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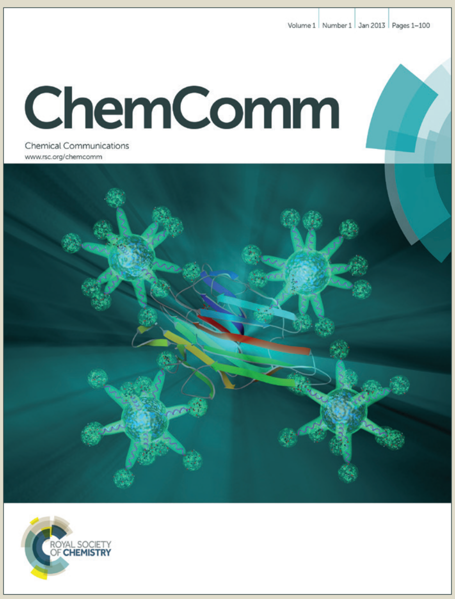

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Efficient labelling of enzymatically synthesized vinyl-modified DNA by an inverse-electron-demand Diels-Alder Reaction

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Many applications in biotechnology and molecular biology rely on modified nucleotides. Here, we present an approach for the postsynthetic labelling of enzymatically synthesized vinyl-modified DNA by Diels-Alder reaction with inverse electron demand using a tetrazine. The labelling proceeds very efficient and supersedes several known approaches.

Conjugation of biopolymers with functional molecules, e. g. affinity tags or fluorescent dyes, is crucial for many applications in molecular biology.[1] Conjugation of a biopolymer with a label is often a very challenging task. Bioorthogonal ligation reactions have been reported, which enable the site specific labelling of modified biopolymers, such as DNA.[2] For example, copper-catalyzed alkyne azide cycloaddition,[3] Staudinger ligation,[4] Diels-Alder reaction,[5] Michael addition,[6] reductive amination[7] or Suzuki cross coupling[8] have successfully been applied to modify DNA. These labelling reactions have in common, that the DNA and the label have to bear fitting reactive groups, which are used for conjugation. The introduction of these reactive moieties into DNA can also be challenging. In nature, DNA is synthesized by DNA polymerases, which catalyze the addition of nucleoside monophosphates to the 3'-end of an existing DNA polymer by using nucleoside triphosphates. Polymerases are also able to incorporate modified nucleotide analogues.[9] Thereby it is possible to introduce reactive moieties in DNA, which can afterwards be exploited for bioorthogonal conjugation. For the labelling of DNA the Diels-Alder reaction is of particular interest. It capitalizes on the selective cycloaddition of alkenes and dienes and does not require catalysts or toxic reagents. For Diels-Alder reactions with inverse electron demand (DARinv) 1,2,4,5-tetrazines[5b] have been used with strained alkenes, such as *trans*-cyclooctenes,[10] norbornenes[5f, 11] or cyclopropenes,[12] giving a dihydropyridazine derivative. Furthermore, it was shown that tetrazines also react with terminal alkenes.[13]

For the postsynthetic modification of enzymatically synthesized DNA via DARinv (Fig. 1A) we envisioned 7-vinyl-7-deaza-2'-deoxyadenosine ($d^{vin}A$) and 5-vinyl-2'-deoxyuridine ($d^{vin}U$) (Fig. 1B) as nucleoside analogues bearing vinyl group at the 7 or 5 position, respectively. The vinyl group is a very small modification that might render nucleosides into dienophiles for labelling by DARinv. In both cases the reactive group points towards the major groove of the DNA duplex and thus, should be accessible by labelling reagents. The nucleosides were synthesized according to known procedures.[14] As reaction partner in the Diels-Alder reaction a known biotinylated tetrazine derivative (Fig. 1B) was used.[13]

To test the ability of the nucleosides to undergo DARinv with tetrazines, we determined the second-order rate constants k_2 (Fig. S1). $d^{vin}A$ undergoes DARinv with a constant k_2 of 0.140 (± 0.001) (L/(mol·s) and $d^{vin}U$ with a rate constant k_2 of 0.010 (± 0.0002) (L/(mol·s). The kinetic rate constant of $d^{vin}U$ is comparable to a recent report.[15] We focused on $d^{vin}A$ in this study, as the DARinv proceeds 14-fold faster than the reaction with $d^{vin}U$. Furthermore, the rate constant of $d^{vin}A$ is about 10-fold higher than that of the reaction of terminal alkenes with tetrazines, which have been reported for efficient labelling.[13] In comparison to other prominent labelling reactions, the DARinv using $d^{vin}A$ proceeds faster than the Staudinger ligation[16] or the strain-promoted azide-alkyne cycloaddition of dibenzo-cyclooctyne.[17] Thus, labelling with $d^{vin}A$ and tetrazines appears to be promising for further applications.

The differences in reactivity of the nucleobases might be explained by their different electronic properties. To gain further insights, we calculated the frontier oribitals of the tetrazine and the vinylated nucleobases and evaluated the energy difference of the interacting orbitals as an estimate for the reactivity.[18] We found, that the energy difference between the interacting orbitals (LUMO+1 of the tetrazine, HOMO of the nucleobases; Fig S2) of the tetrazine and $d^{vin}U$ is larger by 0.55 eV than the difference between the corresponding orbitals when considering d^{vin}A. The smaller energy difference can explain the higher reactivity of d^{vin}A in comparison to d^{vin}U.

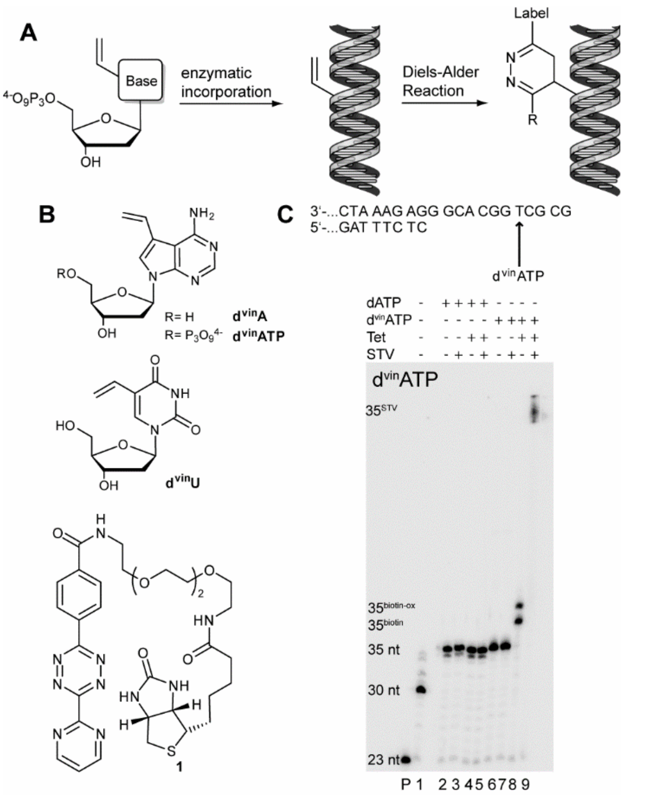

Fig. 1 A: Schematic representation of the labelling strategy. Vinyl-modified dNTP-analogues are enzymatically incorporated into the DNA duplex. The biopolymer is then subjected to DARinv with a tetrazine-modified label. B: Structures of **d^{vin}A**, **d^{vin}ATP d^{vin}U** and tetrazine **1**. C: top: Partial primer template complex used for the primer extension studies. Incorporation positions for d^{vin}ATP is indicated by an arrow. PAGE analysis of PEX and DARinv with d^{vin}ATP, Tet: incubation with tetrazine **4**, STV: incubation with streptavidin, P: primer only, 1: PEX by *KlenTaq* DNA polymerase in the presence of dCTP, dGTP and dTTP, 2: all four natural dNTPs, 3: same as 2 but followed by incubation with streptavidin (STV), 4: same as 2 but followed by incubation with tetrazine **1**, 5: same as 4 but followed by incubation with STV, 6: same as 1 but also in presence of d^{vin}ATP, 7: same as 6 but followed by incubation with STV, 8: same as 6 but followed by incubation with tetrazine **1**, 9: same as 8 but followed by incubation with STV.

Encouraged by these results, we synthesized d^{vin}ATP by phosphorylation as described.[19] We tested the acceptance of the nucleotide analogue by *KlenTaq* DNA polymerase and conducted primer extension reactions (PEX) using a radioactively labeled primer strand. We employed standard conditions for primer extension reactions as they have been reported before.[9a, 9b] The nucleotide analogue was incorporated efficiently into the DNA strand. Full length product was observed in all cases and no stalling of the polymerase during incorporation of the modified nucleotide took place under the employed conditions, indicating efficient processing of the modified building block by the DNA polymerase.

Next, we tested, if the incorporated modification can engage in DARinv in double stranded DNA (dsDNA). The primer extension product was incubated with tetrazine **1**, bearing a biotin moiety. The DNA was used in low concentrations (0.9 µM) while an excess of labelling reagent (1.7 mM) was used. After purification via gel filtration, an aliquot was incubated with streptavidin. All samples were analyzed by denaturing polyacrylamide gel electrophoreses (PAGE). d^{vin}A embedded in dsDNA undergoes DARinv with the biotinylated tetrazine derivative **1**, indicated in the change of the migration of the product by PAGE analysis (Fig. 1C). Further control reactions point out that only when d^{vin}ATP was used as substrate and d^{vin}A was incorporated, the large shift of the product migration was detected by PAGE analysis after subsequent incubation with the biotinylated tetrazine **1** and streptavidin.

Next, we investigated the efficiency of the reaction by varying the reaction time. Interestingly, the starting material was almost completely consumed within 10 minutes. During longer incubation times another band appears that might be derived by the oxidation of the Diels-Alder reaction product, as it is known, that dihydropyridazines easily oxidize to pyridazines in the presence of oxygen.[13]

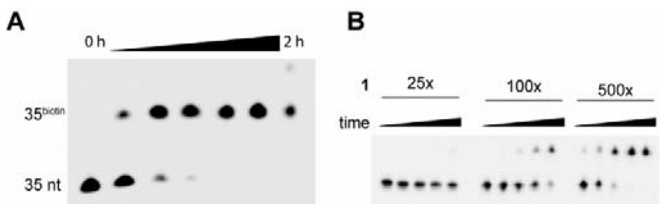

Fig. 2 A: Time course experiment with 1.7 mM tetrazine (samples taken after 0 min, 5 min, 10 min, 20 min, 30 min, 1 h and 2 h). After 10 minutes almost all starting material is consumed. B: Effect of the excess of tetrazine on the reaction speed. 25- (25x, 22 µM) to 500-fold excess (500x, 450 µM) of tetrazine was added. The reactions were stopped after different time points (5 min, 10 min, 30 min, 1 h, and 2 h).

Next, we investigated the dependence of the reaction efficiency on the concentration of the labelling reagent. Therefore, the product of the primer extension reaction in the presence of d^{vin}ATP was incubated with increasing concentrations of tetrazine **1**. As an excess of the tetrazine was applied, the reaction follows a kinetic of pseudo-first order. We stopped each of the reactions after different time points and analyzed the reactions by PAGE. Interestingly, when 22 µM labelling reagent is present, conversion to the reaction product occurred only to a minor extend even after prolonged reaction times (2 h). When 90 µM labelling reagent is used, after 2 hours about 70 % of the starting material has been converted to the reaction product. Applying 450 µM tetrazine **1** about 70 % conversion took place after 30 minutes and after 1 hour no starting material was detected anymore. To gain deeper insights, we performed kinetic studies of the DARinv on the DNA duplex (Fig. S3). We estimated the second order rate constant k_2 of 0.42 (± 0.03) (L/(mol·s). This constant is 3-fold higher than for the nucleoside. This increase in reaction rate might origin from the polar microenvironment in the major groove, in which the Diels-Alder reaction takes place, as it has been reported that Diels-Alder reactions are accelerated in polar solvents.[20] Furthermore we found, that even at low DNA concentration (0.9 µM), applying 450 µM of the tetrazine results in very efficient labelling within 30 min. In comparison, 4 h are required for labelling by copper-catalyzed azide-alkyne cycloaddition in a similar setup when the same concentration of DNA albeit a 1900-fold excess of labelling reagent was used.[9b]

Next, we investigated, if d^{vin}ATP can also be used in the polymerase chain reaction (PCR) by completely substituting natural dATP by d^{vin}ATP (Fig. 3). With the complete exchange of natural dATP by d^{vin}ATP full length product could be observed. The incorporation of 160 modified nucleotides into the DNA duplex indicated by a change of the migration behavior of the DNA product, as monitored by gel electrophoresis analysis.

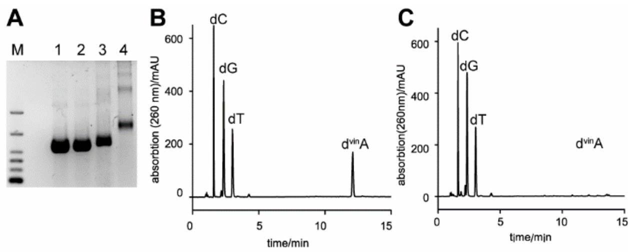

Fig. 3 A: PCR of a 414 bp DNA fragment with dATP completely exchanged by d^{vin}ATP. M: marker, 700 bp, 500 bp, 400 bp, 300 bp, 200 bp, 100 bp, 1: PCR with natural dNTPs, 2: as 1 but incubation with 1.7 mM tetrazine **1** for 1 h at room temperature, 3: PCR with dATP exchanged completely by d^{vin}ATP, 4: as 3 but with incubation of 1.7 mM tetrazine **1** for 1 h at room temperature. PCR reaction without any dATP yielded no product (data not shown). B: HPLC chromatogram of the digested PCR product without incubation with tetrazine 1, C: HPLC chromatogram of the digested PCR product with incubation of tetrazine 1.

Still, the PCR product containing the vinyl groups undergoes DARinv when incubated with the tetrazine for 1 h at room temperature, visible in a large shift of the band. As only one major band appeared after Diels-Alder reaction, one can conclude, that a defined reaction product is formed, indicating, that in most DNA strands a similar number of vinyl groups reacted with the tetrazine. To address the efficiency of the DARinv using the PCR product, we digested the PCR product to the nucleosides. The peak in the HPLC chromatogram corresponding to d^{vin}A completely vanished and a new peak appears at higher retention times (Fig. S4) when the PCR product has been incubated with tetrazine **1**. These findings indicate that all vinyl groups reacted with the tetrazine.

Conclusions

To summarize, here we show that the modified nucleoside triphosphate d^{vin}ATP is efficiently incorporated into DNA by a DNA polymerase. The modified biopolymer can undergo DARinv and can thereby be conjugated. We found that natural dATP can be substituted completely by its modified counterpart in PCR and still, full length product is formed, which can react in DARinv. All in all, we present an efficient tool for labelling and manipulation of enzymatically synthesized DNA.

Notes and references

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Electronic Supplementary Information (ESI) available: [Synthetic details for d^{vin}ATP and experimental details for kinetic measurements, PEX, PCR, labelling with tetrazine, protocol of the digestion of the PCR product to nucleosides]. See DOI: 10.1039/c000000x/

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