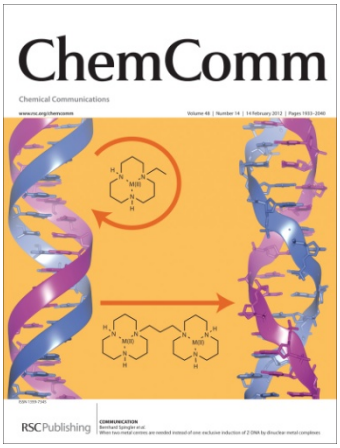

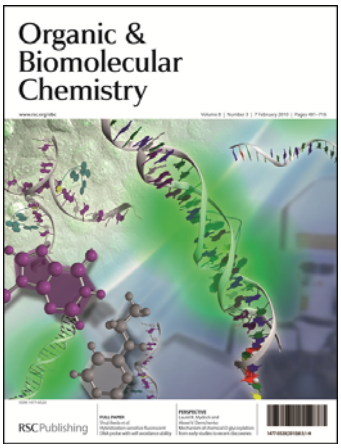

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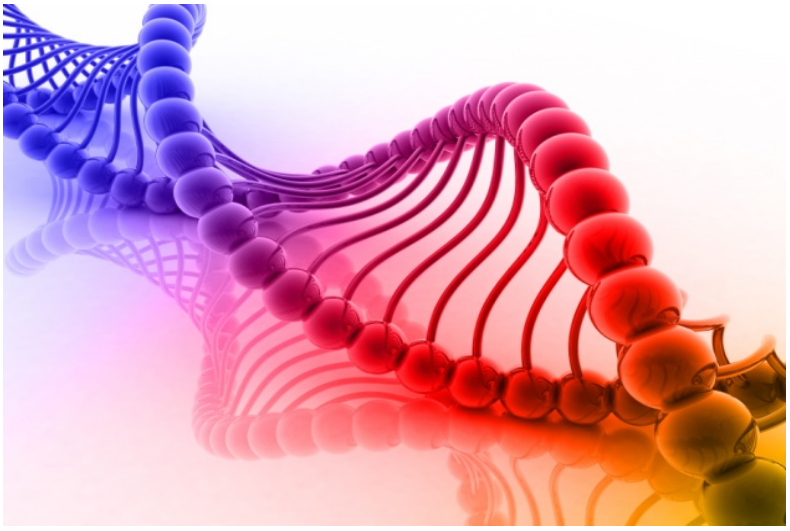

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COMMUNICATION

Templated chemistry for monitoring damage and repair directly in duplex DNA†‡§

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Received 6th June 2012, Accepted 27th June 2012
DOI: 10.1039/c2cc34060g

We report the fluorogenic detection of the product of base excision repair (an abasic site) in a specific sequence of duplex DNA. This is achieved by DNA-templated chemistry, employing triple helix-forming probes that contain unnatural nucleobases designed to selectively recognize the site of a missing base. Light-up signals of up to 36-fold were documented, and probes could be used to monitor enzymatic removal of a damaged base.

Base excision repair (BER) is a major cellular pathway for removal of nucleobases that are damaged by hydrolysis, oxidation, or alkylation.[1,2] The immediate product of this excision is an abasic site. Since abasic sites arise from spontaneous depurination as well as BER, they are perhaps the most commonly found lesion in cellular DNA, and additional cellular mechanisms exist to replace them with coding bases.[1]

Base excision repair pathways are not only important in avoidance of cellular mutations, but also are considered potential therapeutic targets for cancer.[3] Thus methods for monitoring base excision as it occurs in DNA duplexes are of significant interest, both for basic biological study and for evaluating potential inhibitors of these processes. The majority of methods for evaluating base excision involve multiple biochemical steps, and are not amenable to high-throughput screens, or to possible intracellular reporting.[4] A few reports exist of fluorogenic assays based on synthetic duplex DNAs containing quenchers and fluorophores; however, efficiency has been modest, with light-up signals of 4–8-fold,[5,6] and can require multiple enzyme activities.[5]

Here we report the use of designed novel nucleobases coupled with DNA-templated fluorogenic chemistry to selectively recognize abasic sites (resulting from base excision repair) in a targeted sequence of duplex DNA. Nucleic acid-templated chemistry has previously been used in multiple laboratories for detection of undamaged natural sequences of DNA or RNA.[7] It has not been used previously for detection of damaged structures and sequences. Moreover, templated chemistry has been used almost exclusively for detection of single-stranded DNAs and RNAs, and only one report exists of its application in fluorogenic reporting on duplex DNA.[8]

Our design begins with the use of triple helices as targeting motifs, and employs specifically designed modified bases to recognize abasic sites within this triplex. Extensive research has shown that pyrimidine-rich oligodeoxynucleotides can be used to bind to purine-rich sites in duplex DNA.[9] Models of the base triads in such triple helices (Fig. 1B) suggest that larger-than-natural bases with elongated structure might fit into the site where a purine is missing; yet such large nucleobases would, in principle, be sterically blocked from fitting where the prior damaged base (or an undamaged one) exists. Finally, for fluorogenic signaling we adopted the recently described Q-STAR templated chemistry, in which a fluorophore-containing probe is rendered dark by a quenching group attached *via* an α-azidoether linker.[7m,8] When a second probe containing a triarylphosphine group binds adjacent, it triggers accelerated Staudinger reduction of the azide, releasing the quencher and yielding robust fluorescence enhancement.

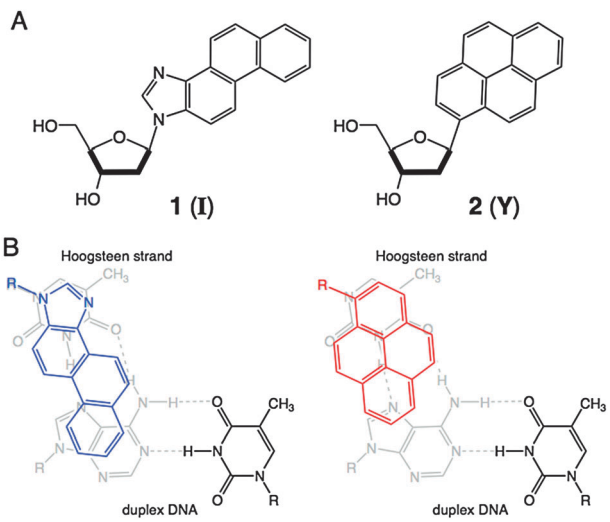

Fig. 1 Structures and design for recognition of abasic sites arising from DNA repair. (A) Nucleosides **I** and **Y**, designed with imidazophenanthrene and pyrene nucleobase replacements respectively. (B) Diagram showing possible fit of extended nucleobases (blue and red) from the Hoogsteen third strand of a triplex into an abasic site in a target duplex (adenine is shown in gray as the potential missing base).

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† The manuscript is dedicated to the memory of H. Gobind Khorana.

‡ This article is part of the 'Nucleic acids: new life, new materials' web themed issue.

§ Electronic supplementary information (ESI) available: Detailed experimental methods and characterization data. See DOI: 10.1039/c2cc34060g

As candidate modified nucleobases for recognizing abasic sites, we designed pyrene (**Y**) and imidazophenanthrene (**I**) (Fig. 1), expecting that (a) their elongated geometry should allow them to reach from the third strand into an abasic site in the purine-rich strand of the duplex, and (b) their large size and hydrophobic surfaces would lead to strong stacking with bases neighboring the abasic site. The tetracyclic nucleoside **I** was previously unknown, while the pyrene nucleoside **Y** has been studied before in other contexts.[10] The β-anomeric pyrene nucleobase has been shown to pair selectively opposite abasic sites in single-stranded DNAs.[10b] This compound has not been studied previously in triple helices, to our knowledge. The synthesis of the imidazophenanthrene nucleoside (**I**) is described in the ESI.§ The second deoxyriboside (β-pyrene, **Y**) was synthesized *via* Pd-catalyzed coupling.[11] The compounds were converted to 5′-dimethoxytrityl, 3′-phosphoramidite derivatives following standard methods.

Oligodeoxynucleotides containing these two modified nucleotides, along with naturally-substituted controls (Fig. 2), were prepared using standard automated DNA synthesis chemistry. The 14-nt oligomers also contained a fluorescein label on a thymine near the 5′ end, and included the quencher-linker at the 5′ end.[8] Oligonucleotides were purified by HPLC and were characterized by MALDI-TOF mass spectrometry (see ESI§). For initiating templated reactions, we also prepared a 3′-conjugated triarylphosphine (TPP)-containing 14mer probe,[7f,m] designed to bind adjacent to the quenched probes and trigger reaction by juxtaposing the phosphine with the quencher/azidolinker group. A final modification employed in all third-strand probes was the use of pseudoisocytosine (ψ) in place of cytosine; this substitution enables triplexes to form stably at neutral pH.[12] A number of 28 bp duplex DNAs were prepared as targets, and contained either a uracil (a model damaged base) at the position being probed, an undamaged adenine, a tetrahydrofuran abasic analog (THF),[13] or a native abasic sugar (Fig. 2).[14] The targets were constructed as hairpins to render them stable and to fix the stoichiometry of the strands.

First we evaluated the binding properties of the modified nucleobases, by looking for evidence of hybridization of the short quenched probes containing them with the different target duplexes. Possible binding transitions were evaluated by thermal melting experiments; the data are shown in Fig. S6 and S7 in the ESI.§ The results show that the unmodified hairpin target has a melting temperature (T_m) of *ca.* 73 °C under these conditions, while the abasic duplexes show two high-temperature melting transitions (~57, 68 °C), presumably due to the disruption of contiguous stable structure by the missing base. When the 14mer probes containing **I** or **Y** were added, a new low-temperature transition (T_m ~ 10–20 °C) characteristic of third-strand binding was seen with the abasic duplexes, but not with the intact duplex (Fig. S6, ESI§). This provides evidence of selective binding of the new probes with the damaged DNAs, indicating possible abasic site recognition by the extended nucleobases. Thermal denaturation experiments with the TPP probe also showed an apparent triplex-binding transition at *ca.* 35 °C (Fig. S7, ESI§), confirming that it binds the duplex as expected.

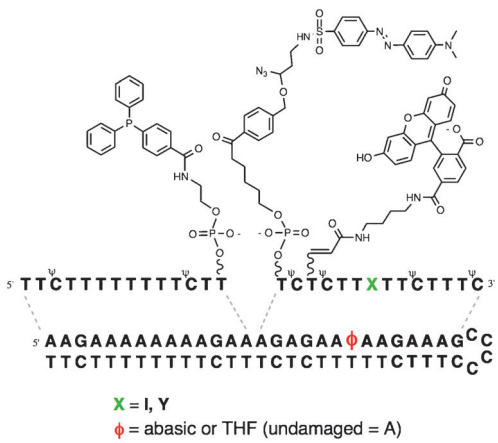

Fig. 2 Probe sequences and structures. Targets are 28 bp hairpin duplexes. Phosphine probe carries triarylphosphine conjugated to the 3′-end as shown. Quenched (Q-STAR) probe is labeled with fluorescein and is conjugated at the 5′-end with a quencher-linker containing a reductively cleavable azidoether group. $^{\psi}$C is pseudoisocytidine, used for triplex formation at neutral pH.

We then proceeded to test whether the probes could carry out selective templated chemistry to yield fluorescence signals with abasic target DNAs. Initial experiments were performed with 200 nM target DNA and 200 nM Q-STAR probe, with an excess of phosphine probe (600 nM) to compensate for any phosphine oxidation that might occur during the reaction.[7e] Time courses of fluorescence emission signals for the reactions at 25 °C are shown in Fig. 3. The data show that both modified probes yield clear fluorogenic signals that increase over 3–4 hours in the presence of target DNA containing either the THF abasic site or the native abasic site. Signals increase by robust factors of 15–18-fold after 3.5 h (probe **I**) and 18–20-fold (probe **Y**); extended experiments demonstrate up to 36-fold enhancement after 12 h (Fig. S8, ESI§). The reactions are clearly templated by the target duplexes, since controls omitting duplexes yielded only a very small background signal after 3.5 h (Fig. 3). Importantly, the reaction is strongly abasic-selective, as the intact adenine-containing target yielded little enhancement (*ca.* 1.5 fold) after this time. Extended reaction times with excess probes also revealed a small degree of isothermal amplification due to slow turnover of the probes on the target (Fig. S9, ESI§). Overall, the results show that the probes **1** and **2**, containing the unnatural bases **I** and **Y**, respectively, can efficiently and selectively recognize missing bases in DNA and report on it with a fluorogenic signal.

Finally, we explicitly tested whether enzymatic repair could be probed by this approach, using uracil, the deaminated product arising from cytosine hydrolysis, as the damaged base. We targeted a duplex containing a U–G mismatch at the variable position, with U situated in the purine-rich strand. Uracil DNA glycosylase (UDG) enzymes remove uracil from such mismatched pairs, leaving abasic sites in their place.[15] Therefore we tested probes both in the presence of the original U–G mismatch and in the presence of added *E. coli* UDG enzyme (see Fig. S10, ESI§). Control experiments using the **Y**-containing Q-STAR probe with the U–G mismatch DNA or with an A–T pair showed only relatively small signals over a 3.5 h time course, confirming that a correct pair or even a mismatched (damaged) base sterically blocks the binding of the unnatural probe. However, when UDG was incubated with the DNA for 30 min and then probes were added, a robust fluorescent signal developed (14-fold enhancement with the **Y** probe in 3.5 h and 10-fold for the **I** probe), consistent with the prior results with chemically synthesized abasic DNAs.

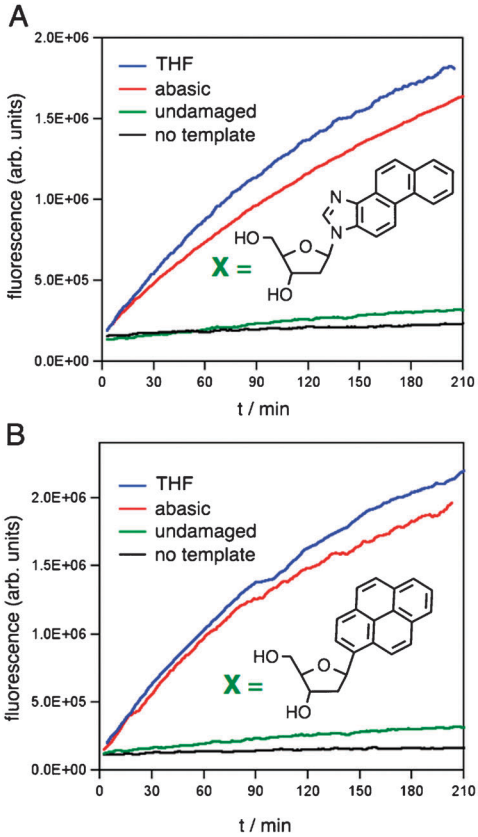

Fig. 3 Fluorogenic signaling of missing bases by probes containing **I** (A) and **Y** (B). Positive signals are seen for either a natural abasic site or the tetrahydrofuran (THF) abasic site mimic. Conditions: 200 nM target DNA and fluorogenic probes; 600 nM TPP trigger probe; 70 mM Na^+, 10 mM Mg^{2+}, 25 °C. Excitation 490 nm; emission 525 nm.

Thus the preliminary experiments show that probes **1** and **2** can detect the enzymatic repair of uracil-containing DNAs that leads to abasic sites formed *in situ*. It is worth noting that the abasic site is an intermediate in base excision repair of multiple forms of DNA damage, and thus we speculate that the current probes may be useful as reporters for diverse enzymatic pathways.

These results provide proof-of-principle that designed nucleobases can be used to recognize the abasic product of base excision repair located directly in duplex DNA. Previous researchers have described modified nucleobases that can recognize a damaged nucleobase,[16] but in the context of single-stranded DNA only. Since damage and its repair occur naturally in the double-stranded context, the current approach offers a biologically relevant strategy for detecting them. In addition, we show that this highly selective recognition can be coupled to a fluorogenic reporting process by applying templated chemistry to this recognition. Although abasic sites have been detected by many approaches previously,[17–19] few have yielded a fluorogenic signal, which simplifies detection by requiring only one step.

We envision this approach as being potentially useful for basic science studies of BER, using pre-made triplex target DNAs as engineered sites of repair. Such probe damaged duplexes, combined with our fluorogenic probes, could also be used in high-throughput screens for inhibitors of various BER enzymes.

This work was supported by the U.S. National Institutes of Health (GM068122).

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