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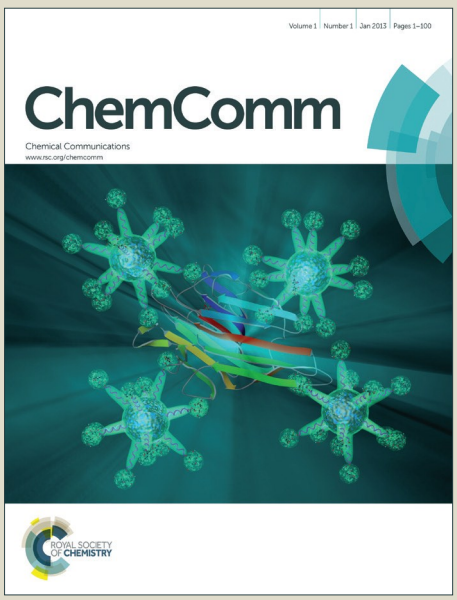

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Journal Name

COMMUNICATION

Furan-PNA: a mildly inducible irreversible interstrand crosslinking system targeting single and double stranded DNA†

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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We here report on the design and synthesis of tailor-made furan-modified peptide nucleic acid (PNA) probes for covalent targeting of single stranded DNA through a crosslinking strategy. After introducing furan-containing building blocks into a PNA sequence, hybridization and furan-oxidation based crosslinking to DNA is investigated. The structure of the crosslinked products is characterized and preliminary investigations concerning the application of these systems to double stranded DNA are shown.

Peptide nucleic acids (PNAs) combine chemical flexibility with the sequence selective recognition of nucleic acid targets and a unique stability towards nucleases and proteases.[1] Given their straightforward conjugation with cell penetrating peptide sequences[2,3] or nuclear localization signals,[4] PNAs have found numerous applications in nucleic acid targeting strategies and have been proposed as biomolecule-based drugs and as probes for diagnostic tools.[5]

PNAs are known to act on nucleic acid targets as steric blockers and have been successfully used in antisense strategies, most notably in the field of correction of aberrant splicing,[6] as well as anti-gene strategies.[7] The advantages of using PNA instead of DNA or other oligonucleotide mimics are linked to its high DNA affinity, which allows properly designed PNA molecules to perform strand invasion of the dsDNA,[8] and sequence-specificity, in particular their ability to discriminate even a single base mismatch. PNAs have recently been shown to perform excellently as anti-miR agents,[9] as probes in applications such as *in vivo* RNA target identification,[10–12] as artificial restriction enzymes,[13] or in templated reactions.[14] One limitation of PNAs is that they are not processed by natural enzymatic machineries, such as RNase-H or RISC, and thus their action relies only on the formation of reversible PNA:DNA or PNA:RNA complexes, which although very stable *in vitro*, can be challenged in cellular and *in vivo* systems by the competition of other biological components, such as proteins. Similarly, the possibility to form only reversible interactions can be useful in the realization of diagnostic tools with great performances in terms of target selectivity, but, on the other hand, the sensitivity of the devices can be influenced by strand dissociation, which can occur during the washing steps that are required in order to remove aspecific interactions.

Besides structural modifications aimed at increasing the stability of complexation, one possible approach to overcome this weakness is to introduce groups in PNA able to perform interstrand crosslinking (ICL). The application of PNAs in ICL strategies has previously been demonstrated by using a wide range of pro-reactive moieties inserted in the PNA strand.[15–19] Unfortunately, in all these approaches the activation steps needed for the production of the reactive species were not compatible with biological conditions (toxic salt, low wavelength / high energy UV irradiation, etc.),[15,16,18,19] and, in some cases, ICL formation was reversible.[15] Crosslinking of PNA without additional reactive units was also investigated, but only under rather harsh conditions such as γ-irradiation.[20]

Our recently developed furan-based DNA interstrand crosslink strategy, where furan can be activated through *in situ* production of singlet oxygen via visible light irradiation of a photosensitizer[21] seems very well suited in all cases where mild and biocompatible activation conditions are required. Additionally, in view of the presence of reactive oxygen species (ROS) inside cancer cells,[22] it is possible to envisage the self-activation of furan-containing probes in these important targets. In this paper we report the incorporation of furan moieties into PNA, and the first evidences of PNA-DNA interstrand crosslinking.

As a model sequence we chose a segment of a PNA previously used to block the expression of the MYCN gene[23] (Fig. 1). The sequence length was chosen to easily evaluate the thermodynamic effect of the insertion of a furan-modified building block by melting temperature measurements (thus with T_m not exceeding 90°C). We decided to introduce the furan modification

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†Electronic Supplementary Information (ESI) available: Probe synthesis, Measurements of Tm values, general protocols for click and crosslink reactions, PAGE experiments, ICL densitometric analyses, Identification of ICL products, NMR spectra and UPLC-MS chromatograms. See DOI: 10.1039/x0xx00000x

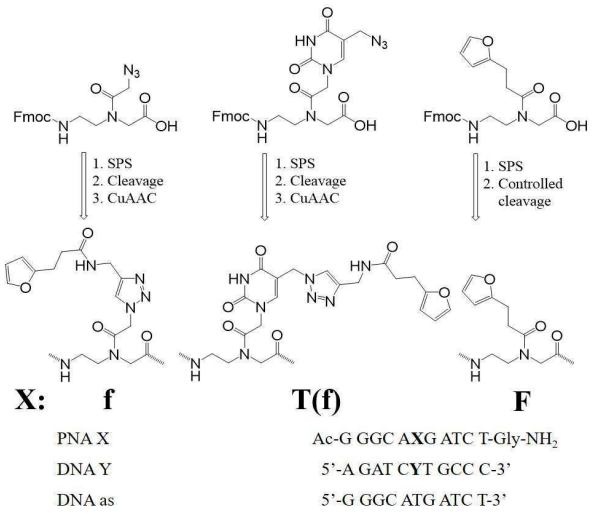

Fig. 1 Monomers and approaches used for obtaining furan-modified PNA probes (top) and sequences of PNA and commercial DNA strands (were Y= A, C, G or T) used in the present study (bottom). SPS: Solid Phase Synthesis.

in the middle of the PNA strand in order to study the formation of the ICL by reaction with the facing nucleobase within the PNA:DNA double-helix, thus avoiding potential reactions with terminal nucleobases which can be unpaired due to breathing of the PNA:DNA complex.

Insertion of the pro-reactive furan moiety during oligonucleotide solid-phase synthesis was achieved in earlier studies.[24–26] However, when moving to peptide synthesis, a series of side reactions was found to occur during the cleavage of the final product from the solid support. A strategy was therefore developed in which the furan moiety was protected against acid-induced damage by the interaction with proximate aromatic group.[27] Direct application of that strategy to the synthesis of a PNA-furan probe was first attempted by incorporation of a furan building block next to a nearby naphthyl modified PNA building block but this approach had to be abandoned due to the extreme insolubility of the final system (data not shown). However, by carefully choosing the cleavage conditions after Fmoc-based solid-phase synthesis (see ESI) we were able to obtain a first furan-PNA (**PNA F**, Fig. 1), in which 2-furylpropionic acid was linked to the PNA backbone, thus substituting the nucleobase. Unfortunately, alkylation of the furan-C4 position by the benzhydrylic carbocation (generated from the Bhoc protective groups) could not be completely avoided. In order to maximize the yields and purity of the desired compounds, we also explored post-synthetic approaches for the introduction of the furan moiety on PNA. Using a monomer containing 5-azidomethyluracil as nucleobase,[28] we initially synthesized a PNA containing this monomer in the central position (**PNA T(N_3)**, depicted in Fig. 2) then cleaved it from the solid-support and linked its azide function to 3-(furan-2-yl)-N-(prop-2-yn-1-yl)propanamide (**2**) by a copper catalyzed 1,3-dipolar cycloaddition (CuAAC). This click reaction proceeded smoothly in solution in 15 minutes, allowing to obtain **PNA T(f)** (Fig. 1 and 2). The same approach was then used for the production of a PNA probe similar to **PNA F**, but using an azide-containing monomer, which was earlier exploited in the solid phase insertion of nucleobase surrogates[29] or the production of molecular beacons,[30] and which allowed subsequent modification in solution to give **PNA f** (Fig. 1).

The effect of the introduction of a furan moiety inside the PNA strand was evaluated through measurement of the melting temperatures (T_m) of the complexes formed with fully complementary as well as mismatched DNA strands. We studied the effect of all four nucleobase permutations facing the modified nucleobase (**DNA A**, **DNA C**, **DNA G** and **DNA T**) in the DNA strand. From the T_m data obtained (Table S1) for the full-match DNA, compared with the results obtained using the unmodified **PNA T** (with X= T), it can be concluded that modification of the C5 position of the uracil nucleobase (**PNA T(f)**) does not affect the stability of the duplex. This indicates that this substitution allows the furan residue to be accommodated into the major groove of the PNA:DNA complex, without generating significant steric hindrance. In contrast, the insertion of furan in **PNA F** and **PNA f**, which leads to the introduction of an abasic site, as expected strongly decreases the stability of the complex regardless of the identity of the opposite nucleobase. This destabilization effect is stronger for **PNA F** than for **PNA f,** suggesting that the longer spacer between the PNA backbone and the aromatic ring in the latter allows the furan moiety to interact with other nucleobases through π-π stacking. This is also corroborated by the surprisingly high melting temperature of **PNA f** when guanine is the opposite base.

The ability of the modified PNAs to undergo ICL with complementary DNA was tested using all four permutations of the facing nucleobase, at 10 μM concentration of each strand. Although, as mentioned above, singlet oxygen has been shown to allow activation of the furan moiety,[19] for this proof-of-principle work N-bromosuccinimide (NBS) was used to trigger the reaction, as described in some of the earlier DNA inter-

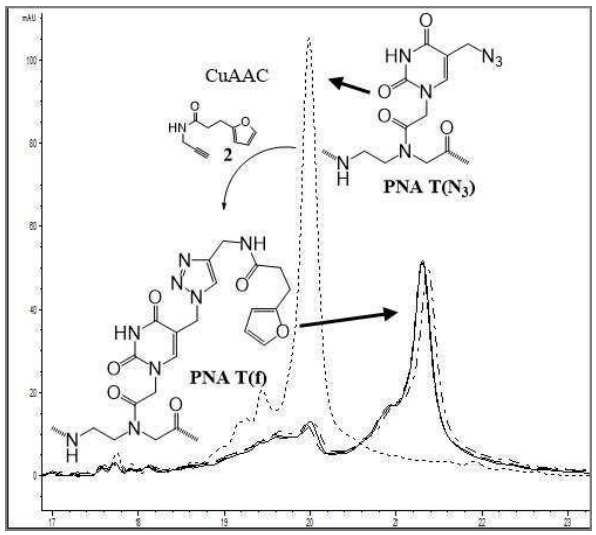

Fig. 2 HPLC profiles of click reaction between **PNA T(N3)** and **2** at t_0 (dotted line), 15 minutes (full line), and 1h (dashed line). In the insert a zoom of the t_R = 17-23 minutes region.

strand crosslinking studies.[23,24] Thus, the PNAs were allowed to anneal with DNA (from 90°C to r.t. in 2h in order to avoid differences derived from kinetics) and then the furan moiety was activated with NBS (4eq, 1eq every 15 minutes). The probe concentration and medium composition for the experiments were initially screened in order to avoid possible artefacts due to the poor solubility of furan-PNA in buffered solution (data not shown). For furan-modified DNA it was earlier described that crosslinking toward guanine and thymine did not occur, while there was a preference in the formation of the crosslink with cytosine and adenine.[25,26] As shown in Fig. 3 in the case of furan-PNA the absence of crosslinking toward guanine and thymine was confirmed while for adenine and cytosine we observed an unexpected reaction selectivity. **PNA f**, with the longer linker, showed better reactivity towards cytosine, while **PNA F** favoured adenine crosslinking. For **PNA T(f)** the visibility of the shifted band was surprisingly found to be dependent on the stain used to develop the PAGE. In particular when using SybrGold (generally a better stain for ICL experiments) a shifted band was detected with cytosine while, when using GelRed, a shifted band was present only for adenine; in both cases considerable smearing of the ssDNA band was visible. However, these bands were found to be linked to the presence of remaining non-crosslinked **PNA T(f):DNA A** duplex species, which were persistent even under denaturing conditions (as demonstrated by an additional experiment where reaction mixtures with and without NBS activation were compared, see ESI, Fig. S4). No conclusive evidence of cross-linking could thus be found for the **PNA T(f):DNA A** combination. This weak to inexistent ICL reactivity of the **PNA T(f)** probe towards adenine can be explained by the effect of the unavailability of the exocyclic nucleobase amino group, which is involved in Watson-Crick recognition, as well as a forced disposition of the furan ring in the major groove, far from any nucleophile of the complementary DNA strand. The small band present in the **PNA T(f):DNA C** experiment, which represents a genuine crosslinked species, can be explained by the lack of Watson-Crick base pairing, which disengages the necessary nucleophiles, while conferring at the same time an increased conformational flexibility (compared to the DNA A case) allowing the furan system to reach the reactive center. An indication of the possible favourable orientation of the furan ring towards the interior of the complex, can be derived from the slight stabilization obtained with **PNA T(f)** when targeting mismatched DNA, if compared to **PNA T** (ΔT_m +0.35°C with DNA G and +1°C in with DNA C and DNA T, see Table S1). Similar results were observed when targeting longer DNA probes (see ESI, Fig. S3).

HPLC purification allowed isolation of the pure ICL products, which were matched with the PAGE gel bands by comparison of their electrophoretic mobility (Fig. S9 and S10). MALDI analysis of the products was difficult, due to the low amount of product and to the presence of the TEEA buffer. MALDI matrices optimized for both DNA and PNA were tested, but only in the latter case it was possible to obtain a clear signal above the noise. As shown in Fig. 4A for **PNA F** to **DNA A** ICL (and Fig. S11 for more examples), no peaks in the adduct region (6.5kDa) were observed but a peak with 131 Da mass difference from the starting PNA was observed. As illustrated in Fig. 4B this signal can be explained by an in-source E1 N-glycosidic fragmentation of the DNA strand induced by the MALDI laser and matrix,[31,32] a process that can be favoured by the extended aromaticity of the system. Since the PNA-DNA ICL adduct holds the properties and behaviour of both species, evaluation of the yield of the crosslink reaction by RP-HPLC was not possible because the best chromatographic conditions for PNA broaden DNA peaks and vice versa.

In contrast to the earlier developed DNA based probes,[33] we expected the here developed PNA-furan probes to offer the possibility to target double stranded DNA. We thus tested their ability to form ICLs in a strand displacement test. We first allowed annealing of the target dsDNA at 20 µM strand concentration. Subsequently the PNA was added to the solution at a final 10 µM concentration and DNA displacement was allowed prior to starting the NBS activation, using a protocol identical to the one adopted when targeting ssDNA. PAGE analysis of the experiments is shown in Fig. 5. As expected, the yield of ICL seems to be lower when compared to ssDNA experiments, which is reasonably related to the competition of the homologous DNA strand for binding to the target DNA. Crosslinking

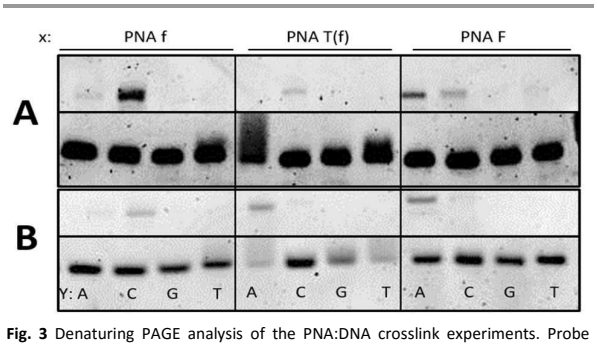

Fig. 3 Denaturing PAGE analysis of the PNA:DNA crosslink experiments. Probe concentration was 10 µM, 4 equivalents NBS activation. PNA: Ac-GGGCA**X**GATCT-Gly-NH_2, DNA: 5'-AGATC**Y**TGCCC-3'; staining of the DNA band was performed with SybrGold (A) or GelRed (B).

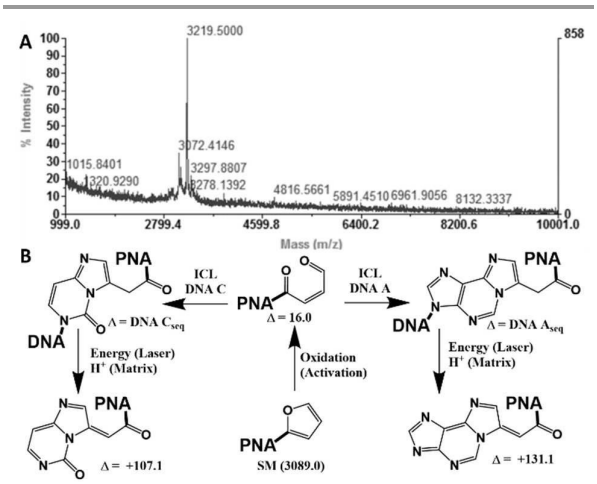

Fig. 4 A - MALDI signal of purified PNA F + DNA A ICL samples (starting PNA MW:3089.0); B - proposed in-source fragmentation products.

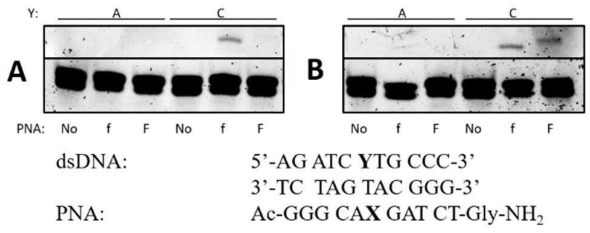

Fig. 5 Denaturing PAGE analysis of the strand displacement crosslink experiments. A) displacement at 25°C; B) displacement at 37°C.

propensity increases when displacement was carried out at 37°C rather than 25°C (respectively Fig. 5B versus 5A) and the preference for targeting **dsDNA C** rather than **dsDNA A** (a four-fold increase of spot intensities as evaluated by densitometric analysis) is in line with the T_m values for the two dsDNA duplexes, being 33.9°C and 50.5°C, respectively. In addition, the lack of reactivity shown by **PNA F** when targeting **dsDNA A** rather than ssDNA A, can be easily explained by the difficulty for this probe to efficiently displace the anti-sense DNA strand from the dsDNA (T_m for PNA F:DNA A is 47.4°C). So in these experiments the ability of the PNAs to perform efficient crosslinking is mainly related to their ability to efficiently compete and displace the homologous DNA strand, rather than to an intrinsic higher reactivity towards the specific base, or to a distortion of the overall structure induced by the presence of a mismatched base.

In conclusion, in this work we show for the first time the synthesis and application of PNA probes to furan-ICL strategies for targeting ss- as well as dsDNA. We here developed protocols for obtaining furan-modified PNA probes in which the furan moiety was inserted either during solid phase synthesis or by a post-cleavage functionalization through a CuAAC reaction. The developed synthetic strategies used for the introduction of the sensitive furan moiety can provide general access to the incorporation of other acid-and alkylation sensitive moieties into PNA. Furthermore, in contrast to earlier developed triplex forming DNA based oligonucleotides, the here developed furan-PNA probes allow targeting of and subsequent crosslinking to ssDNA as well as dsDNA sequences.

Based on the present proof-of-principle results, this type of furan-modified PNA probes thus seems ideally suited for direct application in diagnostic settings for DNA recognition, since covalent crosslinking with the target allows more stringent washing conditions after hybridization in both surface- and solution-based sensing schemes, thus reducing false positives and undesired hybridization artefacts.

An advantage of the combination of PNA probes with furan-based crosslinking in cellular systems is the possibility of the system to escape the DNA repair machinery. In particular, the completely artificial nature of PNA should interfere with the recognition of the ICL site by nucleases, which enable the bypass of the nucleobases involved in the lesion.[34] At the same time the complete change in the H-bond donor-acceptor profile, which occurs after reaction with the activated furan (as also shown in Fig. 4B), should allow to avoid the lesion by-pass and repair steps, where Watson-Crick base pairing is required. Based on the present encouraging findings, further work for optimization of synthesis, structure, and performances in cellular systems of furan-PNA is ongoing.

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