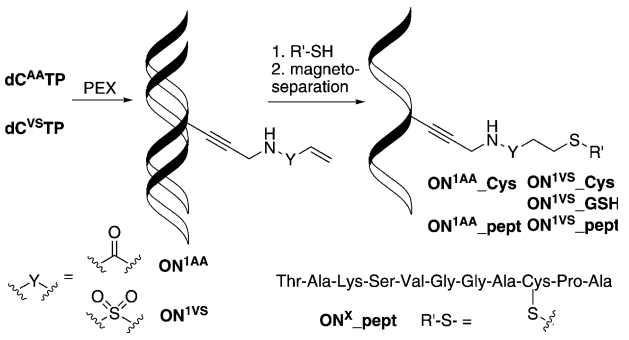


Scheme 2. Michael additions of L-cysteine and glutathione to modified **dC^{X}MP**s. Conditions: i) TEAA buffer (0.3 M, pH 8.3), 25 °C, overnight.

reverse-phase HPLC in 65–93% yields and fully characterized by MS and NMR spectroscopy.

The modified **dC^{X}TP**s (**dC^{AA}TP** and **dC^{VS}TP**) were then tested as substrates for polymerase in primer extension (PEX) experiments (Scheme 3). KOD XL polymerase gave clean full-length ON products which were characterized by PAGE (Figure 2a; Supporting Information, Figure S1; for sequences see Table S1) and MALDI (Supporting Information, Table S2 and Figures S11–S16), confirming that both these **dC^{X}TP**s are good substrates suitable for enzymatic incorporation. Double-stranded DNAs (dsDNAs) containing one Michael-acceptor label were then prepared by PEX and reacted with Cys, GSH, or Cys-containing undecapeptide (pept), followed by magnetoseparation[14] (Scheme 3). As the differences in mobilities on PAGE for conjugates with Cys and GSH were not convincing, the products of conjugation were successfully characterized by MALDI for **ON^{1AA}_Cys**, **ON^{1VS}_Cys**, and **ON^{1VS}_GSH**, but not for **ON^{1AA}_GSH**. The products of conjugation with undecapeptide were well visible on PAGE (Figure 2a; Supporting Information, Figure S2): vinylsulfonamide modified DNA gave almost full conversion to the desired conjugate **ON^{1VS}_pept** (also confirmed by MALDI; Supporting Information, Table S3 and Figures S17–S20), while acrylamide-modified DNA gave only 25% conversion. All these experiments confirm significantly lower reactivity of **ON^{1AA}** compared to vinylsulfonamide **ON^{1VS}**.

Having the reactive modifications and conjugation procedures in hand, we turned our attention to the cross-linking



Scheme 3. Michael additions of L-cysteine, glutathione, and an undecapeptide to modified ONs. Conditions: i) TEAA buffer (0.3 M, pH 8.3), 25 °C, overnight.

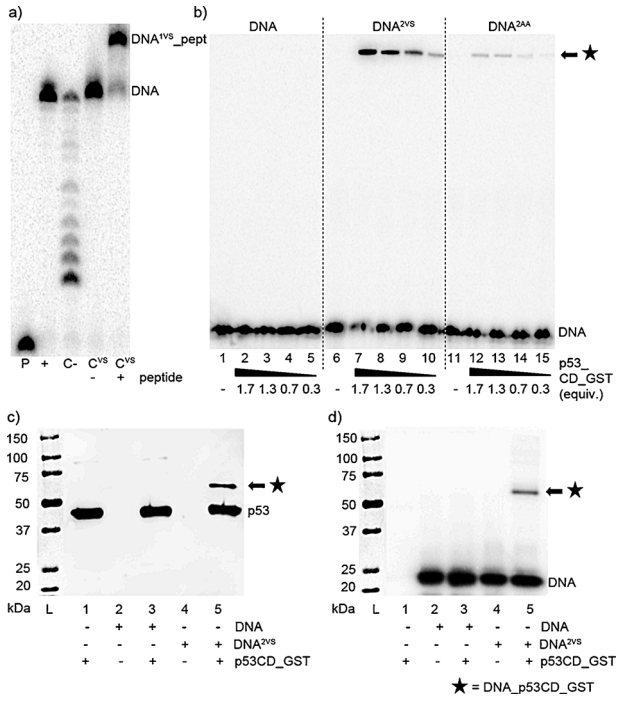


Figure 2. a) Incorporation of **dC^{VS}TP** to DNA (**DNA^{1VS}**) using KOD XL polymerase and its conjugation with undecapeptide (pept). P: Primer; +: natural dNTPs; C-: dTTP, dATP, dGTP; C^{VS}: **dC^{VS}TP**, dTTP, dATP, dGTP; C^{VS} + peptide: **DNA^{1VS}** (4.3 μM), pept (22 mM). b) Conjugation of p53CD_GST and **DNA^{2VS}** (0.15 μM). c) Immunodetection of **DNA^{2VS}_p53CD_GST** conjugate. d) Autoradiograph of 5′-^{32}P-end labeled **DNA^{2VS}_p53CD_GST** conjugate. L: protein standards; 1: p53CD_GST; 2: natural DNA; 3: natural **DNA** + **p53CD_GST**; 4: DNA^{2VS}; 5: **DNA^{2VS}** + **p53CD_GST**. For c) and d): DNA (1 μM), p53CD_GST (0.2 μM). All experiments: pH 7.6, 0 °C/30 min then 25 °C/2 h.

of DNA with a model DNA-binding protein. We have chosen GST-tagged DNA binding (core) domain of tumor-suppressor protein p53[15,16] (p53CD_GST) as a biologically relevant example of a sequence-specific[17] binder to DNA. From the published crystal structures of p53-DNA complexes,[18] we identified two cysteine residues (C275 and C277) in close proximity of the DNA sequence, where the C277 participates on the DNA binding (and thus is presumably available for the

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sequence specific cross-linking), whereas the C275 points to the opposite side and should not cross-link (for the inset from the crystal structure, see the Supporting Information, Figure S3).

To investigate covalent cross-linking between modified DNA and p53, DNA probes (Supporting Information, Table S1) containing p53 recognition sequence,[17] either natural or bearing two MA modifications, were designed and prepared by PEX (**DNA^{2AA}** and **DNA^{2VS}**). Then the DNAs were incubated with different ratios of protein p53 to test its binding activity. The recognition of the binding sequence by p53 protein was monitored by 5% native PAGE (Supporting Information, Figure S4a), confirming that the modifications do not prevent binding of p53. PAGE under denaturing conditions was then performed to see if covalent cross-links have been formed. Figure 2b shows the formation of covalent conjugates represented by the bands with lower mobility (lanes 7–10 and 12–15). Also here, it is clear that DNA^{2VS} is more reactive in Michael addition with p53 (48% conversion in lane 7 compared to only about 5% for DNA^{2AA} in lane 12), and therefore it was selected for all further experiments.

The presence of both p53CD_GST and modified DNA in the covalent adduct was independently confirmed by analysis on 10% SDS PAGE (Figure 2c,d). Western blotting followed by immunodetection of p53 (Figure 2c) confirmed the **DNA^{2VS}_p53CD_GST** conjugate represented by a band with mobility of about 60 kDa (lane 5), while free p53CD_GST moved at about 50 kDa (lanes 1, 3, and 5). Then the same PAGE was performed with 5′-^{32}P-labeled DNA (Figure 2d), where again the characteristic band of **DNA^{2VS}_p53CD_GST** appeared at about 60 kDa (lane 5).

To investigate the specificity of the cross-linking, several control experiments were done. Random-sequence dsDNA (lacking the recognition sequence for p53) bearing one VS-modification (**nonspecDNA^{VS}**) was prepared and incubated with p53CD_GST. Native gel (Supporting Information, Figure S7a) did not show any significant binding of p53 to natural non-specific dsDNA, but a weak band of a complex or conjugate to VS-modified dsDNA was still observed. However, on denaturing PAGE, the conjugate formation was confirmed also for the non-specific sequence (though the yield was lower). This could be explained by transient sequence non-specific DNA binding of the p53 core domain, which searches for its cognate site by hopping along the DNA duplex[19] and is trapped by the reactive MA group.

To rule out some non-specific binding of other cysteines or other amino acids present in p53CD or GST tag and to confirm the cross-linking of C277, a series of control experiments with different p53 constructs and mutants were performed (Figure 3). In the first set of experiments (Figure 3a), we tested the cross-linking with full-length p53 (p53fl, lane 2), untagged p53CD (lane 4), and GST tag alone (lane 5) in comparison with p53CD_GST (lane 3). The cross-linked products have been formed with all p53 constructs (whether or not containing GST tag), whereas the GST itself did not form any cross-link. In the next set of experiments, several mutants of p53CD were prepared and tested: mutant C277S (lacking the key cysteine crucial for the cross-link),

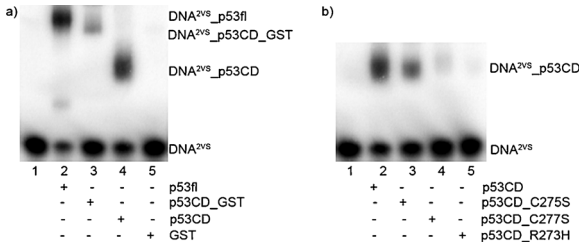

Figure 3. Conjugation of **DNA^{2VS}** with a) p53fl, p53CD_GST, p53CD and GST; b) p53CD mutants C275S, C277S, and R273H. Conditions: DNA (0.15 μM), protein (0.2 μM), pH 7.6, 0°C/30 min then 25°C/2 h.

C275S (lacking a cysteine presumably non-essential for the cross-link), and R273H (which contains both cysteines but does not specifically bind to DNA). Figure 3b shows that the wt p53CD and C275S mutant cross-link with **DNA^{2VS}** (lanes 2 and 3), whereas the C277S and R273H mutants do not form covalent conjugates (lanes 4 and 5). This combination of positive and negative control experiments fully confirms that only DNA-binding proteins containing a suitably positioned cysteine cross-link with the VS-modified DNA.

In conclusion, two types of MA-modified dCTPs bearing reactive acrylamide (AA) or vinylsulfonamide (VS) groups were designed and prepared. The MA modifications did not interfere with DNA polymerases, and the **dC^{X}TP**s were good substrates suitable for PEX synthesis of modified ONs or dsDNA. The MA-modified ONs or DNA readily react with cysteine or cysteine-containing peptides or proteins through Michael addition under physiological conditions. The reactivity of AA-modified nucleic acids is significantly lower than of vinylsulfonamides. The VS-modified DNA was successfully used for covalent cross-linking with different constructs of p53 protein. Therefore, the VS-modified **dN^{VS}TP**s are envisaged as suitable building blocks for the construction of ON or DNA probes for cross-linking to DNA-binding proteins or for the synthesis of irreversible inhibitors of some DNA-modifying enzymes containing a free cysteine (that is, DNA methyltransferases, and so on) or for DNA proteomics. Studies along these lines will continue in our laboratory.

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