ABSTRACT

Title of Document: TARGET ALKYLATION OF SINGLE AND DOUBLE STRAND DNA BY PEPTIDE NUCLEIC ACIDS

Yang Liu, Doctor of Philosophy, 2011

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Quinone methides (QMs) generated *in vivo* can alkylate DNA and function as anti-cancer drugs. Delivery of QMs to target DNA is necessary to reduce the side effects caused by indiscriminate reaction. Previous, DNA was conjugated with a QM and was successfully used to deliver this QM to complementary DNA sequences. Peptide nucleic acids (PNAs) conjugates of QM are now being developed for in vivo application since PNA binds to its complementary DNA or RNA and PNA resists degradation by nucleases and proteases.

The PNA1-QMP1 conjugate is capable of alkylating more than 60% of a complementary ssDNA when added at nearly stoichiometric quantities. No alkylation was observed if non-complementary DNA was treated with the conjugate. PNA1-QMP1 can alkylate a non-complementary DNA only when both the PNA and DNA target bind to a template strand. When no target sequences were present in solution, QM can react with nucleophiles from PNA1 and generate PNA1-QM1 self

adduct. ssDNA can be alkylated by PNA1-QM1 self adduct with a 40% yield. The self adduct can survive after an incubation for 7 days in aqueous solution and preserve half of its original ability to alkylate complementary DNA. The reversibility and stability of the self adduct suggest that it can be used in cells. ssRNA can also be recognized and modified by PNA conjugates with a similar yield as earlier demonstrated with ssDNA. A PNA1-QM1 self adduct may also function as a telomerase inhibitor by alkylating RNA within telomerase.

Polypyrimidine PNAs were prepared to bind to the major groove of duplex DNA selectively and expand the potential targets from single to double strand DNA. A cytosine-rich PNA recognized dsDNA and delivered an electron-rich QMP2 to its target sequences. The polypurine strand within a target dsDNA was alkylated at 37°C with a yield of 26%. PAN-QMP2 also showed strong selectivity toward its fully matched dsDNA over one base mismatch in the triplex recognition site. Successful delivery of a QMP to target single and double strand DNA by PNAs confirms that the use of PNA *in vivo* to target pre-selected sequences is feasible.

TARGET ALKYLATION OF SINGLE AND DOUBLE STRAND DNA BY PEPTIDE NUCLEIC ACIDS

By

Yang Liu

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Professor Douglas Julin
Professor Zvi Kelman

Dedication

To my parents.

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Abbreviations

AEEA - 8-amino-3,6-dioxaoctanoic acid

CHO - chinese hamster ovary

D - diaminopurine

dA - deoxyadenosine

dC - deoxycytidine

dG - deoxyguanosine

DNA - deoxyribonucleic acid

dsDNA - double strand DNA

EDCI - 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide

ESI - electrospray

HATU - *O*-(7-aza-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium

hexafluorophosphate

Hp - N-methyl-3-hydroxypyrrole

Hprt - hypoxanthine phosphoribosyl transferase

hTR - human telomerase RNA

Im - N-methyllimidazole

MALDI - matrix-assisted laser desorption/ionization

MBHA - 4-methylbenzhydrylamine hydrochloride

MES - 2-(N-morpholino)ethanesulfonic acid

Mops - 3-(N-morpholino)propanesulfonic acid

MS - mass spectrum

ODN - oligonucleotide

pcPNA - pseudo-complementary PNA

PCR - polymerase chain reaction

PKA - protein kinase A

PNA - peptide nucleic acid

PNK - T4 polynucleotide kinase

RNA - ribonucleic acid

Py - N-methylpyrrole

RP-HPLC - reverse phase high performance liquid chromatography

QM - quinone methide

QMP - quinone methide precursor

SPPS - solid-phase peptide synthesis

ssDNA - single strand DNA

TBDMSCl - *tert*-butyldimethylsilyl chloride

t-Boc - *t*-Butyloxycarbonyl

TFA - trifluoroacetic acid

TFMSA - trifluoromethanesulfonic acid

TFO - triplex forming oligonucleotide

TRAP assay - telomeric repeat amplication protocol

U^S - thiouracil

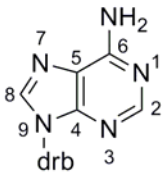
UV-Vis - ultra-violet visible spectroscopy

Z - benzyloxy-carbonyl

Chapter 1 Introduction

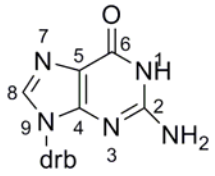
1.1 Importance of DNA alkylation.

Deoxyribonucleic acid (DNA) is the hereditary material used by almost all organisms.[1] The genetic information is stored in the different sequences composed of four bases: adenine, thymine, guanine and cytosine (**Scheme 1.1**). The bases are connected to 2'-deoxyribose sugars and linked by a phosphodiester backbone. Most DNA exists in a duplex form. Two strands of complementary DNA form a double helix structure in an anti-parallel manner.



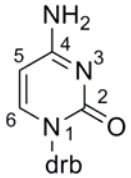
Adenosine

1.1



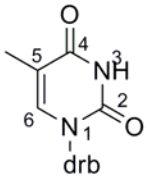
Guanosine

1.2



Cytosine

1.3



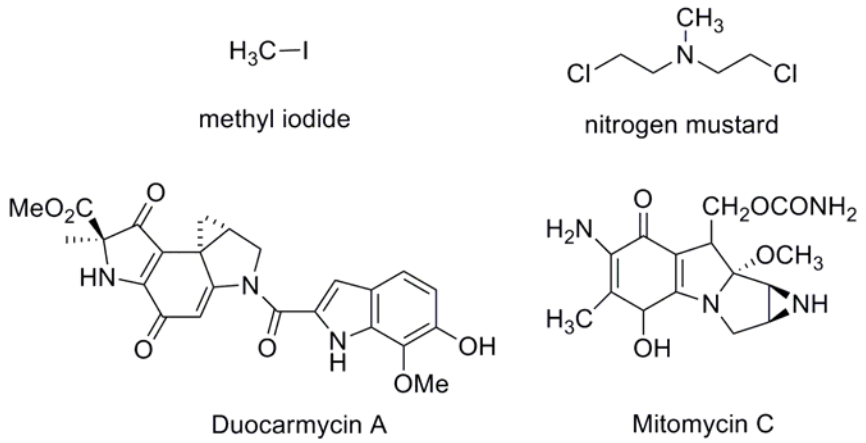
Thymidine

1.4

Scheme 1.1 Structures of DNA bases adenine, thymine, guanine and cytosine

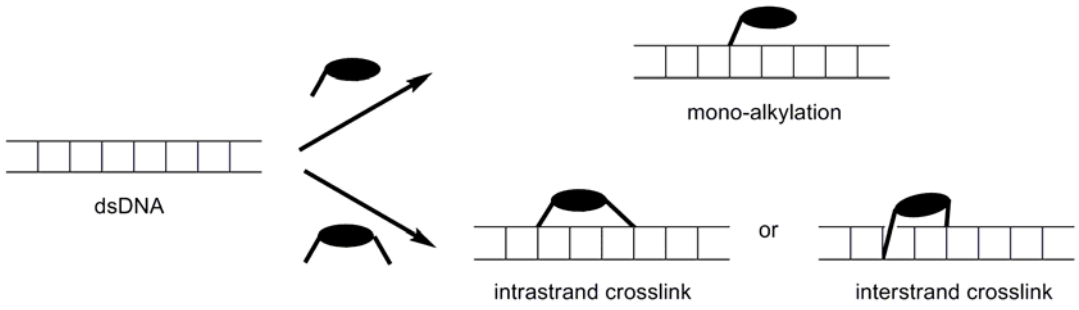
DNA can be damaged by different mutagens like alkylation agents, oxidizing agents and radiation. The rate of DNA damage is 10^3 to 10^6 molecular lesions per cell per day.[2] While this accounts for less than 0.0002% of all human genome, the DNA damage at some genes may cause severe diseases like cancer. However, modification of DNA in cancer cells may inhibit their replication and help to cure the disease. Various alkylation agents from simple methyl iodide and nitrogen mustard

to some natural products like duocarmycin A and mitomycin C can alkylate DNA (**Scheme 1.2**).



Scheme 1.2 DNA alkylation agents

Although the structures of alkylation agents and the DNA adducts are different, there are mainly three types of DNA alkylation – mono-alkylation, intrastrand crosslink and interstrand crosslink. Alkylation agents can react with one single nucleophile from DNA and generate a mono-adduct (**Scheme 1.3**). Some agents like mitomycin C can alkylate two different nucleophiles from DNA to generate intra- and interstrand DNA adducts. Among the three types of DNA adducts, interstrand is the most potent because the crosslink may inhibit the replication of dsDNA.[3]

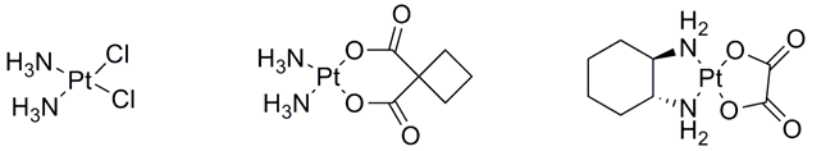


Scheme 1.3 Models of mono- and bis- DNA alkylation

1.2 Reversible and irreversible DNA alkylation agents.

Alkylation agents react with most nucleophiles of DNA. There are both reversible and irreversible DNA alkylation products. Irreversible DNA alkylation induced by compounds like methyl iodide and dimethylsulfate causes permanent DNA damage. These compounds react with most of the nucleophiles of DNA through an S_N2 mechanism. Methyl iodide reacts at the O^6 and N7 positions of dG, N1 and N7 positions of dA, N3, N4 and O^2 positions of dC.[4] Dimethylsulfate reacts with dG N7, dA N1 N3 and dC N3 primarily.[4] The high activity toward DNA and volatile property make these compounds extreme hazardous.

Besides irreversible mono-DNA alkylation, some chemotherapeutic agents such as platinum compounds (**Scheme 1.4**) and mitomycin C can also induce irreversible DNA cross-link adducts.



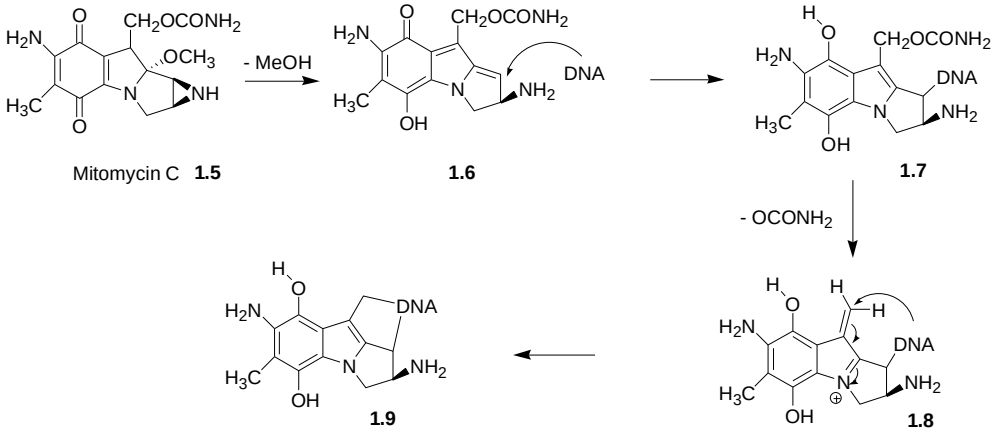
Scheme 1.4 Structures of platinum drugs

Cisplatin is first hydrolyzed, replacing one chloride by a water molecule, the intermediate is then subjected to attack from DNA nucleophiles. It is found that the N7 position of dG is the primary alkylation site.[5] Cisplatin can continue to hydrolyze and alkylate another strand of DNA. The generated interstrand DNA cross-link can ultimately trigger apoptosis.

Although widely used as an anti-cancer drug, cisplatin also causes side effects including kidney damage, nerve damage, vomiting and hearing loss.[6] The second

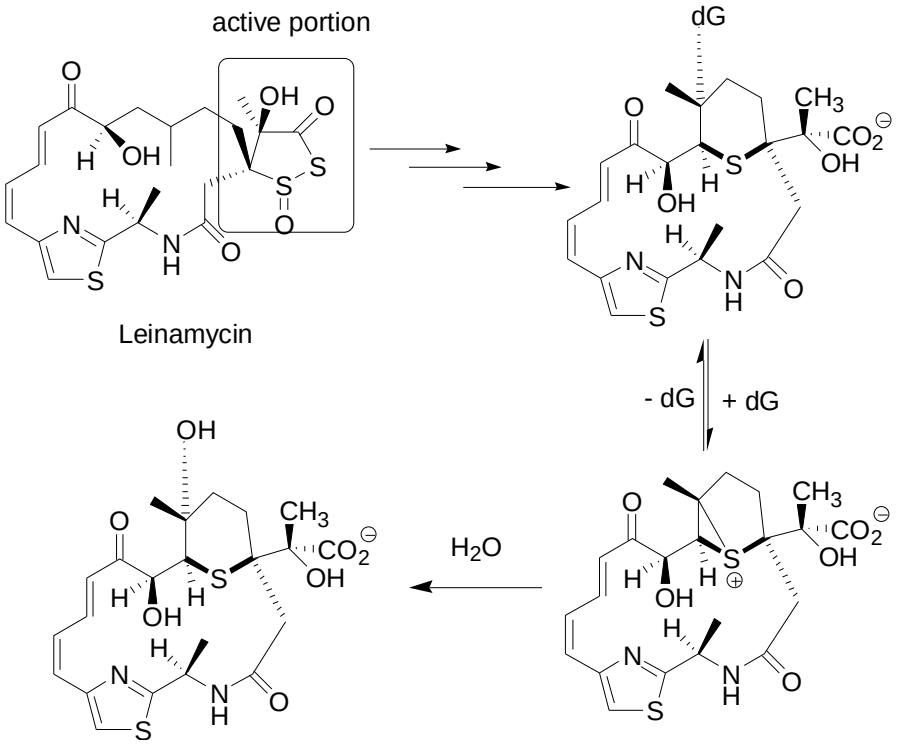
generation carboplatin yields the same active component as cisplatin inside cells but hydrolyze at a much slower rate and reduce the side effects in some patients. To improve the selectivity of platinum compounds toward DNA in tumor cells, cisplatin was conjugated to directing agents like oligonucleotides and acridines.[7, 8] These conjugates increase the sequence selectivity and should reduce off target modification in cells. The effect of directing agents will be discussed in detail later in this chapter.

Mitomycin C (**1.5**) belongs to a family of aziridine-containing natural products and is used as a chemotherapy agent because of its ability to form DNA cross-link (**Scheme 1.5**).[9] Its modification of DNA is dependent on reductive bioactivation to form 2,7-diaminomitosene (**1.6**), a type of quinone methide, as the major intermediate. The mono-adduct **1.7** can continue to eliminate a carbamate and generate a second electrophile center. Intermediate **1.8** can then be attacked by another nuclophile from DNA and form a bis-DNA cross-link adduct. Many solid tumors are lack of oxygen comparing to normal cells. Since mitomycin C is activated by reduction, mitomycin C is more likely to be activated in tumor cells while inhibited by an oxygen-rich environment. Mitomycin C irreversibly alkylates DNA like methyl iodide and dimethylsulfate, but mitomycin C selectively binds to the minor groove of DNA and almost exclusively alkylates DNA at the N^2 position of guanine. To further increase the target selectivity and defeat drug resistance, mitomycin C was conjugated with a lysosomally degradable peptide spacer (Gly-Leu-Gly) and polysaccharide.[10]



Scheme 1.5 Generation of DNA adduct from mitomycin C via QM

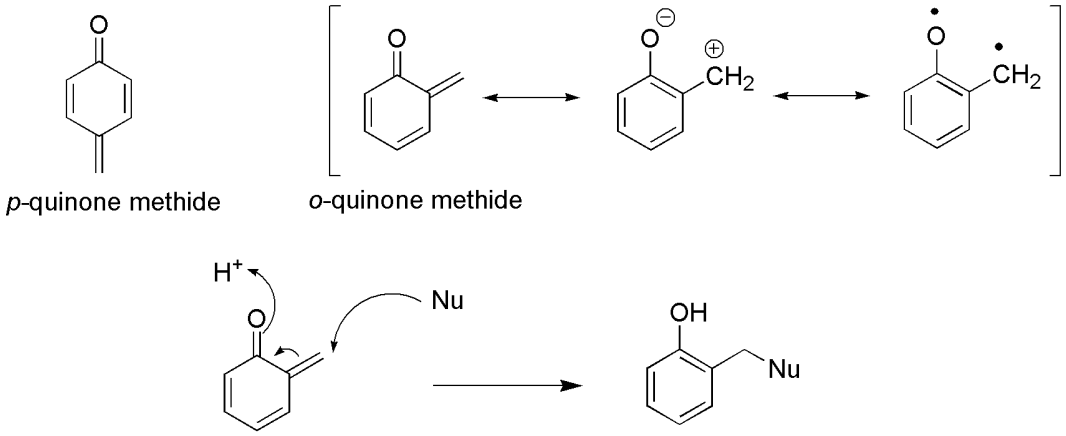
While most of DNA adducts are irreversible, some potent DNA alkylation agents can generate reversible DNA adducts. Leinamycin is a DNA-damaging agent which shows significant antitumor activity (**Scheme 1.6**).[11] Leinamycin is first activated by thiol *in vivo*[12] to form an intermediate that mainly alkylates the N7 position of guanine.[12] The guanine N7 adduct is reversible and can either regenerate an episulfonium ion or be quenched by water. The reversible reaction has the ability to escape trapping with non-target sequences and prolong the life of the active intermediate. Because there are high concentrations of thiols like glutathione and cysteine in cells, leinamycin can be activated in cells.



Scheme 1.6 Alkylation of DNA by leinamycin

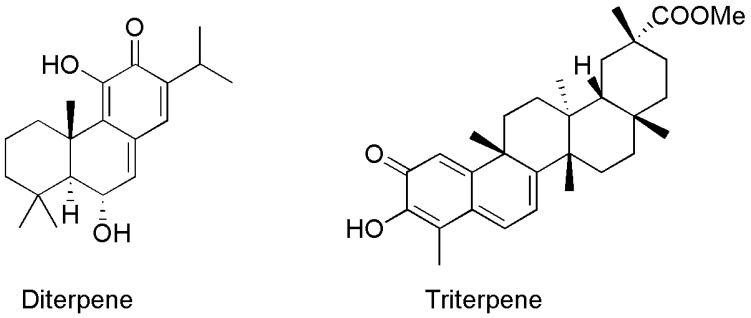
1.3 Quinone Methides (QMs)

The Rokita group used a class of DNA alkylation agents - quinone methides (QMs) to systematically study their reactivity toward various nucleophiles of DNA and the reversibility of each DNA adduct. QMs are electrophilic intermediates that can be generated *in vivo* and react with nucleophiles such as amines, water and thiols (**Scheme 1.7**).[13-17] A nucleophile attacks the exocyclic methylene group and regenerates the phenol moiety. There are two major types of QMs: *o*-QM and *p*-QM. Both *o*-QM and *p*-QM have resonance structures that contain both anionic and cationic centers. The polarized nature of QMs indicate that they can react with both electrophiles and nucleophiles.



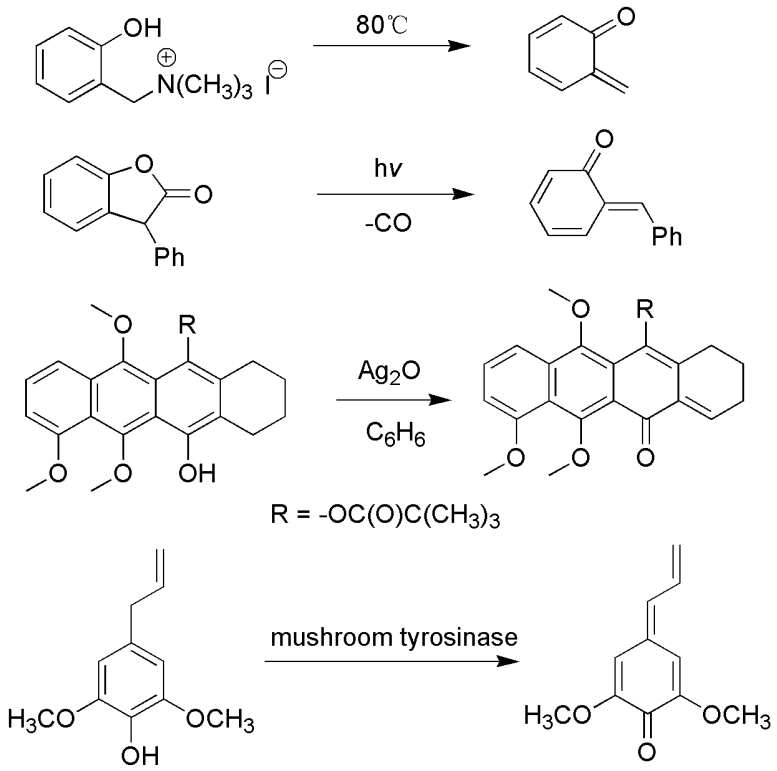
Scheme 1.7 Structure of quinone methide and its adductive product

Generally, QMs are very reactive in aqueous solution and their lifetimes range from 1 ms to several minutes depending on their substituents.[18-21] In contrast, some natural diterpene and triterpene QMs found in plant are stable and persist for days or even months (**Scheme 1.8**).[22] Diterpene shows a strong tumor suppress effect in rats. Triterpene also exhibits high cytotoxicity in a panel of cancer cell lines including human lung carcinoma A-549 and colon carcinoma HT-28.[23]



Scheme 1.8 Examples of natural diterpene and triterpene

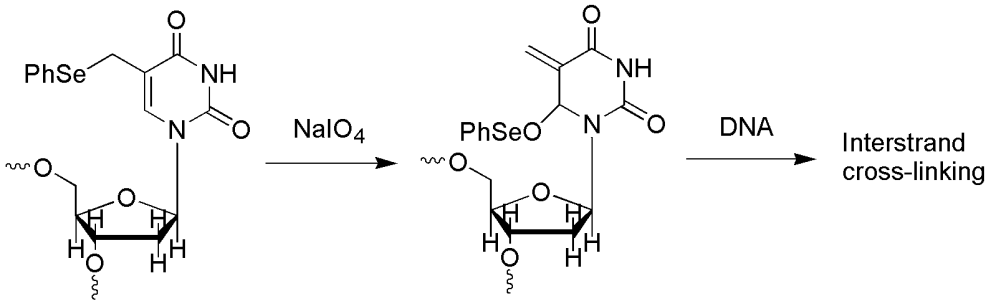
QMs are usually prepared by the activation of qunione methide precursors (QMPs) in situ (**Scheme 1.9**). QMs can be generated from thermal methods,[24] UV light,[25-27] oxidation by Ag_2O and PbO_2,[28, 29] and biological methods.[30-32]



Scheme 1.9 Generation of QMs

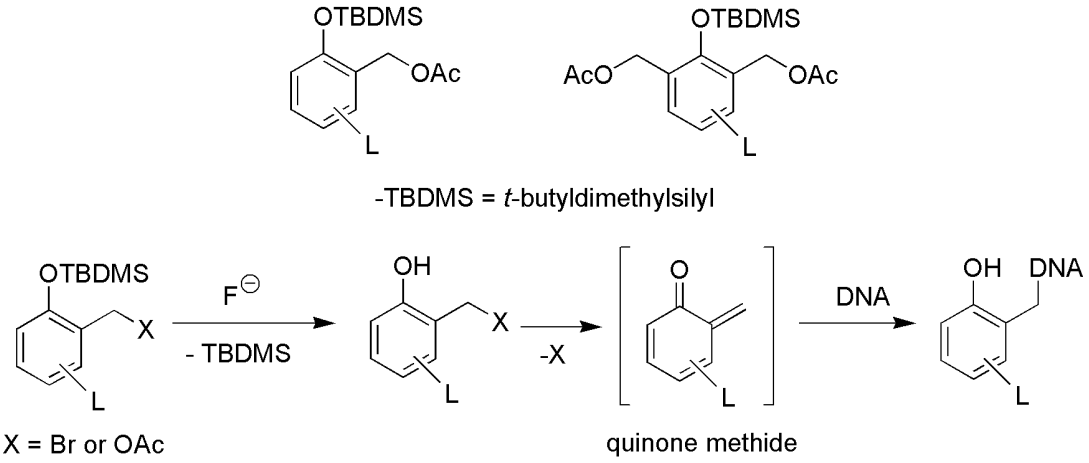
Several groups studied the generation of QM by oxidation and generation of interstrand cross-linking by QM.[33-35] Greenberg group incorporated a phenyl selenide modified nucleotide into a sequence of DNA.[34] After a complementary DNA was annealed with the DNA containing modified nucleotide, $NaIO_4$ or light was used to activate phenyl selenide moiety and generate QM (**Scheme 1.10**). Since the modified nucleotide is incorporated into a sequence, only nucleophiles from its complementary strand and near to the modified base can be alkylated. The

incorporation renders a selectivity toward nucleophiles near to the QM and reduces random alkylation.



Scheme 1.10 Interstrand cross-linking by phenyl selenide derivatives

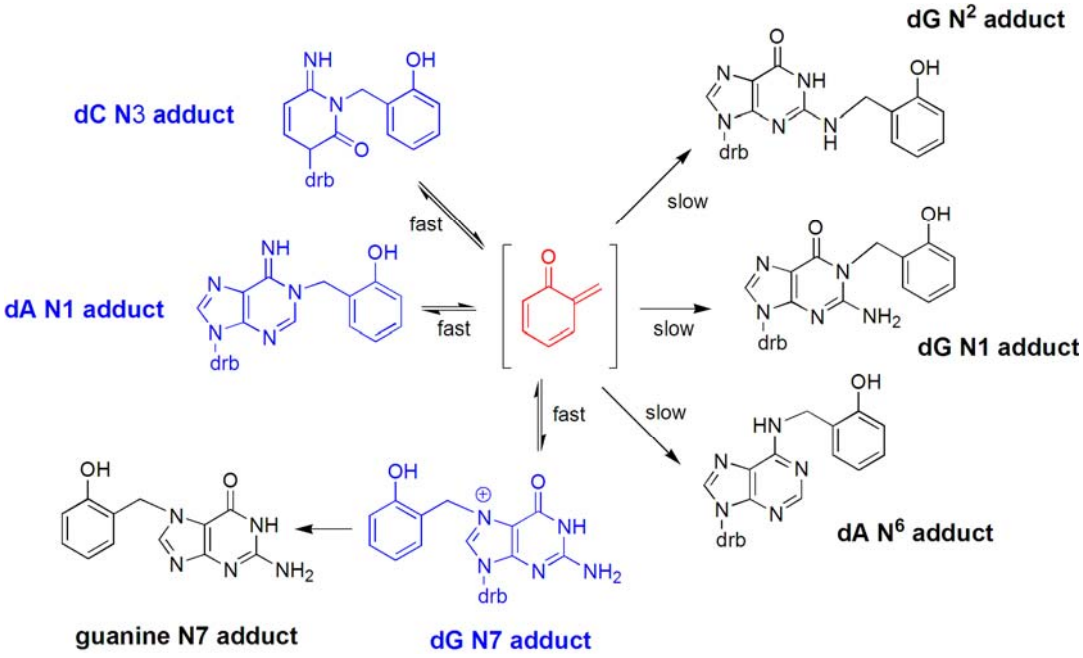
Generation of QM intermediates by fluoride offers a method to prepare QM with almost quantitative yield within hours.[36-38] The hydroxyl group is protected by silyl ether and a good leaving group (X = bromide or acetate) is linked to the benzylic position. The generation of QM is triggered by adding fluoride. The generated QM intermediate can be attacked by nucleophiles from DNA to form DNA alkylation products. When there are two leaving groups, QMs can react with two nucleophiles from either the same strand or different strands of DNA (**Scheme 1.11**).



Scheme 1.11 Generation of QM intermediate by fluoride

1.4 Reversibility of nucleoside-QM adducts

QM intermediate can react with different nucleophiles from DNA and form both reversible and irreversible DNA adducts.[39] The Rokita group found that strong nucleophiles from DNA (dG N7, dC N3 and dA N1) form adducts with QM quickly but the adducts are reversible. These kinetic products can regenerate QM. An oligonucleotide-QM self adduct persists reversibility over at least 8 days in aqueous buffer and can regenerate QM intermediate. In contrast, the lifetime of QM itself is millisecond to second.



Scheme 1.12 Structures of reversible (blue) and irreversible (black) DNA-QM adducts

The generation and stability of different deoxynucleoside adducts were used to study time-dependent alkylation of deoxynucleosides by an unsubstituted *ortho*-QM (**Scheme 1.12**).[39] dA N1 and dC N3 were the major adducts in the first 10 hours. After 50 hours, almost no dA N1 adduct was detected by HPLC and only around 1/3

of dC N3 adduct remained in solution. dG N^2, dG N1 and dA N^6 generated irreversible adducts with QM.[39] The thermodynamic adducts accumulated over time. Eventually, irreversible products dominate in the solution. The kinetic adducts of dA N1, dC N3 and dG N7 can serve as QM reservoirs for the regeneration of the highly transient QM intermediate (**Figure 1.1**).

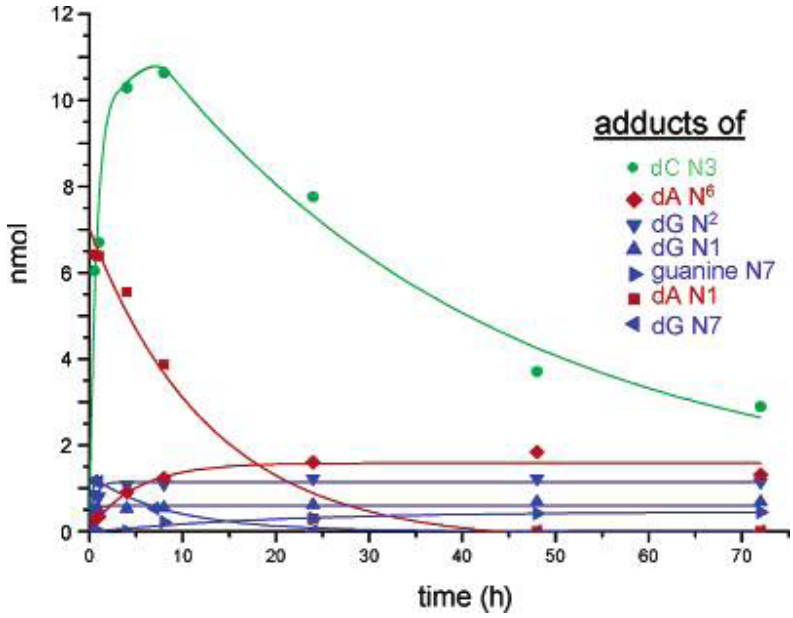
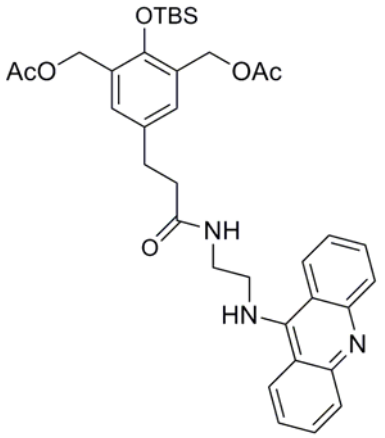


Figure 1.1 Time-dependent evolution of deoxynucleoside alkylation by a quinone methide (copied from ref 39).

Rokita group used a bisQMP-acridine conjugate to generate DNA cross-linking and study the reversibility of DNA adducts (**Scheme 1.13**).[40, 41] Since there are two acetate leaving groups on benzyl ring, the bisQMP can generate two QM intermediate and ultimately alkylate two nucleophiles from DNA. The ratio of DNA cross-linking to mono-alkylation by bisQMP-acridine was much greater than the ratio of cross-linking to mono-alkylation induced by N-mustard.[42, 43] The difference between the two alkylation agents can be rationalized by the reversibility of QM. Since dG N7 is the major alkylation target of QM, the regenerated QM from

reversible dG N7-QM adduct could continually be captured by other nucleophiles from DNA. On contrary, N-mustards generate irreversible adducts and avoids further nucleophiles of other strand to attack the N-mustard and form DNA crosslinking.[44]



BisQMP-acridine conjugate

Scheme 1.13 bisQMP-acridine conjugate

Rokita group also used the mixture of deoxynucleosides (dA, dT, dG and dC) and bisQMP-acridine to study which was the best at extending the lifetime of QM (**Figure 1.2**). Interestingly, only dA extends the life time of QM for more than 100-fold by measuring its ability to generate DNA cross-linking.[40] A single strand DNA containing dA was even more effective in preserving the activity of bisQM than dA. After the single strand DNA was incubated with bisQMP-acridine for 24 hours, incubation with additional 1000-fold excess of 2-mercaptoethanol for 72 hours only suppressed 50% of bisQM's alkylation ability. The result showed that once formed, the regenerated QM from reversible dA-N1-bisQM adducts still prefer the nuclophiles from DNA and not nucleophiles like water in the solution.[40] Reversible

dA-bisQM adducts effectively extend the lifetime of short-lived QM intermediate and improve the alkylation yield of bisQMP.

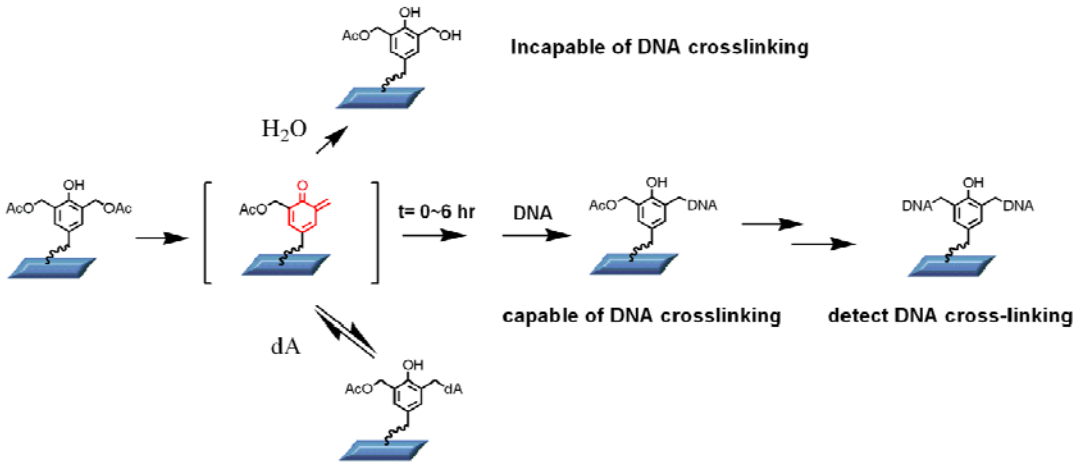


Figure 1.2 Extending the life time of bisQMP by generating dA-bisQM reversible adducts (copied from ref [40]). The blue template represents a acridine intercalator.

1.5 Substituent effect on the stability of QM

Substituents on aromatic ring of QM also influence the stability of QM adducts and alter the kinetics and reactivity of QM reaction with nucleophiles of DNA.[45] Since QM intermediates are typically electron deficit, electron-donating groups will stabilize the intermediate and electon-withdrawing group will destabilize the intermediate. Rokita group used a QM which has substituents on aromatic ring to study the substitution effect on QM (**Figure 1.3**).

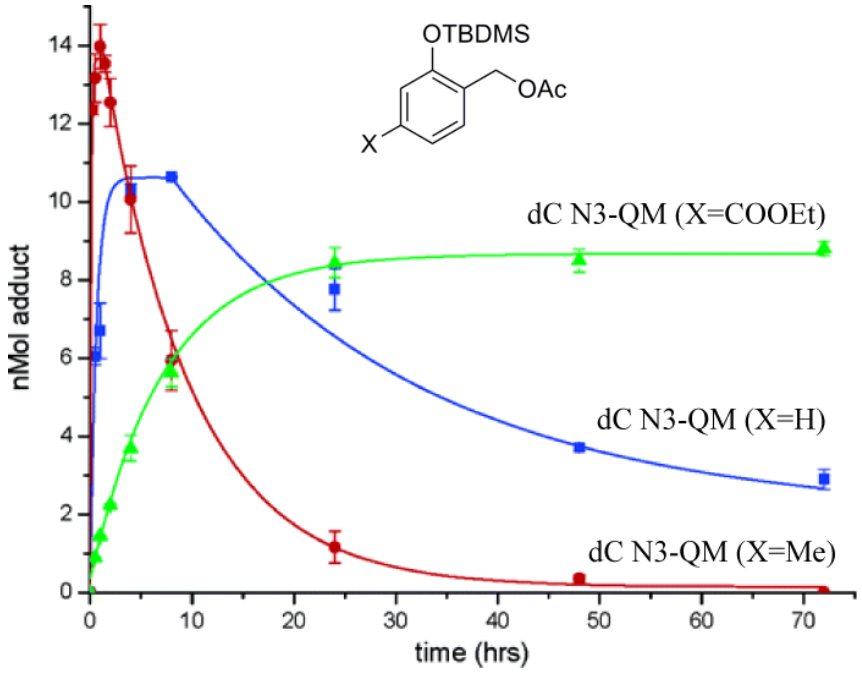


Figure 1.3 Substituent effect on the generation of dC N3-QM adducts (copied from ref[45])

When there is no substitution on the aromatic ring, formation of the dC N3-QM adduct reached a maximum yield after around 10 hours. When an electron-donating methyl substituent was added (X = Me), the dC N3-MeQM adduct formed much quicker than its unsubstituted analogue QM (~ 2 h vs 10 h). Since the electron-donating methyl group stabilizes the QM intermediate, the QM was quickly re-generated and could be quenched by other nucleophiles like water. Twenty five hours later, less than 10% of initial dC N3-MeQM adduct was found by HPLC. When X = COOEt, the re-generation of QM is slow and the dC N3-QM adduct slowly accumulated even after 50 hours since the electron-withdrawing methyl ester destabilize the QM intermediate. By fine tune the substituents on the aromatic ring, we can control the reactivity of QM and design QM for various applications.

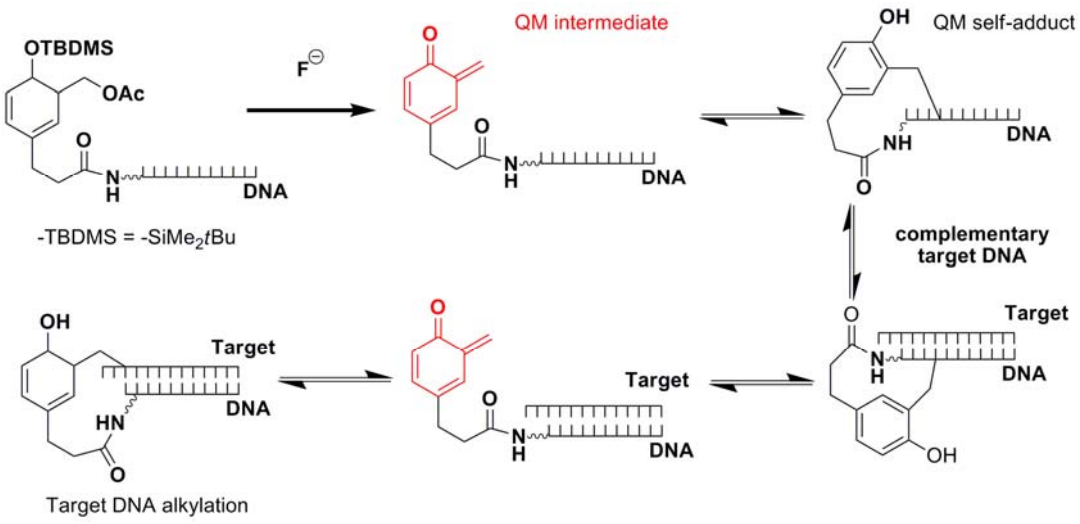
1.6 Target delivery of QMP by various agents

Many chemotherapeutic agents inhibit the replication of cancer cells by alkylating DNA. The lack of selectivity of alkylating agents toward DNA sequences limits the use of DNA alkylation in clinical. Many cellular components like protein and water as well as non-target DNA can also compete with target DNA and react with active QM intermediate. To reduce side reactions, alkylating agents such as N-mustard, psoralen and QM precursors conjugated with site directing ligands have been deployed with great success to alkylate target DNA and induce DNA cross-linking.[46-52] Site directing agents can bind with target sequences by hydrogen bonding, van der Waals or hydrophobic forces.[53-56] DNA alkylating agents are co-localized with the target sequence and modify nearby DNA bases.

DNA intercalators which bind to DNA non-covalently are used as one type of delivery agents. DNA intercalators have been used to deliver DNA alkylating agents to major groove of duplex DNA.[40, 57] Rokita group prepared a bisQMP-acridine conjugate for DNA cross-linking. BisQM directed by acridine can react with guanine N7 specifically since guanine N7, which is one of the strongest nucleophiles of DNA locates in the major groove of DNA. Acridine effectively improves the DNA binding affinity. Conjugation of the bisQMP with acridine significantly increased its cross-linking efficiency by over 50-fold than bisQMP itself.[42]

Although acridine can direct DNA alkylating agents to DNA, it is not sequence specific. To deliver a DNA alkylation agent to a target sequence, oligonucleotides (ODNs) were used as directing agents by forming double or triple helixes with target

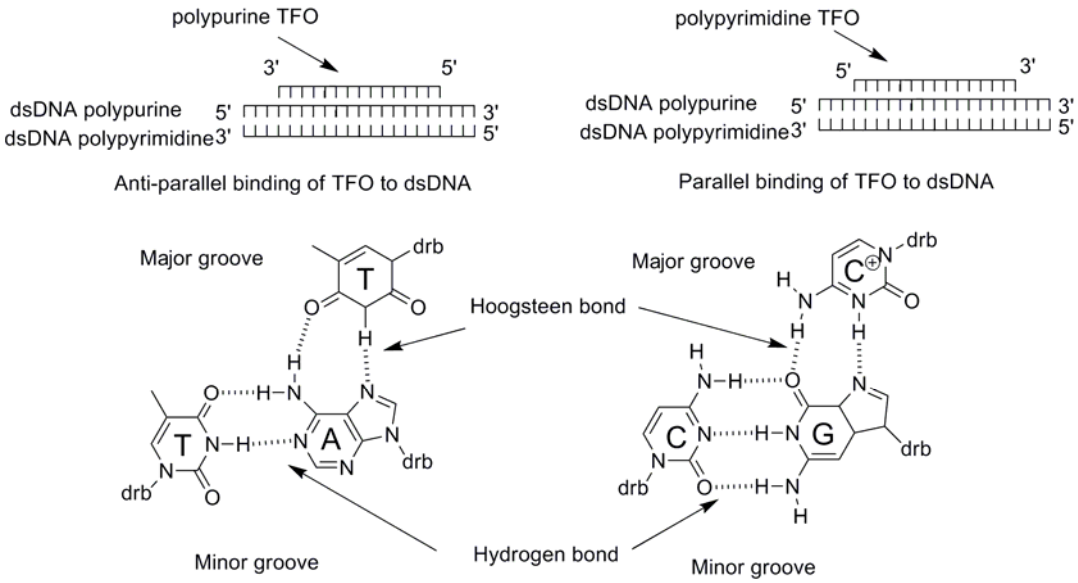
DNA. Directing agents can be easily prepared and have high fidelity toward their complementary sequence. The Rokita group used ssDNA-QMP conjugate to study the target delivery of QMP (**Scheme 1.14**).[37] A ssDNA-QM self-adduct can be formed in the absence of target sequences. The self adduct is reversible and still alkylates target DNA even after eight days pre-incubation in aqueous solution. Meanwhile, non-complementary DNA is neither alkylated by the DNA-QM self adduct nor did it interfere with alkylation of the target sequence. Recognition and alkylation of target sequences make the conjugates of ODNs and DNA alkylation agents a useful strategy in target delivery. The modification of target DNA by reversible self-adduct avoids the use of toxic fluoride and makes the system potentially useful in a cell environment.[37]



Scheme 1.14 Selective delivery of QMP by DNA sequence

DNA triple helix (triplex) structures are also used to direct DNA alkylating agents to their targets. Triplex forming oligonucleotides (TFOs) bind to polypurine strands in the major groove of dsDNA by Hoogsteen pairing.[58] When the TFO is a

polypurine strand, it binds to dsDNA in an anti-parallel manner while a polypyrimidine TFO attaches to dsDNA in a parallel manner (**Scheme 1.15**).



Scheme 1.15 Formation of DNA triplex

Polypyrimidine or polypurine DNA sequences as TFOs have been studied by several groups to deliver DNA alkylation agents like psoralen,[59, 60] transplatin[61, 62] and QMP[63] to target sequences. Psoralen-TFO generated site-specific mutations within mammalian cells by forming DNA crosslinking.[60] Platinum-modified TFO can form a stable triplex structure and generate DNA deoxyguanines or deoxyadenines adducts that are adjunct to the TFO binding site.

The Rokita group conjugated a bisQMP with a polypyrimidine TFO and studied its alkylation of duplex DNA (**Figure 1.4**).[63] Approximate 20% of the purine-rich strand was alkylated while only around 4% for the pyrimidine-rich strand. The difference may indicate that the nuclophiles from the purine-rich strand are more reactive and accessible to the quinone methide.

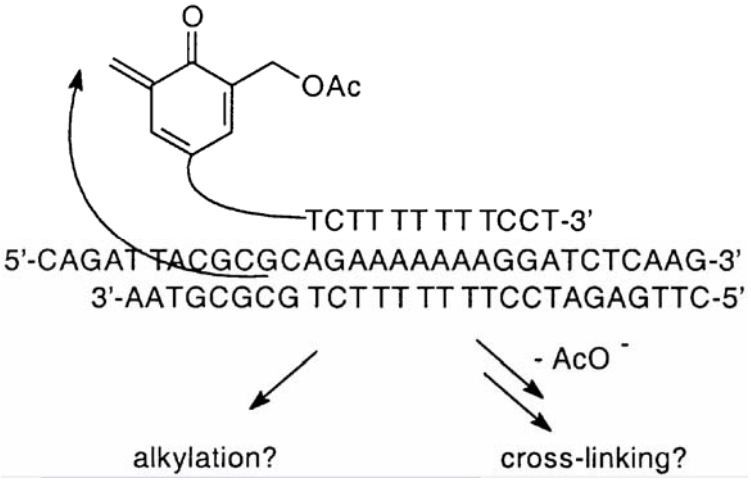
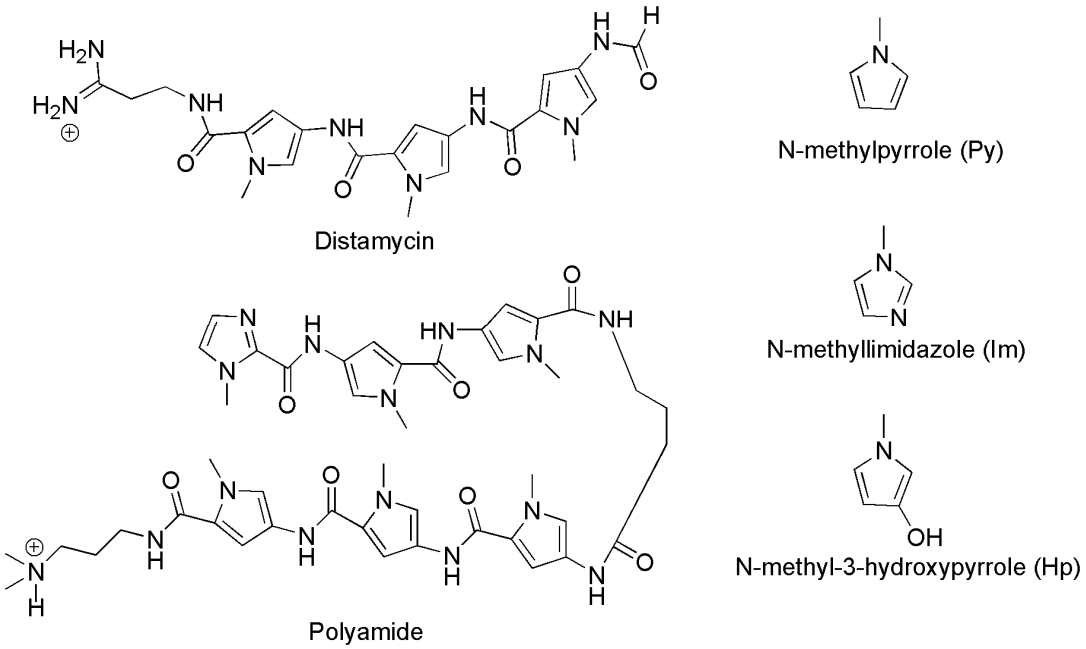


Figure 1.4 Triplex recognition and selective alkylation (from ref[63])

DNA as a directing agent limits its use in vivo since DNA can't pass through the cell membrane. The lability of DNA toward DNase makes it difficult to be deployed *in vivo*. Oligosaccharides can permeate cell and have been used to interfere with transcription.[64, 65] Still, the lack of broad DNA sequences recognition makes carbohydrates a poor candidate to selectively deliver DNA alkylation agents. Pyrrole-imidazole polyamides which are inspired by natural distamycin represent a class of small molecules which can bind to selected DNA sequences as well as pass through the cell membrane (**scheme 1.16**).[47, 49, 66-71]



Scheme 1.16 Structure of distamycin, a pyrrole-imidazole polyamide and Py, Im, Hp

Distamycin contains three N-methylpyrrole (Py) amino acids and binds to the minor groove of DNA at A, T tracts.[72] Py, N-methyllimidazole (Im) and N-methyl-3-hydroxypyrrole (Hp) were linked by amide bonds to recognize bases of DNA. Py/Im targets C/G bases pair and Py/Hp targets A/T.[73] By carefully following the pairing rules, a Py-Im sequence can selectively bind with any predetermined DNA sequences. Several DNA alkylating agents such as seco-CBI,[71] chlorambucil[70] and bisQMP[49] have been conjugated with polyamides. Polyamide-seco-CBI alkylated adenine adjacent to the binding site at a nanomolar concentration.[71] Both chlorambucil and bisQMP conjugates also showed alkylation near the polyamide binding sites. However, reaction at the predicted recognition sequence by bisQMP-polyamide conjugates was low (4%).[49] Generation of bisQM-polyamide self adduct within one hour was confirmed by HPLC and mass spectrometry. The low efficiency of dsDNA alkylation was caused by the

irreversibility of bisQM-polyamide self adduct. The reversibility of self adducts is key to their regeneration and prolonged the life time of QM. Since the self adduct is irreversible, only the initially generated QM can possibly alkylate target DNA sequence. Once the bisQM self adduct is formed, QM can no longer be transferred to DNA.

Since both DNA and polyamide have their limitations for use *in vivo*, new site directing agents which can penetrate the cell membrane, selectively bind to target DNA, remain stable inside of cells and generate the reversible QM self adducts are necessary.

1.7 Peptide nucleic acids (PNAs) as delivery agents

PNA is a DNA mimic with repeating N-(2-aminoethyl)-glycine units as the polymer backbone. A, T, G and C bases are linked onto backbone via a methylene carbonyl groups (**Figure 1.5**).[74] PNA can bind with complementary DNA or RNA by Watson-Crick bases pairing. Unlike DNA, PNAs are shown to resist nucleases and proteases *in vivo* and capable of inhibiting translation *in vivo*.[75] Since PNA has the same bases as DNA, it is reasonable to expect that PNA-QM self adducts should be reversible like DNA-QM self adducts.

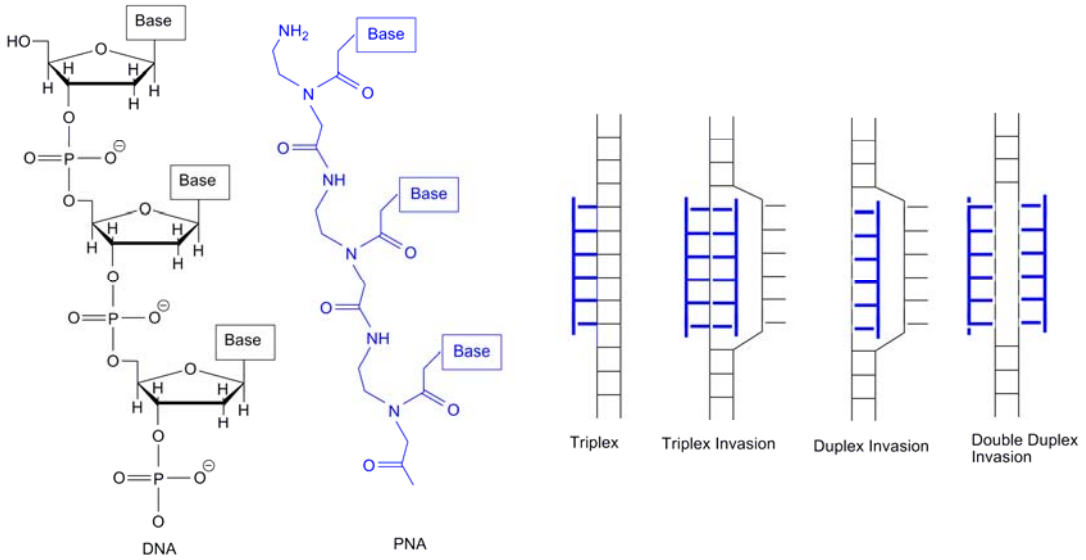


Figure 1.5 Structures of DNA and PNA

PNA can avoid many limitations caused by DNA or polyamides when used *in vivo*. Complementary PNA/DNA binds tighter than a comparable DNA/DNA duplex due to the lack of charge repulsion between the two sequences since the backbone of PNA is neutral. Single base mismatches in PNA/DNA duplex are more destabilized than equivalent one in DNA/DNA.[75]

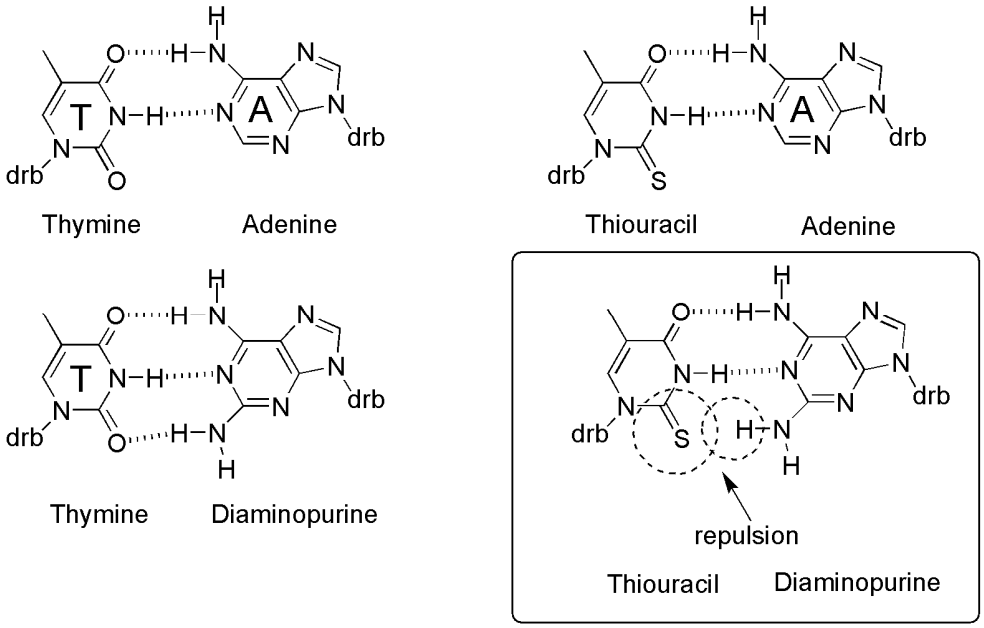
Although most of oligonucleotides exist as a duplex in cells, single-stranded oligonucleotide such as a messenger RNA is available in cells. Single-stranded oligonucleotide can be a good alkylation target. Mixed bases PNA can recognize its complementary DNA and deliver alkylation agents. But the delivery of those alkylation agents to duplex DNA is problematic. A mixed-bases PNA sequence does not show any dsDNA invasion ability alone unless the recognition site sits at the end of dsDNA which is rare in reality.[76]

A polypyrimidine sequence of PNA was first designed to recognize duplex DNA by forming a PNA/DNA_2 triplex (**Figure 1.5**).[75, 77] Unexpectedly, after the PNA bound to the major groove of dsDNA, PNA invaded into dsDNA and generated triplex invasion structure.[77] The PNA/DNA/PNA sandwich structure leaves the polypurine ssDNA in the solution (triplex invasion in **Figure 1.5**). Nielsen showed that three to five positive charged at the end of a PNA sequence can aid the generation of triplex and triplex invasion.[74] The extra charges also increases the solubility of PNA and improve its cellular uptake.[78] Triplex invasion is found to be affected by salt concentration. High salt concentration will suppress triplex invasion since the melting point of dsDNA increases with higher salt concentration. It is always necessary to take the salt concentration in consideration when PNA is used to form triplex or triplex invasion structures.

Both a N-mustard[79] and a psoralen[80] have been conjugated to a polypyrimidine PNA to investigate the delivery of these DNA modification agents to target DNA by PNA. DNA alkylation were found near the PNA binding sites since both triplex and triplex invasion were formed.[79] Compared to PNA itself, the alkylation stabilizes PNA binding in cell and creats a robust method to prevent transcription.

Although polypyrimidine PNA can recognize dsDNA, the recognition sites are restricted to continuous polypurine/polypyrimidine sequences. PNA sequences which can interact with mixed-bases dsDNA is useful to deliver DNA alkylation agents to any sequences. For this reason, pseudo-complementary PNAs (pcPNAs) – diaminopurine (D) and thiouracil (U^S) were designed and synthesized to replace adenine and thymine (**Scheme 1.17**).[81] The extra NH_2 group in D and substitution

of oxygen by sulfur in U^S do not interfere with the formation of hydrogen bonds between T-D and A-U^S while there is a steric clash between the amino group in D and thiol group in U^S. This makes the formation of pcPNA/pcPNA duplex disfavored. Only DNA/DNA and pcPNA/DNA can be formed.



Scheme 1.17 Interactions between PNA bases A, T and pcPNA bases D, U^S

Psoralen was conjugated with pcPNA and was delivered to a target dsDNA within plasmid DNA. Psoralen conjugated with pcPNA induced targeted mutagenesis at a mixed sequence with both episomal and chromosomal DNA without obvious off-target modification.[52, 80, 82] The induced mutations and deletions were at the predicted pcPNA binding site. These data showed that PNA can be useful in delivering DNA alkylating agents to target DNA sequences *in vivo*.

This dissertation investigates the target alkylation of ssDNA, ssRNA and dsDNA by PNA-QMP conjugates. Both the reactivity and stability of PNA-QMP

self adducts and their self adducts were characterized. Formation of PNA triplex and triplex invasion to deliver a QM to dsDNA was accomplished. This study helps to understand the target delivery of a QM to pre-selected sequences by PNA, improving cancer cell targeting, decreasing side effects and the ultimate application *in vivo*.

Chapter 2 Alkylation of ssDNA by PNA1-QMP1 conjugates and their self adducts

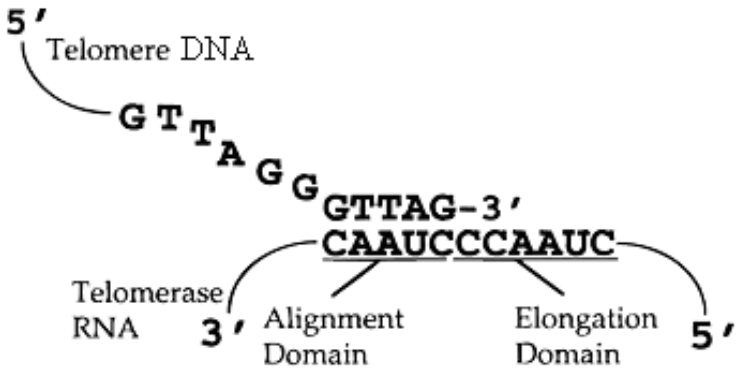
2.1 Introduction

Oligonucleotide-quinone methide precursors (QMPs) have demonstrated effective alkylation of target DNA, but the lability of oligonucleotides to nucleases limits their use in a cell environment. The inability of oligonucleotides to penetrate cell membrane also limits delivery of a QMP to cells. Peptide nucleic acids (PNAs) can bind with their complementary DNA by standard Watson-Crick base pairing and should discriminate between matched and mismatched DNA. PNAs conjugated with positively charged amino acids (lysine and arginine) have been shown to pass the cell membrane.[78, 83, 84] Thus QMP can be delivered to target sequence by conjugating with PNAs. The PNA-QMP conjugate can continue to alkylate target sequences *in vitro* and *in vivo*. In this chapter, the alkylation of predetermined sequences from oligonucleotides in telomerase by PNA-QMP conjugate and PNA-QM self-adduct is studied.

Telomeres contain a highly repeated DNA structures at the ends of linear chromosomes.[85] Human telomeres contain about 5-15 kilobases of a tandem repeat sequence (5'-TTAGGG-3') at the end of telomeres. The sequence provides a method for cells to distinguish the natural termini from random breaks. These structures also help to stabilize DNA during replication.[86] Telomeres keep shortening after each cell cycle.[87] Once the length of telomeres decreases to a

minimum threshold, the protective telomere caps become destabilized and result in replicative senescence or cell death.[88]

In some human cell types such as adult germline cells and stem cells, telomere length is maintained by telomerase during the proliferation of cells.[89] Telomerase is an ribonucleoprotein that adds repeating 5'-TTAGGG-3' to the 3' end of DNA strands in the telomere regions.[90] In most normal cells, telomerase activity is almost undetectable.[91] In contrast telomerase activity is found in most human tumors.[92] The length of the telomeres in tumor cells does not continuously shorten with the successive cell divisions because of the presence of active telomerase. Telomerase is then thought to be an essential factor in the "immortal"[93] property of tumor cells (**Scheme 2.1**). Thereby, telomerase is a potential target for anti-tumor drugs.



Scheme 2.1 Binding of the end of a telomere to human telomerase[94]

Oligonucleotide-based therapeutics are potential methods to develop anti-cancer drugs by binding to the telomerase.[95] There are two major subunits in human telomerase: hTERT and hTR. hTR is a RNA component that acts as a template for replication and hTERT is a protein that catalyzes nucleotide polymerization.[78] The hTR is composed of 451 nucleotides and can serve as

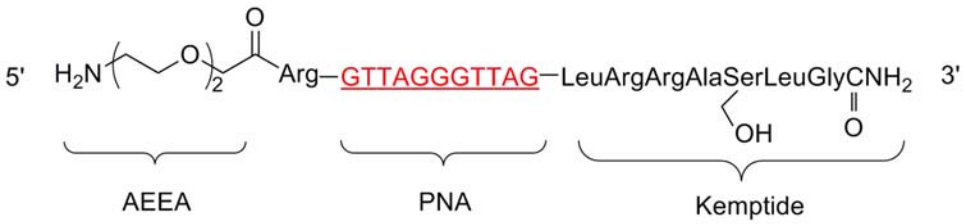
potential PNA binding sites. The Corey group synthesized a series of PNA oligomers to bind progressively to different sites on the RNA template within telomerase.[94] Peptide nucleic acid (PNA) can inhibit telomerase activity with an IC_{50} ranges from 30 uM to 0.001 uM depending on the length of PNA and its binding site. A PNA (5'-GTTAGGGTTAG-3') which binds to nucleotide 45-65 (5'-CUAACCCUAAC) was found to inhibit telomerase activity with IC_{50} = 0.01 uM.[94] We decided to use the 11 bases PNA sequence to selectively deliver QMP to RNA in telomerase because 11 bases PNA is easy to prepare and renders strong binding to RNA in telomerase. We expect QMP to alkylate RNA in telomerase and inhibit the activity of telomerase.

In this chapter we synthesized and characterized a PNA1-QMP1 conjugate and first studied the delivery of QMP1 to DNA which represents the 45-65 bases RNA template in telomerase. After successful recognition of the correct sequence by PNA and alkylation of the sequence by QMP, we continued to examine the delivery of QMP to target RNA in telomerase by PNA. Inhibition of telomerase activity was also studied by telomeric repeat amplication protocol (TRAP assay).

2.2 Result and Discussion

2.2.1 Preparation of PNA1

PNA1 (5'-AEEA-Arg-GTTAGGGTTAG-LeuArgArgAlaSerLeuGly-3'**)** is composed of a 11 base PNA sequence (red), a 7-mer peptide (kemptide) at the 3' end of the PNA and an extra arginine and 8-amino-3,6-dioxaoctanoic acid (AEEA) at the 5' end of the PNA (**Scheme 2.2**).



Scheme 2.2 Structure of PNA1

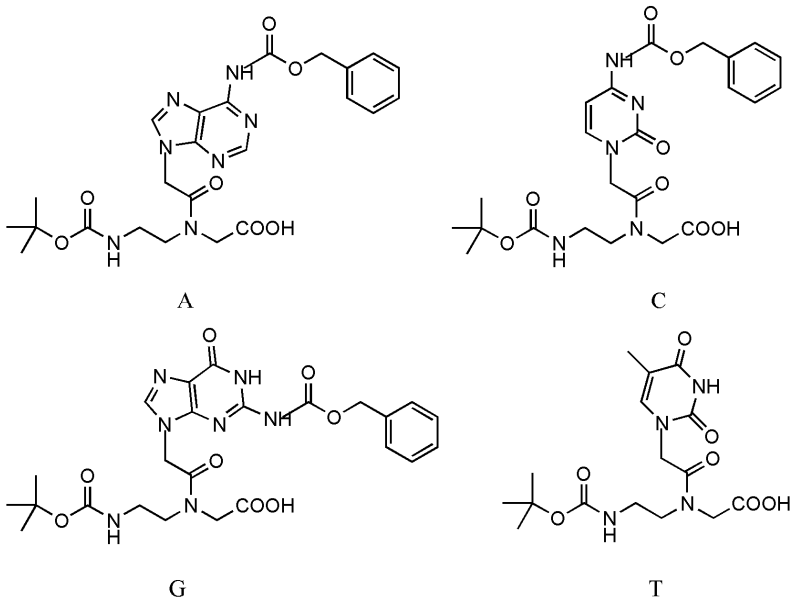
There are several reasons to design the PNA1 sequence:

a) Kemptide[96] is readily phosphorylated by protein kinase A (PKA) at the Ser. Thus the 5'-LRRASLG-3' sequence in PNA1 can function as a phosphorylation site in place of the standard 5'-phosphorylation of DNA in cell environment where the labeling of DNA sequence is difficult.

b) We found that the free amine from arginine at the 5' terminus of PNA sequence can not react with QMP1-succinimidyl (NHS) ester and generate PNA-QMP1 conjugate.[97] The amine group at the 5' terminus of the PNA and QMP1 may be too close to the peptide and PNA backbone and be hindered by the possible self folding of PNA. One AEEA[98] attached at the 5' terminus of the conjugate elongates the linker containing the free amine group and facilitates the conjugate of PNA1 with QMP1.

c) The use of PNA in vivo is hampered by its poor water solubility. Arginine and AEEA improve the solubility of PNA in water.

PNA1 was prepared by solid phase peptide synthesis (SPPS) following previous reported protocols.[78, 97, 99] Benzyloxy-carbonyl (Z) protected PNA monomers which are commercially available were used in SPPS through the *t*-butyloxycarbonyl (*t*-Boc) protocol (**Scheme 2.3**).

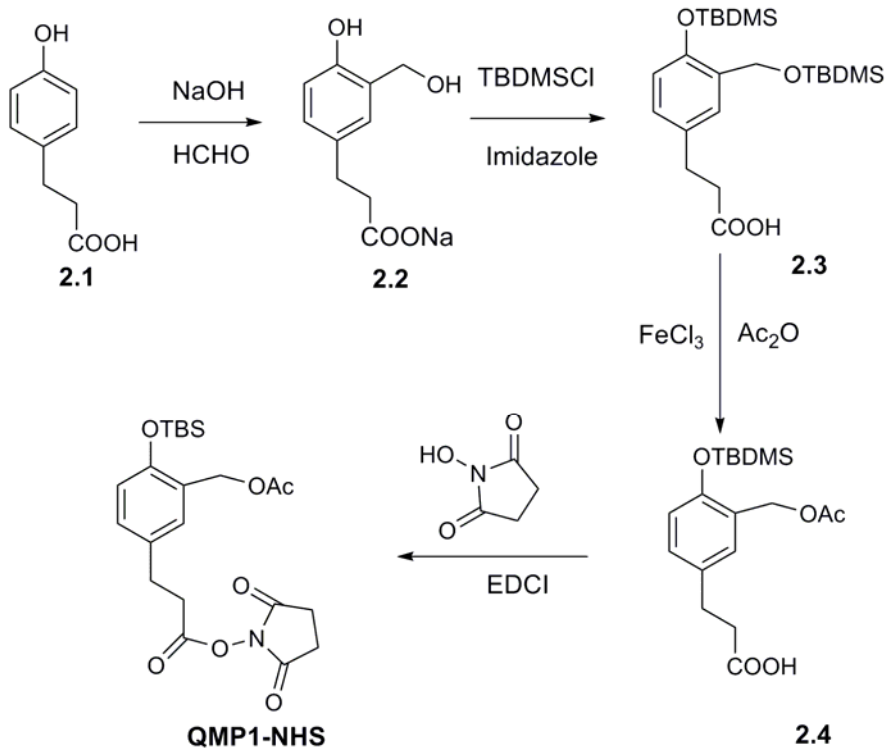


Scheme 2.3 Z-protected PNA- A, T, G and C monomers

The carboxylic acid of a monomer was activated by *O*-(7-aza-benzotriazol-1-yl) -1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and was coupled to the free amine groups from the 4-methylbenzhydrylamine (MBHA) resin. After 15 – 20 minutes of coupling, the non-reacted starting material and by products were washed away by dichloromethane while the added monomer was retained on resin. After washing, the *t*-boc protecting group of the monomer on resin was removed by trifluoroacetic acid (TFA). Another washing step with dichloromethane was performed to remove excess of TFA and its byproducts. The free amine group from the first monomer on resin was ready to react with each successive PNA monomers. By repeating the coupling, deprotecting and washing procedures, the desired peptide and PNA chain was formed on the solid resin. Finally, the PNA1 chain was removed from the resin by treatment with trifluoromethanesulfonic acid (TFMSA). The resulting PNA1 was precipitated by cold ethyl ether, further purified by reverse phase (RP)-HPLC and characterized by mass spectrometry.

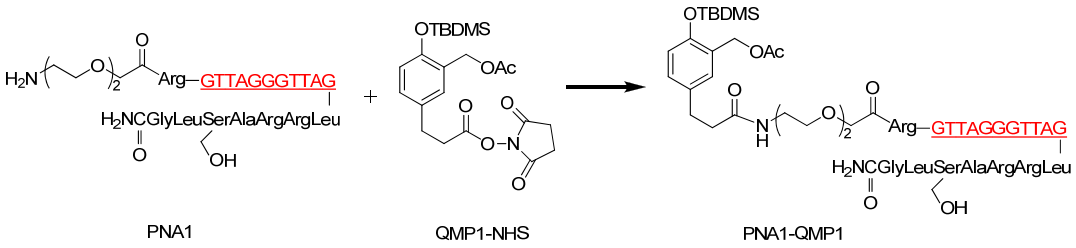
2.2.2 Preparation of PNA1-QMP1 and PNA1-QM1 self-adduct

QMP1-succinimidyl (NHS) ester was synthesized following procedures reported before (**Scheme 2.4**).[37, 97] An aqueous solution of the starting material **2.1** was mixed with 30% HCHO and 10% NaOH, and the final pH was adjusted to 8 by NaOH to generated **2.2**. Aqueous solution containing crude **2.2** was adjusted to pH 2 - 3 by 1M HCl and **2.2** was extracted by ethyl ether. Then the corresponding acid was purified by chromatotron. The hydroxyl groups of **2.2** were then protected by *tert*-butyldimethylsilyl chloride (TBDMSCl) and imidazole. After Lewis acid-catalyzed replacement of the benzylic TBDMS group with acetate, the product **2.4** was coupled with N-hydroxysuccinimidyl (NHS). The final product QMP1-NHS was purified by column chromatography and stored at -20°C. Previously, the QMP1-NHS ester was thought to hydrolyze slowly to its carboxylic acid after a couple of months at -20°C. Interestingly, ^{1}H NMR shows that even after storage at -20°C for 18 months, more than 90% QMP1-succinimydyl ester remained intact.



Scheme 2.4 Synthesis of QMP1-NHS ester

Conjugation of QMP1-NHS ester with PNA1 was achieved as reported previously.[37, 97] The mixture was purified by C18 RP-HPLC (**Scheme 2.5**, **Figure 2.1**). The retention time shifted from 15 minutes (PNA1) to 28 minutes (PNA1-QMP1). This is mainly caused from the hydrophobic TBDMS group. The eluent at 28 minutes was collected and confirmed by MALDI as PNA1-QMP1. PNA1 calculated (*m/z*) was 4144.6, MALDI (*m/z*) found was 4143.3. When HPLC purified PNA1-QMP1 was stored at -20°C for eight months, HPLC showed that more than 80% of PNA1-QMP1 remained intact (**Figure 2.2**, A).



Scheme 2.5 Generation of PNA1-QMP1

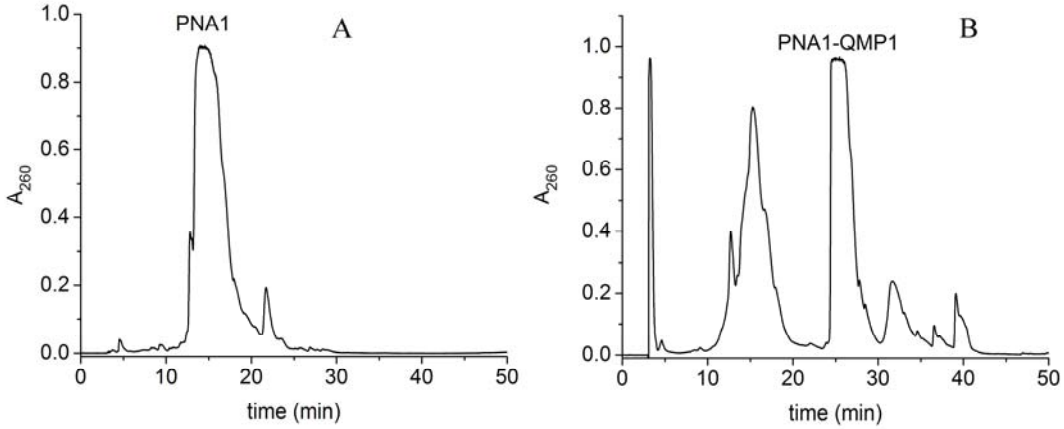
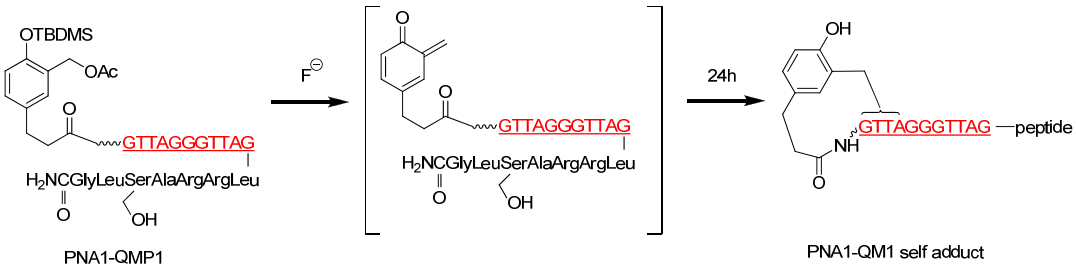


Figure 2.1 RP-HPLC purification of crude PNA1 (A) and crude PNA1-QMP1 (B) linear gradient of 10% to 55% aqueous acetonitrile with 0.1% trifluoroacetic acid (TFA) at 1 ml/min over 30 minutes was used to elute the materials.

The PNA1-QM1 self adduct was prepared by incubating HPLC purified PNA1-QMP1 with potassium fluoride. The PNA1-QM self adduct was purified by RP-HPLC (**Scheme 2.6**, **Figure 2.2**, B). The mass of self adduct was confirmed by MALDI. PNA1-QM1 self adduct calculated (*m/z*) was 4305.8 and MALDI (*m/z*) found was 4304.7.



Scheme 2.6 Generation of PNA1-QM self adduct

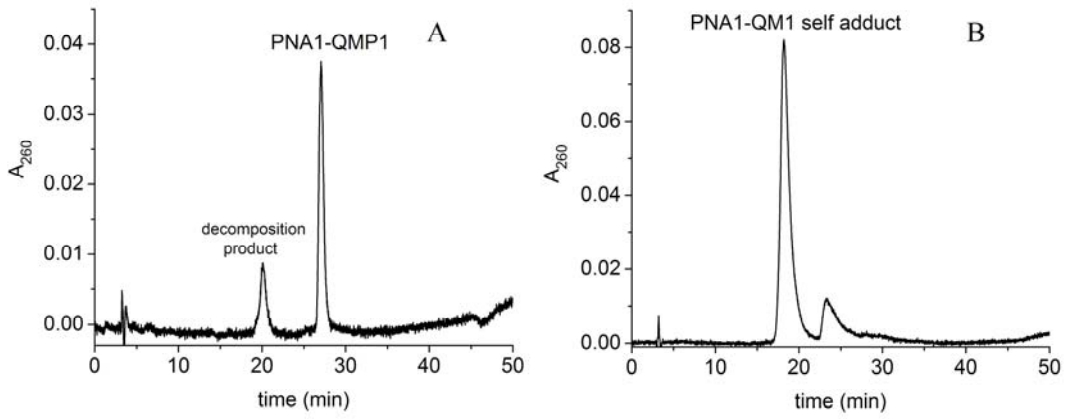
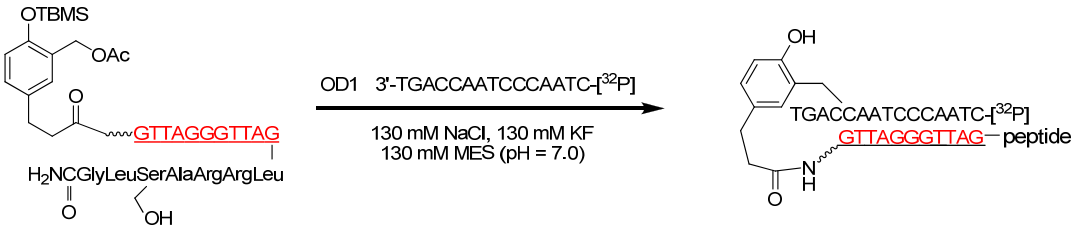


Figure 2.2 RP-HPLC of PNA1-QMP1 and its self adduct (A) PNA1-QMP1 was stored at -20°C for eight months. The decomposition product is PNA1-QMP1 loss of its –TBDMS protecting group; (B) fresh PNA1-QM self-adduct. A linear gradient of 10% to 55% aqueous acetonitrile with 0.1% trifluoroacetic acid (TFA) at 1 ml/min over 30 minutes

2.2.3 Alkylation of ssDNA by PNA1-QMP1

After PNA1-QMP1 was successfully generated, alkylation of ssDNA by PNA1-QMP1 was performed to study whether PNA1 can deliver QMP1 to single strand target sequence. A complementary [^{32}P]-OD1 and 1.1 equivalent of PNA1-QMP1 was incubated in the presence of fluoride at room temperature and then characterized by 20% denaturing gel electrophoresis (**Scheme 2.7**). If OD1 is alkylated by PNA1-QMP1, PNA1-QM1-[^{32}P]-OD1 could be denatured but will be still covalently linked and have a slower electrophoretic mobility than [^{32}P]-OD1. The alkylation yield can be calculated by quantifying the radio-activity of PNA1-QM1-[^{32}P]-OD1 with the sum of the total radio-activity of DNA adduct and unreacted ssDNA on gel.



Scheme 2.7 Alkylation of DNA by PNA1-QMP1

A slowly migrating species was found on denaturing gel electrophoresis which is consistent with interstrand alkylation (**Figure 2.3**). After a 48 hour incubation at room temperature, the PNA1-QM1-[^{32}P]-OD1 product accumulated in a first order process to yield almost 60%. The rate and yield are equal to that reported previously using a DNA-based delivery agent.[37] Less than 2.0% of alkylation was discovered by gel when a non-complementary DNA (OD5, 5'-CGACTGCAGACT-3') was incubated with PNA1-QMP1 (**Table 2.2**, **Figure 2.7**, lane 2). Thus PNA1 can deliver QMP1 to its target sequence selectively. Without the formation of complementary PNA/DNA duplex, PNA1-QMP1 can't alkylate DNA.

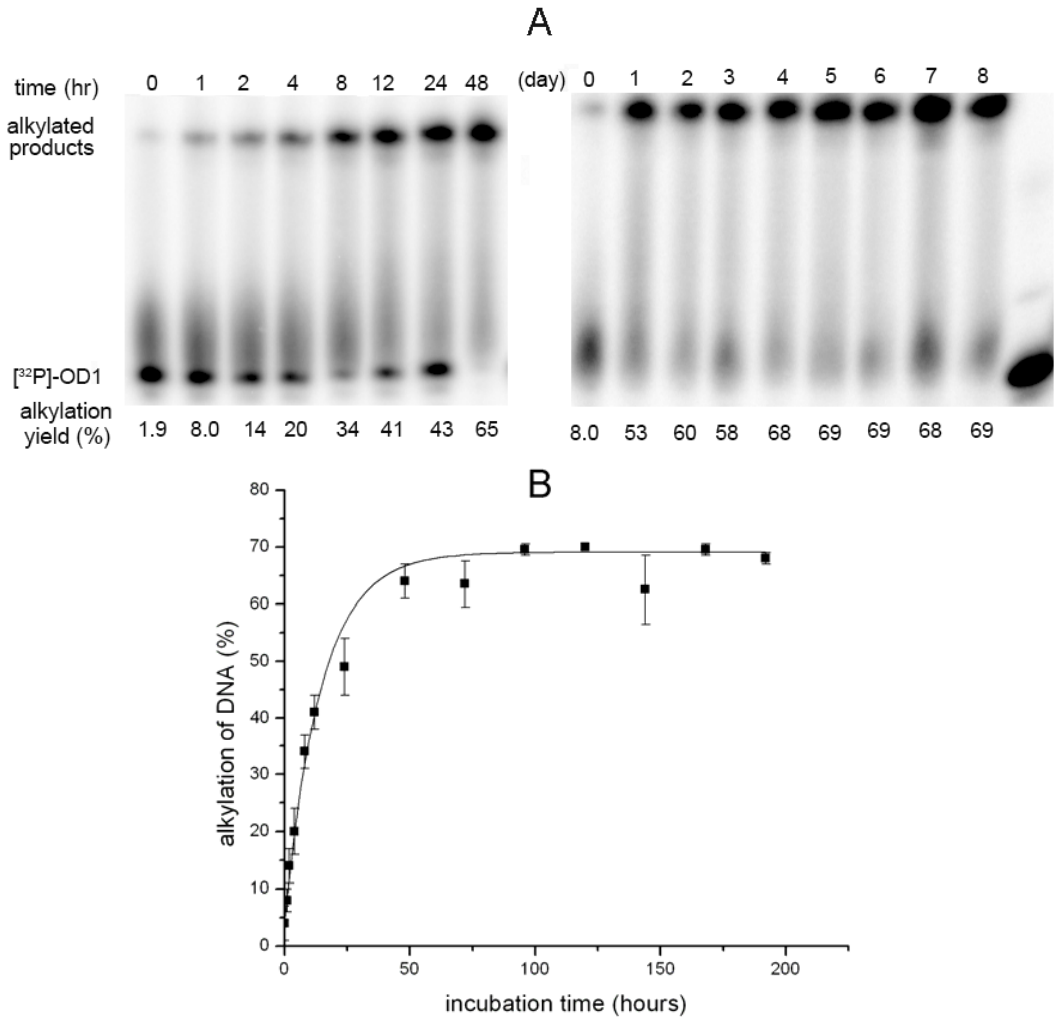


Figure 2.3 DNA alkylation by PNA1-QMP1 conjugate. (A) Gel electrophoresis of DNA alkylation products. [^{32}P]-OD1 (3.0 μM) was mixed with PNA1-QMP1 (3.3 μM) in solution of NaCl (130 mM), MES (130 mM, pH 7.0) and KF (130 mM) for 0 – 8 days. The products were characterized by 20% polyacrylamide denaturing gel. (B) Alkylation of [^{32}P]-OD1 by PNA1-QMP1 was quantified and fit to a first order process, $t_{1/2}$ = 30 h. The error represents the range of two independent determinations.

2.2.4 Alkylation of DNA by radio labeled PNA1-QMP1

The ultimate goal of this project is to deploy the PNA1-QM1 self adduct into cell environments. We decided to radio label the PNA1 sequence and [^{32}P]-PNA1 can function as a marker to help to trace the exact reaction spot in the future *in vitro* and *in vivo* research. For our target delivery of QMP1 by PNA1 project, [^{32}P]-PNA1-QM1-OD1 should have the same electrophoretic mobility as PNA1-QM1-[^{32}P]-OD1. This can confirm that species moves slower than [^{32}P]-OD1 on gel electrophoresis are the DNA adducts we expected.

Radio-labeling of PNA1-QMP1 by cAMP-dependent protein kinase A (PKA) and [^{32}P]-adenosine-5'-triphosphate (ATP) followed a published procedure.[100] A C18 Sep-Pak cartridge was used to purify [^{32}P]-PNA1-QMP1 by removing buffer and ATP.

Alkylation of target ssDNA was evaluated by incubating complementary OD1 with [^{32}P]-PNA1-QMP1. The highest band on gel electrophoresis is labeled [^{32}P]-PNA1-QMP1 conjugate since it carries only one charge and barely moves. Products of the same electrophoretic mobility were appeared in the middle of gel regardless of which oligomer contained the label. [^{32}P]-labeled PNA1-QMP1 showed a similar alkylation yield (63% *vs* 51%) and the alkylation rate of target DNA sequence by [^{32}P]-PNA1-QMP1 was comparable to alkylation of [^{32}P]-DNA by PNA1-QMP1 (**Figure 2.4**).

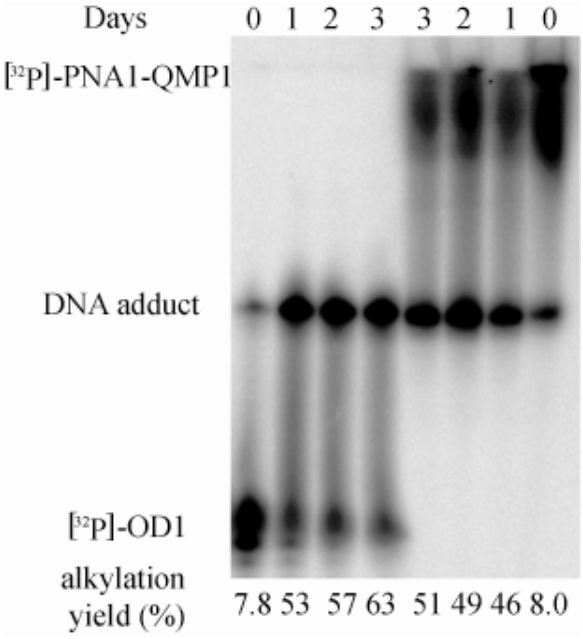


Figure 2.4 Alkylation of ssDNA by PNA1-QMP1. Alternative [^{32}P]-labeling of the target OD1 and PNA1-QMP1 generates the same product with an intermediate electrophoretic mobility. PNA1-QMP1 (3.3 μM) was incubated with OD1 (3.0μM) in solution of NaCl (130 mM), MES (130 mM, pH 7.0) and KF (130 mM) for 0 – 3 days at room temperature.

2.2.5 Discrimination of a single base mismatch in DNA alkylation

The ultimate utility of the quinone methide conjugate relies on its ability to discriminate between target and off-target sequences. A related DNA-QM conjugate had previously showed target alkylation of its complementary DNA, but the response to a single mismatch between a conjugate and its target had not been examined. To measure the extent of this selectivity, two DNA targets which have one base mismatch (OD2, OD3) were prepared (**Table 2.1**). OD2 has a T/T mismatch at the 5' end of PNA1 and OD3 has a G/T mismatch in the middle of PNA1.

PNA1-QMP1	3'-GLSARRL-GATTGGGATTG-Arg-AEEA-QMP1-5'
OD1	5'-CTAACCCTAACCAGT-3'
OD2	5'-CTAACCCTA**T**CCAGT-3'
OD3	5'-CTAAC**T**CTAACCAGT-3'

Table 2.1 Sequences of PNA1-QMP1 conjugate, PNA complementary DNA and DNAs with one-base mismatch. The mismatched base is underlined.

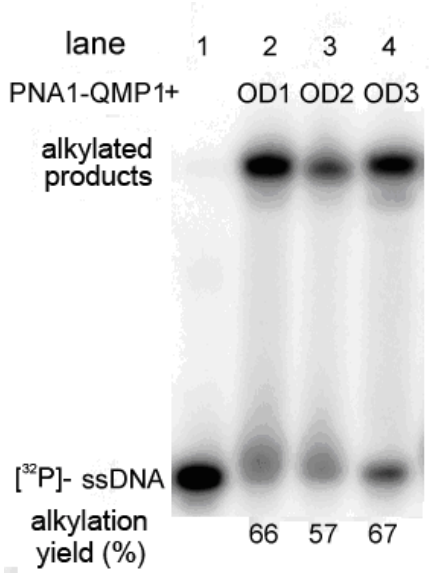


Figure 2.5 Alkylation of OD1,OD2 and OD3 by PNA1-QMP1. Lane 1: control. ssDNA (lane 2, [^{32}P]-OD1 (3.0 μM); lane 3, [^{32}P]-OD2 (3.0 μM); lane 4, [^{32}P]-OD3 (3.0 μM)) was mixed with PNA1-QMP1 (3.3 μM) in buffer containing NaCl (130 mM), MES (130 mM, pH 7.0) and KF (130 mM) for 2 days

The PNA1-QMP1 conjugate was still fully capable of alkylating OD2 or OD3 in the absence of OD1 as expected since association between OD2 or OD3 and PNA1-QMP1 is thermodynamically favored over the alternative of single-stranded structures (**Figure 2.5**). OD3 and complementary OD1 showed equivalent alkylation yield by PNA1-QMP1 while OD2's alkylation yield is slightly lower than OD1. Although a single mismatch in the middle of OD3 is expected to decrease the melting point of PNA1/OD3, the PNA1/OD3 duplex can still form and deliver QMP1 close to nucleophiles from DNA. When the mismatch occurred at the end of

PNA1/OD2 duplex, QMP1 had higher chances to rotate freely in the solution than tightly binding PNA1/OD3 and thus had lower alkylation yield.

To further address the effect of single base mismatch on target sequence alkylation, we incubated different ratios of OD1 and OD2 with PNA1-QMP1 and analyzed the target alkylation yield (**Figure 2.6**). Alkylation of OD2 decreased by 70% after addition of one equivalent of OD1 and was essentially blocked after addition of 5 equivalents of OD1. Conversely, one equivalent of OD2 suppressed alkylation of OD1 by only 16%, and even 10 equivalent of OD2 only suppressed alkylation of OD1 by half.

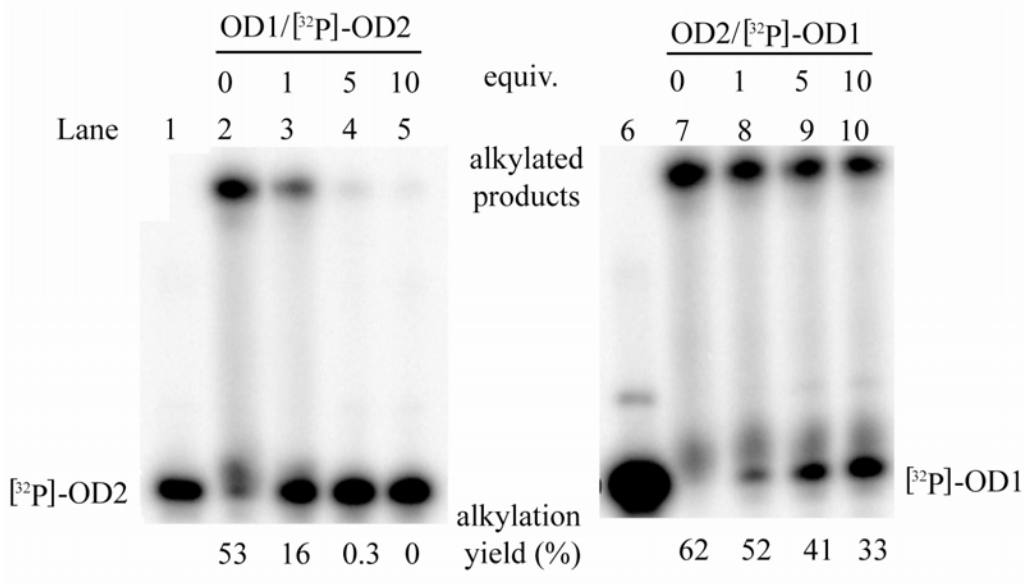


Figure 2.6 Competition for alkylation by PNA1-QMP1 targets containing no mismatches (OD1) and a single mismatch (OD2). Selectivity for [^{32}P]-OD2 was measured in the presence of 0 – 10 equivalents of OD1 and 1.2 equivalents of PNA1-QMP1. Conversely, the selectivity for [^{32}P]-OD1 was measured in the presence of 0 – 10 equivalents of OD2 and 1.2 equivalents of PNA1-QMP1. Reaction mixtures were incubated for 192 h in the presence of NaCl (130 mM), MES (130 mM, pH 7.0) and KF (130 mM) under ambient conditions

Both the location and type of mismatches formed between the conjugate and its target variants 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. The lone T/T mismatch limited alkylation of OD2 to 25% of the total reaction of OD1 and OD2 at equimolar concentrations (**Figure 2.6**, lane 3 *vs.* lane 2). This level of discrimination is likely higher than that expected from a DNA conjugate since mismatches appear to be more destabilizing in PNA/DNA than DNA/DNA duplex.[75]

2.2.6 Template-dependent alkylation of a non-complementary target

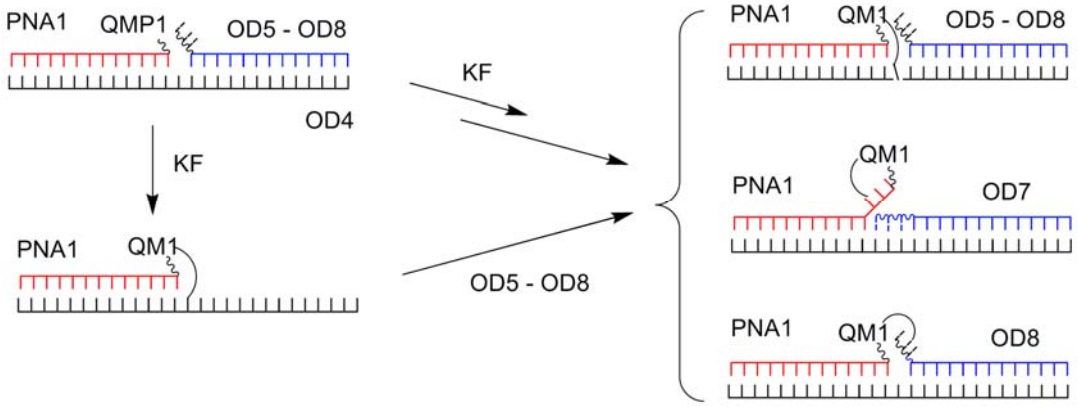
Alkylation of a non-complementary nucleotide sequence by the QMP1 conjugate will expand the utility of PNA1-QMP1 conjugates. Previously, it was shown that two ssDNA can be assembled via bis-PNA template.[101] Because unrelated sequences can be co-localized and oriented for reaction by hybridization to a template strand, alkylation of a non-complementary target sequence can be achieved with a template strand (**Scheme 2.8**).

Template OD4 is complementary to PNA1 and has extra 13 free bases which serve as the binding site of the third strand of DNA. A series of DNA sequences (OD5 – OD8) that differ in only their 3'-termini can bind to OD4 by Watson-Crick pairing. OD5 – OD8 are non-complementary to PNA1 and serve as PNA1-QMP1 alkylation targets. OD5 – OD8 were co-localized with PNA1-QMP1 since both DNA and PNA were bound to OD4. We used PNA1-QMP1, the template strand

OD4 and OD5 – OD8 to identify the requirement for alkylation of OD5 – OD8 (**Table 2.2**).

PNA1-QMP1	3'-GLSARRL -GATTGGGATTG-Arg-AEEA-QMP1-5’
OD4	5'-CTAACCCTAAC CAGTCTGCAGTCG-3'
OD5	3'-TCAGACGTCAGC-5'
OD6	3'-GTCAGACGTCAGC-5'
OD7	3'-TTGGTCAGACGTCAGC-5'
OD8	3'-GCTGTCAGACGTCAGC-5'

Table 2.2: Sequences of PNA1-QMP1 conjugate and DNA sequences



Scheme 2.8 Template directed alkylation of a non-complementary target

In the absence of its targets, PNA1-QMP1 merely alkylated the template strand OD4 (**Figure 2.7**, lane 1). When there is no template sequence OD4, no interstrand alkylation of any non-complementary sequences OD5 – OD8 occurred (**Figure 2.7**, lane 3 & 4; **Figure 2.8**, lane 3 & 6). In the presence of the template OD4, only about 1% of alkylation was observed between PNA1-QMP1 and a target (OD5) that aligned one residue away from the conjugate. Less than 2% alkylation was observed for another target (OD6) that bound directly adjacent to PNA1-QMP1 (**Figure 2.7**, lane 5 & 6). Extending a target strand by three nucleotides (OD7) to overlap and compete with PNA1-QMP1 for association with the template similarly limited the yield of

alkylation (2 - 4%, **Figure 2.8**, lanes 1 and 2). Only when a target (OD8) contained a non-complementary 3 bases extension that remained free to react with the transient QM of PNA1-QMP1 can generated ~ 40% DNA alkylation (**Figure 2.8**, lane 5).

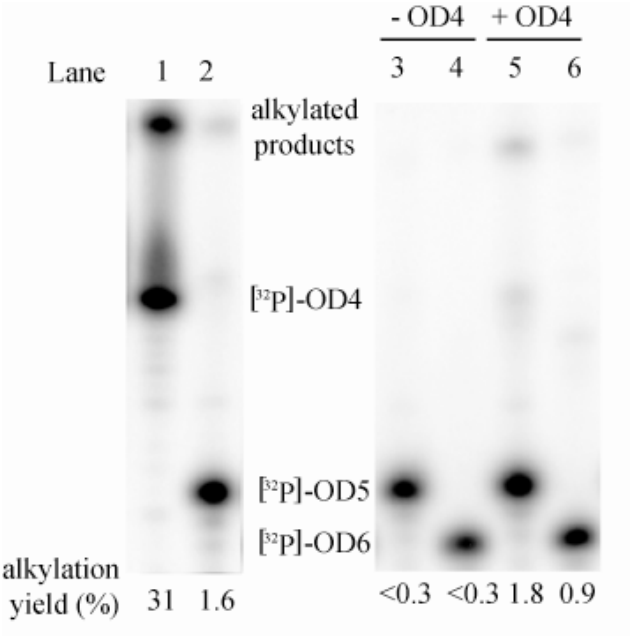


Figure 2.7 Alkylation of OD4, OD5 and OD6 by PNA1-QMP1. [^{32}P]-OD4 (3.0 μM, lane 1) and [^{32}P]-OD5 (3.0 μM, lane 2) were incubated with PNA1-QMP1 (3.3 μM) in NaCl (130 mM), MES (130 mM, pH 7.0) and KF (130 mM) for 5 days under ambient conditions before analysis. [^{32}P]-OD5 (3.0 μM, lane 3) and [^{32}P]-OD6 (3.0 μM, lane 4) were incubated with PNA1-QMP1 (3.3 μM) under equivalent conditions for 2 days prior to analysis. [^{32}P]-OD5 (3.0 μM, lane 4) and [^{32}P]-OD6 (3.0 μM, lane 6) were incubated with PNA1-QMP1 (3.3 μM) under equivalent conditions for 2 days prior to analysis.

The reversibility of QM reaction was also studied by first generating the PNA1-QM1-OD4 adduct. Alkylation of OD4 alone by PNA1-QMP1 produced a yield of ~31% (**Figure 2.7**, lane 1). This PNA1-QM1-OD4 adduct then served as a reservoir of QM since QM transfer was still observed after either target OD7 and OD8 was added 192 h subsequent to incubation of PNA1-QMP1, OD4 and fluoride (**Figure 2.7**, lanes 1 and 4; **Scheme 2.8**). 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 OD7 versus OD8 remained unchanged. The decrease in alkylation yield after pre-generation PNA1-QM1-OD4 reflects the regeneration of QM and the weakly competing reactions of water and weak nucleophiles of DNA. Whether target reaction derives from the initial generation of QM or from subsequent regeneration by reversible DNA adducts, a 3 bases single-stranded and non-complementary region adjacent to the PNA conjugate gave maximum yields of target alkylation.[102]

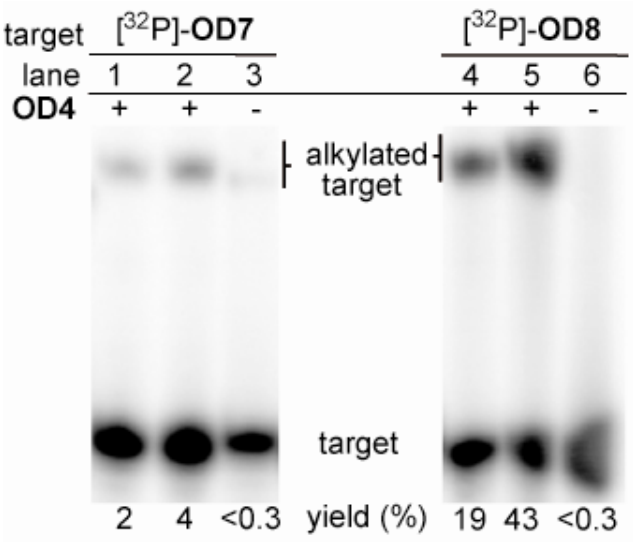


Figure 2.8 Template-dependent alkylation of target DNA. PNA1-QMP1 (3.3 μM) and the template OD4 (3.0 μM) were incubated for 192 h under ambient conditions in MES (130 mM pH 7) and NaCl (130 mM) after reaction was initiated by addition of KF (130 mM). OD7 (lane 1) and OD8 (lane 4) were then added to this mixture and incubated for another 192 h prior to electrophoretic analysis. Alternatively, PNA1-QMP1, OD4 and either OD7 (lane 2) or OD8 (lane 5) were mixed together under equivalent conditions prior to addition of KF and incubation for 192 h. PNA1-QMP1 and either OD7 (lane 3) or OD8 (lane 6) were also mixed together in the absence of the template OD4 under equivalent conditions prior to addition of KF and incubation for 192 h.[102]

2.2.7 Alkylation of ssDNA by a PNA-QM self-adduct

Alkylation of a target DNA sequence by an oligo-QM self adduct avoids the requirement of toxic fluoride to initiate the reaction. Thus the self adduct could be used in vivo without need of an additional trigger. Previously, the Rokita group showed that both a DNA-QM self adduct[37] and a pyrrole-imidazole-QM self adduct[49] can be generated, but only DNA-QM self adduct is reversible and can continue to alkylate a target sequence. It is useful to study whether PNA1-QM1 self adduct can be generated and alkylate target ssDNA.

We used RP-HPLC to monitor the generation of PNA1-QM1 self adduct (**Figure 2.9**) and examined the reversibility of the self adduct by its ability to alkylate a target sequence. Upon addition of fluoride, deprotection of the silyl group intermediate (DP) was generated and detected at 260 nm on HPLC (**Figure 2.9**, 0 h). After four hours, all the PNA1-QMP1 was converted to DP and the PNA1-QM self-adduct. Twelve hours later, only the PNA1-QM1 self adduct was observed. All of these intermediates and final self adducts were collected, lyophilized and successfully characterized by MALDI. The formation of self adduct proceeded at a rate similar to that observed previously for the generation of DNA-QM self adduct.[37] This shows that the kinetics of oligo-QM self adduct generation is only affected by the nucleophiles. At least for PNA and DNA, the backbone does not affect the generation of self adducts.

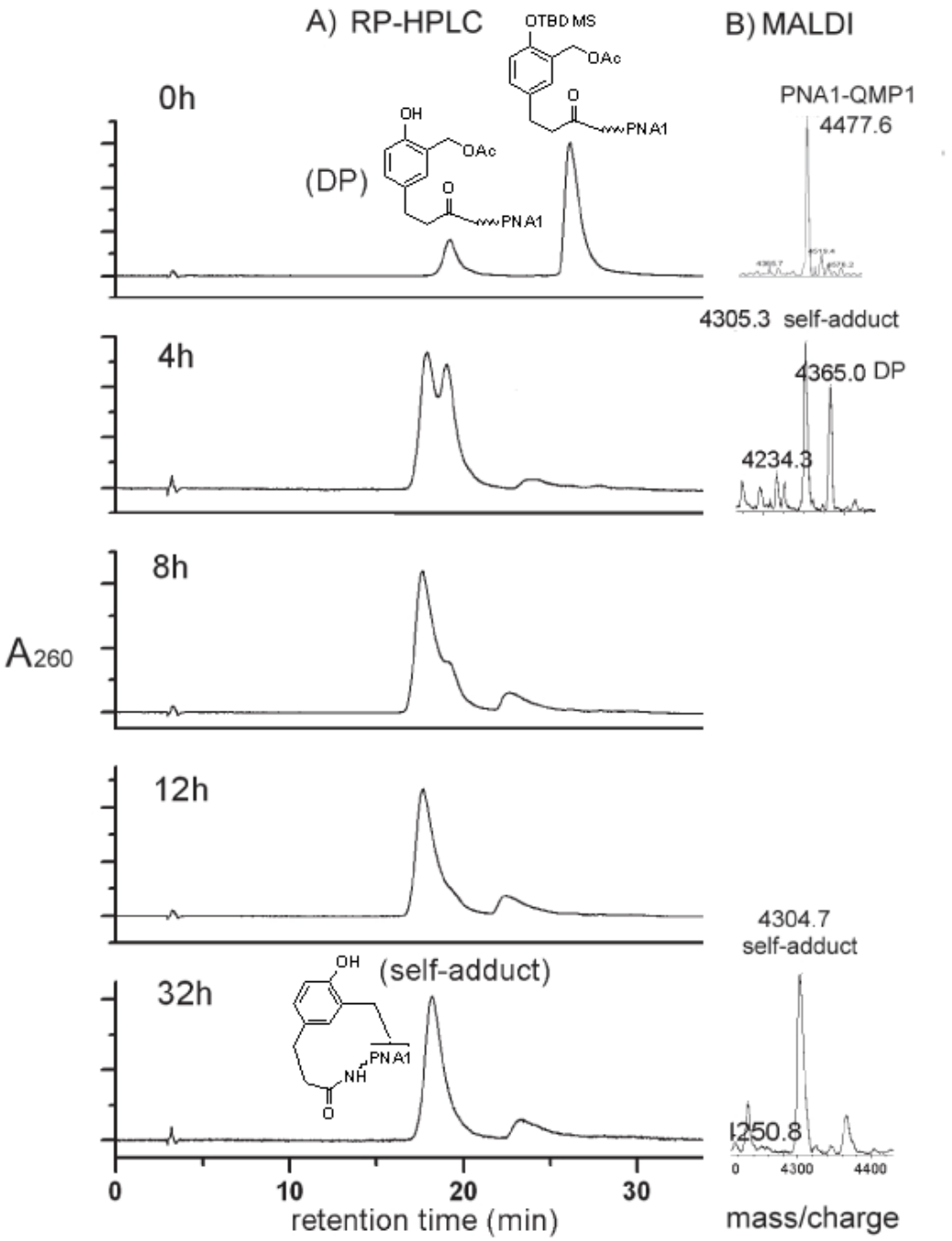
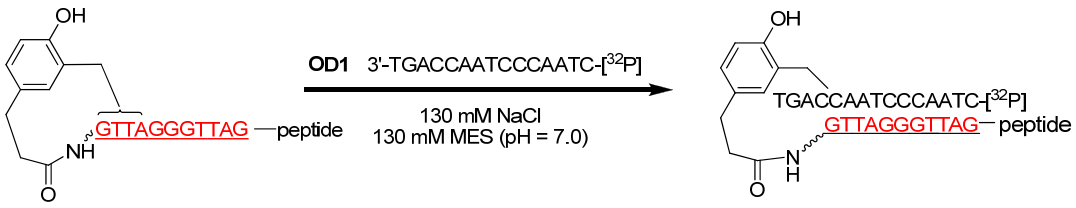


Figure 2.9 Generation of the PNA1-QM1 self adduct. A) PNA1-QMP1 was mixed with KF (130 mM) in a solution of NaCl (130 mM), MES (130 mM, pH 7.0). Mixtures were analyzed by RP-HPLC after 0 – 32 hours. B) Materials from different peaks (0 h: 25 – 27 min was PNA1-QMP1; 4 h, 8 h: 18 – 21min was DP and PNA1-QM1 self adduct; 32 h: 18 – 20 min was PNA1-QM1 self adduct)from RP-HPLC was collected, lyophilized and characterized by MALDI.

After the generation of PNA1-QM1 self adduct, the delivery of a QM1 to target target DNA by PNA1 was examined by incubating the self adduct (1.1 equiv.) generated *in situ* with [^{32}P]-OD1 in buffer at room temperature. After various time, a high molecular weight species corresponding to PNA1-QM1-DNA adducts was found on 20% denaturing gel electrophoresis (**Scheme 2.9, Figure 2.10**).

Although the interstrand cross-linking induced by the self adduct is slower than that induced by PNA1-QMP1 ($t_{1/2}$ = 30 h *vs.* $t_{1/2}$ = 10 h), no chemical signal is needed to initiate the alkylation, and this will allow the self adduct to be used in vivo in the future. DNA alkylation by the PNA1-QM self adduct was around 40% after 4 days whereas equivalent of PNA1-QMP1 generated > 60% alkylation yield. The difference of alkylation rate is because QM generated from QMP1 within a duplex can participate in intra- and interstrand alkylation directly. In contrast, release of the QM from a reversible self adduct may require a structural reorganization of its complex before interstrand alkylation may occur. Meanwhile, all the QMP1 can be converted to QM to participate DNA alkylation whereas the PNA-QM self adduct contains both reversible and irreversible self adduct.



Scheme 2.9 Alkylation of OD1 by PNA1-QM1 self adduct

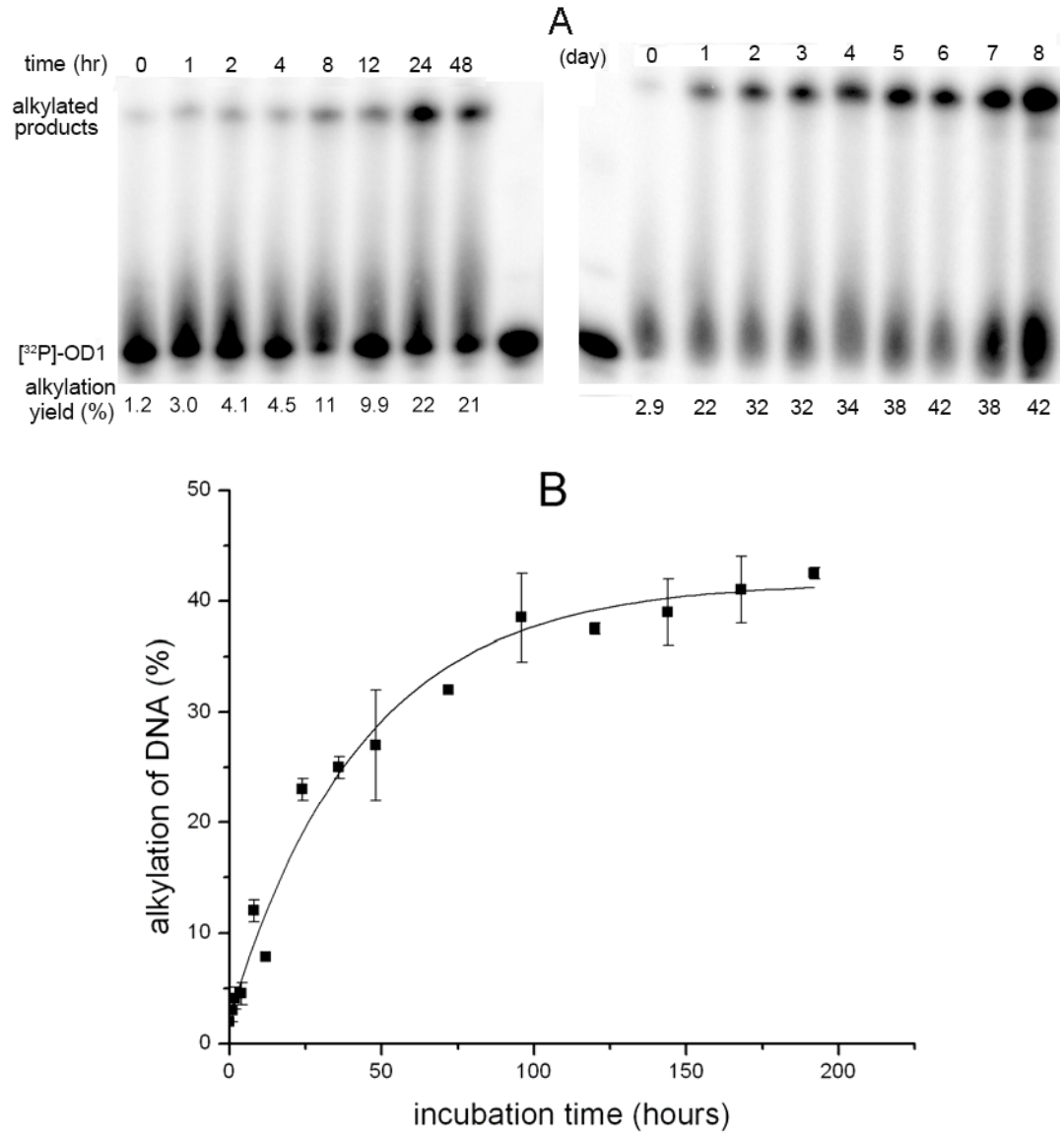


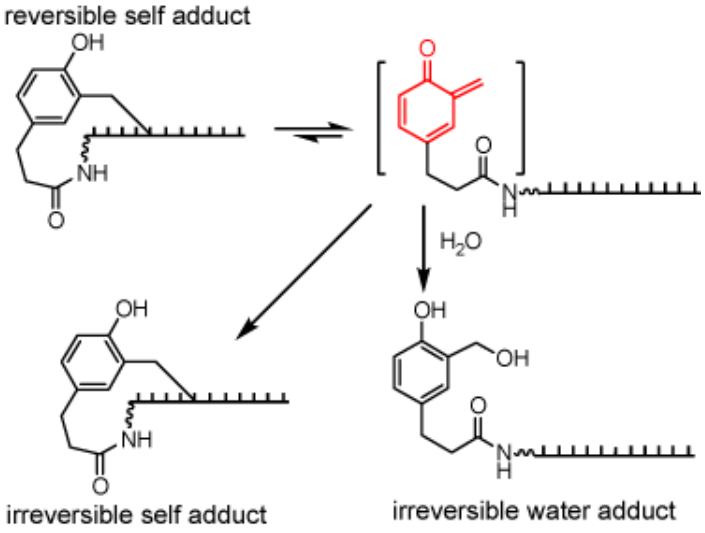
Figure 2.10 DNA alkylation by PNA1-QM1 self adduct. (A) Gel electrophoresis of DNA alkylation products. The self-adduct was prepared in situ by addition of KF (130 mM) to a solution PNA1-QMP1 (3.3 μM) in MES (130 mM pH 7) and NaCl (130 mM) and incubated under ambient conditions for 24 h. The target [^{32}P]-OD1 (3.0 μM) was then added and incubation was continued for the indicated time. The products were characterized by 20% polyacrylamide denaturing gel. (B) Alkylation of [^{32}P]-OD1 by PNA1-QM1 self adduct was quantified and fit to a first order process. The error represents the average of two independent determinations.

Previously, we showed that PNA1-QMP1 can be radio-labled by PKA and [^{32}P]-ATP. [^{32}P]-PNA1-QMP1 can continue to alkylate target sequence (**Chapter**

2.2.4, **Figure 2.4**). We then radio labeled PNA1-QM1 self adduct by PKA and [^{32}P]-ATP following the same procedure to prepare [^{32}P]-PNA1-QMP1. After [32P]-PNA1-QM1 self adduct was incubated with OD1 for 8 days, no alkylation of OD1 by [^{32}P]-PNA1-QM1 was found on gel (data not shown). We think that reversible PNA1-QM1 self adduct might be converted to irreversible products by either nucleophiles from PNA1 itself or from nucleophiles in buffer during the radio labeling procedure. Thus the irreversible [^{32}P]-PNA1-QM1 lost its ability to further alkylate target DNA.

2.2.8 Stability of PNA1-QM1 self adduct

The final yield of target alkylation is affected by the percentage of reversible self adduct since the reversible self adduct keeps regenerating QM intermediate and is quenching by weak nucleophiles to form irreversible products.[39] When PNA1-QMP1 was treated with fluoride, intrastrand alkylation of PNA1 by QM1 form reversible adducts which are dominate since strong nucleophiles react with QM1 quicker than weaker nucleophiles.. After longer time incubation, the reversible self adducts may slowly redistribute to their irreversible adducts (**Scheme 2.10**). Study the stability of PNA1-QM1 self adduct will help the understanding of self adduct storage conditions and its shelf life when potentially used as a anti-cancer drug in the future.



Scheme 2.10 Generation of a QM intermediate from the reversible PNA-QM self adduct and its irreversible trapping by water and weak nucleophiles from PNA[102]

Stability of self-adduct in aqueous solution was evaluated by measuring the ability of OD1 alkylation by the self-adduct after its pre-incubation in aqueous solution for 0 – 7 days (**Figure 2.11**). More than 50% of self-adduct's original alkylation ability was maintained (26% vs 44%) after a seven day pre-incubation of the PNA1-QM self adduct in aqueous solution at room temperature prior to addition of [^{32}P]-OD1. This means that half of the reversible self adduct was quenched after incubation without target sequences. Meanwhile, still half of the reversible self adduct was resistant to quenching and retained its ability to generate a QM intermediate and alkylate target DNA. This is consistent with previous data from our group that DNA-QM self adducts retain half of their alkylation ability after 8 days incubation in aqueous solution at room temperature.[37]

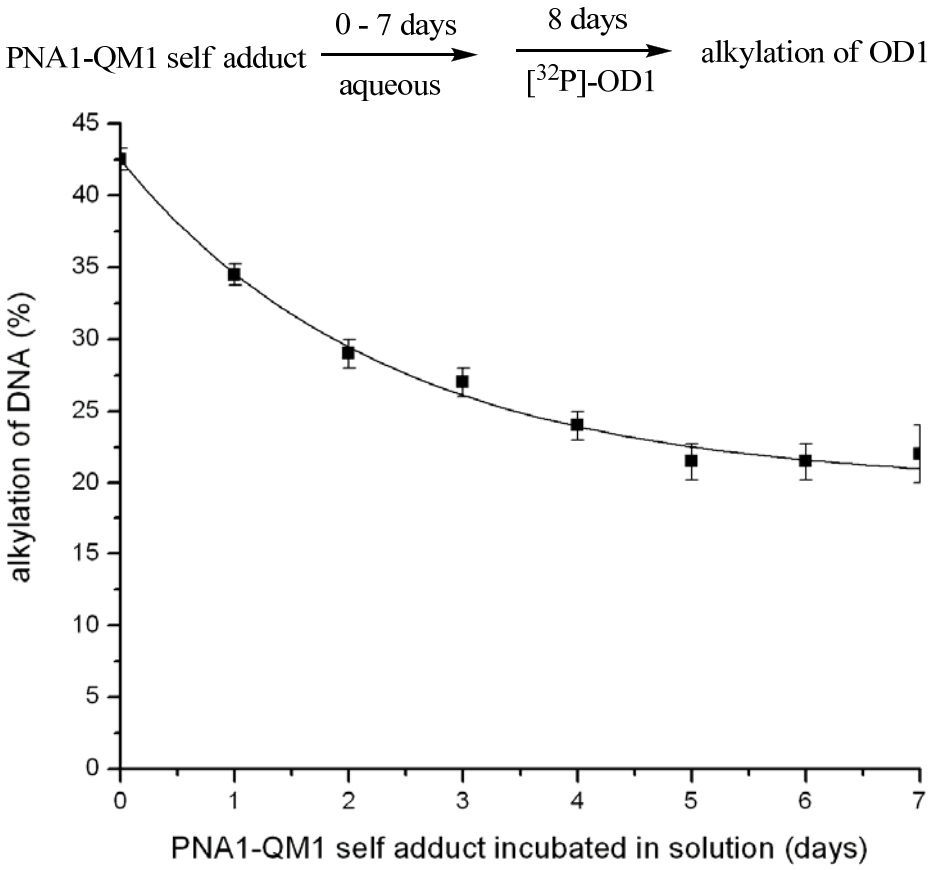


Figure 2.11 DNA alkylation by the PNA1-QM1 self adduct. The self adduct was prepared *in situ* by addition of KF (130 mM) to a solution PNA1-QMP1 (3.3 μM) in MES (130 mM pH 7) and NaCl (130 mM) and incubated under ambient conditions for 24 h. The self adduct was incubated under the same conditions for additional 0 – 7 days prior to addition of target [^{32}P]-OD1 (3.0 μM). Incubation was then continued for 8 days and the alkylated product was quantified. The average of two independent determinations was plotted and fitted to a first order process. The error represents the range of the observed yields.

Although the exact nucleophiles which quench the QM1 intermediate are unclear, we expected irreversible adduct between QM and water contributes to the loss of self adduct activity. Mass spectroscopy showed that even after eight days pre-incubation in buffer under room temperature, PNA1-QM1 self adduct remained dominant (**Figure 2.12**).

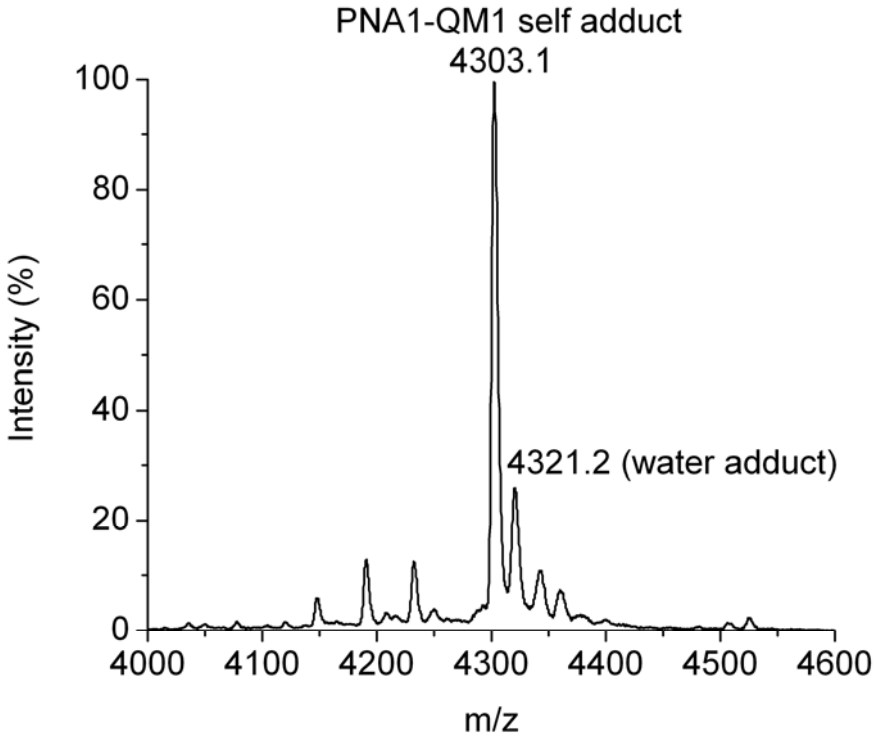
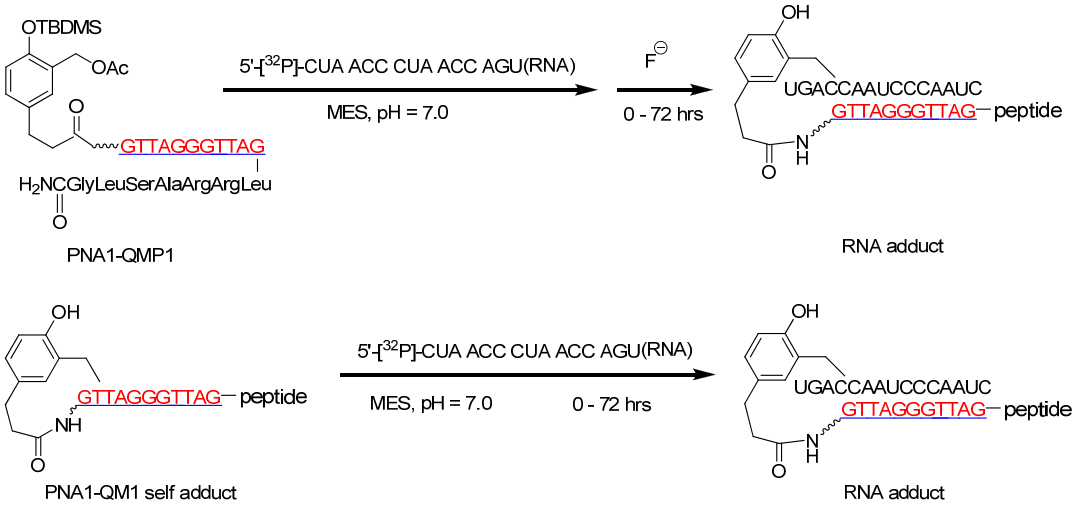


Figure 2.12 MALDI characterization of PNA1-QM1 self adduct after eight days incubation. The self adduct (5 μM) was generated by incubation of PNA1-QMP1 in solution of NaCl (130 mM), MES (130 mM, pH 7.0) and KF (130 mM) for 24 hours. The mixture was incubated at room temperature for an additional 8 days and then purified by C18 Sep-Pak cartridge, lyophilized, re-dissolved in water and characterized by MALDI. PNA1-QM1 self adduct calculated m/z: 4305.8, found: 4303.1; PNA1-QM1 water adduct calculated m/z: 4323.8, found: 4321.2

2.2.9 Alkylation of ssRNA by PNA1-QMP1 and PNA1-QM1 self adduct

Our ultimate goal is to utilize the PNA1-QM1 self adduct to induce target RNA alkylation in telomerase and inhibit the function of telomerase. Previously, Corey group found that a 11 base PNA (5'-GTTAGGGTTAG-3') can effectively bind to RNA in telomerase.[94] We studied the target alkylation of ssRNA *in vitro* before studying the alkylation of RNA in telomerase by self adduct (**Scheme 2.11**).



Scheme 2.11 Alkylation of ssRNA by PNA1-QMP1 and PNA1-QM1 self adduct.

Unfortunately, the PNA/RNA duplexes could not be denatured by gel electrophoresis under the conditions used to denature PNA/DNA duplex. To differentiate covalently linked PNA1-QM1-RNA adduct from PNA1/RNA duplex, the duplex need to be denatured. More formamide was added to help the denaturing since formamide can stabilize ssRNA in solution and electrophoresis. Previously, 3 µL PNA/DNA sample was mixed with 3 µL formamide before loading onto gel. In the revised procedure, 3 µL PNA/RNA sample was mixed with 7 µL formamide before loading onto gel. After loading, gel was running at 50 - 55°C instead of 40 - 45°C to fully denature PNA/RNA duplex (**Figure 2.13**).

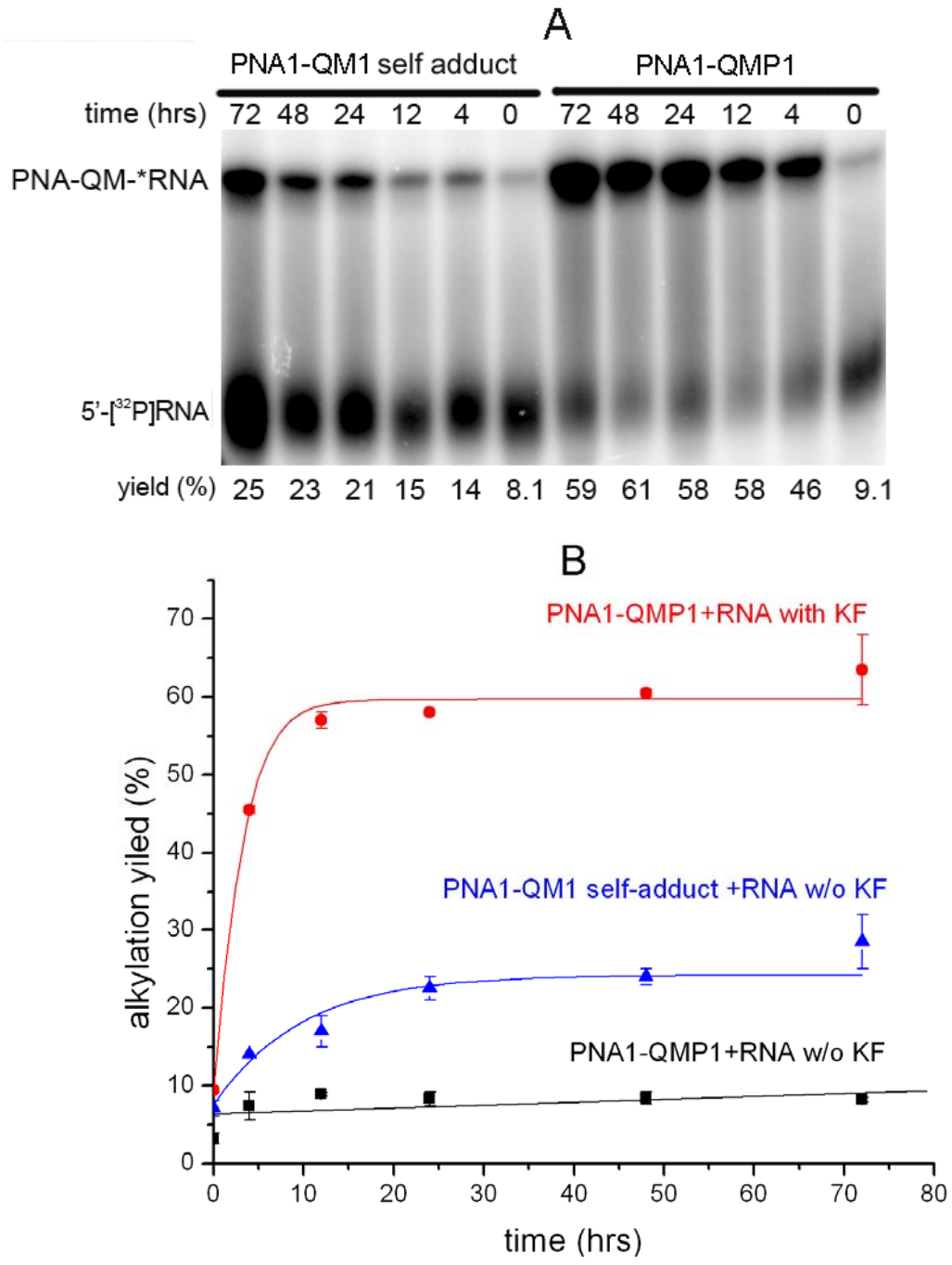


Figure 2.13 (A) Gel electrophoresis of RNA alkylation by PNA1-QM1 self adduct and PNA1-QMP1. [^{32}P]-RNA (3.0 μM) was mixed with PNA1-QM1 self adduct or PNA1-QMP1 (3.3 μM) in solution of NaCl (130 mM) and MES (130 mM, pH 7.0) for 0 – 72 h. (B) Alkylation of [^{32}P]-RNA by PNA1-QMP1 with fluoride, PNA1-QMP1 without fluoride and PNA1-QM1 self adduct were quantified and plotted. The error represents the average of two independent determinations.

Both PNA1-QMP1 and PNA1-QM1 self adduct could alkylate target RNA. The alkylation rate of RNA was quicker than DNA ($t_{1/2}$ = 2.2 h *vs.* 10 h), and the alkylation yield of RNA by PNA1-QMP1 was ~60% which is similar to the alkylation yield of ssDNA. The alkylation rate of RNA by PNA1-QM1 was also found be quicker than alkylation rate of DNA (RNA $t_{1/2}$ = 8 h *vs.* DNA $t_{1/2}$ = 30 h). Quicker alkylation of RNA than DNA by PNA1-QMP1 might because of the structure of RNA. Different conformation of PNA1/RNA from PNA1/DNA may contribute the fast alkylation of RNA.

2.2.10 Telomeric repeat amplication protocol (TRAP assay) used to monitor the inhibition of telomerase activity

We are interested in inhibiting telomerase activity by alkylation of RNA in telomerase with PNA1-QM1 self adduct since the RNA can be recognized and modified by reversible PNA1-QM1 self adduct. We collaborated with Prof. Xiang Zhou's research group (Wuhan University) and used a TRAP assay to test the inhibition of telomerase by the PNA1-QM1 self adduct. There are two steps in a TRAP assay. In the first step, telomerase adds telomeric repeats (GGTTAG) onto the 3' end of the substrate oligonucleotide and generates a series of oligonucleotides which are only different by various numbers of telomeric repeats. In the second step, the products are amplified by polymerase chain reaction (PCR) and visualized. When telomerase is active, a DNA ladder will appear on gel electrophoresis. When the activity of telomerase is inhibited, the adding of telomeric repeats will stop and a short or no ladder can be found on gel.[103, 104]

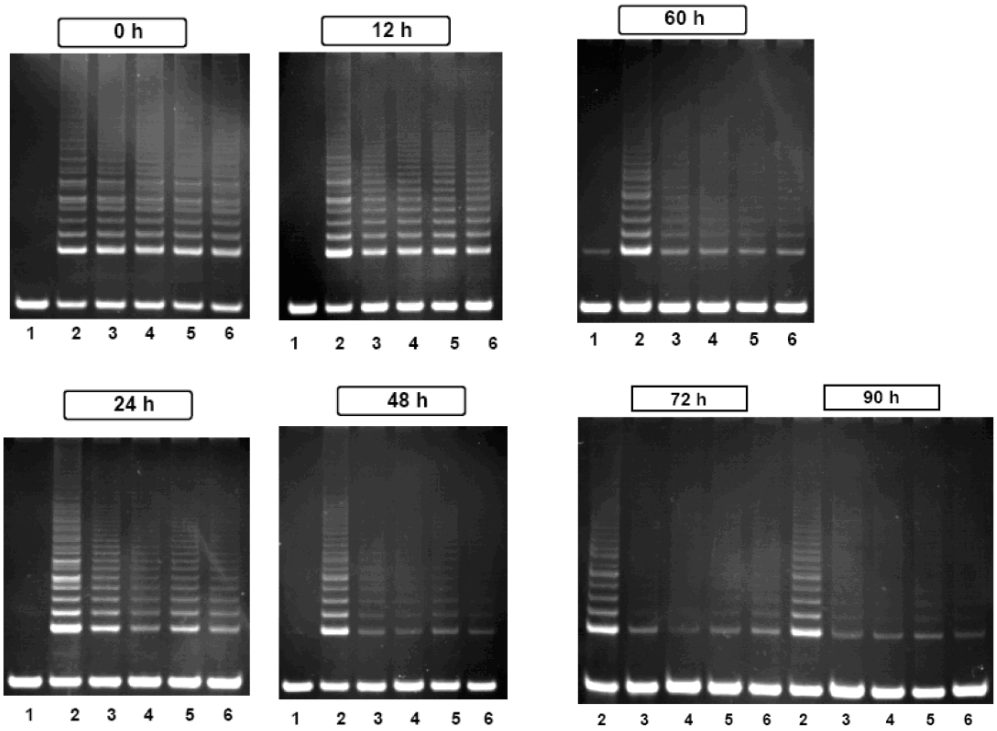


Figure 2.14 Characterizing the activity of PNA1 and PNA1-QM1 self adduct toward telomerase by TRAP assay (Data from Dan Zhang in Prof. Zhou's lab). Different amount of PNA1 or PNA1-QM1 self adduct was mixed with telomerase for 0 – 90 hours before the telomerase was extracted and used in TRAP assay. The telomerase was extracted by phenol/chloroform and used in TRAP assay. For every time point, lane 1: negative control (no telomerase was used in step one); lane 2: positive control (telomerase was not treated by PNA1 or PNA1-QM1 self adduct and used directly); lane 3: PNA1 250 nM; lane 4: PNA1 500 nM; lane 5: PNA1-QM1 self adduct 250 nM; lane 6: PNA1-QM1 self adduct 500 nM.

When telomerase was pre-treated with PNA1 or PNA1-QM1 self adduct for 0 hours, telomerase activity did not change from the positive control (**Figure 2.14**, 0 h, lane 3 – 6 *vs* lane 2). In all cases, telomerase added telomeric repeats at the end of the oligonucleotide. Neither PNA1 nor the PNA1-QM1 self adduct affect the telomerase activity. After 72 hours pre-incubation of telomerase with PNA1 or self adduct, both PNA1 and PNA1-QM1 self adduct inhibited the activity of telomerase (**Figure 2.14**, 72 h, lane 3 – 6). The inhibition of telomerase activity by PNA1 itself

could be caused by the tight binding between PNA1 and telomerase RNA.[94] We did not determine whether alkylation of the RNA in telomerase by the self adduct had any effects since PNA1 itself binds with RNA and inhibits telomerase activity. A PNA which binds to RNA in telomerase weaker than PNA1 and won't inhibit the telomerase activity itself is necessary to observe the effect of RNA alkylation induced by PNA-QM self adduct.

2.2.11 Alkylation of ssDNA by a short PNA2-QMP1

The Corey group has shown that a PNA with eight bases (5'-CAG TTA GG-3') binds to telomerase RNA but the binding is weaker than the 11-base PNA1 (IC_{50} = 0.4 μM *vs.* 0.01 μM) for inhibition of the telomerase activity.[94] Ideally, PNA2 itself won't inhibit the telomerase activity while PNA2-QM self adduct can alkylate RNA in telomerase and inhibits telomerase activity. PNA2 (5'-AEEA-Arg-CAGTTA GG-Arg-3'), PNA2-QMP1 and the PNA2-QM1 self-adduct were thereby prepared following previously used procedures to prepare PNA1 and PNA1-QMP1 and arranged for the inhibition of telomerase activity (**Figure 2.15**). Target alkylation of RNA in telomerase by PNA2-QM1 self adduct requires the selective binding of PNA2 with RNA. Before alkylating RNA in telomerase, the target delivery of QMP1 by PNA2 to its complementary DNA OD1 was first studied to examine the DNA target binding ability of PNA2 (**Figure 2.16**).

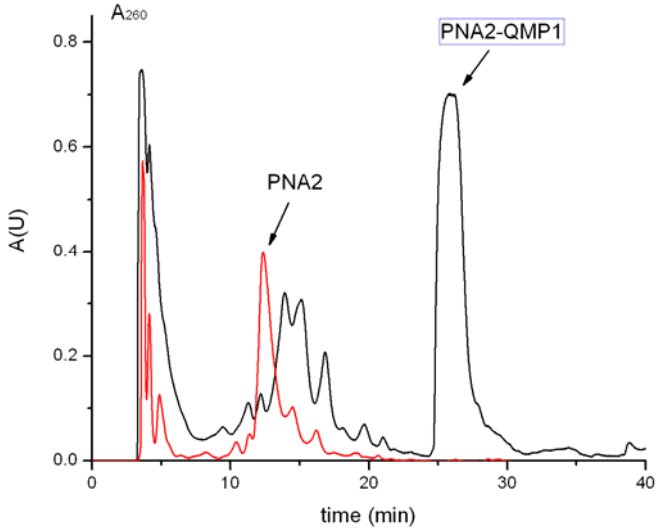


Figure 2.15 HPLC purification of crude PNA2 (red) and crude PNA2-QMP1 (black). Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% trifluoroacetic acid (TFA) at 1 ml/min over 30 minutes. 12 – 14 minutes eluents from crude PNA2 (red) and 25 – 28 minutes eluents from crude PNA2-QMP1 (black) were collected.

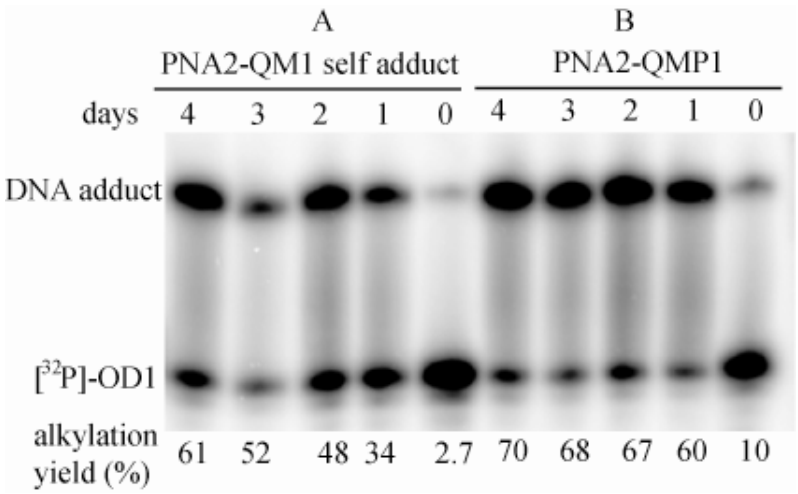


Fig 2.16 A) Alkylation of [^{32}P]-OD1 by PNA2-QM1 self adduct. (A) The self adduct was prepared in situ by addition of KF (130 mM) to a solution PNA2-QMP1 (3.3 μM) in MES (130 mM pH 7) and NaCl (130 mM) and incubated under ambient conditions for 24 h. The target [^{32}P]-OD1 (3.0 μM) was then added and incubation was continued for additional 0 - 4 days. The products were characterized by 20% polyacrylamide denaturing gel. (B) Alkylation of [^{32}P]-OD1 by PNA2-QMP1. [^{32}P]-OD1(3.0 μM) was mixed with PNA2-QMP1 (3.3 μM) in solution of NaCl (130 mM), MES (130 mM, pH 7.0) and KF (130 mM) for 0 - 4 days. (time 0 means 10 – 15 min before loading samples onto gel).

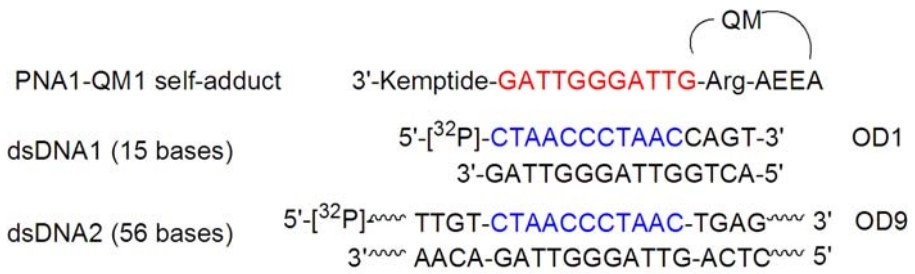
Alkylation of OD1 by PNA2-QMP1 and PNA2-QM1 self adduct was used to examine the effective binding of DNA by PNA2 (**Figure 2.16**). Both PNA2-QMP1 and PNA2-QM1 self adduct can alkylate target OD1. Alkylation of OD1 by PNA2-QMP1 reached maximum yield at ~70% after two days which is similar to the alkylation induced by PNA1-QMP1. Reducing PNA bases from 11 to 8 doesn't affect the alkylation yield and rate. Although PNA2-QMP1 alkylate OD1 a little quicker than PNA2-QM self adduct, the trend is not as obvious as that for PNA1-QMP1 and PNA1-QM self adduct. The alkylation yield and rate by PNA2 have no difference from PNA1. PNA2-QM1 alkylated ~60% of OD1 after 4 days compared to ~40% alkylation yield of OD1 from PNA1-QMP1 self adduct. The high alkylation yield of OD1 by PNA1-QM1 self adduct suggests that this self adduct might be a good alkylation agent toward RNA in telomerase.

TRAP assay was used to study the telomerase activity inhibition by PNA2 and PNA2-QM1 self adduct. Preliminary data found that PNA2 itself doesn't inhibit the telomerase activity (Dan Zhang from Prof. Xiang Zhou's lab, unpublished data). While no inhibition of telomerase activity was found after the telomerase was treated with PNA2-QM1 self adduct. One possible reason for this result is that PNA2-QM1 reacts with RNA in telomerase not quickly enough and might be quenched by other nucleophiles. Novel QMP which can alkylate oligonucleotides faster than QMP1 may be used to alkylation RNA in telomerase and inhibit telomerase activity. The exact reason why PNA2-QM1 self adduct does not inhibit telomerase is unclear and will require future studying. New PNA which binds tighter than PNA2 but weaker

than PNA1 and electron-rich QMP which reacts with RNA quicker than QMP1 may be able to inhibit telomerase activity by alkylating RNA in telomerase.

2.2.12 Alkylation of dsDNA by PNA1-QMP1

Although our initial goal is to recognize single strand oligonucleotide by PNA1, use of PNA1 as a directing agent to deliver QMP to dsDNA *in vitro* and *in vivo* is useful since cellular DNA exists mainly in its duplex form. Successful alkylation of dsDNA requires the co-localization of QMP1 with target dsDNA. PNA might invade into dsDNA and bind with its complementary strand since PNA/DNA binds tighter than corresponding DNA/DNA duplex. It is also obvious that the invasion of dsDNA will be easier if the binding site locates at the end of dsDNA than in the middle of a duplex. So two duplex DNAs were chosen as the PNA1-QMP1 alkylation target. A 15-base dsDNA1 has the PNA1 binding site at the end of DNA and a 56-base dsDNA2 has the same binding site in the middle of dsDNA. dsDNA was then incubated with PNA1-QMP1 and the PNA1-QM1 self adduct to study the alkylation of OD1 or OD9 in dsDNA (**Scheme 2.12**).



Scheme 2.12. Sequences of PNA1-QM1 self adduct, dsDNA1 and dsDNA2. OD9 = 5'-GGTGGCCATTTTTTGTCTAACCCTAACTGAGAAGGGCGTAGGCGCCGTG CTTTTGC-3'

dsDNA1 was incubated with PNA1-QM self adduct in 12 - 192 mM NaCl for 4 days. Since the melting point of dsDNA increases with higher salt concentration, PNA1-QM1 is more difficult to invade into dsDNA at 192 mM NaCl than at 12 mM NaCl solution. After a 4 day incubation, a slow electrophoretic mobility species represents the target ssDNA alkylation was found on gel and unexpected, the alkylation yields of 25 – 30% were independent of NaCl concentration was used (**Figure 2.17**). This is because the PNA1 binding site locates at the end of dsDNA and the invasion of PNA1 may be less affected by salt concentration as previously thought.

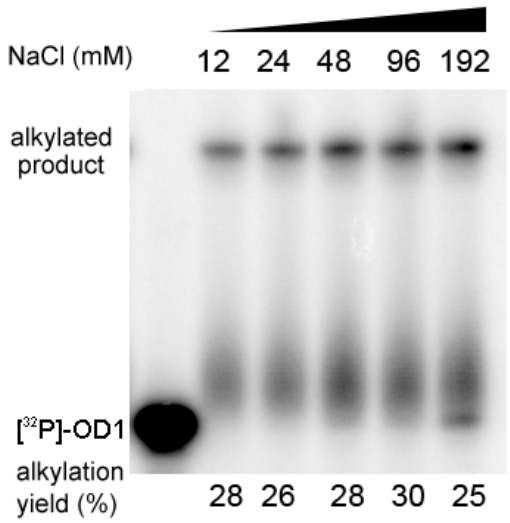


Figure 2.17 Alkylation of dsDNA1 by a self adduct. The PNA1-QM self adduct (3.3 μM) and dsDNA1 (3.0 μM) were incubated in MES (12 mM, pH 7.0) and NaCl (12 mM – 192 mM) for 4 days.

Both PNA1-QMP1 and PNA1-QM1 self adduct were next incubated with a 56 bases dsDNA2 in a solution containing 10 mM sodium salt to further study whether the location of target sequences within dsDNA has any effect on DNA alkylation (**Scheme 2.12**, **Figure 2.18**). Incubation of dsDNA2 with PNA1-QMP1 resulted in

less than 2% of alkylation (lane 2 - 6), and incubation of this dsDNA with PNA1-QM1 self adduct resulted in an alkylation yield of 8.0% (**Figure 2.18**, lane 11). When the salt concentration was increased to 50 mM, less than 2% of the dsDNA2 was alkylated by PNA1-QM1 self adduct (data not shown).

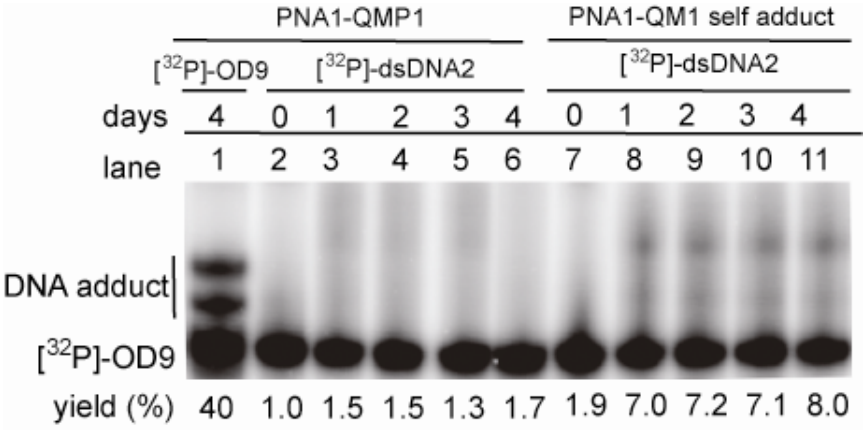


Figure 2.18 Alkylation of dsDNA2 by PNA1-QMP1 and the PNA1-QM1 self adduct. PNA1-QMP1 (3.3 μM) in solution containing 5 mM NaCl and 5 mM NaF was incubated with [^{32}P]-OD9 (3.0 μM) for 4 days (lane 1); [^{32}P]-dsDNA2 (3.0 μM) for 0 – 4 days (lane 2 – 6). PNA1-QMP self adduct (3.3 μM) in buffer containing 10 mM NaCl was incubated with [^{32}P]-dsDNA2 (3.0 μM) for 0 – 4 days (lane 7 – 11).

The different of alkylation yield between dsDNA1 and dsDNA2 by PNA1-QM1 self adduct (~30% *vs.* 8.0%) can be explained by the difference of DNA invasion ability. A previous study from Smolina showed that a mixed base PNA can induce dsDNA invasion only when the target site is at the end of dsDNA.[76] For the PNA1-QM1 self adduct, it invades to a lesser extend in dsDNA2 than in dsDNA1 since the target binding site locates in the middle of dsDNA2. Other PNA sequences which will be studied in the next chapter will be used to recognize dsDNA.

2.2.13 Alkylation of plasmid dsDNA by PNA1-QMP1

Although a mixed sequence of PNA is not expected to invade dsDNA when the target sequence is not located at the end of the dsDNA,[76] we incubated PNA1-QMP1 with plasmid DNA containing target sequence because the super coiled structure of plasmid DNA can aid invasion of PNA.[105, 106]

In order to monitor the target sequence alkylation, we incubated [^{32}P]-PNA1-QM1 self adduct with a 3421 bases plasmid DNA – pUC-human telomerase RNA (pUC-hTR) containing the target sequence for 4 days under room temperature or 37°C. After incubation, the mixture was digested by restriction enzyme BsiEI and separated by 2% agarose gel (**Figure 2.19**). Each DNA fragment band was cut from the agarose gel and measured for ^{32}P by scintillation counting to characterize the target DNA alkylation (**Figure 2.19**). The restriction fragment containing the target DNA was expected to be more strongly radio-labeled since this alone should be alkylated.

After cutting each band from the gel, none of the bands contained radio-activity (< 1000 dpm by scintillation counter) compared to > 800,000 dpm radio-active [^{32}P]-PNA1-QMP1 used in reaction. More than 95% of radio-activity corresponding to [^{32}P]-PNA1-QMP1 was retained at the loading point of the gel since PNA1-QMP1 has only one charge and barely moved on gel electrophoresis. The result showed that [^{32}P]-PNA1-QMP1 did not alkylate a plasmid DNA containing the 11-base target sequence..

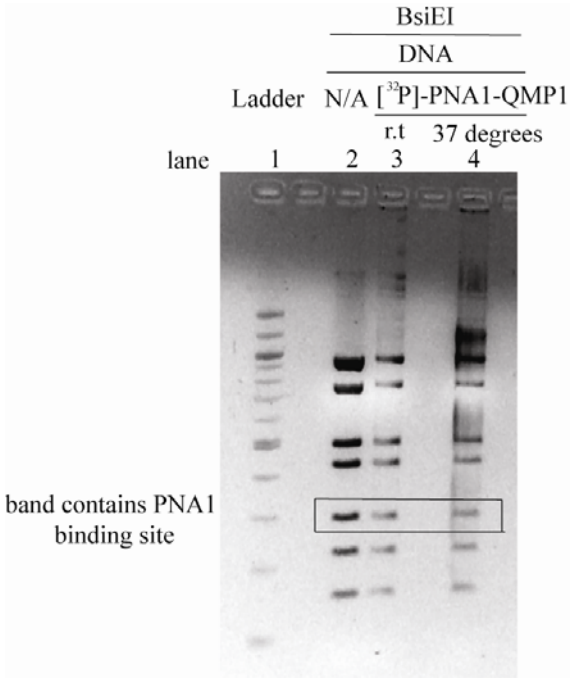


Figure 2.19 Agarose gel of BsiEI digested pUC-hTR plasmid DNA. Lane 1: 1 kb DNA ladder; lane 2: plasmid DNA digested by BsiEI; lane 3: After plasmid DNA was incubated with [^{32}P]-PNA1-QMP1 for 4 days at room temperature, the resulting solution was digested by BsiEI; lane 4: After plasmid DNA was incubated with [^{32}P]-PNA1-QMP1 for 4 days at 37°C, the resulting solution was digested by BsiEI. The PNA1 binding site was indicated in gel.

Lack of dsDNA invasion by PNA1 limits PNA1-QMP1 to localize and alkylate target duplex sequences in cells. Polypyrimidine PNA sequences may be necessary to form triplex complexes with dsDNA and potentially deliver QMP to target dsDNA.[77, 107, 108] Preparation and use of such a polypyrimidine PNAs to deliver QMP to target duplex sequences is described in the next chapter.

2.3 Summary

Delivery of a QMP to target sequences such as ssDNA, ssRNA and dsDNA by mixed bases PNAs were studied to evaluate the recognition of oligonucleotides by PNA. Both PNA1-QMP1 and PNA2-QMP1 can effectively alkylate their

complementary single strand DNAs. The rates and yields of reaction mimic those observed for earlier derivatives based on DNA conjugates.[37] PNA1-QMP1 also discriminates fully complementary sequences from ones containing T/T or G/T mismatch sequences. The sensitivity toward one base mismatch makes PNA a good delivery agent for targeting DNA. The life time of QM is greatly prolonged by forming PNA1-QM1 self adduct compare to the short lived quinone methide. Even after an 8 day incubation under ambient conditions, the self adduct retained more than half of its alkylation ability. The reversible PNA1-QM1 self adducts through intrastrand alkylation provides a biocompatible conjugate for delivery of a quinone methide for target specific alkylation. The reversibility of PNA-QM self adduct makes it applicable *in vivo* to alkylate target sequences without the use of toxic fluoride. A random DNA sequence can be held adjacent to PNA1-QMP1 by deploying a DNA template. The random DNA can then be alkylated by QMP1. When the random DNA is just adjacent or one base away from PNA1-QMP1, no alkylation was observed. A three extra free bases from target sequence at the position close to PNA and the template DNA are required to generate up to 40% target alkylation.

Since one of our goals is to inhibit telomerase activity by alkylating RNA within telomerase, target alkylation of ssRNA which has the same sequence as RNA in telomerase was studied. Target ssRNA was alkylated by either PNA1-QMP1 conjugate or PNA1-QM1 self adduct successfully. The inhibition of telomerase activity by TRAP assay could not be differentiated from the inhibition induced by the binding of PNA1 itself. A second PNA (PNA2) which binds to target RNA weaker

than PNA1 was then constructed. Neither PNA2 nor PNA2-QM1 self adduct inhibited telomerase activity. New PNA which binds to RNA within telomerase weaker than PNA1 while stronger than PNA2 may be required to study the effect of target RNA alkylation in telomerase.

The target alkylation was then expanded to dsDNA to study the future application in cell environment since DNAs are mainly present in a duplex form in cellular conditions. PNA1-QMP1 and PNA1-QM1 invaded into dsDNA1 in which the target sequence is at the end of the duplex DNA. Salt concentration (12 – 192 mM) does not affect the DNA invasion and alkylation. When the target site locates in the middle of a 56 bases duplex DNA or a plasmid DNA, no DNA invasion and alkylation were found. These results showed that a target DNA binding or dsDNA invasion is requirement to generate target DNA alkylation. Other deliver methods such as the generation of triplex structure are needed to deliver QMP to dsDNA when the target locates in the middle of dsDNA.

2.4 Materials and methods

Materials. Solvents, starting materials, and reagents of the highest commercial grade were used without further purification. The pUC-hTR plasmid DNA was provided by Prof. Carol Grieder. Single-stranded DNAs and RNA were purchased from IDT (Coralville, IA). Aqueous solutions were prepared with distilled, deionized water with a resistivity of 18.0 MΩ. Water used in handling RNA was prepared by the laboratory of Prof. Sarah Woodson. Boc-protected PNA monomers were obtained from Applied Biosystems (Foster City, CA). 4-Methylbenzhydrylamine (MBHA) resin (0.7

mmol/g), boc-protected amino acids, O-(benzotriazol-1-yl)-N, N, N', N'-tetramethyluronium hexafluorophosphate (HBTU) and 2-(7-aza-1H-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 Peptide International (Louisville, KY). Kaiser test kit was obtained from Aldrich (St. Louis, MO). T4 polynucleotide kinase (PNK), cAMP-dependent protein kinase (PKA) and restriction enzymes were obtained from New England Biolabs (Ipswich, MA). [γ-^{32}P]-ATP was purchased from Perkin-Elmer (Waltham, MA). Micro bio-spin columns with bio-gel P-6 was from Bio-Rad (Hercules, CA). SepPak C18 cartridge was from Waters (Milford, MA). GeneJET plasmid miniprep kit was from Fermentas (Glen Burnie, MD).

PNA Synthesis. PNA-peptide chimera (PNA1, 5'-AEEA-Arg-GTTAGGGTT AG-LeuArgArgAlaSerLeuGly-3') was synthesized on MBHA resin by manual solid-phase peptide synthesis as described by Corey group[99] and my Master's thesis.[109] 1.5 mL cleavage solution (1 part thioanisole, 1 part *m*-cresol, 2 parts TFMSA and 6 parts neat TFA) was used to remove the oligomer from 50 mg resin. The solution was cooled at -20°C before using. Freshly opened TFMSA provided the best result.[109] TFMSA that had turned brown or black provided poor cleavage efficiency.

PNA1 and PNA2 were purified by reverse phase HPLC (RP-HPLC) with a C18 column (Varian Microsorb-MV, 300Å pore, 250 mm) at 1 ml/min flow rate with a gradient of the acetonitrile (0.1% TFA) from 10% to 55% in H_2O (0.1% TFA) over

30 minutes.. PNA1 was characterized by MALDI-TOF (Shimadzu Axima-CFR) and PNA2 was characterized by ESI-MS. PNA1 calculated (*m/z*) was 4144.6, MALDI (*m/z*) found was 4143.3. $[PNA2+3H^{+}]^{3-}$ calculated (*m/z*) was 895.3 and ESI-MS (*m/z*) found was 895.2. Calculated $[PNA2+2H^{+}]^{2-}$ was 1342.5 and ESI-MS (*m/z*) found was 1342.7

Synthesis of QMP1-NHS ester.

3-[3-Hydroxymethyl-4-hydroxyphenyl]propionic acid (2.2)[97]

Aqueous NaOH (10%, 5 ml) was added to 3-(4-hydroxyphenyl)propionic acid (2.0 g, 12 mmol) and pH of the solution was adjusted to 8. Formaldehyde (37%, 5 mL) was added to the resulting solution and stirred at 50° C for 17 hours. The yellow solution was poured into 100 ml acetone. The orange oil was collected from the bottom of the flask. The oil was then mixed with 5 ml methanol and poured into 150 ml acetone to form a white precipitate. The solid was collected by filtration.

The solid was then dissolved in 10 ml water and adjusted to pH 3 with 1 M HCl. The aqueous solution was extracted with 50 ml ether 3 times. After the removal of ether by rotoevaporator, the remaining liquid was purified by chromatotron (hexane : ethyl acetate = 2 : 1 and 0.5% HOAc) and yielded product **2.2** as a faint yellow solid (500 mg, 21.2%). ^{1}H NMR (D_2O) δ 7.04 (s, 1H), 6.96 (s, H), 6.72 (s, H), 4.48 (s, 2H), 2.72 (t, *J*=8, 2H), 2.52 (d, *J*=8, 2H). The ^{1}H NMR is consistent with the literature.[37]

3-[3-*tert*-Butyldimethylsilyloxymethyl-4-*tert*-butyldimethylsilyloxyphenyl] propionic acid (2.3)[42]

Imidazole (1.0 g, 15 mmol) was added to a solution of *tert*-butyldimethylsilyl chloride (TBDMSCl, 1.50 g, 10 mmol) and compound **2.2** (500 mg, 2.55 mmol) in 12 ml of DMF. The mixture was stirred at room temperature for 15 hours, diluted with brine (100 mL), and extracted with 200 mL ether for 3 times. The organic phases were combined, dried over $MgSO_4$, and concentrated under reduced pressure. The product was redissolved in 10 ml MeOH and 1.50 g of potassium carbonate was added to the methanol solution. The solution was stirred for 3 h and adjusted with 0.2 M HCl to pH = 3. The mixture was then diluted with water and extracted with 100 ml ether for 3 times. The organic phases were combined, washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by silica gel chromatotron (hexane: ethyl acetate= 19:1) to yield **2.3** as a colorless oil (400 mg, 37%). ^{1}H NMR ($CDCl_3$) δ 7.26 (s, 1H), 6.93 (d, *J*= 8.0, 1H), 6.65 (d, *J*= 8.0, 1H), 4.17 (s, 2H), 2.87 (t, *J*= 8.0, 2H), 2.63 (t, *J*= 8.0, 2H), 0.98 (s, 9H), 0.18 (s, 6H). The ^{1}H NMR is consistent with the literature[37]

3-[3-Acetoxymethyl-4-*tert*-butyldimethylsilyloxyphenyl] propionic acid (2.4)[42]

Solid ferric chloride (10 mg, 0.62 mmol) was added to a solution of **2.3** (200 mg, 0.83 mmol) in acetic anhydride (3 mL) at 0 °C. The reaction mixture was stirred for 30 minutes and then extracted with 150 mL ether three times. The organic phases were combined, washed with water and saturated aqueous $NaHCO_3$, dried with $MgSO_4$, and concentrated under reduced pressure. The residue was subjected to silica gel flash chromatography (hexane: ethyl acetate= 10:1) and yielded **2.4** as a colorless liquid (44 mg, 27%). ^{1}H NMR ($CDCl_3$) δ 7.13 (s, 1H), 7.03 (d, *J*= 8.0, 1H),

6.74 (d, *J*= 8.0, 1H), 5.06 (s, 2H), 2.87 (t, *J*= 8.0, 2H), 2.63 (t, *J*= 8.0, 2H), 0.97 (s, 9H), 0.21 (s, 6H). The ^{1}H NMR is consistent with the literature.[37]

***N*-Succinimidyl-3-(3-acetoxymethyl-4-*tert*-butyldimethylsilyloxyphenyl) propionate (QMP1-NHS ester)**[37]

N-Hydroxysuccinimide (46 mg, 0.42 mmol) was added to a DMF solution (5.0 mL) of **2.4** (40 mg, 0.11 mmol). This mixture was cooled to 0 °C and combined with 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDCI, 80 mg, 0.42 mmol). The resulting yellow solution was then stirred for 20 hours from 4 °C to room temperature, diluted with brine, and extracted with 30 mL ether for 3 times. The organic phases were combined and washed with brine, dried over $MgSO_4$, and concentrated under reduced pressure. The resulting residue was subjected to silica gel flash chromatography (hexane: ethyl acetate, 3:1) to yield QMP1-NHS as a white solid (24 mg, 47%). ^{1}H NMR ($CDCl_3$) δ 7.14 (s, 1H), 7.05 (d, *J*= 8.0, 1H), 6.75 (d, *J*= 8.0, 1H), 5.07 (s, 2H), 2.97 (t, *J*= 8.0, 2H), 2.88 (t, *J*= 8.0, 2H), 2.82 (s, 4H), 2.08 (s, 3H), 0.97 (s, 9H), 0.21 (s, 6H). ^{1}H NMR is consistent with the literature[37]

Preparation of PNA and QMP1 conjugate (PNA1-QMP1, PNA2-QMP1**)**.

QMP1-NHS (1 mg) in 100 µl CH_3CN/DMF (v/v = 2/1) was mixed with PNA1 or PNA2 (100 µl, 5.0 mM) in MOPS (250 mM, pH 7.5) and incubated for 24 hours at room temperature. The desired product was purified by RP-HPLC using the same condition as that used for purification of PNA1. PNA1-QMP1 calculated (*m/z*) was 4478.9 and MALDI (*m/z*) found was 4477.6. $[PNA2\text{-}QMP1+3H^+]^{3-}$ calculated (*m/z*)

was 1007.1 and ESI-MS found (*m/z*) was 1006.5. PNA2-QMP1 calculated (*m/z*) was 3018.4 and MALDI (*m/z*) found was 3018.0.

Preparation of PNA-QM self adducts. PNA1-QMP1 or PNA2-QMP1 (20 μM, 100 μl) were mixed with 200 μl KF (1.0 M) in MES (25 mM, pH 7.0) at room temperature. After 24 hours, the self adduct was purified by RP-HPLC using the same condition used for purification of PNA1. PNA1-QM1 self adduct calculated (*m/z*) was 4305.8 and MALDI (*m/z*) found was 4304.7. PNA2-QM1 self adduct calculated (*m/z*) was 2845.2 and MALDI (*m/z*) found was 2845.0.

Radio-labeling DNA, RNA and PNA. The 5' terminus of ssDNA was labeled by T4 PNA and [γ-^{32}P]ATP. PNA1-QMP1 was labeled by cAMP-dependent protein kinase A (PKA, New England Biolab, Ipswich, MA) and [γ-^{32}P]-ATP as described previously.[110] Typically, an aqueous solution of PNA1-QMP1 (1.5 μL, 50 μM) was mixed with PKA (2500 units), BSA (2.5 μL, 10 mg/ml), ATP (5 μL, 25 μM, 25 Ci) and buffer (10 μL) containing 50 mM MES (pH 6.9), 10 mM $MgCl_2$, 0.5 mM EDTA and 1 mM DTT to total 50 μL. After incubation at 37°C for 30 minutes, the reaction was desalted using a SepPak C18 reverse-phase cartridge (Waters, Milford, MA). After the labeled PNA conjugate was loaded onto a cartridge, water containing 0.1% TFA was used to wash the buffer away. PNA was retained on the C18 cartridge but could be washed from resin by 50% acetonitrile in water (0.1% TFA). The eluent was dried with a Speed-Vac.

Annealing dsDNA.

1.0 equivalent of the 5'-[^{32}P]-radio labeled oligonucleotide (3.0 μM) and 1.1 equivalent of its unlabeled complementary DNA (3.3 μM) were annealed in a solution of MES (20 mM, pH 7) and NaCl (20 mM) in a 1.5 mL centrifuge tube. The tube was incubated in 90°C water bath for 5 minutes and was cooled to room temperature over more than 3 hours. The final dsDNA concentration is 3.0 μM.

Target alkylation of DNA and RNA.

ssDNA (3.0 μM) or dsDNA (3.0 μM) were mixed with PNA1-QMP1 (3.3 μM) in NaCl (130 mM), MES (130 mM, pH 7.0) and KF (130 mM) for different lengths of time. Identical conditions except for the exclusion of fluoride were used when using the self-adduct (3.3 μM) to alkylate ssDNA (3.0 μM). Reactions were quenched by adding 3 μL of formamide (0.05% bromophenol blue and 0.05% xylene cyanol FF in formamide) and loaded to the gel directly. Products were analyzed by 20% denaturing polyacrylamide gel electrophoresis (PAGE) using 7 M urea, 89 mM Tris-borate pH 8, and 5 mM EDTA. A temperature of 40 – 45°C was maintained during electrophoresis. Gels were visualized by a Molecular Dynimics Phosphorimager (Sunnyvale, CA). The yield was measured by the ratio of radio-activity from high molecular weight band over the sum of the radio-activity from high molecular weight band and ssDNA band on PAGE. All the reactions were repeated twice and the mean number was calculated as final alkylation yield.

Mini-prep for plasmid DNA

A single colony in 5 mL LB broth containing ampicillin (50 μg/ml) was gown with shaking (250 RPM) at 37°C. After overnight incubation, the mixture was centrifuged at 5000 g for 5 minutes at 4°C. After the supernant was carefully removed, 1 mL resuspension solution (GTE buffer containing RNase A) was added to suspend the pellet. This mixture was mixed by vortex and then transferred to a 1.5 mL centrifuge tube. 250 μL lysis solution (0.2 M NaOH, 1% sodium dodecyl sulphate) was added to the tube and the solution was mixed by inverting the tube for 5 – 6 times. Finally, 350 μL neutralization solution (20 – 50% guanidinium chloride) was added and the tube was inverted for another 5 – 6 times. Solution was transferred to a spin column (Fermentas) and was centrifuged at 16100 g for one minute. 500 μL wash solution (water diluted by ethanol) was then added to the filter and centrifuged at 16100 g for one minute. The flow through was discarded. Another 500 μL wash solution (water diluted by ethanol) was added to the filter and centrifuged at 16100 g for one minute. The flow through was discarded. 50 μL water was added to the filter and a new tube was used. The mixture was then centrifuged at 16100 g for another 2 minutes and the flow through in new tube was the plasmid DNA wanted. The concentration of DNA was determined by UV/Vis at 260 nm.

Chapter 3 Alkylation of double strand DNA by PNA-QMP conjugates

3.1 Introduction

DNA is typically found as a duplex of anti-parallel strands in nature. Target alkylation of duplex DNA (dsDNA) may inhibit the DNA replication and kill tumor cells. In chapter two, mixed bases PNA designed to alkylation single strand DNA (ssDNA) can alkylate dsDNA but only when the target site locates at the end of a dsDNA. This requirement restricts the utility of PNA in delivering alkylation agents like QMP to target duplex sequences *in vivo*. Oligonucleotides which can recognize dsDNA are instead necessary to deliver DNA alkylation agents to target sequences.

A polypyrimidine oligonucleotide can bind to a polypurine strand of DNA in the major groove of dsDNA and form triplex.[58] Triplex forming oligonucleotides (TFOs) have been used to deliver DNA modification agents such as transplatin,[61, 62] N-mustard[79] and psoralen[50, 52, 80] to dsDNA. Results showed that site-specific gene modification occurred near target binding sites. Although the target sequences are restricted to polypurine/polypyrimidine, these sequences are not rare in mammalian genomes.[111, 112]

Seidman studied the use of DNA as a TFO to deliver a psoralen to the Chinese hamster ovary (CHO) cell lines (**Figure 3.1**).[113] The hypoxanthine phosphoribosyl transferase (Hprt) gene in CHO cells contains a polypurine:polypyrimidine sequence adjacent to exon 5 that can serve as a TFO binding site.[114] A seventeen base TFO can bind with its target dsDNA (**Figure 3.1**) and psoralen-TFO conjugate induced DNA interstrand crosslinking near TFO binding site.[115]

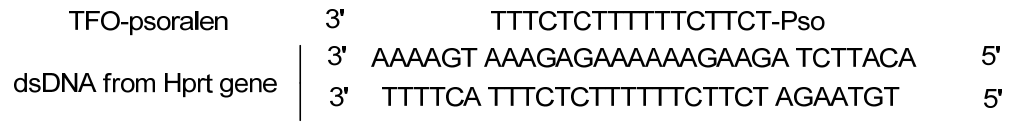
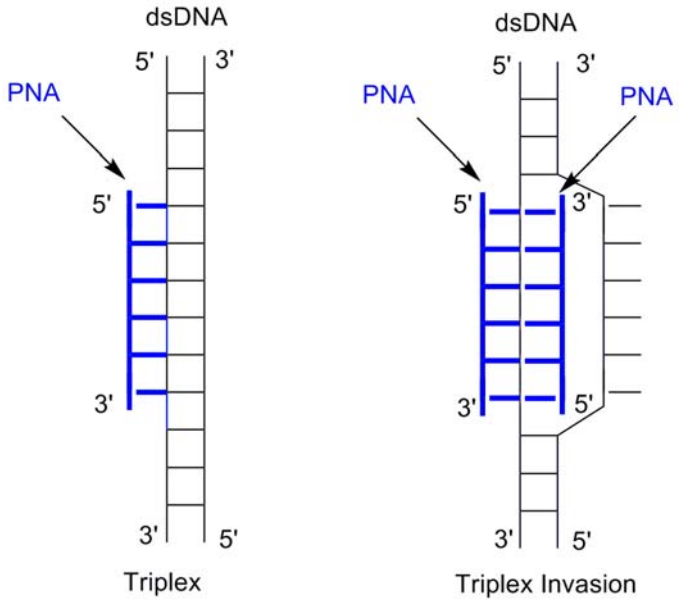


Figure 3.1 Sequences of the triplex target in the CHO Hprt gene and the TFO-psoralen conjugate (pso = psoralen)

DNA as a TFO has its limitations. Normally, magnesium must be present to relieve the electron repulsion between a TFO and its target dsDNA. A pH lower than 5.0 and TFOs of more than fifteen bases are required to form stable triplex structure.[58, 116] PNA is a good replacement of DNA as a TFO. Ten to fifteen bases of polypyrimidine PNA can form triplex structure with target dsDNA since the backbone of PNA is neutral and lack of electron repulsion.[108] However, a PNA·DNA_2 triplex is a kinetic product and will be converted to a PNA_2·DNA to form triplex invasion structures (**Scheme 3.1**).[107, 108] Unlike mixed-bases PNA of chapter two, triplex invasion by polypyrimidine PNA can occur in the middle of a dsDNA. Thus polypyrimidine PNA is more applicable than mixed-bases PNA in targeting dsDNA.



Scheme 3.1 Structures of triplex and triplex invasion by polypyrimidine PNA (blue) and dsDNA (black)

3.2 Result and Discussion

3.2.1 Selection of polypyrimidine sequences

Psoralen-TFO has shown to modify Hprt and induce gene knock in effect.[115] Target delivery of a QMP to Hprt gene by forming PNA·DNA_2 triplex will help to understand the recognition of dsDNA by polypyrimidine PNA. We decided to synthesize a 14 bases PNA3 (5'-AEEA-Arg-TTCTTTTTTCTCTT -LeuArgArgAlaSerLeuGly-3') containing three arginines to deliver QMP to a target dsDNA OD10-OD11 from Hprt gene (**Scheme 3.2**). The introduction of three positive charges (lysine or arginine) at either end of PNA has a ~14-fold affinity improvement in triplex formation.[108] Since lysine has an additional free amine group at its side chain, the amine group would interfere with the coupling of PNA with QMP-NHS ester, arginine was used. Three arginines within PNA3 will render three positive charges (pKa of guanidinium group = 12.48) in neutral and acidic

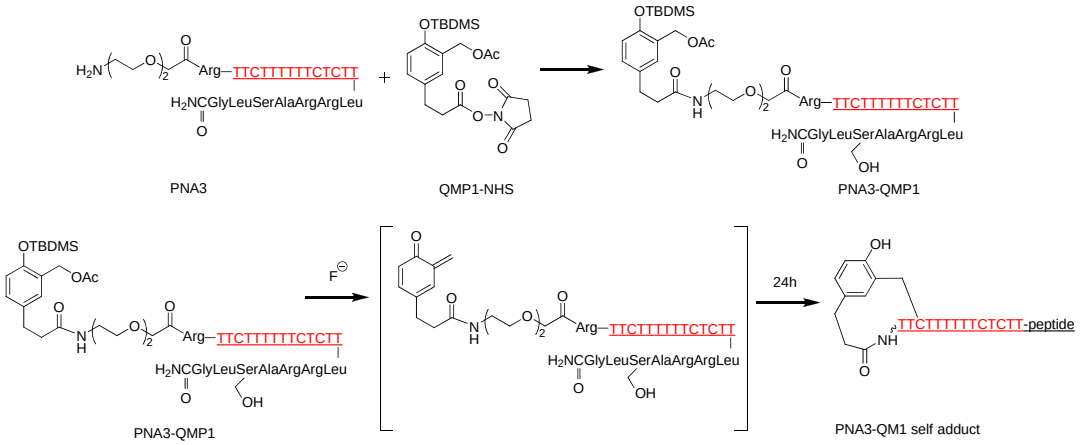
conditions and avoid the reaction with QMP-NHS ester. AEEA at 5' terminus of PNA aids the conjugation of QMP-NHS ester with PNA3 (chapter 2).

PNA3	5'-AEEA-Arg-TTCTTTTTTCTCTT-LRRASLG-3'
OD10	5'-ATTCTAGAAG AAAAAAGAGAATGAA-3'
OD11	5'-TTCATTCTCTTTTTTCTTCTAGAAT-3'

Scheme 3.2 Sequences of PNA3 and its binding sequences in Hprt gene OD10 and OD11

3.2.1 Preparation of PNA3-QMP1 and PNA3-QM self adduct

PNA3 was synthesized by manual solid phase peptide synthesis following the standard procedure used in chapter 2.[97] The PNA3-QMP1 and PNA3-QM1 self adducts were prepared following the same protocol reported previously (**Scheme 3.3**).[97]



Scheme 3.3 Generation of PNA3-QMP1 and PNA3-QM1 self adduct

3.2.2 Stability of PNA3-QMP1 in aqueous solution

PNA-QMP will generate irreversible water adducts and lose its alkylation ability. To determine the storage conditions for PNA-QMP conjugates, the stability of PNA3, PNA3-QMP1, PNA3-QM1 self adduct and old PNA3-QMP1 was monitored by

RP-HPLC (**Figure 3.2**). PNA3 remained intact after storage at -20°C for three months as evident from its homogeneity on RP-HPLC (**Figure 3.2**, A). Less than 5% of PNA3-QMP1 decomposed after three days storage at -20°C. Materials eluted at 19 minute represents intermediate that lost TBDMS protecting group (**Figure 3.2**, B). The PNA3-QM1 self adduct was also stable after storage at -20°C for one month as one peak on HPLC (**Figure 3.2**, C). Interestingly, more than 90% of HPLC purified PNA3-QMP1 decomposed after approximately three months storage at -20°C (**Figure 3.2**, D). Peak at 19.2 minutes corresponding to the PNA3-QM1 intermediate without TBDMS protecting group was shown on HPLC. Less than 10% of PNA3-QMP1 remained intact. Previously, HPLC showed that more than 80% of a mixed base PNA1-QMP1 conjugate was stable in aqueous solution even after eight months storage at -20°C. The decomposition of PNA3-QMP1 is quicker than the mixed base PNA1-QMP1. It is unclear why PNA3-QMP1 is more prone to decompose. PNA3-QMP1 from different preparations was characterized by HPLC to rule out the possibility that there were some impurities in the original PNA3-QMP1 solution which may catalyze the decomposition of PNA3-QMP1. The same results were found again, almost all PNA3-QMP1 conjugated decomposed. The result showed that PNA3-QMP1 decompose spontaneously but slowly. The conjugate should be prepared freshly for subsequent experiments.

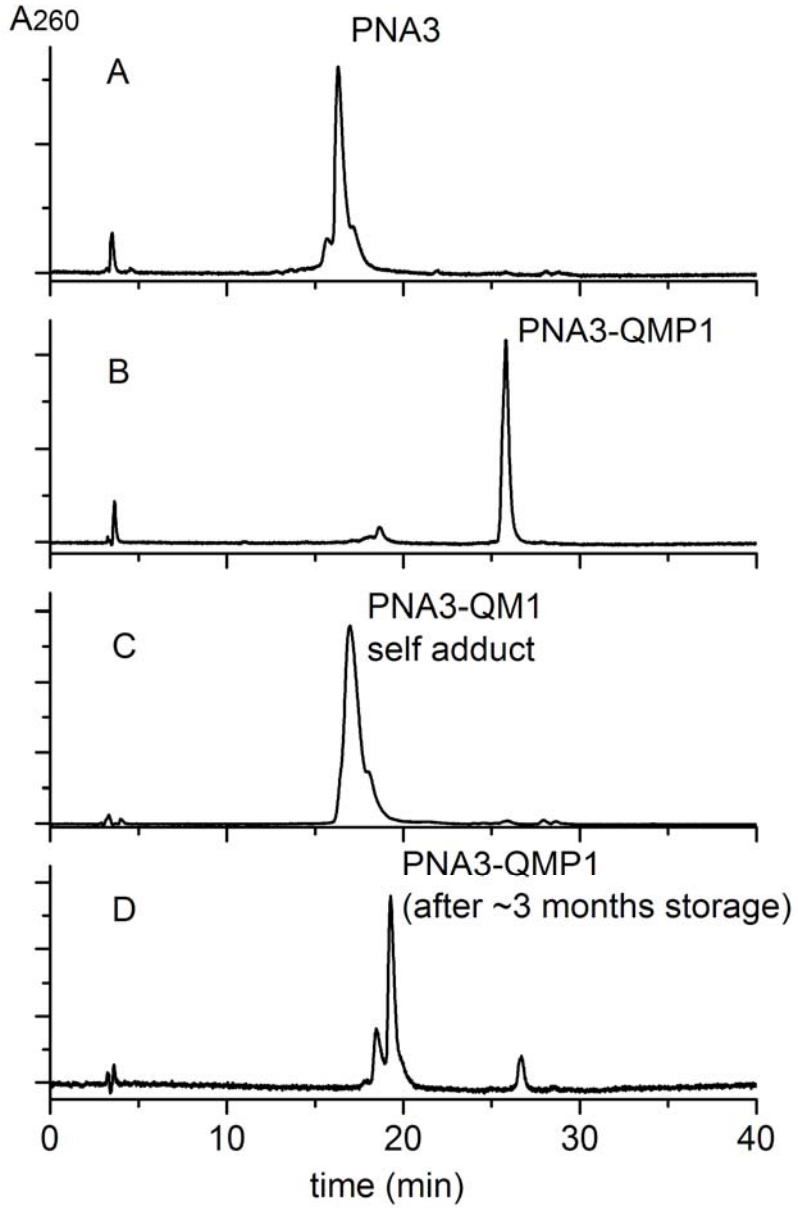
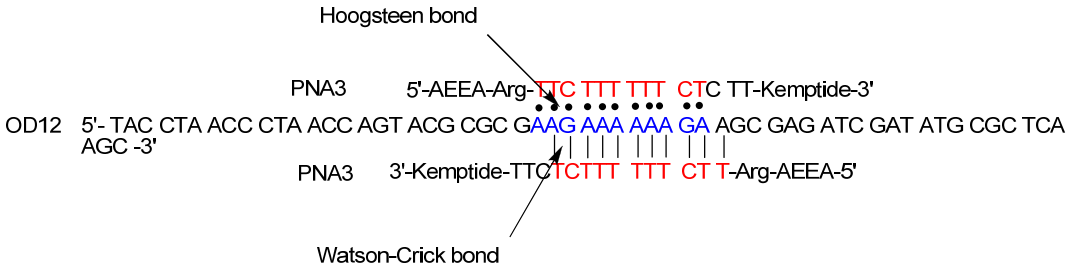


Figure 3.2 HPLC characterization of PNA3, PNA3-QMP1, PNA3-QM self adduct and aged PNA3-QMP1. (A) HPLC purified PNA3 was stored at -20°C for three months; (B) HPLC purified PNA3-QMP1 aqueous solution was stored at -20°C for three days; (C) HPLC purified PNA3-QM1 self adduct was stored at -20°C for one month; (D) HPLC purified PNA3-QMP1 aqueous solution was stored at -20°C for three months. All HPLC runs used the same condition: linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min

3.2.3 Denaturing of PNA/DNA duplex

Our ultimate goal was to use the PNA3-QM1 self adduct to alkylate target dsDNA by forming triplex structures in cell. As mentioned in chapter two, successful denaturing of PNA/DNA duplexes is necessary to differentiate non-covalently bound PNA/DNA from covalently bound PNA-QM-DNA. Un-denatured PNA/DNA would have the similar electrophoretic mobility as PNA-QM-DNA. Soon after we prepared PNA3, we found that the PNA3/OD10 duplex could not be denatured on gel. Low mobility species were showed when OD10-OD11 was incubated with PNA3 in the absence of QMP1. Nielsen showed that the Tm of a 10 base polypyrimidine PNA/DNA duplex was more than 70°C and increased approximately 10°C for every additional polypyrimidine base.[117] The PNA3/OD10 mixture was pre-heated to either 70°C or 90°C for 2 minutes before loading onto a denaturing gel, but the low mobility bands representing PNA/DNA duplex were still found after electrophoresis.

To further address the problem, we used single strand OD12 instead of OD10 to study the denaturing of PNA/DNA (**Scheme 3.4**). The number of triplex forming bases in OD12 was 11 as compared to 14 bases in OD10 to reduce the triplex binding affinity. Since dG N7 is a primary alkylation site by QMP,[63] several deoxyguanosines were placed at both ends of triplex binding site in OD12 to facilitate target alkylation.



Scheme 3.4 Possible triplex structure of PNA3/OD12 complex. "Dot" means Hoogsteen bond and "vertical line" means Watson-Crick bond

PNA3 was then incubated with [^{32}P]-OD12 to study the conditions for successful denaturing. Theoretically, PNA3 can form both a PNA3/OD12 duplex and a PNA3/OD12/PNA3 triplex structure (**Scheme 3.4**). After [^{32}P]-OD12 (3.0 μM) was mixed with PNA3 (3.3 μM) for 3 days, products were characterized by 20% denaturing gel. The PNA3/OD12 duplex was not fully denatured by denaturing gel. When the final solution was pre-heated at 90°C for 2 minutes, still more than 50% of high molecular weight band representing non-denatured PNA/DNA duplex was observed after gel electrophoresis.

Phenol-chloroform extraction was next tested to solve the denaturing problem. PNA functions like a protein because its neutral amide backbone[74] and PNA will be extracted by phenol-chloroform. Phenol extraction was performed 2 - 4 times to fully remove PNA from PNA/DNA complexes. The aqueous solution was finally dried by Speed-Vac. Results were inconsistent. Sometimes, the PNA/DNA could was denatured, no low mobility bands were found after gel electrophoresis. Sometimes, low mobility bands representing un-denatured PNA/DNA complexes were found after gel electrophoresis.

A competition experiment was then performed to aid the denaturing of PNA/DNA duplex. Although PNA3 binds to OD12 very tight, additional OD12

might compete with [^{32}P]-OD12 to generate PNA3/OD12 duplex and displace [^{32}P]-OD12 into the solution thus aid the denaturing of PNA3/[^{32}P]-OD12 duplex. Displacement of [^{32}P]-OD12 was characterized by gel electrophoresis (**Figure 3.3**). After [^{32}P]-OD12 was mixed with PNA3 for 24 hours, 0 – 200 equivalents of non-radio labeled OD12 was added to the solution and incubated for another 2 days.

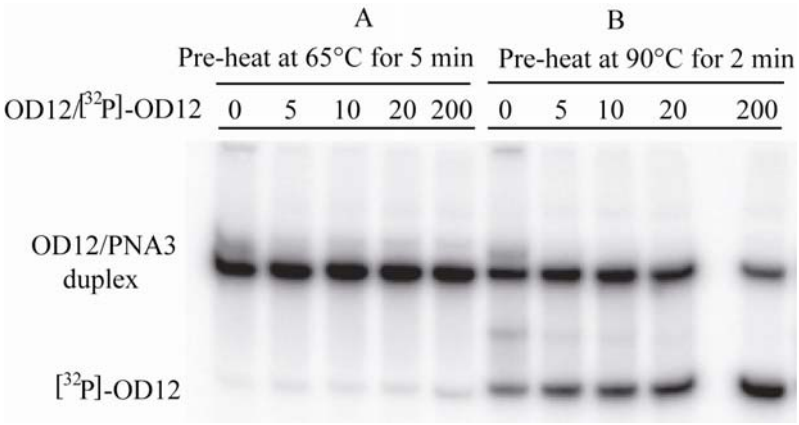
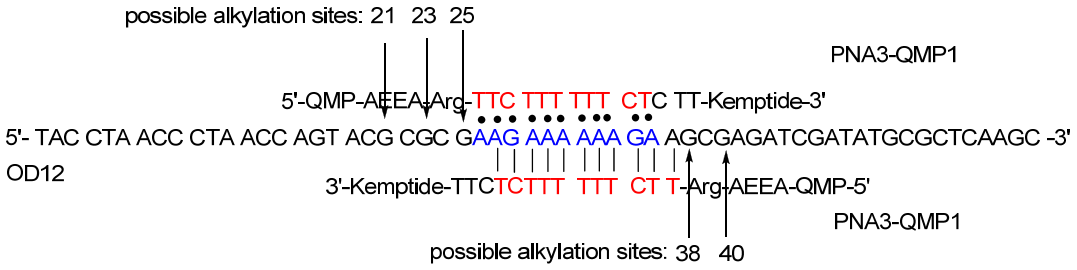


Figure 3.3 Competition-aided DNA denaturation. [^{32}P]-OD12 (50 nM) was incubated with PNA3 (3.0 μM) in solution containing 10 mM sodium chloride at room temperature for 24 hours. Various amounts of OD12 were added and the solution was incubated for two additional days. Each 5 μL of final solution was then mixed with 5 μL of formamide and pre-heated before loading onto the 20% denaturing gel. (A) pre-heat at 60°C for 5 minutes; (B) pre-heat at 90°C for 2 minutes.

Almost all the [^{32}P]-OD12 still bound to PNA3 after heating at 60°C with 200 eq. of OD12 competitor for 5 minutes (**Figure 3.3**, A). Low mobility species representing PNA3/[^{32}P]-OD12 duplex remained at ~30% by denaturing gel even after 2 minutes pre-heating at 90°C (**Figure 3.5**, B). This shows that once a PNA/DNA duplex forms, it is stable at even 90°C.

Because the alkylation of dG N7 site is labile to piperidine treatment and induce strand scission at alkylation site, piperidine treatment was used as an alternative method to characterize DNA alkylation. DNA fragment found on gel can

be used to monitor the occurrence of DNA alkylation. After [^{32}P]-OD12 (50 nM) was incubated with PNA3-QMP1 (3.0 μM) and fluoride for 24 hours, the mixture was subjected to piperidine treatment (**Scheme 3.5**, **Figure 3.4**).



Scheme 3.5 Possible alkylation sites for PNA3-QMP1 if PNA_2/DNA structure formed.

Five minutes incubation at 60°C is enough for A+G foot-printing of ssDNA (**Figure 3.4**, lane 1). Fifteen to thirty minutes incubation used in the same protocol caused over reaction as evident by the disappearance of stating material ([^{32}P]-OD12) on gel (**Figure 3.4**, lane 2 - 4).[118] When PNA3 was incubated with [^{32}P]-OD12, no cleavage by piperidine treatment was detected on gel (**Figure 3.4**, lane 6). More than 90% of PNA/OD12 duplex remained non-denatured after 30 minutes incubation at 90°C as evident by a slow mobility species detected after electrophoresis. This is consistent with the conclusions that either no alkylation occurred or PNA/DNA duplex can not be denatured under these conditions. When the mixture of OD12 and PNA3-QMP1 was treated with piperidine, three fragments were observed and corresponded to 25', 23' and 21' on OD12 (**Figure 3.4**, lane 8). Although only single strand OD12 and PNA3-QMP1 were incubated, a triplex PNA/DNA/PNA structure can still be formed (**Scheme 3.5**). After formation of triplex, PNA3-QMP1 alkylated three deoxyguanosines adjunct to one terminus of triplex binding site. Alkylation at 38' and 40' positions of OD12 were also expected since PNA3 can bind

to OD12 in an anti-parallel manner. Because the possible DNA fragments from 38' and 40' alkylation and piperidine treatment will still bind with PNA3, the PNA-DNA fragments had similar electrophoretic mobility as non-denatured PNA/DNA duplex (HMW bands in **Figure 3.6**). These data showed that PNA3/OD12 can't be denatured on gel and piperidine treatment can be used to characterize the target alkylation of OD12 by PNA3-QMP1 conjugate. PNA3 can bind to single strand OD12 in a PNA_2·DNA manner and deliver QM to OD12.

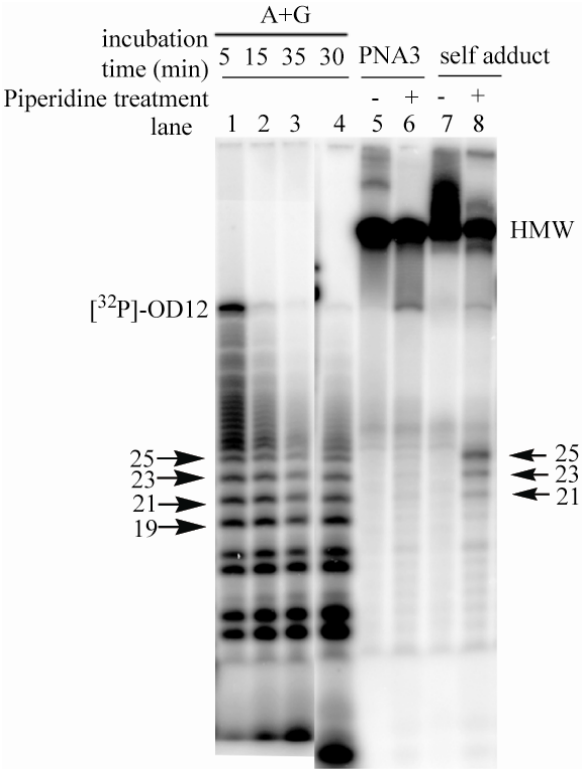
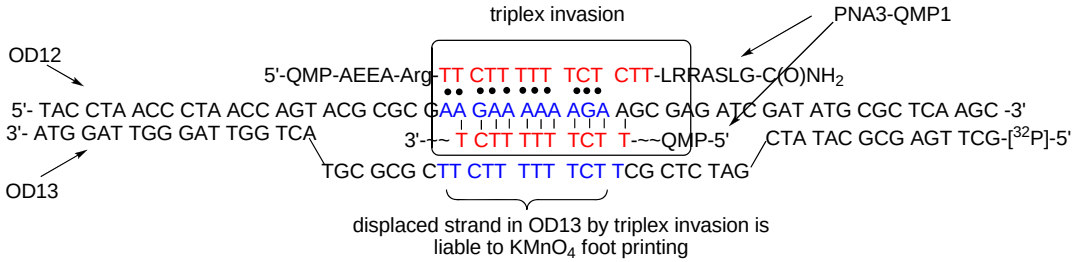


Figure 3.4 Characterization of DNA alkylation by piperidine cleavage. OD12 A+G standard (lane 1 – 4). Samples were incubated with formic acid at 60°C for different times before piperdine cleavage: 5 minutes (lane 1); 15 minutes (lane 2); 35 minutes (lane 3); 30 minutes (lane 4); after [^{32}P]-OD12 (100 nM) was incubated with PNA3 (3.3 μM) in $NaHPO_4$ (20 mM, pH 6.5) and NaF (5 mM) for 3 days, samples were either loaded onto the gel directly (lane 5) or treated with piperidine before loading onto the gel (lane 6); [^{32}P]-OD12 (100 nM) was incubated with PNA3-QMP1 (3.3 μM) in $NaHPO_4$ (20 mM, pH 6.5) and NaF (5 mM) for 1 day. This sample was either loaded onto the gel directly (lane 7) or treated with piperidine before loading onto the gel (lane 8).

3.2.4 Alkylation of dsDNA by PNA3-QMP1 and the PNA3-QM1 self adduct

Our goal is to selectively deliver QMP1 to the major groove of polypurine and polypyrimidine dsDNA by forming PNA/DNA_2 triplex. The only method to characterize the alkylation of target ssDNA by PNA3-QMP1 is piperidine treatment. We then used piperidine treatment to study the alkylation of dsDNA ([^{32}P]-OD12-OD13) by PNA3-QMP1 or PNA3-QM1 self adduct. After dsDNA was incubated with PNA3-QMP1 or PNA3-QM1 self adduct for 3 days, samples were subjected to piperidine treatment. More than 90% of the radio-activity was retained at high molecular weight band corresponding to the non-denatured PNA/DNA duplex and no DNA cleavage was found on gel after piperidine treatment. Experiments were repeated several times and similar results were found (gel not shown).

One reason that alkylation of dsDNA failed could be the lack of PNA binding to dsDNA. Finding out whether target binding occurred will help the designing of future experiments. If PNA/DNA/DNA triplex forms, PNA will continue to generate a triplex invasion product (**Scheme 3.1**) and displace part of the polypyrimidine DNA strand from duplex (**Scheme 3.6**, OD13). We used permanganate foot-printing to study the triplex invasion by PNA (**Scheme 3.6, Figure 3.5**). When there is no triplex invasion, thymine in duplex DNA are not susceptible to $KMnO_4$ and no strand break can be found on gel. If triplex invasion occurs, thymine from the polypyrimidine strand DNA will be displaced from duplex and accessible to $KMnO_4$. Piperidine treatment will then induce strand break at the oxidized thymine.[119] The displaced sequence from polypyrimidine DNA can be characterized by $KMnO_4$.



Scheme 3.6 Strand displacement by triplex invasion

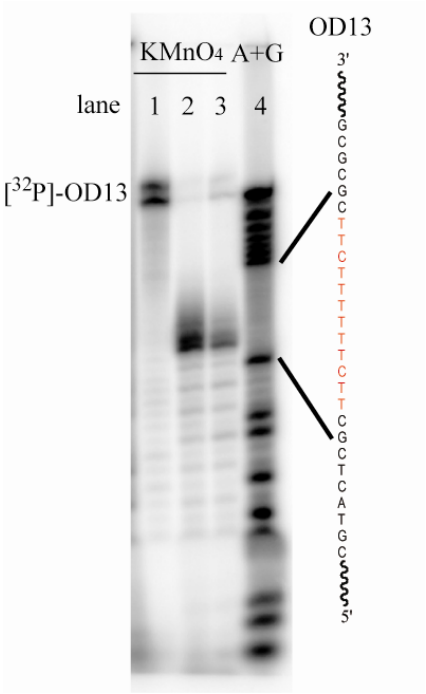


Figure 3.5 $KMnO_4$ probing of triplex invasion by PNA3-QMP1 and PNA3. OD12-[^{32}P]-OD13 was subjected to $KMnO_4$ probing (lane 1); [^{32}P]-OD13-OD12 (50 nM) was incubated with PNA3-QMP1 (1.5 μM) in $NaHPO_4$ (10 mM, pH 6.5) and NaF (5 mM) for 1 day, the solution was then subjected to $KMnO_4$ probing (lane 2); [^{32}P]-OD13-OD12 (50 nM) was incubated with PNA3 (1.5 μM) in buffer containing $NaHPO_4$ (10 mM, pH = 6.5) and NaF (5 mM) for 1 day and then treated with $KMnO_4$ probing (lane 3); [^{32}P]-OD13 A+G standard (lane 4).

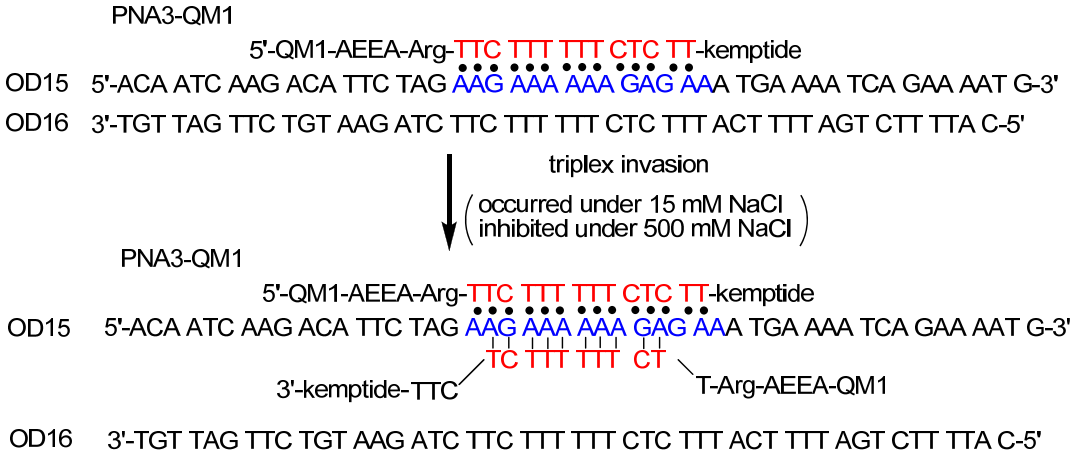
Thymine residues were not susceptible to $KMnO_4$ oxidation in the absence of PNA suggesting that all thymines were held within the duplex (**Figure 3.5**, lane 1). DNA strand breaks corresponding at thymines were evident when dsDNA was

incubated with 30 eq. of PNA3-QMP1 or PNA3, (**Figure 3.5**, lane 2 & 3). This means both PNA3-QMP1 and PNA3 can invade dsDNA.

Since strand displacement was detected, triplex binding must have occurred. The lack of alkylation of dsDNA by PNA3-QMP1 may be from others reasons. The low alkylation yield was difficult to detect after piperidine treatment may contribute to the failure of dsDNA alkylation detection. If triplex invasion can be inhibited while only triplex was formed, the PNA·DNA_2 triplex might be successfully denatured by gel and piperidine treatment might be avoided. Low alkylation yield might be visualized on gel.

3.2.5 Inhibition of triplex invasion by increasing the salt concentrations

High salt concentration may inhibit triplex invasion since the Tm of duplex DNA increases with higher salt concentration while the Tm of PNA/DNA duplex is barely affected.[74] Since both the formation of triplex and triplex invasion might be affected by high salt concentration, maximizing the formation of triplex while inhibiting triplex invasion is preferred. OD15-OD16 containing a 14 base triplex recognition site was used. PNA3 and the PNA3-QM1 self adduct were incubated with [^{32}P]-OD15-OD16 under salt concentrations of 15 – 500 mM to study dsDNA alkylation and inhibition of triplex invasion (**Scheme 3.7, Figure 3.6**).



Scheme 3.7 Triplex invasion by PNA3-QM1.

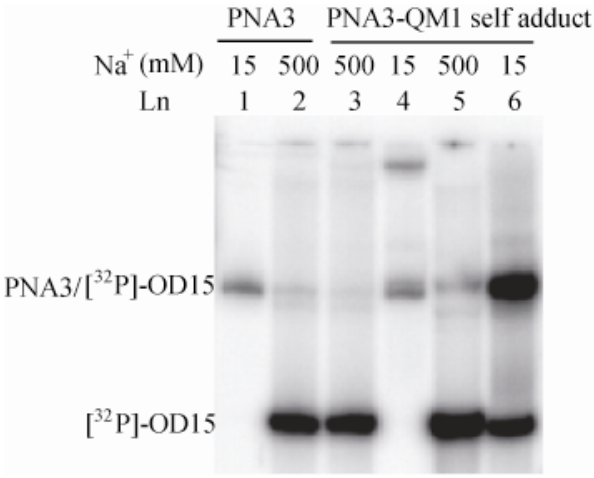


Figure 3.6 Inhibition of DNA invasion by various salt concentrations. [^{32}P]-OD15-OD16 duplex (50 nM) was incubated with PNA3 (3.0 μM) or PNA3-QM1 (3.0 μM) with 15 or 500 mM NaCl for 2 days under room temperature. Sample from lane 1 - 4 were loaded onto gel directly. Samples from lane 5 and lane 6 were pre-heated at 90°C for 20 minutes without formamide prior to loading onto gel

As shown on lane 1 and lane 4, when the salt concentration is low (15 mM sodium), no free [^{32}P]-OD15 was seen by gel. All the radio-activity was retained at the low mobility species representing the PNA3/OD15 complex. The result showed that both PNA3 and the PNA3-QM self adduct can form triplex invasion. Once the PNA3/OD15 duplex forms, it can't be denatured by gel. The low radio-activity from

lane 1 and 4 compared with other lanes was due to the precipitation of [^{32}P]-OD15. On the contrary, no high molecular weight bands corresponding to PNA/DNA duplex persisted in the denaturing gel when samples were incubated with 500 mM salt concentration (**Figure 3.6**, lane 2 and 3). The high salt concentration (500 mM) inhibits the DNA invasion and no PNA3/OD15 duplex was formed. It is unknown whether the PNA/DNA/DNA triplex was formed at lane 2 and 3 prior to gel analysis. Even if the triplex forms, it will dissociate under the denaturing conditions of this gel electrophoresis experiment.

We also incubated dsDNA and the PNA3-QM1 self adduct in solution containing 500 mM NaCl to investigate the alkylation of dsDNA. Since the non-covalent denaturation is possible under these conditions, low mobility species on gel would be DNA alkylation product. After a 3 day incubation, no low mobility band representing DNA alkylation product was observed (**Figure 3.6**, lane 3). Additionally, no evidence of alkylation was detected after piperidine treatment (gel not shown). This unexpected result might be due to the failure of generating stable triplex under high salt concentration (500 mM NaCl). Nielsen showed that formation of PNA/DNA/DNA triplex can be partially inhibited by 500 mM – 1M salt concentration.[107]

3.2.6 Alkylation of DNA with eight bases complementary to PNA3.

Previous experiments showed that neither 11 (PNA3/OD12) nor 10 bases complementary (PNA3/OD15) duplex can be denatured on gel. Failure of denaturing duplex makes the detecting DNA alkylation products on gel impossible.

Although piperidine treatment can be used to characterize the target alkylation, it can't characterize alkylation when the alkylation yield is low or the adduct can be reversed by piperidine. A DNA which can form duplex with PNA3 and the duplex can be denatured by gel was necessary before alkylation could be detected. We deployed OD14 which has eight bases complementary to PNA3 and studied the denaturing of PNA3/OD14 duplex (**Scheme 3.8**, **Figure 3.7**, lane 1). This PNA3/OD14 duplex was successfully denatured by 20% denaturing gel.

PNA3 5'-AEEA-Arg-TTCTTTTTTCTCTT-LeuArgArgAlaSerLeuGly-3'

OD14 5'-TATCGCAAAAAGAACAGT-3'

Scheme 3.8 Sequences of PNA3 and OD14

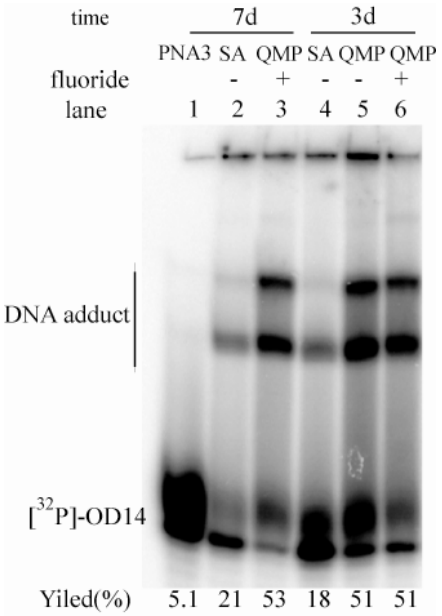


Figure 3.7 Alkylation of OD14 by PNA3-QMP1 and PNA3-QM1 self adduct at room temperature. All the reactions contained [^{32}P]-OD14 (3.0 μM) in MES (130 mM, pH 7.0). Lane 1: PNA3 (3.3 μM) in buffer for 7 days; lane 2: HPLC purified PNA3-QM self adduct (3.3 μM) in buffer for 7 days; lane 3: PNA3-QMP1 (stored under -20°C for 3 months, 3.3 μM) and NaF (130 mM) in buffer for 7 days; lane 4: HPLC purified PNA3-QM1 self adduct (3.3 μM) in buffer for 3 days; lane 5: PNA3-QMP1 (stored under -20°C for 3 months, 3.3 μM) in buffer for 3 days, no NaF was added; lane 6: PNA3-QMP1 (stored under -20°C for 3 months, 3.3 μM) and NaF (130 mM) in buffer for 3 days. "SA" in lane 2 and 4 were self adduct.

Successful denaturing of PNA3/OD14 during gel electrophoresis makes the direct detection of OD14 alkylation possible (**Figure 3.7**). The PNA3-QM1 self adduct can effectively alkylate target OD14 with a yield of ~20% after three days (**Figure 3.7**, lane 2 & 4). Although most of PNA3-QMP1 lost its TBDMS group after its storage at -20°C for three months (**Figure 3.2**), the resting mixture still alkylated target sequence OD14 with a yield of more than 50%. This yield is similar to that produced by alkylation of ssDNA with a mixed base PNA1-QMP1 (~60%). Interestingly, no difference in the alkylation yield was detected if fluoride was present or absent (**Figure 3.7**, lane 5 & 6). Multiple slow mobility bands were detected on gel. This suggests that PNA3 may form a PNA3/OD14/PNA3 triplex and the QMP1 can be delivered by both PNA3 strands to alkylate OD14.

To fully identify the relationship between target alkylation and the formation of triplex structure, we studied the alkylation of [^{32}P]-OD14 by PNA3-QM1 self adduct. The PNA3-QM1 self adduct can alkylate up to 20% target ssDNA after 72 – 96 hours (**Figure 3.8**). PNA3-QM1 self adduct produces multiple bands with OD14 as seen by gel electrophoresis.

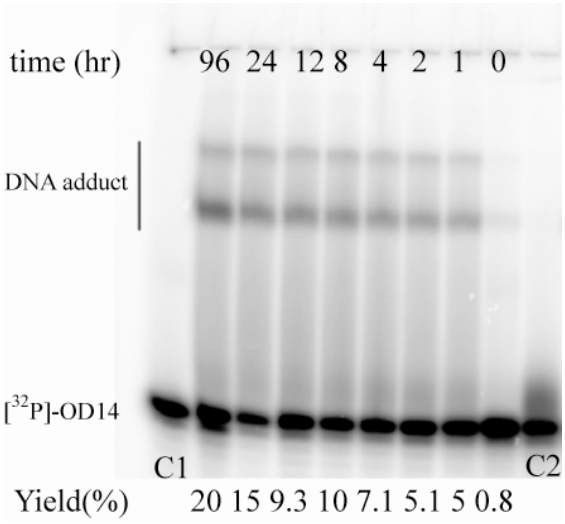


Figure 3.8 Alkylation of [^{32}P]-OD14 by the PNA3-QM1 self adduct. C1: [^{32}P]-OD14; C2: [^{32}P]-OD14 (3.0 μM) was incubated with PNA3 (3.3 μM) in NaCl (130 mM), MES (130 mM, pH 7.0) and NaF (130 mM) for 96 hours; other lanes: After PNA3-QMP1 (3.3 μM) was incubated in NaCl (130 mM), MES (130 mM, pH 7.0) and NaF (130 mM) for 24 hours, [^{32}P]-OD14 (3 μM) was added and incubated for additional 0 - 96 hours

3.2.7 Generation of PNAs with 8 and 9 base complementarity to their targets

The results above with OD14 demonstrated that an 8 base oligonucleotide complementary to PNA3 was alkylated by PNA3-QM1 self adduct. The PNA/DNA complex was successfully denatured by gel so that the alkylation could be detected. PNA4 and PNA5 have an eight polypyrimidine bases were prepared to deliver a QMP to target dsDNA (**Scheme 3.9**). PNA4 can form a triplex with OD10-OD11. Since PNA4 is 7 bases complementary to OD10, the PNA4/OD10 duplex should be denatured on gel. The non-covalently bound PNA4/OD10 was expected to undergo denaturation and target DNA alkylation can be easily detected by gel without the interference of non-denatured PNA4/OD10 duplex.

PNA5-QMP 5'-QMP-AEEA-Arg-**A**-TTCTTTTT-Arg-3'

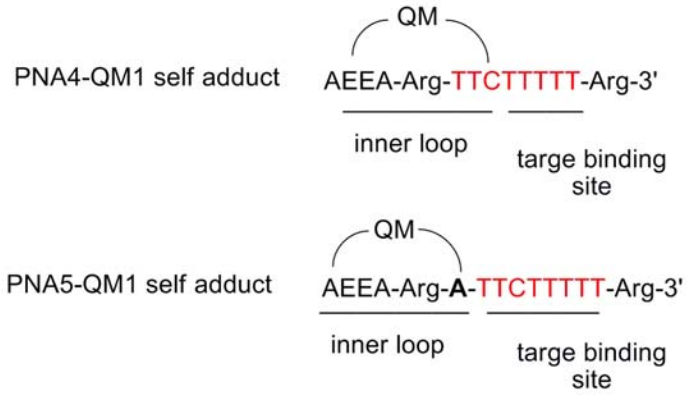
PNA4-QMP 5'-QMP-AEEA-Arg-TTCTTTTT-Arg-3'

OD10 5'-ATTCTAGAAG AAAAAAGAGAATGAA-3'

OD11 5'-TTCATTCTCTTTTTTCTTCTAGAAT-3'

Scheme 3.9 Sequences of PNA4, PNA5 and OD10, OD11

In chapter one, we introduced the lack of reaction of thymine with QM. Since there is only one dC – the potential PNA4-QM1 self adduct formation site in PNA4, PNA4-QM1 self-adduct greatly changed the integrate structure of PNA4. The possible internal loop formed by QM1-AEEA-Arg-TTC of PNA4 could change the triplex recognition site from 8 bases TTCTTTTT to 5 bases TTTTT and which may compromise recognition of dsDNA. Previously our group showed that dA can effectively capture and release QM intermediate to increase the lifetime of QM.[40] So we also prepared PNA5 which has one extra dA at 5' terminal of its PNA backbone (**Scheme 3.10**). Ideally, dA in PNA5 can effectively trap and release the QM intermediate and would not affect the binding properties of the original polypyrimidine bases.



Scheme 3.10 Inner loops formed by PNA4-QM1 and PNA5-QM1 self adducts

PNA4 and PNA5 were successfully prepared by solid phase peptide synthesis, purified by HPLC and confirmed by mass spectrometry.[97] PNA-QMP conjugates and PNA-QM self adducts were prepared, purified and characterized following the same methods used to prepare the PNA1-QMP1 conjugate and its self adduct (HPLC and MS spectrums are shown in Appendix **Figure 6 - 15**).

Both PNA4-QMP1 and PNA5-QMP1 were incubated with OD10-OD11 at room temperature to study the target alkylation of dsDNA by PNA-QMP1. No alkylation of DNA was observed by gel electrophoresis although PNA/DNA duplex can be denatured on gel. We suspect that no PNA/dsDNA triplex was formed in solution and is consistent with the need for a polypyrimidine PNA longer than 10 bases to form PNA/dsDNA triplex.[108] Shorter PNAs (8 - 10 bases) may require a high percentage of cytosine to form PNA/DNA_2 triplex.[79] A PNA of 8 bases (PNA4) was too short to form a triplex.

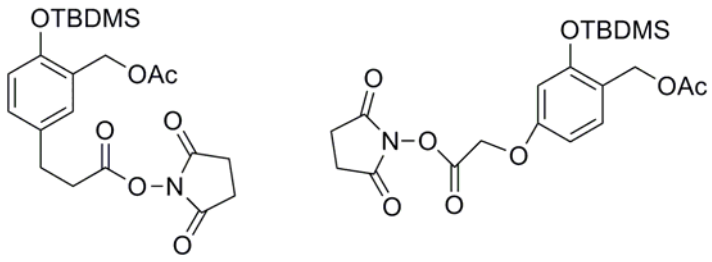
3.2.8 Preparation of a cytosine rich PNA (PNA6) and its QMP1 conjugate

In our attempts to deliver a QM by PNA recognition to a dsDNA, we had three major problems: 1) dsDNA probably did not form a triplex with an 8 base recognition region of PNA4; 2) PNA/DNA complementary region larger than 10 bases could not be denatured for detecting alkylation; 3) A 14 bases dsDNA target may form triplex with PNA, but the triplex invasion was continued to be formed, no dsDNA alkylation was observed. To solve these problems, we prepared a new PNA sequence (PNA6) and used a different QMP (QMP2) to alkylate target dsDNA. PNA6 should fulfill two conditions: 1) effectively form PNA/DNA/DNA triplex with target dsDNA

because it has a 11 base triplex binding site and is a cytosine rich sequence (6 cytosines); 2) PNA/DNA duplex can be denatured by gel since PNA6 is only 5 bases complementary (anti-parallel) to its binding DNA – OD17 (**Scheme 3.11**). The novel QMP2 was prepared by Chengyun Huang from Rokita group recently as an electron-rich QMP2 (**Scheme 3.12**).[120] One carbon attached to the aromatic system in QMP1 is substituted by an oxygen atom inQMP2. The extra oxygen stabilizes the QM intermediate and accelerates generation of QM.[45]

PNA6-QMP2	5'-QMP-AEEA-Arg-CTTCCT CTTCC-Arg-Arg-3'
OD17	5'-~~~AACG GAAGGAGAAGG AAAA~~~-3'
OD18	3'-~~~TTGC CTT CC TCTTCC TTTT~~~--5'
OD19	5'-~~~AAGA GAAGGAGAAGG AAAA~~~-3'
OD20	3'-~~~TTCT CTT CC TCTTCC TTTT~~~--5'

Scheme 3.11 Sequences of PNA6, target dsDNA OD17-OD18 and OD19-OD20

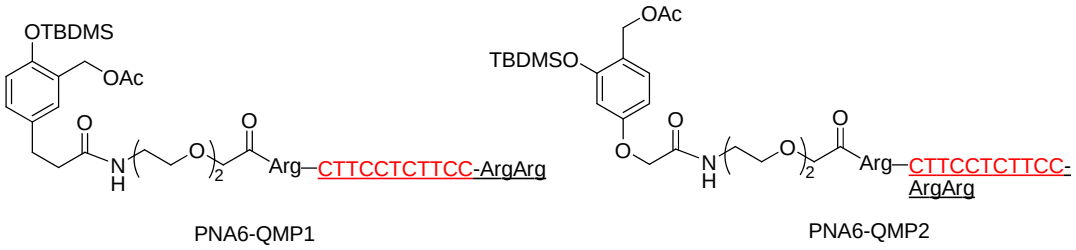


Scheme 3.12 Structure of QMP1 and QMP2

Recently, Chengyun Huang demonstrated that the novel DNA-QMP2 conjugate and its self adduct can form triplex with a polypurine:polypyrimidine duplex DNA target and the alkylation of target sequences was more rapidly than the traditional DNA-QMP1 and its self adduct.[120] QMP2 conjugated with PNA6 was expected to

alkylate its target (OD17-OD18 and OD19-OD20) and the adduct should be easily characterized by denaturing gel since PNA6 was only 5 bases that are complementary to OD17.

Both PNA6-QMP1, PNA6-QMP2 and their self adducts (**Scheme 3.13**) were prepared following the same methods to generate PNA1-QMP1 and its self adduct. All the conjugates were successfully purified by HPLC and characterized by MALDI (Appendix).



Scheme 3.13 Structures of PNA6-QMP1 and PNA6-QMP2

3.2.9 Alkylation of duplex DNA by PNA6-QMP1 and PNA6-QMP2.

We chose two duplex DNAs, OD17-OD18 and OD19-OD20 as targets to study the target sequence alkylation (**Scheme 3.12**). Both dsDNAs have the same 11-base recognition site to PNA6. OD19-OD20 is from the natural NFKB1 gene and has an adenine base adjunct to recognition site. Since guanine N7 was found to be a primary alkylation site of QMP1[121], the guanine near the triplex binding site may boost the yield of DNA alkylation. Target dsDNA OD17-OD18 has a guanine base at the adjunct of PNA6 binding site was also studied (**Scheme 3.11**). PNA6-QMP1, PNA6-QMP2 and their self adducts were incubated with target [^{32}P]-OD17-OD18 and [^{32}P]-OD19-OD20 for 4 days at either room temperature or 37°C (**Figure 3.9**).

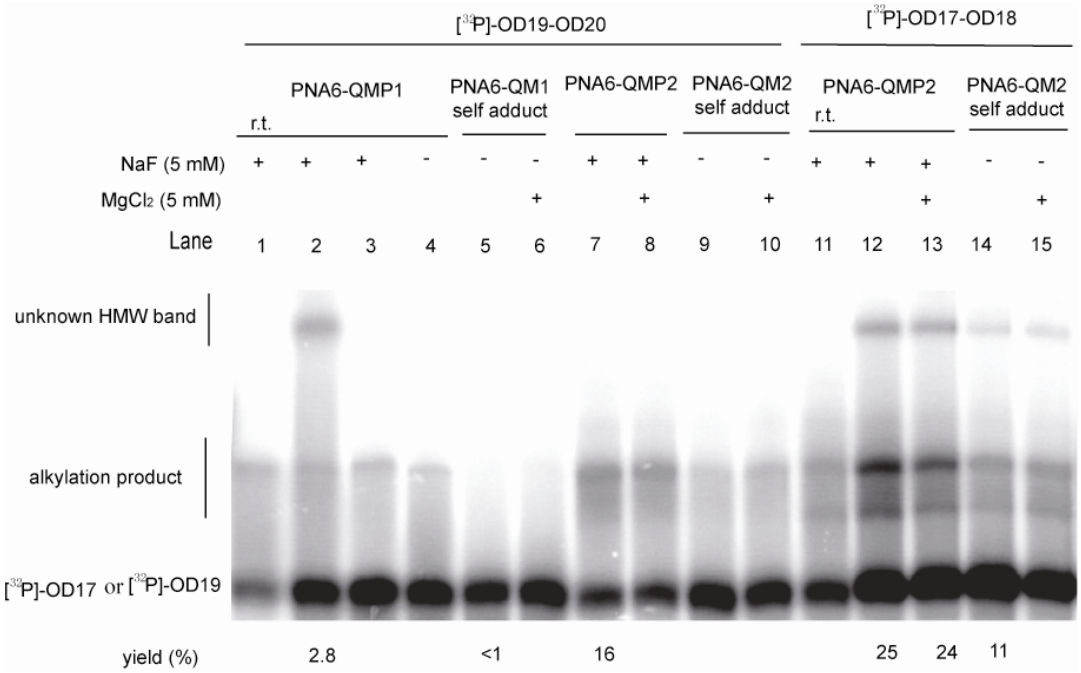


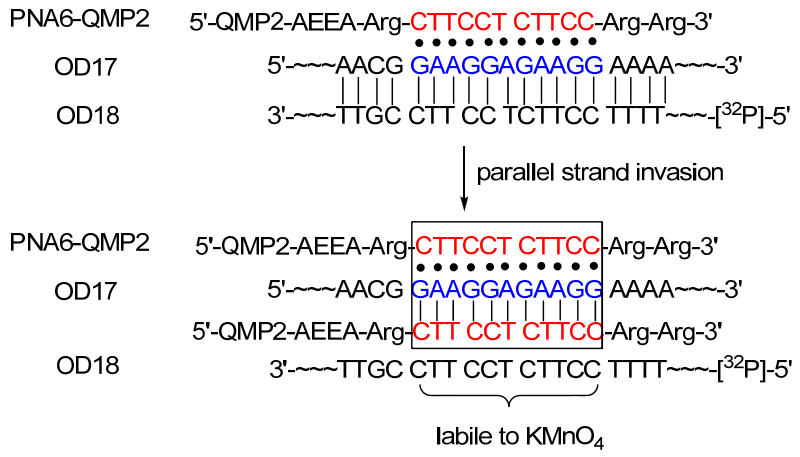
Figure 3.9 Alkylation of dsDNA by PNA6-QMP1 and PNA6-QMP2 conjugates. PNA6 conjugates (5 μM) and dsDNA (50 nM) were incubated in 160 mM NaCl and 10 mM $NaHPO_4$ (pH 6.0) at 37°C for 4 days except lane 1 and 11 were incubated at room temperature for 4 days.

As expected, PNA6/DNA complexes can be denatured by gel analysis (**Figure 3.9**, lane 5 & 6). PNA6-QM1 self adduct did not alkylate target DNA as evident by the lack of low mobility products (lane 5, 6, 9 & 10). Almost no alkylation products by PNA6-QMP1 were generated at either room temperature or 37°C (lane 1, 2). In contrast, PNA6-QMP2 provided alkylation bands (**Figure 3.9**, lane 7, 8). When a guanine is adjacent to the binding site of [^{32}P]-OD17-OD18, [^{32}P]-OD17 was alkylated in ~25% yield (**Figure 3.9**, lane 12 - 13). Although the PNA6-QM2 self adduct can also alkylate [^{32}P]-OD17-OD18, the yield is less than PNA6-QMP2 (11% vs 25%, lane 14 *vs.* lane 12). Also, unlike the requirement for generating DNA triplex, PNA/DNA_2 triplex does not need $MgCl_2$ to relieve repulsion between

backbones as is necessary for DNA·DNA_2 triplex.[114] DNA alkylation was independent of $MgCl_2$ (lane 12 *vs.* lane 13).

Two reasons might explain the higher alkylation yield from QMP2 than QMP1. First, generation of QM2 and the alkylation of DNA by QM2 are quicker than QM1. The quick generation of PNA6-QM2-DNA adduct will stabilize the triplex structure. Secondly, the linker locates at the *para*- position of TBDMS in QM1 while the link in QM2 locates at the *meta*- position of TBDMS. Oxygen at the *meta*- position can form resonance structure and stabilize QM2.[45]

$KMnO_4$ foot-printing was used to probe the strand displacement after triplex invasion (**Figure 3.10**). After PNA6-QMP2 (250 nM) was incubated with OD17-[^{32}P]-OD18 (50 nM) in buffer at room temperature for 4 days, the mixture was subjected to $KMnO_4$ treatment. The resulting cleavage caused by the displacement of thymine from OD18 showed that strand invasion occurred. Considering PNA6 has only 5 bases that are complementary to OD17, Tm of 5 bases complementary PNA/DNA is not high enough to support triplex invasion. PNA6 likely generates a parallel PNA/DNA duplex with OD17 but not traditional anti-parallel PNA/DNA duplex (**Scheme 3.14**). This type of structure has been mentioned by Nielsen group before.[107]



Scheme 3.14 Parallel strand invasion by PNA6-QMP2

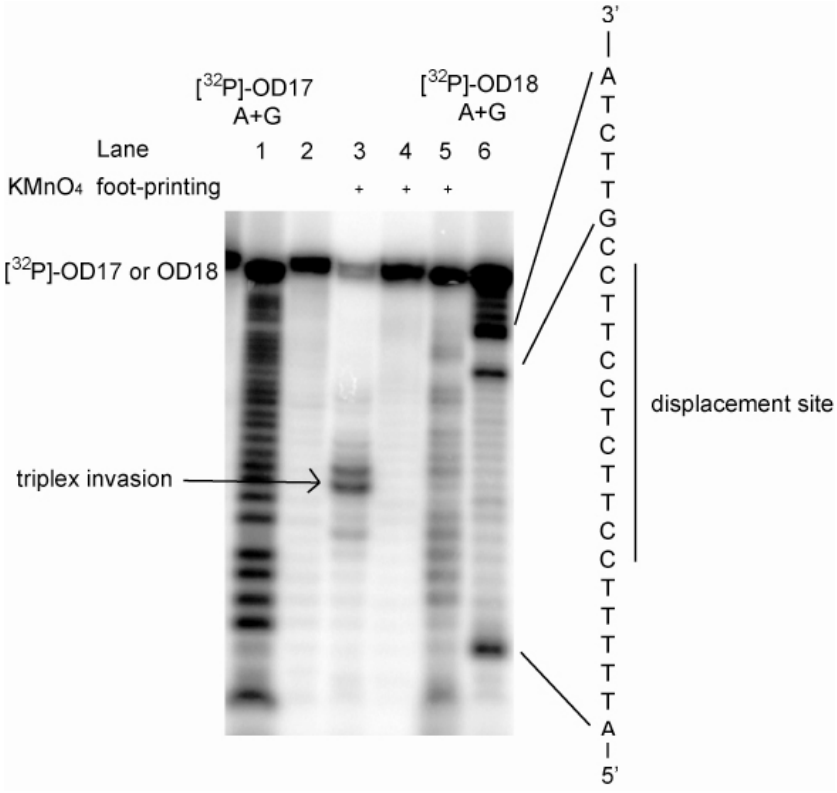


Figure 3.10 Foot-printing of displacement of OD18 to check for strand invasion. [^{32}P]-OD17 A+G ladder (lane 1); PNA6-QMP2 (0.25 μM) was incubated with [^{32}P]-OD17-OD18 (50 nM) in 160 mM NaCl, 10 mM $NaHPO_4$ (pH 6) and NaF (5 mM) at room temperature for 4 days and then was treated with piperidine (lane 2); OD17-[^{32}P]-OD18 (50 nM) was incubated with PNA6-QMP2 (0.25 μM) (lane 3) or without PNA6-QMP2 (lane 4) in 160 mM NaCl, 10 mM $NaHPO_4$ (pH 6) and 5 mM NaF at room temperature for 4 days, samples were probed by $KMnO_4$ foot-printing; $KMnO_4$ treatment of [^{32}P]-OD18 (lane 5); [^{32}P]-OD18 A+G ladder (lane 6).

3.2.10 Time dependence of dsDNA alkylation by PNA6-QMP2

The time dependence of PNA6-QMP2 reaction was measured to determine the rate of dsDNA alkylation (**Figure 3.11**). Neither the PNA6-QM2 self adduct nor PNA6-QMP1 alkylated dsDNA (**Figure 3.11**, lane 1 & 2). 50 equivalent of PNA6-QMP2 alkylated dsDNA at 37°C. 48 hours are required to reach a maximum alkylation yield of ~27% (**Figure 3.11**, lane 7). Multiple slowly migrating species were found on denaturing gel suggesting that alkylation may have occurred on several alkylation sites (**Figure 3.11**). This can be explained by the several binding patterns. PNA6-QMP2 and DNA can generate both PNA/DNA_2 (triplex) and PNA/DNA/PNA (parallel strand invasion) as shown in **Scheme 3.14**.

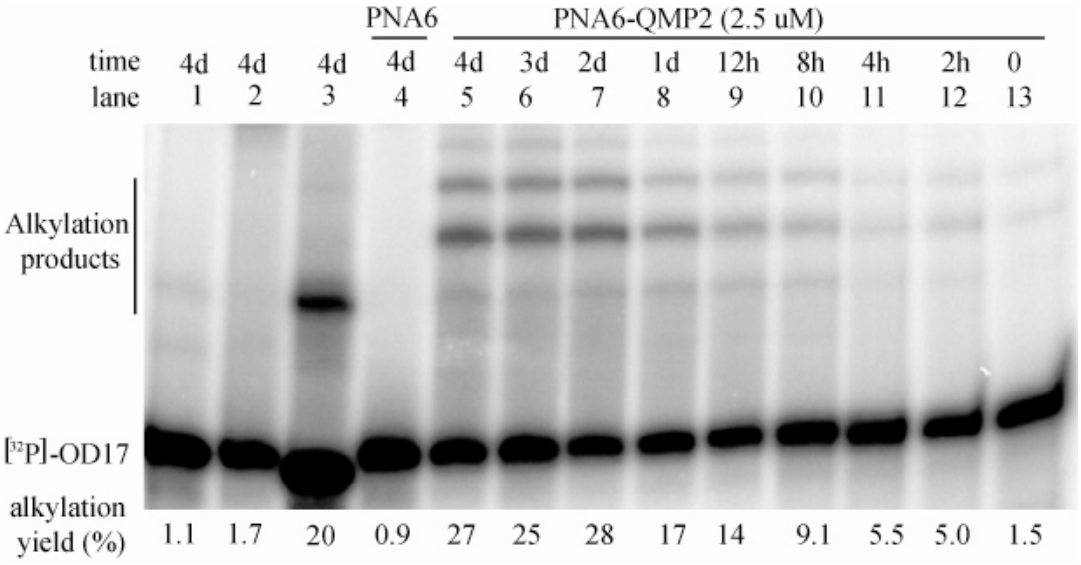


Figure 3.11 Alkylation of dsDNA by PNA6-QMP2. [^{32}P]-OD17 (2.2 μM) was incubated with PNA6-QMP2 (2.5 μM) in 10 mM sodium phosphate (pH 6), 50 mM NaCl and 100 mM NaF at 37°C for 4 days (lane 3). [^{32}P]-OD17-OD18 duplex (50 nM) was incubated in same buffer as used in lane 3 at 37°C for 4 days with PNA6-QM2 self adduct (2.5 μM) (lane 1); PNA6-QMP1 (2.5 μM) (lane 2); PNA6 (2.6 μM) (lane 4). [^{32}P]-OD17-OD18 duplex (50 nM) was incubated in buffer at 37°C for 0 - 4 days with PNA6-QMP2 (2.5 μM) (lane 5 - 13). Final mixtures were mixed with formamide and loaded onto the 20% denaturing gel directly.

3.2.11 Effect of a single-base mismatch on dsDNA alkylation by PNA6-QMP2

Selective delivery of QMP2 to target sequences by PNA requires discrimination of its target dsDNA effectively from off-target DNAs. One base mismatch at either the end or middle of the binding site on duplex DNA target was used to test the specificity of PNA6 (**Scheme 3.15**). Both the OD17E-OD18E and OD17M-OD18M duplex have the same triplex recognition site as OD17-OD18 except each duplex DNA present one C/T or T/T mismatch of Hoogsteen pairing (**Figure 3.12**).

Scheme 3.15 PNA6-QMP2 and the sequences of its targets containing one mismatch

When ssDNA was incubated with PNA6-QMP2 in the presence of fluoride, the ssDNA can be alkylated with a yield ranging from 20% to 65% (**Figure 3.12B** lane 4 - 6). Multiple bands generated with excess PNA-QMP suggested that that multiple PNA/DNA alkylations could be formed during reaction. Fully matched OD17 (parallel match, not complementary) was alkyated with the greatest yield (65%) and similar to that of the fully complementary ssDNA by PNA1-QMP1 (~70%). The

presence of a mismatch at the end of binding site (OD17E) only slightly decreased the alkylation yield (55%). Although the single strand OD17M could be alkylated by PNA6-QMP2, its yield of 20% is significantly lower than that of OD17 (65%). These results showed that the place of the mismatch inside the triplex recognition site greatly affects the alkylation yield despite the alkylation of all the single strands.

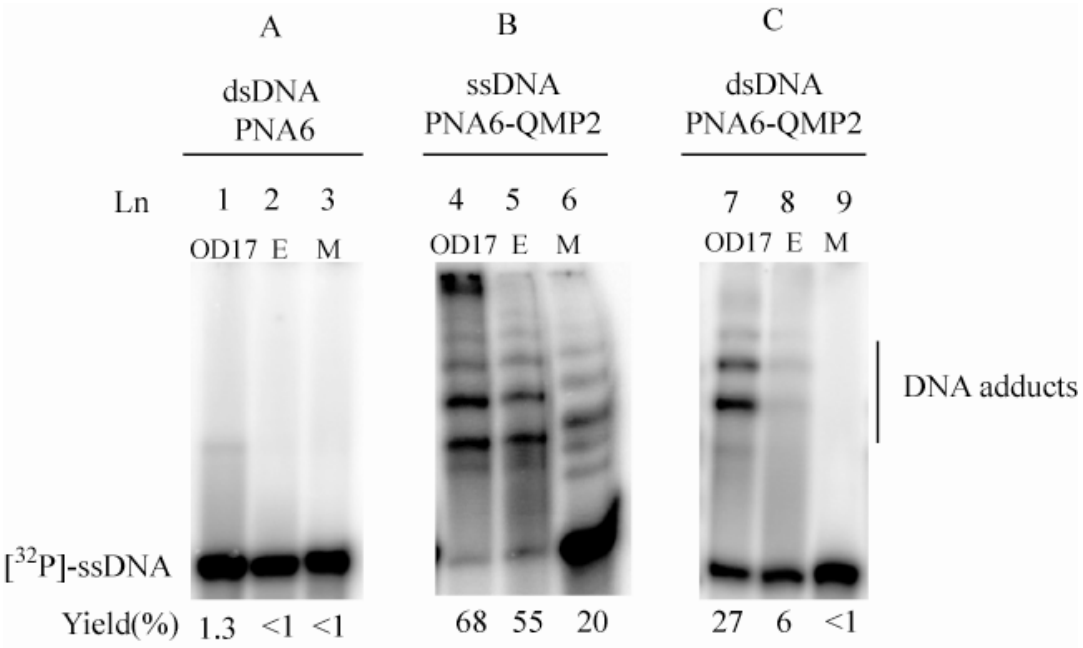


Figure 3.12 Alkylation of ssDNA and dsDNA by PNA6-QMP2. E means OD17E, M means OD17M. (A) duplex DNA (50 nM) (lane 1: OD17-OD18; lane 2: OD17E-OD18E or lane 3: OD17M-OD18M) was incubated with PNA6 (2.5 μM) in buffer (50 mM NaCl, 100 mM NaF and 10 mM $NaHPO_4$ pH 6.0) at 37°C for 3 days. (B) ssDNA (0.2 μM) (lane 4: OD17; lane 5: OD17E or lane 6: OD17M) was incubated with PNA6-QMP2 (10 μM) in buffer (50 mM NaCl, 100 mM NaF and 10 mM $NaHPO_4$ pH 6.0) at 37°C for 3 days. (C) duplex DNA (50 nM) (lane 7: OD17-OD18; lane 8: OD17E-OD18E or lane 9: OD17M-OD18M) was incubated with PNA6-QMP2 (2.5 μM) in buffer (50 mM NaCl, 100 mM NaF and 10 mM $NaHPO_4$ pH 6.0) at 37°C for 3 days.

The effect of a single base mismatch is more obvious in alkylation of dsDNA than the alkylation of ssDNA by PNA6-QMP2 (lane 7 - 9). Under conditions that supported alkylation, 27% of fully matched OD17-OD18 duplex was alkylated

compared to a 6% of alkylation yield with a C/T mismatch at the end of triplex recognition site (lane 8). When a mismatch was present in the middle of the triplex recognition site, almost no target DNA alkylation was detected (lane 9). These data showed that the third strand PNA6 is very sensitive to its recognition site, even one base mismatch greatly surpress the formation of triplex. The sensitivity of this system should make the PNA useful in delivering QMP2 to target sequences in cells in the future.

3.3 Summary

Target alkylation of dsDNA should help the use of PNA-QMP under cell conditions in the future. In a 14 base polypyrimidine PNA sequence containing only 3 cytosines (PNA3), PNA3-QMP1 conjugates decomposed after eight months at -20°C. Thermodynamically stable triplex invasion products are the final products when polypyrimidine PNA was incubated with dsDNA. Complementary polypyridmine PNA/DNA which has more than 8 Watson-Crick bases can not be denatured by denaturing conditions used by gel electrophoresis. When eight to nine bases PNA were used to deliver QMP to target dsDNA, no target dsDNA alkylation was observed by gel. The failure of alkylation was possibly due to the poor binding of PNA to dsDNA.

Successful delivery of an electron-rich QMP2 by PNA6 was detected by gel. PNA6 contained 11 bases to form Hoogsteen bonds with its target dsDNA and 5 bases within PNA6 can generate Watson-Crick bonds with the polypurine strand of DNA. This PNA/DNA complex was successfully denatured by gel electrophoresis.

Interestingly, this PNA6 can still invade into dsDNA and form a parallel PNA/DNA structure. Only the electron rich QMP2 conjugated with PNA6 could alkylate target dsDNA (~25%). Traditional QMP1 conjugated with PNA6 showed little alkylation (< 10%) toward dsDNA under the same conditions. The successfully alkylation of dsDNA by PNA6-QMP2 could be applied to the cell environment where a recognition site exists.

3.4 Materials and methods

Materials Materials used in this chapter is the same as those used in chapter two. QMP2 was synthesized by Chengyun Huang in Rokita lab.

PNA preparation: PNAs were synthesized following standard solid phase peptide synthesis techniques described in chapter two. PNAs were purified by reverse phase HPLC (RP-HPLC) with a C18 column (Varian Microsorb-MV, 300Å pore, 250 mm) at 1 ml/min flow rate with a gradient of the organic phase increasing from 10% to 55% in 30 minutes. PNAs were characterized by MALDI-TOF and ESI-MS (**Table 3.1**).

Name	Calculated mass (*m/z*)	Found (*m/z*)
PNA3	4755.2 (MALDI)	4753.4
PNA4	2589.7 (MALDI)	2590.6
$[PNA5+3H^+]^{3+}$	956.0 (ESI)	956.2
PNA6	3469.9 (MALDI)	3471.5

Table 3.1 Mass spectral analysis of PNA3 – PNA6

Preparation of PNA and QMP1 or QMP2 conjugate: Procedures for conjugating PNAs and QMPs were the same as those described in chapter two. Conjugates were purified by HPLC and confirmed by MALDI.

Preparation of PNA-QM or PNA-QM2 self adduct: **PNA-QMP1** (**Table 3.2**). Procedures were the same as used in chapter two. The self adducts were purified by HPLC and confirmed by MALDI.

Name (precursor)	Calc. (*m/z*)	Found (*m/z*)	Name (self adduct)	Calc. (*m/z*)	Found (*m/z*)
PNA3-QMP1	5090.7	5091.8	PNA3-QM	4916.4	4918.4
PNA4-QMP1	2924.2	2925.0	PNA4-QM	2749.9	2747.9
PNA5-QMP1	3199.5	3198.0	PNA5-QM	3025.2	3024.0
PNA6-QMP1	3805.4	3805.6	PNA6-QM	3631.1	3630.2
PNA6-QMP2	3809.4	3808.8	PNA6-yQM	3635.1	3636.3

Table 3.2 Mass spectral analysis of PNA-QMP conjugates and PNA-QM self adducts

Target alkylation of DNA

ssDNA (2.2 μM) or dsDNA (50 nM) was mixed with PNA-QMP1 in solution of NaCl (50 mM), NaF (100 mM) and $NaHPO_4$ (10 mM, pH 6.0) for the indicated time at room temperature. When ssDNA (2.2 μM) was incubated with PNA-QMP2 (2.5 μM) under the same buffer conditions mentioned above, reactions were carried out at 37°C. When the self-adduct (3.3 μM) was used to alkylate ssDNA (3.0 μM), the reaction contained only NaCl and $NaHPO_4$ (pH 6). Reaction (5 μL) was stopped by adding of 10 μL formamide (0.05% bromophenol blue and 0.05% xylene cyanol

FF in formamide). Products were analyzed by 20% denaturing polyacrylamide gel electrophoresis (PAGE) using 7 M urea, 89 mM Tris-borate pH 8, and 5 mM EDTA. A temperature of 50 – 55°C was maintained during electrophoresis. Gels were visualized by a Molecular Dynimics Phosphorimager (Sunnyvale, CA). Yield was calculated by dividing the radio activity of slow mobility bands with the overall radio activity of that line on gel.

Phenol-chloroform extraction

Reaction samples (5 μL) were diluted to 100 μL by H_2O and 100 μL phenol extraction solution (phenol/chloroform/isoamyl alcohol = 25/24/1) was added to the reaction sample. The resulting mixture was vortexed for thirty seconds and was centrifuged at 16100 g for 5 minutes. The organic solution (bottom layer) was carefully removed by pipette. After repeating the extraction for 2 – 4 time, the DNA in aqueous phase was dried by Speed-Vac and ready for future use.

Piperidine Cleavage[118]

Reaction samples (10 μL) were suspended in 50 μL of 10% v/v piperidine and incubated at 90°C for 30 minutes. After incubation, the samples were dried in a Speed-Vac for 30 minutes. The dried samples were dissolved with 50 μL water and dried in Speed-Vac again for 30 minutes to remove the residual piperidine.

$KMnO_4$ Foot printing

Reaction samples (10 μL, containing PNA-QMP and dsDNA) in buffer (10 mM $NaHPO_4$ pH 6, 10 – 150 mM NaCl, 5 – 100 mM NaF) were mixed with 2 μL $KMnO_4$ (20 mM) for 1 minute at room temperature, and the reactions were quenched by adding 2 μL 2-mercaptoethanol. The resulting solutions were dried by Speed-Vac and subjected to piperidine treatment.

Maxam A+G lane sequencing reaction (DNA A+G ladder)

[^{32}P]-DNA (1 μL) and 2 μL calf thymus DNA (1 mg/ml) were mixed with 4 μL formic acid (10% v/v) and incubated at 60°C for 10 minutes. The samples were then dried by Speed-Vac and treated with piperidine.

Chapter 4 Conclusions

Alkylation of target DNA is essential to improve the drug selectivity in tumor cells and reduce their side effects. Previously, alkylation of target DNA by a oligonucleotide – quinone methide precursor (QMP) conjugate and its self adduct was achieved *in vitro*.[37] Generally, the requirement of UV, heat or chemicals to initiate alkylation restricts the use of DNA alkylating agents in cell environment. The reversibility of the oligonucleotide-QM self adduct supports potentially use *in vivo* since no additional trigger is needed to initiate target alkylation. Oligonucleotides as site-directing agents have limitations due to their lack of stability *in vivo* and cellular penetration.

This dissertation has focused on studying delivery of QMP to single and double strand DNA by peptide nucleic acids (PNAs). PNAs are resist to nucleases *in vivo* and can pass through cellular membrane when conjugated with positive peptides. These properties make PNA superior to DNA as delivery agent. PNA can bind with it complementary DNA or RNA with Watson-Crick base pairing rules. PNA-QM self adduct is expected to be reversible since PNA has the same bases as DNA, thus PNA could provide *in vivo* alkylation from a self adduct as DNA had acted *in vitro*. The reversibility, stability and potential cell penetrating ability make PNA a good target directing agent to deliver QMP in cells.

A single strand DNA can be alkylated by PNA-QMP with ~70% alkylation yield while no alkylation occurred when PNA-QMP was incubated with off-target sequences. When PNA-QMP was incubated with its complementary DNA and an

one base mismatch sequences, the conjugate showed strong selectivity toward its complementary sequence.

To deliver QMP to target DNA in cells, toxic fluoride can't be used to initiate the alkylation. PNA-QM self adduct can avoid the use of fluoride when the self adduct is reversible. The reversibility of a PNA-QM self adduct was confirmed by incubating the self adduct with target single strand DNA. The self adduct was able to alkylate its complementary DNA with a ~40% alkylation yield. After 7 days pre-incubation in aqueous solution, only half of reversible self adduct was quenched by irreversible reaction and another half was still able to alkylate its target sequence.

For the first time, successful target alkylation of RNA by PNA-QMP and its self adduct were shown. Alkylation of RNA makes PNA-QM self adduct potentially applicable in alkylating RNA in telomerase and inhibiting telomerase activity to kill tumor cells. TRAP assay showed that both PNA and PNA-QM self adduct can inhibit telomerase activity. Although the inhibition of telomerase activity from PNA binding with RNA in telomerase can not be differentiate from inhibition induced by RNA alkylation, at least PNA can effectively deliver QMP to a target sequence within telomerase.

Polypyrimidine PNA was also used to deliver QMP to duplex DNA (dsDNA) by forming triplex structures. Ten to fourteen bases polypyrimidine PNAs were shown to bind with their target DNA and form triplex. After the formation of $PNA{\cdot}DNA_2$ triplex, PNAs continue to invade into dsDNA and form triplex invasion structure. Once triplex invasion formed, PNA/DNA duplex has more than 10

complementary bases can not be denatured on and the target DNA alkylation can not be detected by denaturing gel.

An eleven base polypyrimidine PNA6 which has six cytosines was able to deliver an electron-rich QMP2 to target dsDNA. The cytosine rich PNA6 showed target selectivity toward dsDNA. A single mismatch in the target dsDNA can totally inhibit its alkylation. The high sensitivity of PNA toward dsDNA makes PNA a good directing agent to deliver QMP2 *in vivo*.

This dissertation studied the requirements of PNA to successful delivery of QMP to target ssDNA, ssRNA and dsDNA. A mixed base PNA can target ssDNA and ssRNA by forming duplex while polypyrimidine PNA is required to form a triplex structure for recognition of a target dsDNA. Furthermore, the requirement of an electron-rich QMP2 has been shown in alkylating dsDNA. From these results, corresponding PNA sequences can be designed to target different DNA or RNA. The PNA-QMP conjugate is now ready for the ultimate *in vivo* test.

Appendix

Instructions for the following tables: These sheets mark each step of the sequence--giving the reagents used and the times required. Working down a column, each block can be checked off as that particular step is completed. When a column is finished, goes to the top of next column.

resin 100 mg (0.7 mmol/g, 70 μmol) was swelled in DMF for three hours								
3-GlyLeuSerAlaArgArgLeu-PNA-Arg-QMP								
AA monomers	G	Capping	L	S	A	R	R	L
95% TFA/ m-cresol (2 × 4 min)	×	×						
DCM wash (3 × 0.5 min)	×	×						
Kaiser test		×						
DMF wash (1 × 1 min)		×						
DCM wash (2 × 0.5 min)		×						
Coupling (20 min)		Capping						
DMF wash (2 × 1 min)								
DCM wash (2 × 1 min)								
Kaiser test								
Next column								

Table 1. Procedures for peptide synthesis.

Resin-kemptide 60 mg (0.7 mmol/g, 42 μmol) was swelled in DMF for three hours														
3-GlyLeuSerAlaArgArgLeu-GATTGGGATTG-Arg-AEEA-NH_2-5’														
PNA monomers	G	Capping	A	T	T	G	G	**G**	G	**G**	A	T	T	G
95% TFA/ m-cresol (2 × 4 min)		×						×		×				
DCM wash (2 × 0.5 min)		×						×		×				
NMP wash (1 × 0.5 min)		×						×		×				
DCM wash (2 × 0.5 min)		×						×		×				
Kaiser test		×								×				
DMF wash (1 × 1 min)		×						×						
DCM wash (2 × 0.5 min)		×						×		×				
Coupling (20 min)		Capping												
DMF wash (2 × 1 min)														
DCM wash (2 × 1 min)														
Kaiser test	×													
Next column														

Table 2. Procedures for PNA synthesis. When a continuous “G” was synthesized, a second time coupling was performed to ensure the coupling efficiency (blue in PNA monomers).

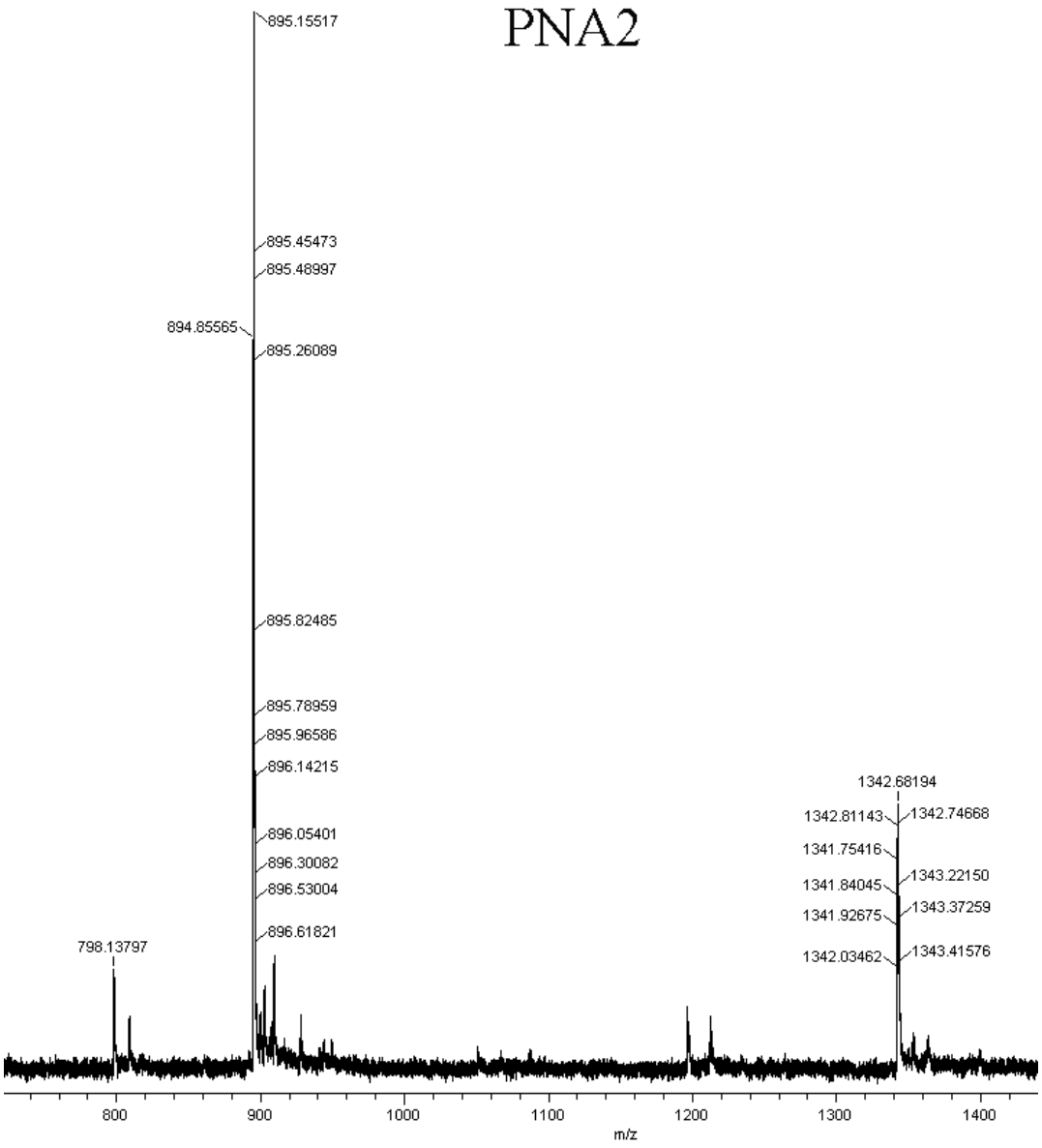


Figure 1. ESI-MS of PNA2. $[PNA2+3H^{+}]^{3-}$ calculated (*m/z*) was 895.3 and ESI-MS (*m/z*) found was 895.2. Calculated $[PNA2+2H^{+}]^{2-}$ was 1342.5 and ESI-MS (*m/z*) found was 1342.7.

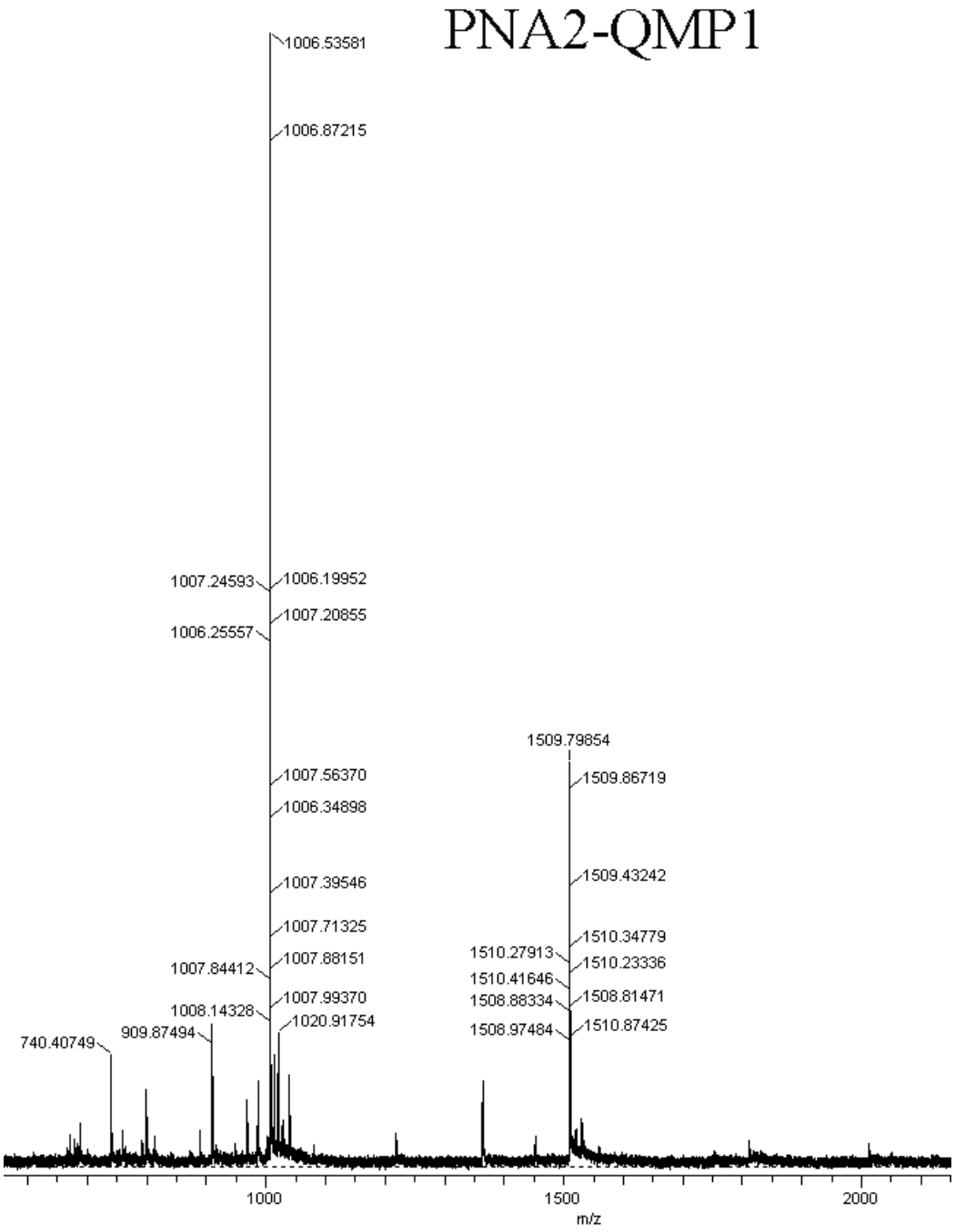


Figure 2. ESI-MS of PNA2. $[PNA2\text{-}QMP1+3H^{+}]^{3-}$ calculated (*m/z*) was 1007.1 and ESI-MS found (*m/z*) was 1006.5. $[PNA2\text{-}QMP1+2H^{+}]^{3-}$ calculated (*m/z*) was 1510.2 and ESI-MS found (*m/z*) was 1509.8.

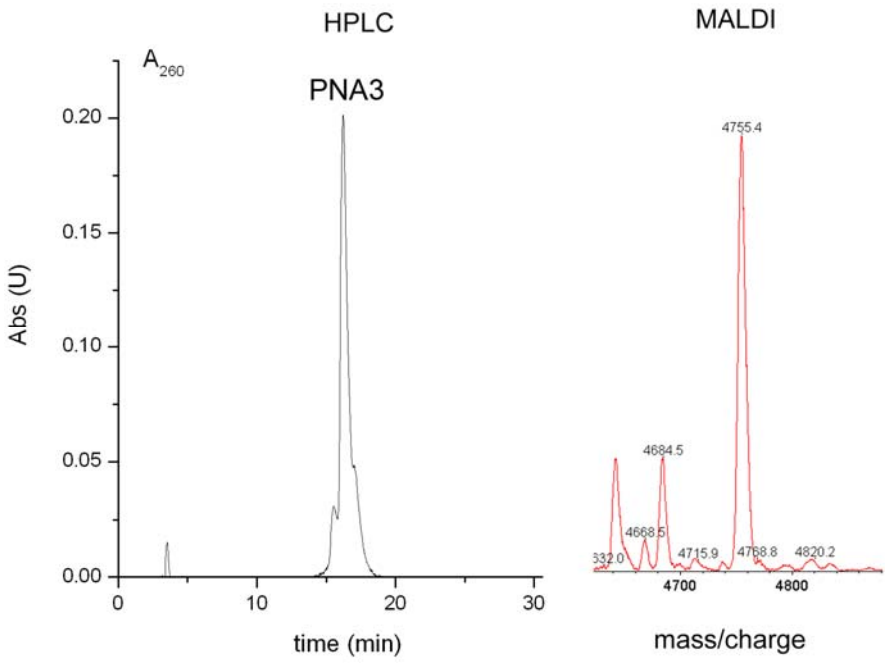


Figure 3. HPLC and MALDI spectrum of PNA3. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% trifluoroacetic acid (TFA) at 1 ml/min over 30 minutes, 16 – 18 minute eluent was collect, lyophilized and characterized by MALDI. PNA3 calculated (*m/z*) is 4755.2 and MALDI found (*m/z*) was 4755.4

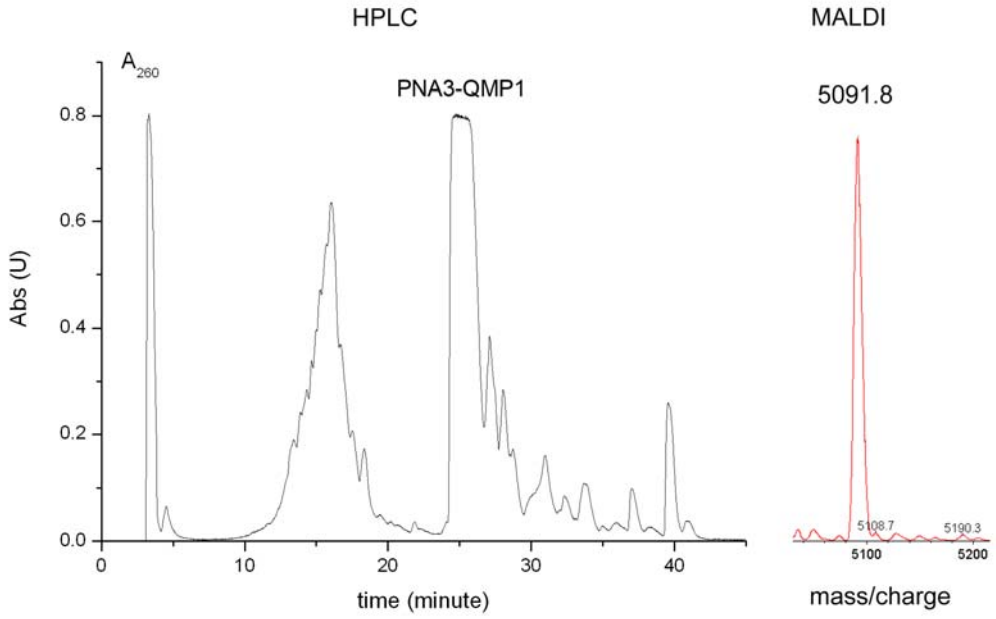


Figure 4. HPLC and MALDI spectrum of PNA3-QMP1. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min. The 26 - 28 minutes eluents from HPLC was collected and examined by MALDI. PNA3-QMP1 calculated (*m/z*) is 5090.7 and MALDI found (*m/z*) was 5091.8.

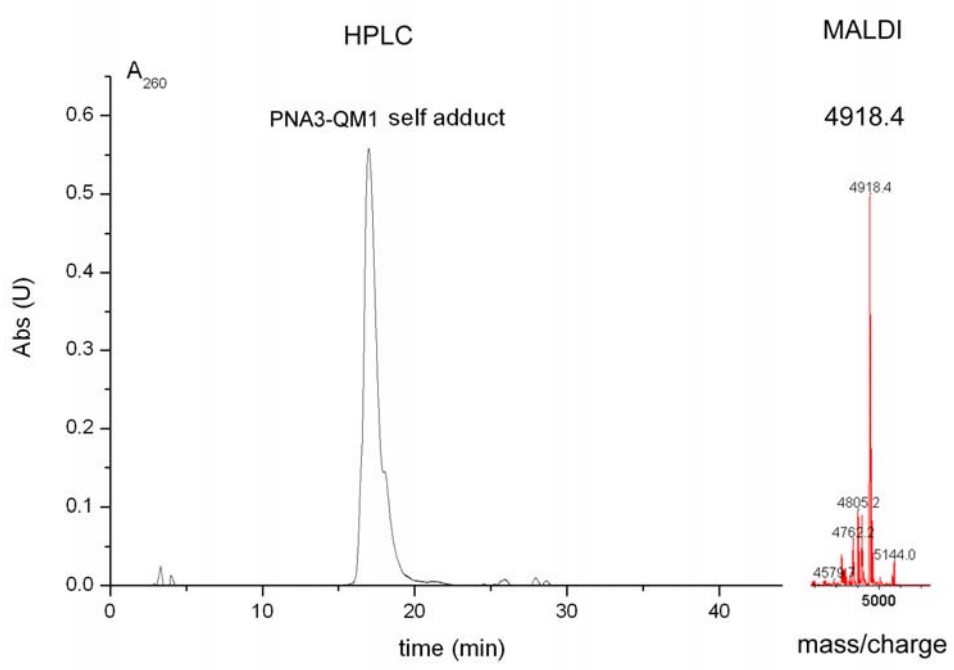


Figure 5. HPLC and MALDI spectrum of PNA3-QM1 self adduct. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min. The 16 - 20 minutes eluents from HPLC was collected and examined by MALDI. PNA3-QM1 calculated (*m/z*) is 4916.4 and MALDI found (*m/z*) was 4918.4.

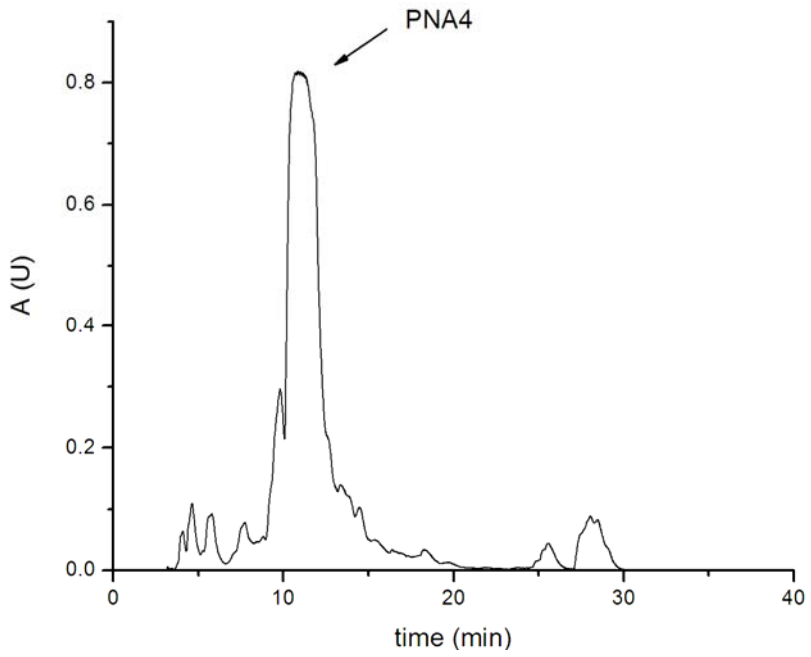


Figure 6. HPLC spectrum of PNA4. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

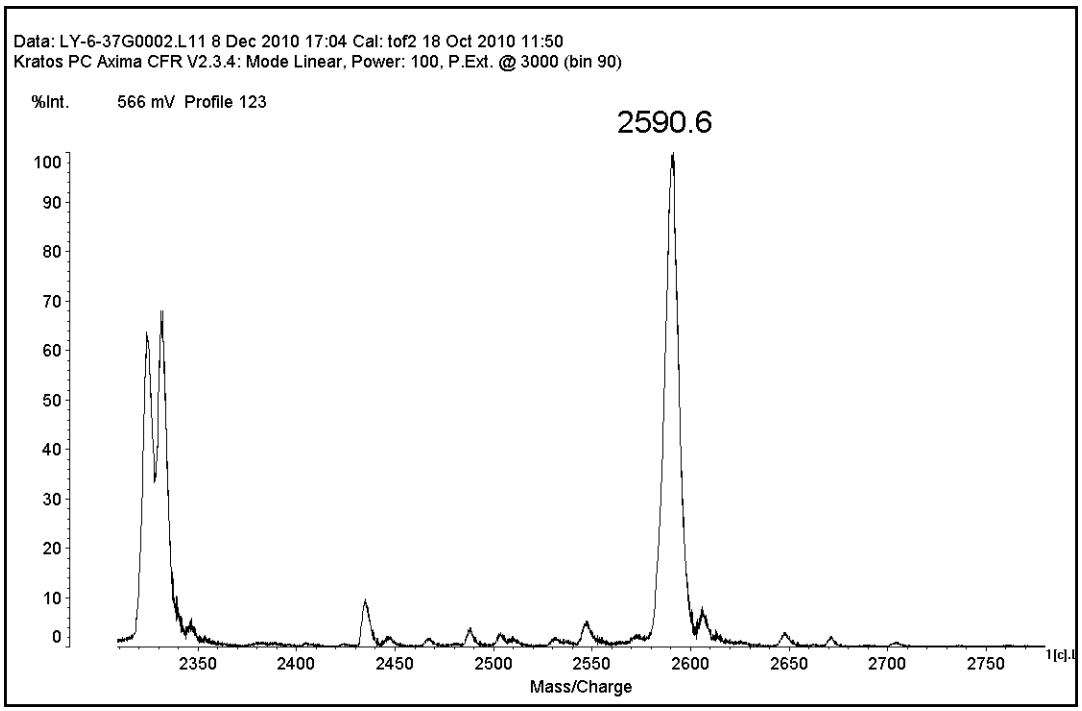


Figure 7. MALDI spectrum of PNA4. PNA4 calculated (*m/z*) is 2589.7 and MALDI found (*m/z*) was 2590.6.

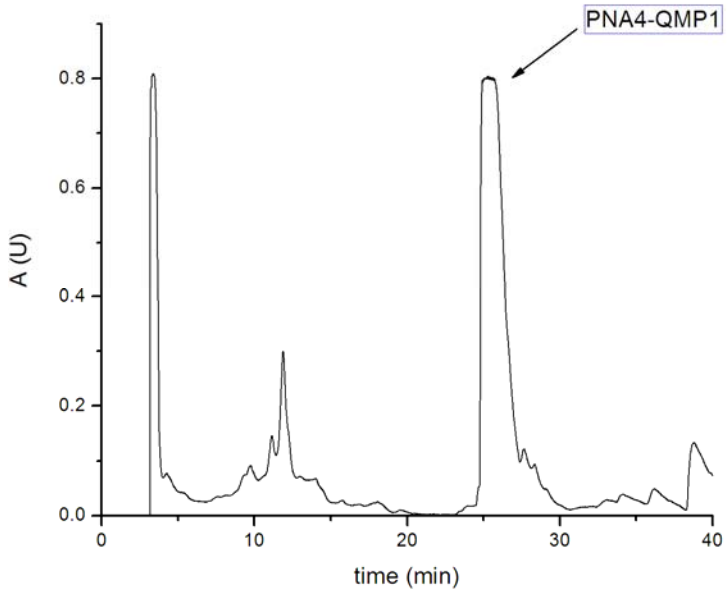


Figure 8. HPLC spectrum of PNA4-QMP1. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

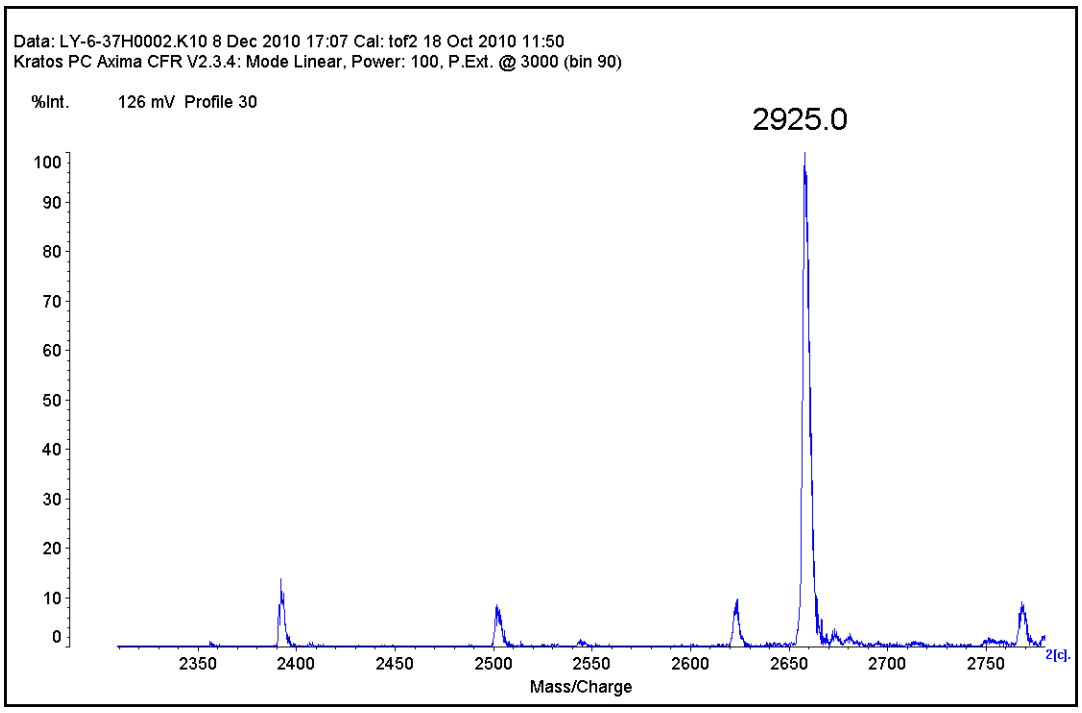


Figure 9. MALDI spectrum of PNA4-QMP1. PNA4-QMP1 calculated (*m/z*) is 2924.2 and MALDI found (*m/z*) was 2925.0.

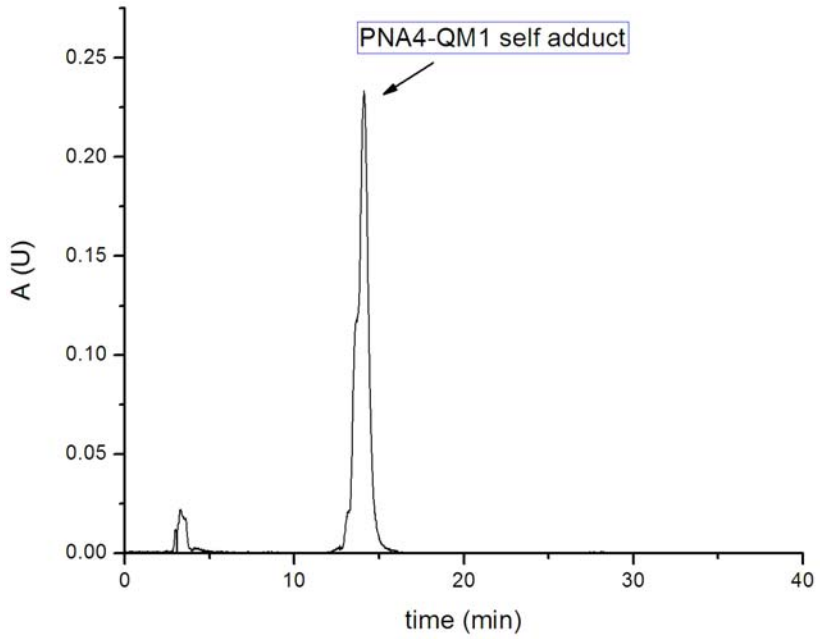


Figure 10. HPLC spectrum of PNA4-QM1 self adduct. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

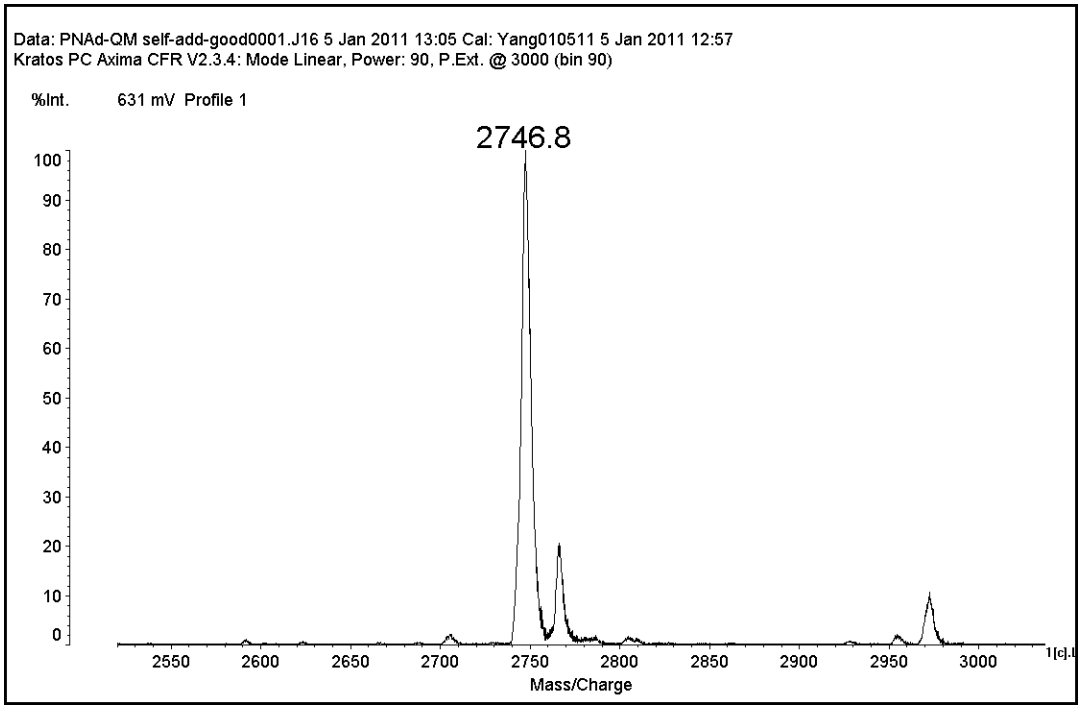


Figure 11. MALDI spectrum of PNA4-QM1 self adduct. PNA4-QM1 self adduct calculated (*m/*z) is 2749.9 and MALDI found (*m/*z) was 2746.8.

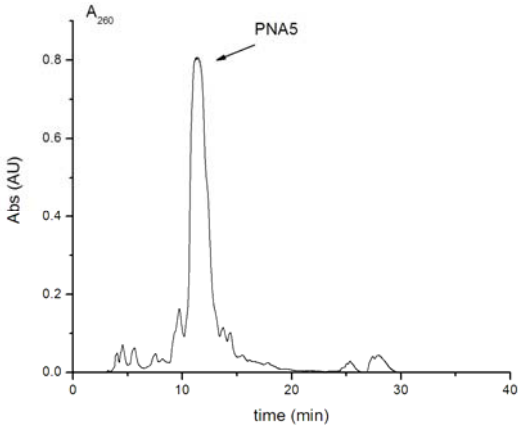


Figure 12. HPLC spectrum of PNA5. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

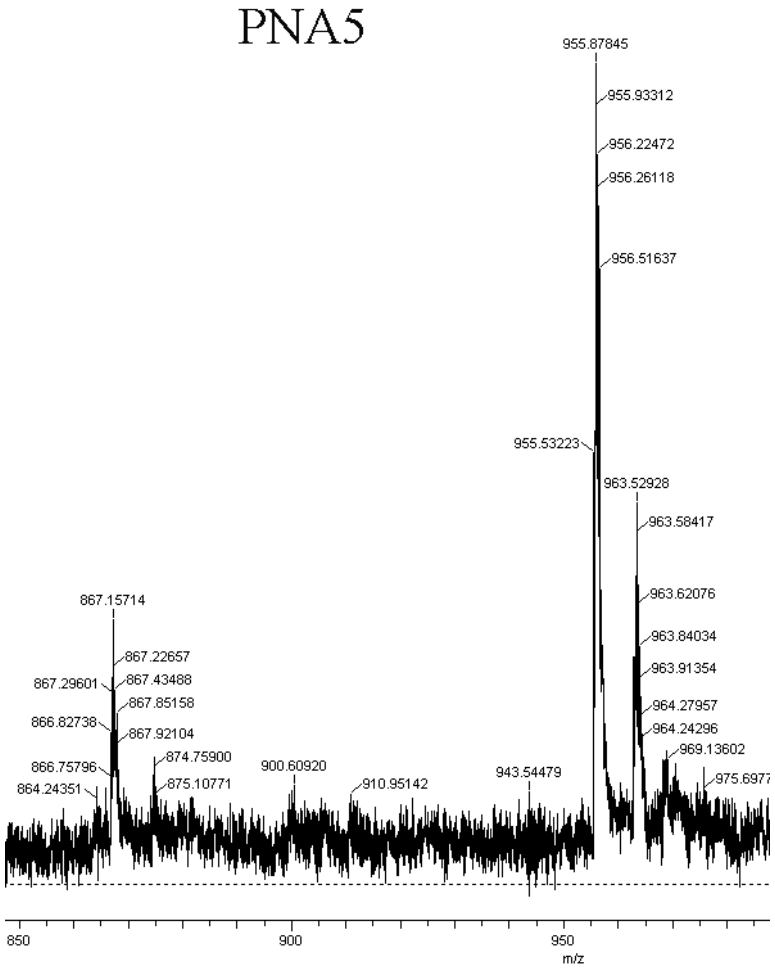


Figure 13. ESI-MS of PNA5. $[PNA5\text{-}QMP1+3H^{+}]^{3-}$ calculated (*m/z*) was 956.0 and ESI-MS found (*m/z*) was 956.2.

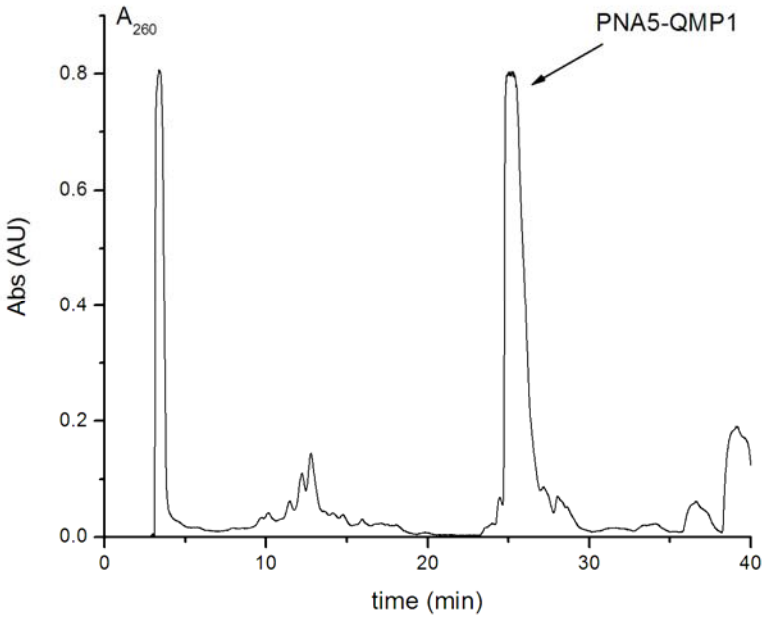


Figure 14. HPLC spectrum of PNA5-QMP1. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

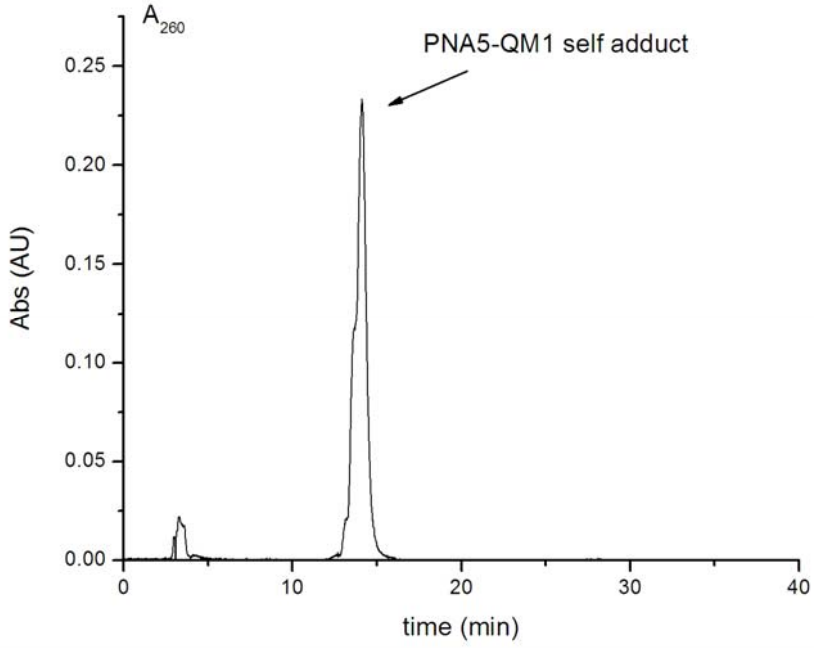


Figure 15. HPLC spectrum of PNA5-QM1 self adduct. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

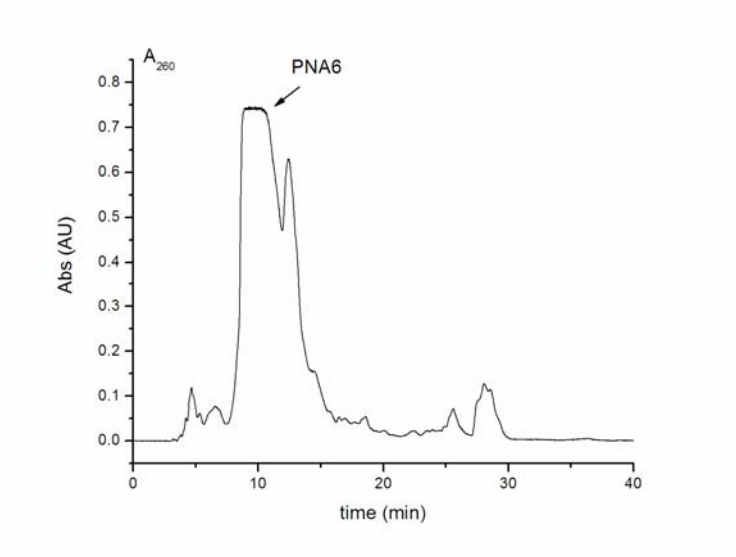


Figure 16. HPLC spectrum of PNA6. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

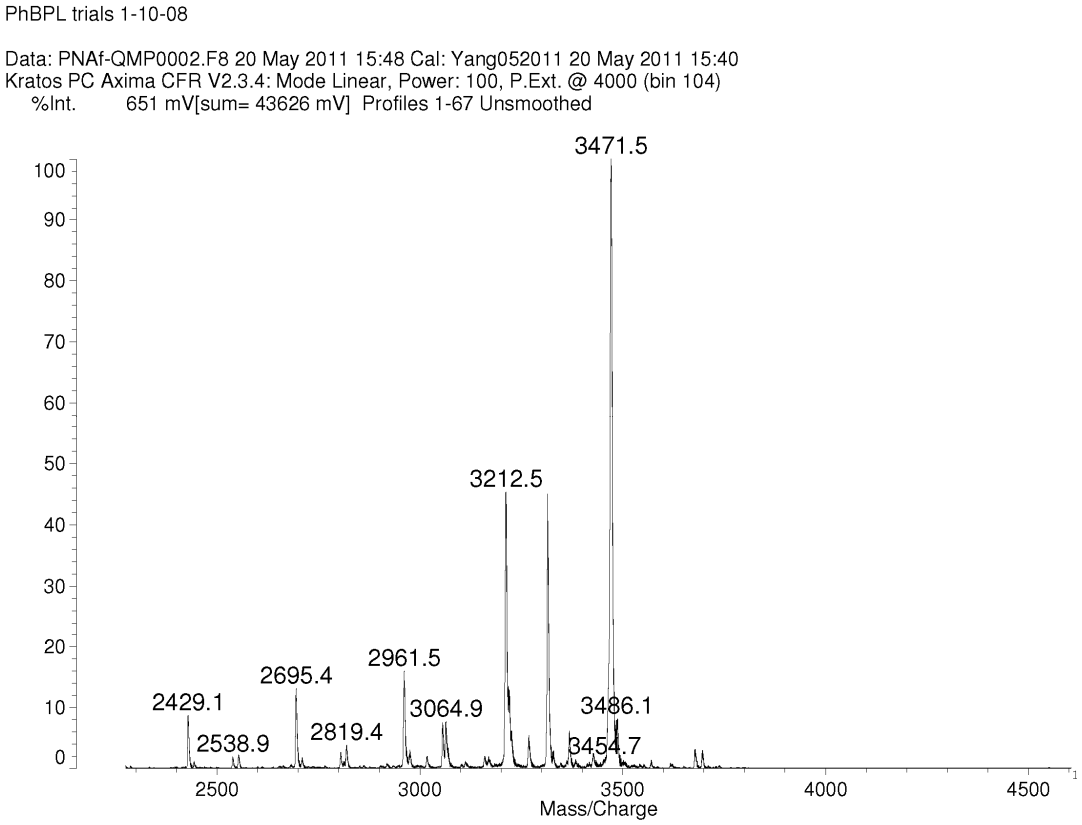


Figure 17. MALDI spectrum of PNA6. PNA6 calculated (*m/z*) is 3469.9 and MALDI found (*m/z*) was 3471.5.

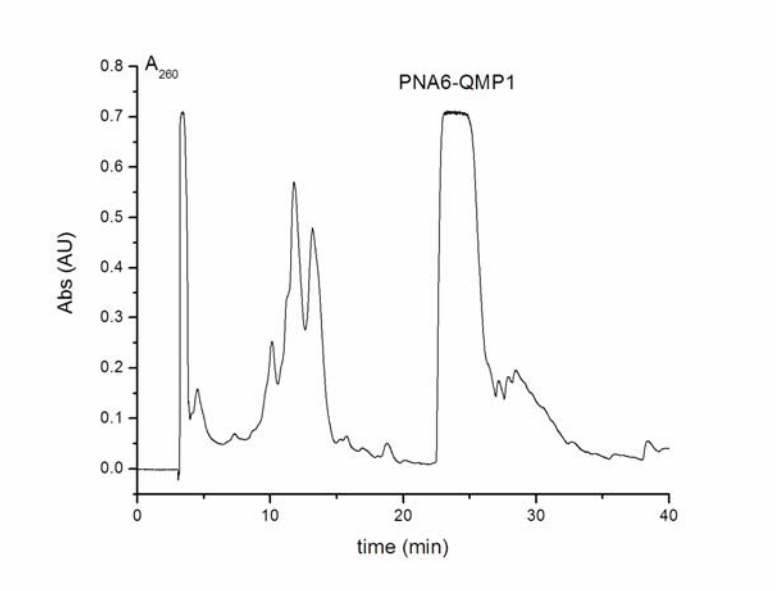


Figure 18. HPLC spectrum of PNA6-QMP1. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

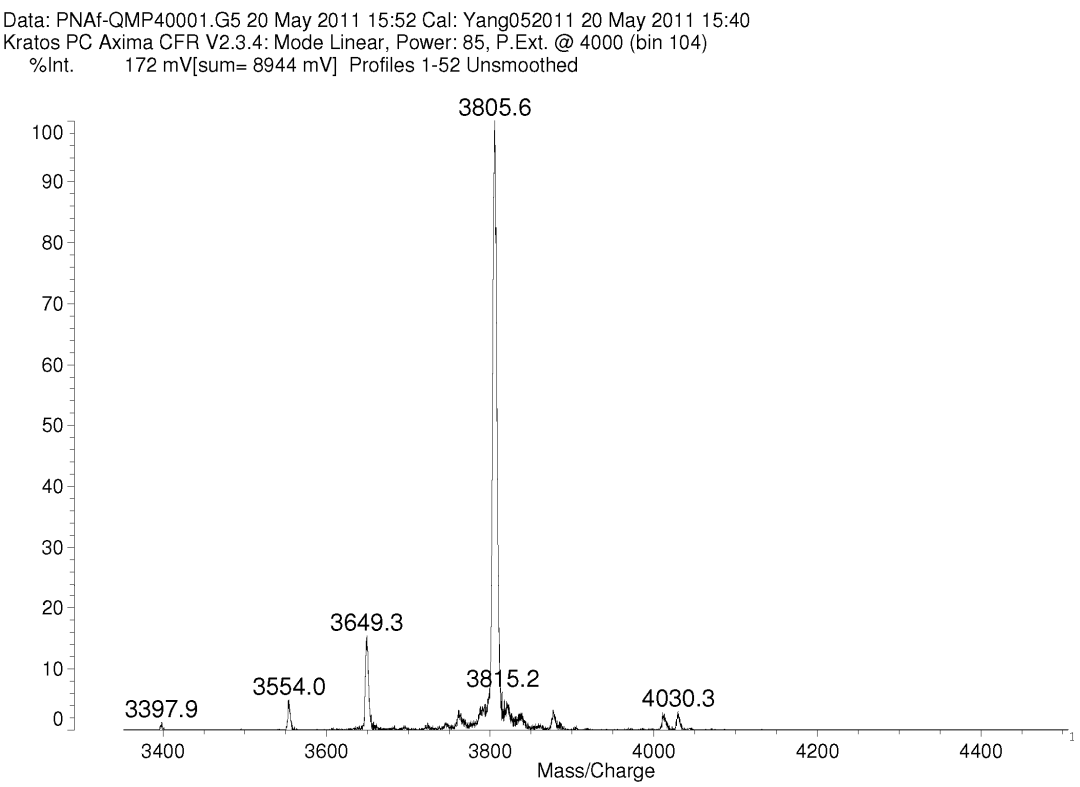


Figure 19. MALDI spectrum of PNA6-QMP1. PNA6-QMP1 calculated (*m/z*) is 3805.3 and MALDI found (*m/z*) was 3805.6.

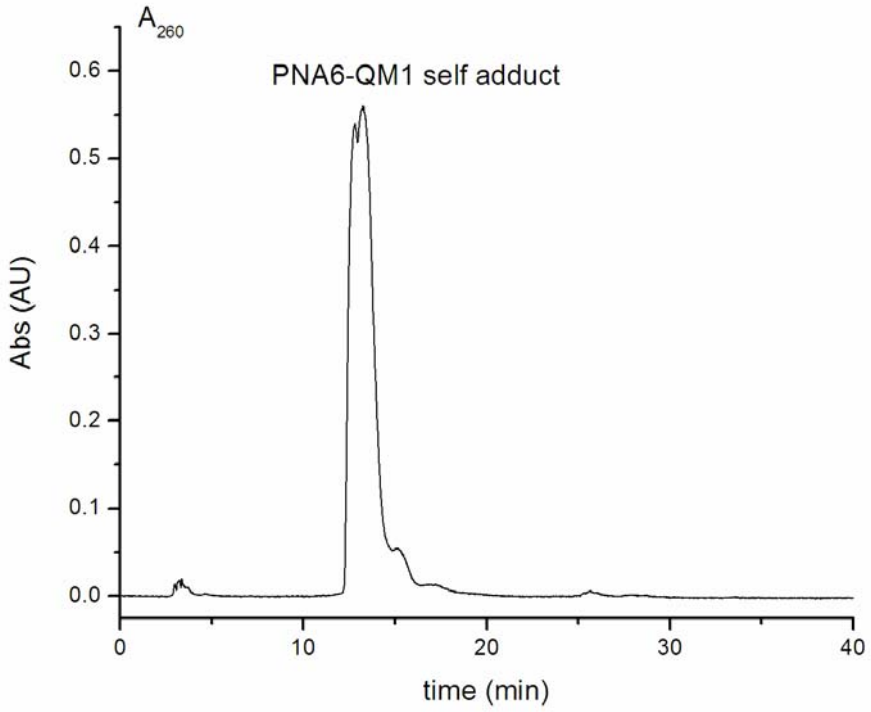


Figure 20. HPLC spectrum of PNA6-QM1 self adduct. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

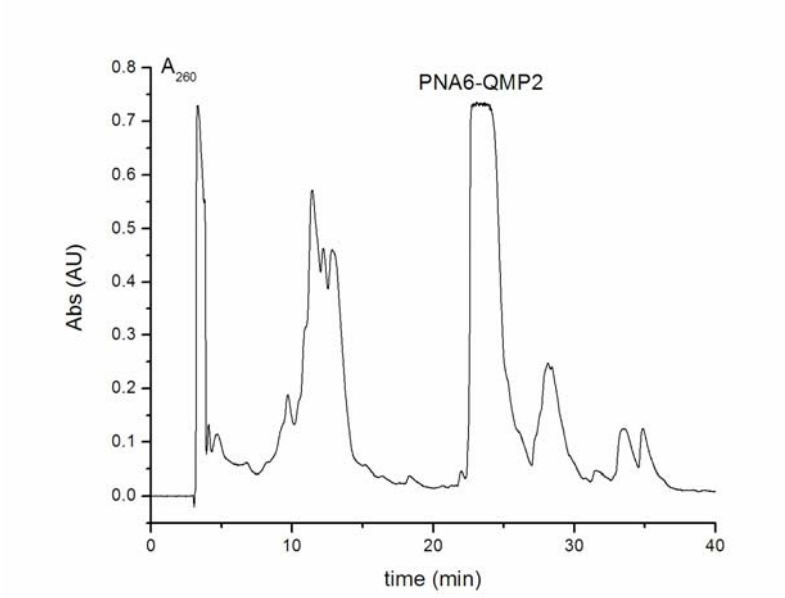


Figure 21. HPLC spectrum of PNA6-QMP2. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

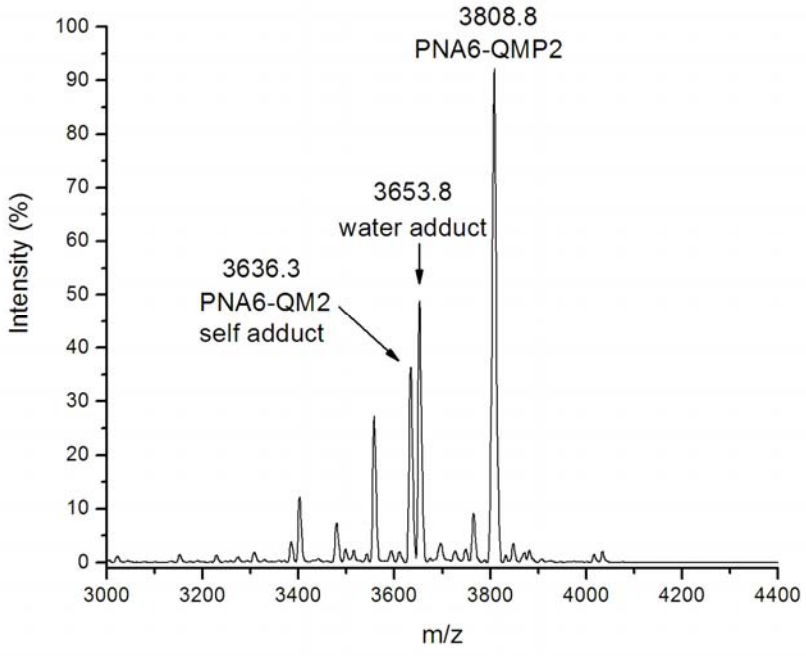


Figure 22. MALDI spectrum of PNA6-QMP2. PNA6-QMP2 calculated (*m/z*) is 3809.4 and MALDI found (*m/z*) was 3808.8. PNA6-QM2 self adduct calculated (*m/z*) is 3635.1 and MALDI found (*m/z*) was 3636.3. PNA6-QM2 water adduct calculated (*m/z*) is 3653.1 and MALDI found (*m/z*) was 3653.8.

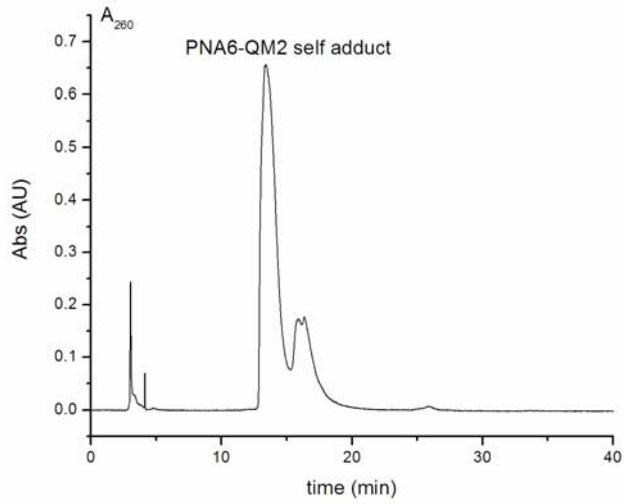


Figure 23. HPLC spectrum of PNA6-QM2 self adduct. Linear gradient of 10% to 55% aqueous acetonitrile with 0.1% TFA at 1 ml/min.

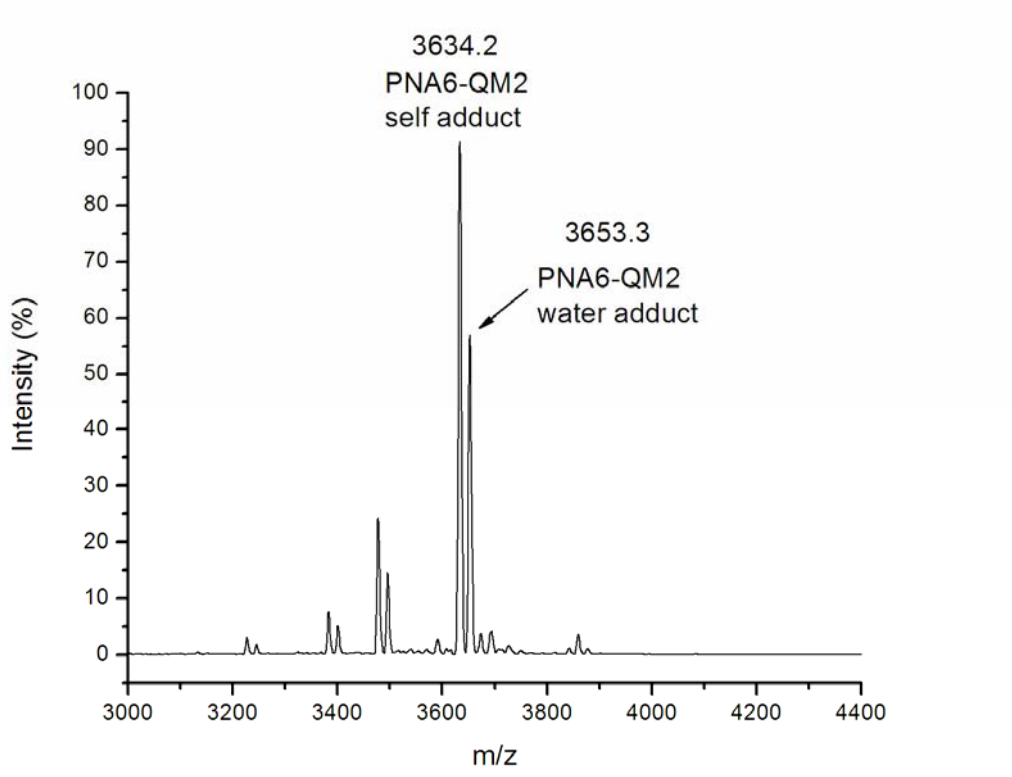


Figure 24. MALDI spectrum of PNA6-QM2 self adduct. PNA6-QM2 self adduct calculated (*m/z*) is 3635.1 and MALDI found (*m/z*) was 3634.2. PNA6-QM2 water adduct calculated (*m/z*) is 3653.1 and MALDI found (*m/z*) was 3653.3.

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