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Inducible Alkylation of DNA by a Quinone Methide–Peptide Nucleic Acid Conjugate

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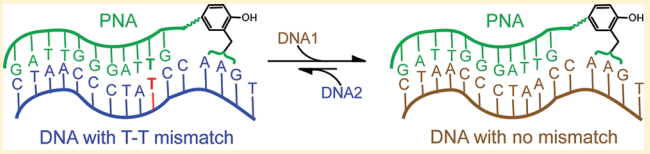
Supporting Information

ABSTRACT: The reversibility of alkylation by a quinone methide intermediate (QM) avoids the irreversible consumption that plagues most reagents based on covalent chemistry and allows for site specific reaction that is controlled by the thermodynamics rather than kinetics of target association. This characteristic was originally examined with an oligonucleotide QM conjugate, but broad application depends on alternative derivatives that are compatible with a cellular environment. Now, a peptide nucleic acid (PNA) derivative has been constructed and shown to exhibit an equivalent ability to delivery the reactive QM in a controlled manner. This new conjugate demonstrates high selectivity for a complementary sequence of DNA even when challenged with an alternative sequence containing a single T/T mismatch. Alternatively, alkylation of noncomplementary sequences is only possible when a template strand is present to colocalize the conjugate and its target. For efficient alkylation in this example, a single-stranded region of the target is required adjacent to the QM conjugate. Most importantly, the intrastrand self-adducts formed between the PNA and its attached QM remained active and reversible over more than 8 days in aqueous solution prior to reaction with a chosen target added subsequently.



Numerous techniques in biochemistry rely on alkylation for stabilizing target–probe interactions despite the often low efficiency and unpredictable selectivity of this reaction. Complications associated with alkyaltion are not easily averted since increasing a reagent's reactivity may compromise selectively, and conversely increasing its selectivity may compromise reaction efficiency. One approach to overcome these challenges involves use of masked electrophiles that can be activated for alkylation after induction by heat, light, or a chemical signal. This strategy provides the potential for temporal and even spatial control of reaction and has been adopted for a range of topics including gene targeting from cell free models to cell culture. Psoralen has been applied with great success in such applications.[1−4] As expected, little biological effect is observed until the system is exposed to the wavelength of light that is absorbed by psoralen.

Induction of alkylation by an external signal becomes most difficult when attempting to extend its use for therapeutic applications in an organism. Perhaps the most promising design for selective alkylation within animals derives from conjugates that are activated exclusively in the environment created by their intended target. In this case, no external signal is required to initiate the desired reaction. Derivatives of the natural product CC-1065 exhibit such activation upon binding to the minor groove of duplex DNA,[5,6] and this activity can be directed to a chosen nucleotide sequence by its conjugation to oligonucleotide derivatives or pyrrole–imidazole polyamides.[7,8] Aziridine and vinylpurine derivatives demonstrate a similarly enhanced reactivity when bound to duplex DNA[9−11] although some concern persists on the ability of cellular thiols to compete for alkylation of these derivatives in vivo. Alternatively, the reversible reactivity of quinone methides (QM) may be used to construct sequence-specific alkylating agents. This transient and highly reactive electrophile is protected from undesired alkylation by its reversible capture to form intrastrand self-adducts.[12,13] Even highly nucleophilic thiols are relatively ineffective in quenching transient formation of QM within these oligonucleotide conjugates. Intermolecular, rather than intramolecular, transfer of the QM only dominates their reaction after the self-adducts associate with their complementary targets.[12,14]

Originally, quinone methide precursors (QMP) protected with a silyl group were conjugated to DNA-based directing agents for synthetic ease and general stability. The silyl group provided control of QM production under a nonphysiological signal, fluoride (Scheme 1). The potential for unprotected QM derivatives to serve in vivo applications became apparent once the persistence and reversibility of their self-adducts were identified. In contrast to the short half-life (~2.5 h) of a model QM adduct of G N7,[15] oligonucleotide self-adducts remain capable of QM transfer for many days through multiple cycles of QM generation and trapping.[12] Consequently, QM self-adducts may serve as sequence specific alkylating agents that require no more activation than their association with a chosen target. Of course, application of this discovery for biological systems necessitates substitution of the DNA component with an equivalent sequence directing ligand that is compatible with cellular conditions. Our first attempt in replacing the DNA ligand relied on a pyrrole–imidazole

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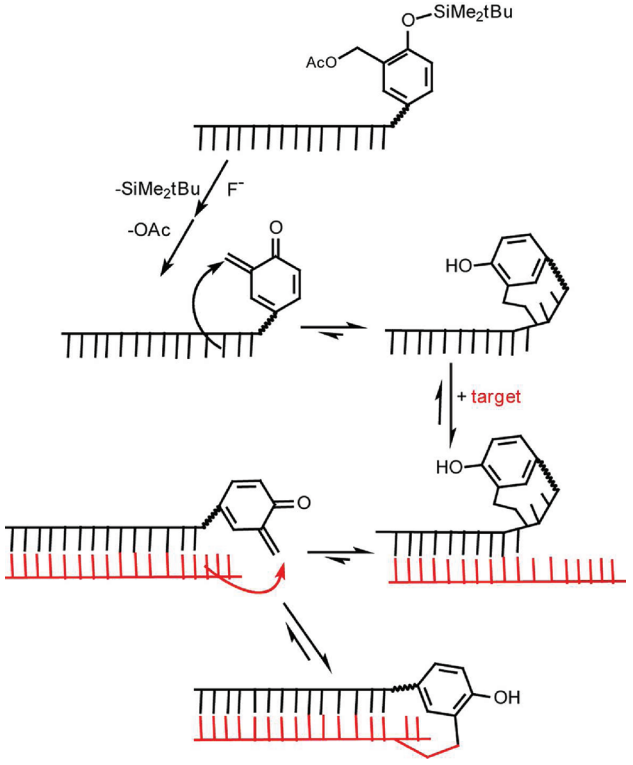
Scheme 1. Generation of a Quinone Methide Conjugate, Its Subsequent Reversible Formation of a Self-Adduct through Intrastrand Reaction, and Ultimate Interstrand Transfer of the Quinone Methide for Target Alkylation



polyamide conjugate.[16] This successfully delivered QM alkylation to a targeted sequence of duplex DNA, but its yield of alkylation was very low. Further analysis revealed that self-adduct formation within this conjugate was not reversible and thus not capable of maintaining a reservoir of QM for target reaction.

We now report successful use of peptide nucleic acids (PNA) as a sequence directing component of QM self-adducts. PNA is an attractive replacement of DNA for directing QM alkylation since its nucleobases are identical to those in DNA, and therefore they both share the same high efficiency for capture and release of the transient QM. In addition, PNA supports a range of binding modes with nucleic acids that may be used in future applications to target single- and double-stranded nucleic acids in vivo.[17] Stability of a PNA−DNA heteroduplex is higher than an equivalent DNA−DNA duplex due in part to the lack of electrostatic repulsion between the nonionic PNA and anionic DNA.[18] Also, the unnatural peptide-like backbone of PNA resists biological degradation and further conjugation can dramatically increase its cellular uptake.[19−21] PNA has already proven effective in delivering alkylating agents such as a nitrogen mustard and psoralen to specific genes, and in both examples, the reactive component significantly enhanced the reagents' potency.[3,22]

MATERIALS AND METHODS

General Materials and Methods. Solvents and reagents of the highest commercial grade were used without further purification. Oligonucleotides were purchased from IDT (Coralville, IA). Boc-protected PNA monomers were obtained from Applied Biosystems (Foster City, CA). 4-Methylbenzhydrylamine resin (MBHA, 0.7 mmol/g), Boc-protected amino acids, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), and 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from Advanced ChemTech (Louisville, KY). Boc-8-amino-3,6-dioxaoctanoic acid (Boc-AEEA) was purchased from Peptides International (Louisville, KY). Kaiser test kits were obtained from Aldrich (St. Louis, MO). Aqueous solutions were prepared with distilled, deionized water with a resistivity of 18 MΩ. PNA derivatives were characterized by mass spectrometry using an Axima-CFR MALDI-TOF instrument (Shimadzu, Columbia, MD) with a matrix of 3,5-dimethoxy-4-hydroxycinnamic acid (Fisher Scientific, Fairlawn, NJ). DNA was labeled at the 5′-terminus using [γ-^{32}P]-ATP (Perkin-Elmer, Waltham, MA) with T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) following standard procedures.

PNA Synthesis. The peptide-PNA (pPNA, 5′-AEEA-Arg-*GTTAGGGTTAG*-LeuArgArgAlaSerLeuGly-3′, italic letters represent PNA bases) was synthesized on MBHA resin by manual solid-phase synthesis as described previously.[23] pPNA was purified by reverse phase HPLC (RP-HPLC) using a C18 column (Microsorb-MV, 300 Å pore, 250 mm, Varian, Palo Alto, CA) and a linear gradient of 10−55% aqueous acetonitrile with 0.1% trifluoroacetic acid (TFA) at 1 mL/min over 30 min. The desired pPNA was confirmed by its m/z of 4143.3 (calcd 4144.6).

pPNA Conjugation to the Quinone Methide Precursor (pPNA-QMP) and Formation of Its Quinone Methide Self-Adduct (pPNA-QM). The *N*-succinimidyl ester of the quinone methide precursor (QMP) was prepared as described previously,[12] dissolved (1 mg) in CH_3CN/DMF (100 μL, v/v = 2/1), and incubated with pPNA (100 μL, 5.0 mM) in MOPS (250 mM, pH 7.5) for 24 h at room temperature. The desired conjugate was purified by RP-HPLC using the conditions described above and confirmed by mass spectrometry (m/z found: 4481.4; calcd: 4478.9). This conjugate pPNA-QMP (20 μM)) was deprotected in MES (25 mM pH 7.0) by addition of KF (1.0 M) to form the self-adduct pPNA-QM. After this mixture was incubated at room temperature for 24 h, the self-adduct was again purified by RP-HPLC as described above. Mass spectrometric data were consistent with the desired product (m/z found: 4305.1; calcd: 4305.8).

Radiolabeling pPNA-QMP. pPNA-QMP was labeled by cAMP-dependent protein kinase A (PKA, New England Biolab, Ipswich, MA) and [γ-^{32}P]-ATP as described previously.[24] Typically, an aqueous solution of pPNA-QMP (1.5 μL, 50 μM) was mixed with PKA (2500 units), BSA (2.5 μL, 10 mg/mL), ATP (5 μL, 25 μM, 25 μCi), and buffer (10 μL) containing 50 mM MES (pH 6.9), 10 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM DTT. After incubation at 37 °C for 30 min, the reaction was desalted using a SepPak C18 reverse-phase cartridge (Waters, Milford, MA).

Alkylation of Target DNA. Single-stranded DNA (3 μM) was typically treated with pPNA-QMP or its self-adduct (3.3 μM) in MES (130 mM, pH 7.0), NaCl (130 mM), and KF (130 mM) under ambient conditions for the indicated times. Reaction was quenched by addition of a formamide solution (3 μL, 0.05% bromophenol blue and 0.05% xylene cyanol FF). Products were separated by denaturing (7 M urea) polyacrylamide gel electrophoresis (20%) with 89 mM Tris-borate pH 8 and 5 mM EDTA. A temperature of 40−45 °C was maintained during electrophoresis. Radiolabeled materials were detected

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and quantified by a Molecular Dynamics Phosphorimager (GE Healthcare, Piscataway, NJ). Alkylation yields are reported as the average of two or more independent determinations of the ratio (%) of product over the sum of product and remaining starting material.

Melting Temperature for PNA·DNA Heteroduplexes. Duplex stability of the matched duplex pPNA·OD1 and mismatch containing duplex pPNA·OD2 was determined by pre-annealing stoichiometric mixtures of the appropriate strands (3 μM) in MES (130 mM, pH 7.0), NaCl (130 mM), and KF (130 mM) by heating samples to 90 °C and then allowing them to cool to 20 °C over 30 min. The samples' absorbance at 260 nm was monitored while their temperature was raised 0.5 °C/min, and the T_m values were estimated as the maxima in change of absorbance over the temperature range 20−90 °C. Values are reported as the average of two independent determinations, and the error is the range of these data.

RESULTS AND DISCUSSION

Design and Preparation of the pPNA-QMP Conjugate. Previous studies with DNA-QM conjugates indicated that most any sequence is capable of forming self-adducts and transferring its QM to a complementary target.[13] Self-adducts are likely formed as a heterogeneous mixture of species as a result of QM capture by the various strong nucleophiles present in the attached sequence-directing strand. Systematic evaluation of such strands lacking A, G, or C alternatively demonstrated that no individual nucleobase was critical for generating self-adducts or effecting their subsequent alkylation of complementary strands.[13] Only T remains inert to this QM, and a QMP conjugate with $(T)_{10}$ is the sole derivative observed to date that does not have the capacity to form a self-adduct.[12] Thus, there was great freedom in selecting an initial system for evaluating the utility of PNA for deliver of a QM.

We began with sequences based on the ribonucleoprotein telomerase. This target had already been successfully inhibited by a series of PNAs that lack covalent reactivity,[25] and consequently our model studies were designed to expedite future applications that target telomerase activity in cell culture. This will allow direct comparisons between targets of DNA and RNA using PNA derivatives that rely on noncovalent interactions with those that additionally express the covalent and reversible reactivity of QM. The 3′-terminus (C-terminus) of our PNA conjugate was extended with Kemptide to provide a site for ^{32}P-labeling (Table 1).[26] Similarly, the 5′-terminus (N-terminus) was extended with Arg for coupling to the QMP. These extensions were additionally expected to enhance the solubility of the otherwise neutral PNA.

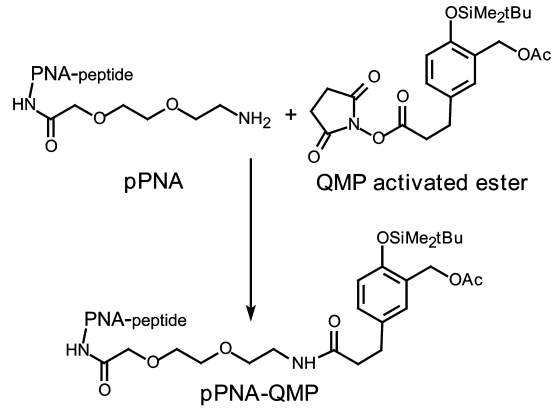
Table 1. Sequences of DNA Targets and the pPNA-QMP Conjugate[a]

pPNA-QMP	3′-GlyLeuSerAlaArgArgLeu-*GATTGGGATTG*-Arg-AEEA-QMP-5′
OD1	5′-CTAACCCTAACCAGT-3′
OD2	5′-CTAACCCTA**T**CCAGT-3′
OD3	5′-CTAACCCTAAC CAGTCTGCAGTCG-3′
OD4	3′-TCAGACGTCAGC-5′
OD5	3′-GTCAGACGTCAGC-5′
OD6	3′-TTGGTCAGACGTCAGC-5′
OD7	3′-GCTGTCAGACGTCAGC-5′

[a]PNA is designated in italics. The residue (T) forming a mismatch is underlined and bold.

The peptide-PNA (pPNA) conjugate was prepared by routine solid-phase synthesis using Boc-protected monomers as described previously.[23] However, no coupling between the *N*-hydroxysuccinimide ester of QMP and the α-amino group of the terminal Arg in pPNA was observed by RP-HPLC after 3 days under conditions that generate an equivalent conjugate in 1 day between the same activated ester and 5′-aminohexyl-oligonucleotides.[12,13] After extending the 5-terminus with an additional short ethylene glycol linker (AEEA),[27,28] coupling with the activate ester of QMP proceeded at a rate similar to that observed previously with oligonucleotides (Scheme 2).[12] Over 90% of both oligomers coupled to the QMP within 1 day.

Scheme 2. Synthesis of pPNA-QMP



Alkylation of a DNA Target Strand by the pPNA-QMP Conjugate. Silyl protection of the QM is convenient for initial manipulation of its conjugate, but it is not appropriate for future in vivo studies. Instead, the reversible self-adduct described below or alternative precursors designed for activation through redox chemistry or enzymatic hydrolysis can be envisioned for cellular applications.[29−31] Still, the fluoride-initiated reaction of pPNA-QMP provided a rapid method to confirm the ability of PNA to deliver a QM intermediate to its target and determine the general susceptibility of a PNA−DNA heteroduplex to interstrand alkylation. Although this reaction was anticipated, a recent series of studies with other electrophiles revealed a surprising sensitivity to the helical structure formed by DNA−DNA and DNA−RNA duplexes.[11,32,33] Addition of 1.1 equiv of pPNA-QMP to its [^{32}P]-labeled DNA complement (OD1) in the presence of fluoride generated a high molecular weight species as detected by denaturing gel electrophoresis that is consistent with interstrand alkylation (Figure 1A and Figure S1). This product accumulated in a first-order process to a yield of almost 60% after 48 h (Figure 1B). Its composition as a heteroduplex of DNA and PNA was confirmed by alternative [^{32}P]-labeling of the pPNA-QMP. Products of equivalent electrophoretic mobility were generated in similar yields regardless of which oligomer contained the label (Figure 1A). Both the rate and yield of target alkylation by the PNA-based reagent affirm that PNA is equal or superior to DNA for directing alkylation by a QMP,[12] although neither yet acts fast enough for in vivo applications.

Target Discrimination by the pPNA-QMP Conjugate. The ultimate utility of the quinone methide conjugate will depend on its ability to discriminate between target and non-target sequences. A related DNA-QM conjugate had previously demonstrated a high level of specificity for complementary versus noncomplementary DNA targets, but the response to a single mismatch between a conjugate and its target had not

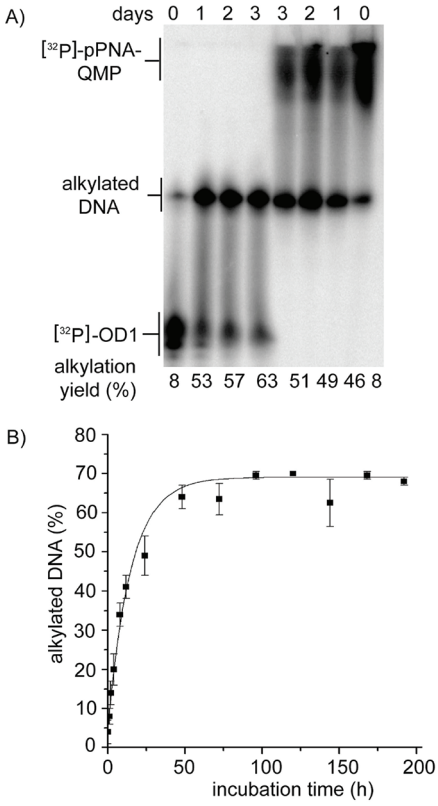


Figure 1. DNA alkylation by the PNA conjugate pPNA-QMP. (A) Alternate [^{32}P]-labeling of the target OD1 and pPNA-QMP generates the same product with an intermediate electrophoretic mobility after initiating reaction with fluoride and incubating the mixture for the indicated time under ambient conditions. (B) Alkylation of [^{32}P]-OD1 with pPNA-QMP was quantified and fit to a first-order process. The error represents the range of two or more independent determinations (see Figure S1).

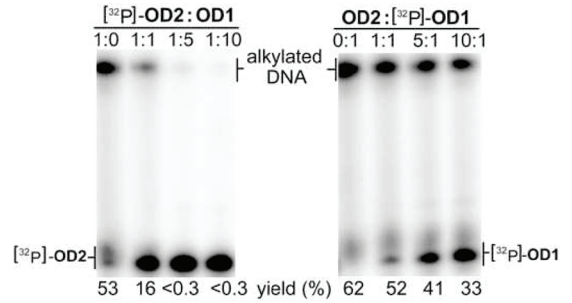


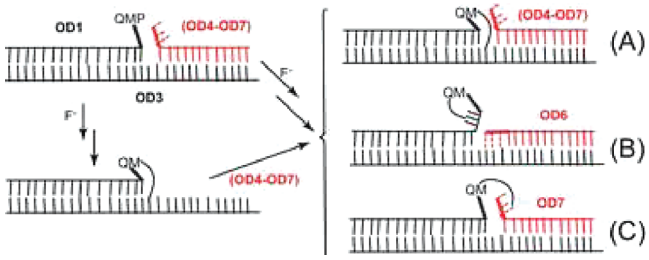
Figure 2. Competition for alkylation by pPNA-QMP between targets containing no mismatches (OD1) and a single mismatch (OD2). Selectivity for [^{32}P]-OD2 was measured in the presence of 0–10 equiv of OD1 and 1.1 equiv of pPNA-QMP. Conversely, the selectivity for [^{32}P]-OD1 was measured in the presence of 0–10 equiv of OD2 and 1.1 equiv of pPNA-QMP. Reaction mixtures were incubated for 192 h in the presence of MES (130 mM, pH 7.0), NaCl (130 mM), and KF (130 mM) under ambient conditions.

been examined.[12] Based on the reversibility of the QM chemistry, thermodynamics rather than kinetics was expected to dominate reaction selectivity.[34] To measure the extent of this control, a second DNA target (OD2) was prepared that differs from the complementary target OD1 by a single A to T substitution (Table 1). The pPNA-QMP conjugate was still fully capable of alkylating OD2 in the absence of OD1 as expected since association between OD2 and pPNA-QMP is thermodynamically favored over the random coiled structures (Figure 2). However, alkylation of OD2 decreased by 70% after addition of one equivalent of OD1 and was essentially blocked after addition of 5 equiv of OD1. Conversely, 1 equiv of OD2 suppressed alkylation of OD1 by only 16%, and even 10 equiv of OD2 suppressed alkylation of OD1 by little more than 47% (Figure 2).

Both the location and type of mismatches formed between a conjugate and its target may affect the selectivity of alkylation, but the high potential for distinguishing between fully and partially complementary sequences is clearly apparent from this single example. Alkylation of the fully complementary target OD1 was favored by 4 to 1 over that of the strand with a single T/T mismatch OD2 when both were present in equimolar concentrations (Figure 2). Again, this level of discrimination is consistent with the greater stability of the fully complementary pPNA·OD1 (T_m = 69 ± 1 °C) versus pPNA·OD2 (T_m = 63 ± 1 °C).

Template-Dependent Alkylation of a Noncomplementary Target. Alkylation of a chosen nucleotide sequence does not necessarily require the construction of a complementary QM conjugate. Instead, unrelated sequences can be colocalized and oriented for reaction by hybridization to a template strand. This principle serves as the basis for many applications including nonenzymatic ligation of DNA strands[35−38] and optical detection of DNA using a binary set of reactive probes.[39−41] A series of DNA sequences (OD4–OD7) that differ in only their 3′-termini were used with a template strand (OD3) and pPNA-QMP to identify the requirements for similar reaction of a QM with these adjacent targets (Table 1 and Scheme 3). In the absence of such targets,

Scheme 3. Template-Directed Alkylation of a Noncomplementary Target



pPNA-QMP merely alkylated the template strand OD3 (Figure S2). If instead the template was absent from a mixture of the conjugate and any of its noncomplementary targets OD4–OD7, no interstrand alkylation was detected (lanes 3 and 6, Figure 3; Figure S2). Even in the presence of the template OD3, very little alkylation (1%) was observed for a target (OD4) that aligned one residue away from the conjugate pPNA-QMP. A very low yield of alkylation (<2%) was also observe for another target (OD5) that bound directly adjacent to pPNA-QMP (Figure S2). Extending a target strand by three nucleotides (OD6) to overlap and compete with pPNA-QMP for association with the template similarly limited the yield of alkylation (2–4%, lanes 1 and 2, Figure 3).

A typical yield of ~40% alkylation was achieved once the target (OD7) contained a noncomplementary extension that remained free to react with the transient QM generated by pPNA-QMP (lane 5, Figure 3). The relative yield of the various products designated A, B, and C in Scheme 3 is again expected to reflect the thermodynamics rather than kinetics of reaction

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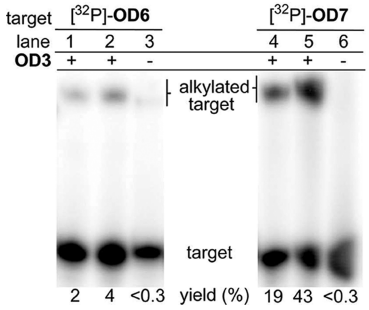

Figure 3. Template-dependent alkylation of target DNA. pPNA-QMP (3.3 μM) and the template OD3 (3.0 μM) were incubated for 192 h under ambient conditions in MES (130 mM pH 7) and NaCl (130 mM) after reaction was initiated by addition of KF (130 mM). OD6 (lane 1) and OD7 (lane 4) were then added to this mixture and incubated for another 192 h prior to electrophoretic analysis. Alternatively, pPNA-QMP, OD3, and either OD6 (lane 2) or OD7 (lane 5) were mixed together under equivalent conditions prior to addition of KF and then incubated for 192 h. pPNA-QMP and either OD6 (lane 3) or OD7 (lane 6) were also mixed together in the absence of the template OD3 under equivalent conditions prior to addition of KF and then incubated for 192 h.

since the QM component is consumed and regenerated many times to allow for equilibration during the 8 day incubation.[15] The reversibility of QM reaction is also illustrated by transfer of the tethered QM well after all of its protected precursor (QMP) has been consumed. The alkylated product formed by pPNA-QMP and OD3 alone (31%, Figure S2) likely served as a reservoir of QM since QM transfer was still observed after either target OD6 and OD7 was added 192 h subsequent to incubation of pPNA-QMP, OD3 and fluoride (lanes 1 and 4 of Figure 3; Scheme 3). The yield of target alkylation under these conditions was ~50% less than that generated when all strands were present at the initiation of QM formation. However, the relative reactivity of OD5 versus OD6 remained unchanged. The decrease in alkylation yield after preincubation of OD3 and pPNA-QMP likely reflects the slow progress of weakly competing reactions of water and certain weak nucleophiles of DNA that irreversibly consume the QM as described further under discussion of the self-adduct longevity.[12,15] Whether target reaction derives from the initial generation of QM or from subsequent regeneration by reversible DNA adducts, the maximum yields of target alkylation are highest when it contains a single-stranded region adjacent to the PNA conjugate.

Generation and Application of the pPNA-QM Self-Adduct. The primary applications of conjugates such as pPNA-QMP will rely on their ability to form self-adducts reversibly. These self-adducts, like the conjugate-template adduct above, may transfer their QM to a chosen target without the need for an external chemical or photochemical signal to trigger formation of the reactive intermediate. Only the target sequence itself should effectively compete for the transient QM and support interstrand transfer at the expense of regenerating the intrastrand self-adduct as demonstrated with a previous DNA-based conjugate.[12] Even high concentrations of thiols that should serve as trapping agents for the transient QM do not effectively quench intra- and interstrand reaction involving the self-adducts.[12,14]

The PNA analogue described here was designed with the expectation that it would act as a faithful mimic of the conjugate based on DNA. Formation of the pPNA-QM self-adduct was monitored by RP-HPLC under conditions that separated the initial pPNA-QMP, its deprotected product, and the self-adduct (Figure S3). Both deprotection of the silyl group and formation of the self-adduct proceeded at rates similar to those observed previously for the DNA conjugates.[12] Under these conditions loss of the silyl group is relatively fast, and consequently formation of the QM intermediate is likely rate determining. Complete conversion to the self-adduct required ~24 h in the presence of 130 mM potassium fluoride in 130 mM MES pH 7 under ambient conditions, and all species were confirmed by MALDI-TOF (Figure S4). When necessary, the self-adduct was generated in situ prior to use or stored after isolation from RP-HPLC. The pPNA-QM self-adduct lost less than 10% of its ability to alkylate a complementary target after 6 months of storage at −20 °C.

Target Promoted Alkylation by a pPNA-QM Self-Adduct. The reversibility of alkylation by the QM of this study ensures that the ultimate profile of products should reflect the thermodynamics of reaction even if kinetic products form initially.[14,34] Reversible systems should also adapt to new equilibria if constituents or conditions change as previously described in a variety of combinatorial models based on dynamic and covalent chemistry.[42,43] Noncomplementary targets that do not affect the self-adduct are not expected to induce changes in the equilibria of QM adducts. Only favorable interactions between the self-adduct and its target allow for interstrand transfer of the QM. This transfer, however, does not change the essential QM regeneration and recapture of the interstrand products.[12,34] When OD1 was introduced into a solution of the pPNA-QM self-adduct, interstrand alkylation followed a first-order process although more slowly ($t_{1/2}$ of 30 h, Figure 4 and

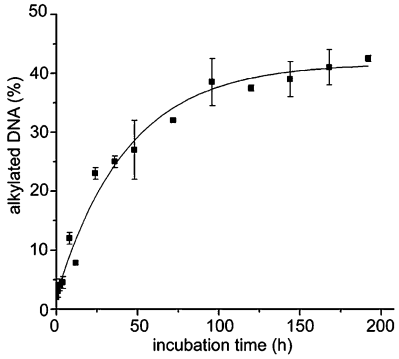

Figure 4. DNA alkylation by the pPNA-QM self-adduct. The self-adduct was prepared in situ by addition of KF (130 mM) to a solution pPNA-QMP (3.3 μM) in MES (130 mM pH 7) and NaCl (130 mM) and incubated under ambient conditions for 24 h. The target [^{32}P]-OD1 (3.0 uM) was then added, and incubation was continued for the indicated time before quantifying the alkylated product (see Figure S5). The average of two independent determinations was plotted and fit to a first-order process. The error represents the range of the observed yields.

Figure S5) than the equivalent first-order process of the pPNA-QMP conjugate ($t_{1/2}$ of 10 h, Figure 1). Additionally, a 1.1 equiv of the self-adduct generated a 40% yield of target alkylation whereas equivalent conditions supported a yield of >60% using the precursor pPNA-QMP. The difference in kinetics is easily rationalized by the ability of the initial QM formed by pPNA-QMP within a duplex to partition directly between intra- and interstrand alkylation. In contrast, QM formed by the self-adduct may require a structural reorganization of its

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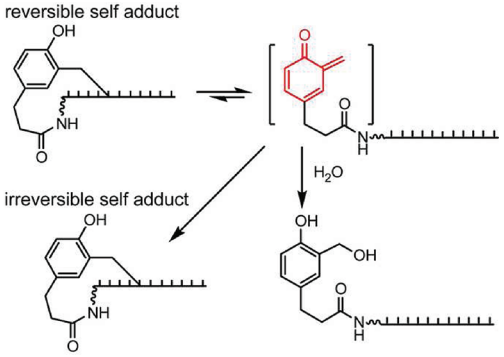
complex before interstrand alkylation can predominate (Scheme 1). Such a reorganization could also explain the relative yields of target alkylation if the conformation of the self-adduct resulted in a nascent QM that was more susceptible to irreversible quenching than the QM intermediate generated from within a duplex. Additionally, a reduced affinity for the complementary target is likely for self-adducts formed from QM capture by nucleophiles near the center of the PNA strand that would likely maximally interfere with Watson−Crick base pairing to a target.

Longevity of a Dynamic pPNA-QM Self-Adduct. The rate and final yield of target alkylation can be influenced by the profile of species comprising the self-adduct as tested previously by DNA forming hairpin and random coiled structures.[13] Early studies on a low molecular weight model QM illustrated a rapid formation of reversible nucleobase adducts through reaction with strong nitrogen nucleophiles such as dG N7, dA N1, and dC N3 that also act as strong leaving groups for QM regeneration.[15] However, these kinetic products were replaced over hours with thermodynamic products formed by irreversible trapping of the steady-state population of QM with water and weak nitrogen nucleophiles of DNA including the 2-amino of dG and 6-amino of dA that act as weak leaving groups for suppression of QM regeneration.[15] Accordingly, intrastrand alkylation of pPNA-QM was expected to form similar reversible adducts with strong nucleophiles most rapidly. These would then slowly redistribute to the corresponding irreversible derivatives formed by QM reaction with water and the weak nucleophiles above (Scheme 4).

Scheme 4. Irreversible Trapping of a Self-Adduct



The maximum yield of target alkylation decreased by 50% after incubating the pPNA-QM self-adduct for 168 h prior to addition of OD1 (Figure 5). This suggests that half of the self-adduct population was susceptible to quenching. Interestingly, the remaining fraction appeared resistant to quenching and remained capable of regenerating and transferring its tethered QM to OD1 even after many days in aqueous solution. The source of this persistent activity is not yet clear, but very similar results were observed earlier with an analogous DNA conjugate.[12] The time-dependent loss of activity is more easily explained and even expected by irreversible reaction between the QM and weak nucleophiles within the DNA. Mass spectral analysis indicated that the self-adducts of pPNA-QM remained dominant even after the extended preincubation described in Figure 5 (Figure S7). The product formed by addition of water to the transient QM was also anticipated, but it remained a minor component.

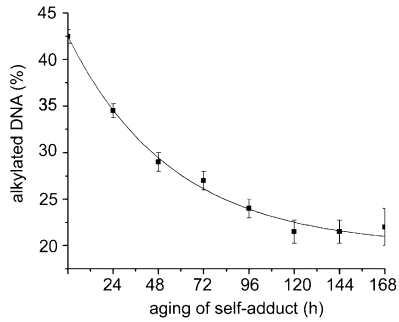


Figure 5. Persistence of the pPNA-QM self-adduct. The self-adduct was formed as described in Figure 4 and then incubated under ambient conditions for an additional 0−168 h prior to addition of [^{32}P]-OD1. Incubation was then continued for 168 h before quantifying the alkylated product (see Figure S6). The average of two independent determinations was plotted and fit to a first-order process. The error represents the range of the observed yields.

CONCLUSION

Reversible alkylation supported by quinone methide chemistry has the potential to avoid many of the difficulties, such as poor selectivity and irreversible consumption, that are typically associated with covalent reagents. The self-adduct formed by pPNA-QMP through intrastrand alkylation provides the first biocompatible conjugate for delivery of a quinone methide to alkylate a chosen sequence. The rates and yields of reaction mimic those observed for earlier derivatives based on DNA conjugates. Both demonstrate persistence in solution and reasonable yields of interstrand QM transfer even when used at near stoichiometric concentrations with its target. The PNA derivative in particular demonstrated a high degree of target discrimination. Only the kinetics of reaction remain to be improved for this strategy of selective alkylation to be ready for cellular applications. This final obstacle is currently being addressed by using QMs with electron-donating substituents that promote rapid and reversible generation of the QM intermediate.[44]

ASSOCIATED CONTENT

Supporting Information

Electrophoretic analysis of target alkylation (Figures S1, S2, and S5) and pPNA-QM self-adduct aging (Figure S6); RP-HPLC characterization of pPNA-QM self-adduct formation (Figure S3); MALDI-TOF detection of the pPNA-QMP conjugate and its derivatives (Figures S4 and S7). This material is available free of charge via the Internet at http://pubs.acs.org.

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