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Sequence selective tagging of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) using PNAs

In Seok Hong^{a,b}, Marc M. Greenberg^{a,*}

^a*Johns Hopkins University, Department of Chemistry, Baltimore, CA 21218, United States*

^b*Kongju National University, Department of Chemistry, 182, Shinkwan-dong, Kongju, Chungnam 314-701, Korea*

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Sequence selective tagging of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) using PNAs

In Seok Hong^{a,b}, Marc M. Greenberg^{a,*}

E-mail: mgreenberg@jhu.edu; phone: 410-516-8095; fax: 410-516-7044

^a Johns Hopkins University, Department of Chemistry, Baltimore, CA 21218, United States

^b Kongju National University, Department of Chemistry, 182, Shinkwan-dong, Kongju, Chungnam 314-701, Korea

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ABSTRACT

8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) is a commonly formed DNA lesion that is useful as a biomarker for oxidative stress. Methods for detecting 8-oxodGuo at specific positions within DNA could be useful for correlating DNA damage with mutational hotspots and repair enzyme accessibility. We describe a method for covalently linking ("tagging") peptide nucleic acids (PNAs) containing terminal nucleophiles under oxidative conditions to 8-oxodGuo at specific sites within DNA. Several nucleophiles were examined and the ϵ -amine of lysine was selected for further studies. As little as 10 fmol of 8-oxodGuo were detected by gel shift using ³²P-labeled target DNA and no tagging of dG at the same site or 8-oxodGuo at a distal site was detected when potassium ferricyanide was used as oxidant in substrates as long as 221 bp.

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DNA lesions have been implicated in a variety of diseases, most notably cancer, as well as aging.¹⁻⁵ Selective detection of nucleic acid damage is of interest because the lesions are potential biomarkers.^{6,7} 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) is a mutagenic lesion derived from dG, the most readily oxidized native nucleotide.^{8,9} 8-OxodGuo is even more readily oxidized than dG and serves as the precursor to a number of other mutagenic lesions.¹⁰⁻¹⁴ Consequently, there is significant interest in using 8-oxodGuo as a biomarker, which requires developing methods for its quantitation in DNA.^{15,16} Detecting damaged nucleotides at specific positions within DNA is potentially useful for a variety of reasons, including as an early risk assessment by providing correlations between mutagenic

hotspots and the formation/persistence of specific lesions at those same positions. Advances are being made on this problem, and we wish to describe our efforts in this regard.

Sensitive methods for detecting DNA lesions exist and new approaches have been reported in recent years.¹⁷⁻¹⁹ Mass spectrometry (MS) is a powerful tool for detecting modified nucleotides,²⁰ and MSⁿ sequencing is useful for mapping the location of a damaged nucleotide in a short oligonucleotide.^{21,22} Nanopore sequencing is another elegant method for detecting lesions as specific positions within DNA, and significant advances have been made in recent years.²³⁻²⁵ Even more recently, deep sequencing has been combined with restriction enzymes to ascertain DNA lesion location.²⁶

Our research group has developed methods for quantifying 8-oxodGuo in DNA by taking advantage of the ability to trap ("tag") an oxidized form of it with nitrogen nucleophiles first reported by the Burrows group (Scheme 1).²⁷⁻³⁰ When used in conjunction with qPCR, as little as 14 amol of 8-oxodGuo was detected using this strategy.³¹ In this Letter we combine chemical tagging with sequence selective probing by modified peptide nucleic acids (PNAs) to detect 8-oxodGuo at specific positions.

Pseudocomplementary peptide nucleic acids (pcPNAs) were synthesized via standard methods on solid phase supports to enable targeting single or double stranded DNA.^{32,33} Consequently, a series of decameric pcPNAs containing 2-thiouracil (U^S) and 2,6-diaminopurine (D) instead of thymine and adenine were prepared (Figure 1). The pcPNAs

Scheme 1. Nucleophilic tagging of 8-oxodGuo under oxidative conditions.

contained lysines (Lys) at their carboxamide termini (equivalent to the 3'-termini of oligonucleotides) to enhance solubility. The amino termini (5'-termini) contained 1 of 5 tags (or no tag) that were either bonded directly to the pcPNA unit or to an intervening lysine. The tags were either 1,3-diamine containing amino acids (**1**, **2**) (synthesized according to published methods)^{34,35} that were meant to mimic a diamine portion of spermine that traps the oxidized 8-oxodGuo, or Lys (**3**, **4**).²⁸ Finally, a single pcPNA (**5**) was prepared to bind the complementary DNA strand.

A. 5'-Tag-(Lys)_n-GU^SD GDU^S CDC U^S-Lys-CONH₂

5'-Tag-Lys-GU^SD GDU^S CDC U^S-Lys-CONH₂

5'-H₂N-(Lys)_n-GU^SD GDU^S CDC U^S-Lys-CONH₂
3 n = 1; **4** n = 2

B. 5'-H₂N-Lys-DGU^S GDU^S CU^SD C-Lys-CONH₂
5

Figure 1. pcPNA sequences used in this study. (A.) pcPNA sequences targeted to 8-oxodGuo containing strand. (B.) pcPNA sequence targeted to complementary strand. (C.) Structures of diaminopurine (D) and 2-thiouracil (U^S) nucleobases.

Figure 2. Chemically synthesized DNA substrates used in this study. Red sequences correspond to PNA binding sites.

Figure 3. Selective tagging of 8-oxodGuo in (A.) **9** or **12** (B.) **10** or **13**. Reaction conditions: [DNA] = 10 nM, [pcPNAs] = 100 nM, phosphate (pH 7.3, 10 mM), [K₃Fe(CN)₆] = 1 mM. Hybridization was carried out at 95 °C for 5 min and the tagging reaction was carried out at 25 °C for 30 min.

Initial experiments were designed to determine whether there was an optimal distance between the binding sequence and 8-oxodGuo for tagging (**6-8**). Oxidation by Na₂IrCl₆ (0.1 mM) and two equivalents of PNA **1b** and **5** provided slightly higher tagging yields with all 3 substrates than did **1a** under identical conditions.³⁶ In addition, tagging a 8-oxodGuo that is directly adjacent to the binding sequence (**6**) by **1a** (43%) and **1b** (47%) was greater than when 1 (7) or 2 (8) A•T intervening base pairs were present (31-39%). While these data indicate the optimal spacing between the lesion and PNA binding site, they also reveal that neither probe will distinguish between 8-oxodGuo generated at different positions of consecutive dGs. Nonetheless, efficient tagging (>80%) of 8-oxodGuo in **6** was maintained by 100 nM **1b** and **5** when the substrate was as low as 0.5 nM in a 20 µL reaction, indicating that we could detect as little as 10 fmol of the lesion.³⁶

Figure 4. Buffer effect on 8-oxodGuo tagging by **4** in **9** and the respective single-stranded oligonucleotide (**12**). Reaction conditions: [DNA] = 10 nM, [4] = 100 nM, phosphate (pH 7.3, 10 mM), [K₃Fe(CN)₆] = 1 mM. Hybridization was carried out at 95 °C for 5 min and the tagging reaction was carried out at 25 °C for 30 min. % Tagging ± Std. Dev. are based on 3 replicates.

8-OxodGuo tagging by a broader range of probes was explored using a series of 51 bp duplexes containing the same decameric binding site (**9**, **10**), as well as the corresponding single stranded oligonucleotides (**12**, **13**). Duplex tagging was carried out with and without complementary strand binding **5**. The use of target DNA containing 8-oxodGuo at a site distal from the PNA binding (**10**) region revealed that tagging initiated via Na₂IrCl₆ oxidation was nonspecific (data not shown). Substituting K₃Fe(CN)₆ (1 mM) for the iridium oxidant

eliminated this problem (Figure 3B). Tagging by the probes was typically more effective on single stranded (12-44%) DNA than duplex targets (6-44%) (Figure 3A). Only **2** (12%) yielded considerably lower tagged product yields than the other probes (33-44%) when reacting with single-stranded target. In addition, in experiments with duplex substrate (**9**) binding the complementary strand by **5** was unnecessary under the high temperature (95 °C) hybridization conditions. Consequently, subsequent experiments were carried out without **5**.

Figure 5. Selectivity of 8-oxodGuo tagging by **4**. Reaction conditions: [DNA] = 10 nM, [**4**] = 100 nM, phosphate (pH 7.3, 10 mM), [$K_3Fe(CN)_6$] = 1 mM, [Na_2IrCl_6] = 0.1 mM. Hybridization was carried out at 95 °C for 5 min and the tagging reaction was carried out at 25 °C for 30 min. % Tagging $\pm$ Std. Dev. are based on 3 replicates.

Although tagging yields by PNAs **1b** (44%) and **2** (44%) were modestly greater than those using **4** (33%), the more facile synthesis of the latter, which does not require chemical synthesis of the corresponding amino acid led us to optimize 8-oxodGuo detection using it. Carrying out the tagging reaction on **9** or the respective single-stranded oligonucleotide (**12**) at higher pH vastly increased the product yield to over 80% at pH 8.8 (Figure 4). Tagging selectivity was examined at these optimized pH conditions using $K_3Fe(CN)_6$ (1 mM) and Na_2IrCl_6 (0.1 mM) (Figure 5). Use of either oxidant provided $\geq 80\%$ of the tagged product with the matched sequence (**9**). However, the stronger iridium oxidant yielded $\geq 25\%$ of tagged material with the mismatched 8-oxodGuo substrate (**10**) or the 51 bp DNA lacking the lesion (**11**). Fortunately, no tagging of **10** or **11** was observed when ferricyanide was employed.

Figure 6. PNA sequences used in this study.

Having established conditions for selective tagging of 8-oxodGuo containing DNA, we explored selective detection using longer PNAs containing biotin (**17-20**, Figure 6) for potential applications in pull-down experiments, and longer lengths of DNA. The PNA sequences (10-16 bases long) were based upon **4**

and an additional Lys was added at the carboxy (3') terminus to which the biotin was bonded. Tagging of "matched" (**14**), "mismatched" (**15**), and non-8-oxodGuo containing (**16**) sequences were examined (Figure 7). Excellent selectivity was observed using PNAs that recognize 10, 12, and 14 nucleotides. No tagging was detected for either the "mismatched" sequence (**15**) or the DNA lacking 8-oxodGuo (**16**), and the yield increased to more than 90% with the longer PNAs indicating that incorporating biotin was not detrimental for binding/tagging. However, increasing the PNA length further (**20**) resulted in nonspecific tagging (Figure 7).

Figure 7. Effect of PNA length on 8-oxodGuo. Reaction conditions: [DNA] = 10 nM, [PNA] = 100 nM, phosphate (pH 8.8, 10 mM), [$K_3Fe(CN)_6$] = 1 mM. Hybridization was carried out at 95 °C for 5 min and the tagging reaction was carried out at 25 °C for 30 min.

Finally, 8-oxodGuo detection by a 12mer PNA (**18**) was demonstrated in 132 bp and 221 bp PCR fragments (Figure 8). The PCR fragments contained the same DNA sequences used to determine the selectivity of longer PNAs (**14-16**). (See Supplementary Material for the complete sequences.) Tagging yields in excess of 80% were observed using both fragments. Moreover, no reaction with either the mismatched sequence (**15**) or one lacking 8-oxodGuo (**16**) was detected.

Figure 8. 8-OxodGuo tagging by **18** in PCR products containing DNA sequences **14-16**. (A.) 132 bp sequence. (B.) 221 bp sequence. (See Supplementary Material for complete DNA sequences.) Reaction conditions: [DNA] = 50 nM, phosphate (pH 8.8, 10 mM), [$K_3Fe(CN)_6$] = 1 mM. Hybridization was carried out at 95 °C for 5 min and the tagging reaction was carried out at 25 °C for 30 min.

In conclusion, 8-oxodGuo was selectively detected at a single position within DNA using PNAs containing amines at their amino (5') termini. Adduct formation may involve reaction with more than one nucleophile on the PNA. ^{32}P -Labeling enables us to detect 10 fmol. Selective detection was demonstrated in DNA fragments as long as 221 bp. We anticipate that coupling (pc)PNA probes with an amplification method, such as qPCR will significantly reduce the detection limits and enable us to probe for 8-oxodGuo at unique positions in isolated genomic DNA.³¹

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