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Sequence-specific covalent capture coupled with high-contrast nanopore detection of a disease-derived nucleic acid sequence**

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Abstract

Hybridization-based methods for the detection of nucleic acid sequences are important in research and medicine. Short probes provide sequence specificity, but may not provide a durable signal. Sequence-specific covalent cross-link formation can anchor probes to target DNA and also may provide an additional layer of target selectivity. Here we develop a new cross-linking reaction for the covalent capture of specific nucleic acid sequences. This process involves reaction of an abasic site in a probe strand with an adenine residue in the target strand and was used for the detection of a disease-relevant T→A mutation at position 1799 of the human BRAF kinase gene sequence. Ap-containing probes are easily prepared and display exquisite specificity for the mutant sequence under isothermal assay conditions. DNA duplexes generated in these studies were quantitatively measured using both denaturing gel electrophoresis and a protein nanopore.

Keywords

DNA cross-linking; SNP; nucleic acid sequence capture; nanopore; abasic site

Methods for the detection of DNA and RNA sequences are important in research and medicine and many different approaches undoubtedly will be required to meet the various

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needs of research and clinical laboratories.^[1] Logically, many strategies for the detection of nucleic acid sequence rely on Watson-Crick hybridization of a probe strand to target DNA or RNA in the sample.^[2] However, the non-covalent, inherently reversible nature of nucleic acid hybridization presents challenges because signal can be compromised by partial denaturation of the probe-target duplex during analysis (e.g. washing). The use of longer probes (>20 nucleotides, nt) increases stability of probe-target complexes but degrades sequence specificity.^[2]

Covalent cross-links can be used to stabilize target-probe complexes.^[3] and, in some cases, provide an additional layer of target selectivity beyond that afforded by Watson-Crick hybridization.^[4] In the work described here, we developed a new cross-linking reaction that may be useful for the covalent capture of specific nucleic acid sequences. The cross-linking probes used in these studies are prepared in a one-step procedure from inexpensive commercial reagents and achieve exquisite sequence specificity under isothermal assay conditions. Cross-linked DNA duplexes generated in these studies were quantitatively measured using denaturing gel electrophoresis and a protein nanopore.

The cross-linking process developed here involves covalent reaction of an abasic (Ap) site in the probe strand with a deoxyadenosine residue in the target strand (Figure 1A).^[5] Importantly, Ap-containing probe strands are easily generated by treatment of the corresponding 2'-deoxyuridine-containing oligodeoxyribonucleotide with the enzyme uracil DNA glycosylase (UDG).^[5,6] We set out here to determine whether the dA-Ap cross-linking reaction could be exploited for selective detection of a single-base polymorphism (SNP) in a human gene sequence. SNPs are the smallest differences that can exist in nucleic acid sequence, yet have immense importance in biology and medicine.^[1,7] Seeking proof-of-concept, we focused on detection of a T→A mutation at position 1799 of the *BRAF* kinase gene sequence that encodes a cancer-driving valine→glutamic acid substitution at amino acid 600 of the protein (V600E).^[8] The anticancer drug vemurafenib (zelboraf) specifically inhibits the V600E kinase.^[8]

We designed an Ap-containing oligonucleotide probe to cross-link with A1799 in the mutant *BRAF* sequence (Figure 1C). Formation of the dA-Ap cross-link has been observed^[5] when an adenine residue was positioned one nucleotide (nt) to the 3'-side of the Ap site on the opposing strand (Figure 1B) but, until now, the sequence specificity of this cross-linking reaction has not been characterized. Incubation of the mutant *BRAF* target-probe duplex **A** in HEPES buffer (50 mM, pH 7) containing NaCl (100 mM) at 37 °C gave a $7.3 \pm 2.0\%$ yield of a slowly-migrating band on a denaturing polyacrylamide gel, consistent with that expected^[5] for the cross-linked duplex (Figure 2A, lane 1, and Figure S2). In contrast, the wild-type target-probe duplex **B** gave a relatively low yield of a slowly-migrating band ($2.3 \pm 0.6\%$, Figure 2A, lane 5).

Encouraged by the selective cross-linking of the Ap-containing probe with the mutant *BRAF* target sequence, we sought a second-generation Ap-containing probe that would decrease the background signal associated with cross-linking to the wild-type *BRAF* sequence. The exact location of the cross-link generated between our first-generation probe and the wild-type sequence was uncertain, but we suspected that flexibility introduced by the T-T

mispair^[9] enabled cross-link formation between the Ap site and the directly opposing guanine residue.^[10] Accordingly, we prepared a new probe strand containing an adenine residue on the 3'-side of the Ap site, such that the probe was complementary to the *wild-type BRAF* sequence (duplex **D**, Fig. 2B). We were gratified to find that background cross-link formation between the second-generation probe and the wild-type *BRAF* sequence in duplex **D** *decreased to undetectable levels* (Figure 2B, lane 5). We were further pleased to find that the desired cross-link formation between the second-generation probe and the mutant *BRAF* target sequence in duplex **C** *increased dramatically* to $85\pm3\%$ (Figure 2B, lane 1 and Figure S2). Iron-EDTA footprinting experiments confirmed that cross-link attachment in duplex **C** was to the “mutant” adenine residue at position 1799 (Figure S3).

We next set out to determine whether the cross-linking reaction can be used to quantitatively measure the fraction of mutant versus wild-type *BRAF* sequence present in a sample. In this experiment, mixtures containing various proportions of mutant and wild-type duplexes were denatured by warming to 50–70 °C in the presence of the second-generation Ap-probe, cooled, incubated at 37 °C, and the cross-link yield assessed by gel electrophoretic analysis. A clear connection between cross-link yield and the fraction of mutant duplex in the samples was observed (Figure 3).

We then examined use of the α -hemolysin (α -HL) protein nanopore for single-molecule detection of this cross-linked probe-target duplex. The α -HL ion channel can be used to create a device in which a nanoscale pore (1.4 nm wide)^[11] spans a lipid bilayer that separates two chambers of aqueous electrolyte.^[12] Application of an electric potential induces a readily measured ion current and the sequence and structure of nucleic acids can be analyzed based upon the characteristic current blocks produced when they are driven into the pore by the electrophoretic potential.^[13] For the nanopore experiments, we prepared a third-generation Ap-containing probe strand with a dC₃₀ overhang on the 3'-end (Figure 4). The poly-dC extension was employed to increase the rate at which the α -HL nanopore captures the duplexes and to facilitate rapid unzipping of (uncross-linked) duplexes in the nanopore.^[13,14] Separate gel electrophoretic analysis demonstrated that cross-link yields were not affected by the dC₃₀ overhang (Figures S4 and S5). In a device employing a single α -HL nanopore embedded in the lipid bilayer, analysis of the mixture generated by combination of the Ap probe with the mutant *BRAF* target sequence (duplex **E**) revealed several distinct current signatures. We observed very short current blocks consistent with the translocation of single-strands ($I/I_0=13.2\pm0.3\%$; $\tau=150\pm30\ \mu\text{s}$) and uncross-linked duplexes ($I/I_0=12.2\pm0.9\%$; $\tau=12\pm0.3\ \text{ms}$; Fig. 4).^[12] More importantly, we observed persistent current blocks ($I/I_0 = 13.2\pm0.3\%$, Figure 4A) consistent with capture of a cross-linked duplex in the nanopore. Following capture of the cross-linked duplex, current flow could be restored only when the voltage polarity of the nanopore device was reversed, causing the cross-linked duplex to “back out” of the channel. When the voltage polarity was reset, the open pore was again able to record current signatures associated with the nucleic acid species in the mixture (Figure 4A). Analysis of the mixture generated by combination of the Ap probe with the wild-type *BRAF* gene sequence (duplex **F**) revealed no persistent current blocks, only short current blocks consistent with the translocation of single-strands and uncross-linked duplexes (Figures 4B and S7). The current signature of cross-linked duplex is

unmistakably different from that of the uncross-linked DNA, thus providing a high-contrast signal for detection of the *BRAF* mutation. When multiple α -HL ion channels were embedded in the lipid bilayer, the analysis of mixtures derived from the mutant duplex **E** revealed a series of incremental current decreases consistent with sequential, irreversible blocking of individual pores by the cross-linked duplex (Fig. 5).^[14] By counting the number of events with each type of current signature, the nanopore can be used for the quantitative analysis of mixtures containing both mutant and wild-type *BRAF* sequences (Figures S8 and S9).

Our results introduce a new hybridization-induced, programmable cross-linking reaction that can be used for the sequence-specific covalent capture of nucleic acids. The probe-target complexes generated in this manner could be detected by typical fluorescence,^[2] colorimetric,^[15] or electrochemical methods;^[16] however, we showed here that nanopore technology combined with sequence-specific cross-linking chemistry has the potential to provide a high contrast – in essence digital – signal for single-molecule sensing of nucleic acid sequences.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Covalent capture of specific nucleic acid sequences by interstrand cross-link formation.

Panel A. Covalent cross-link formation by reaction of Ap aldehyde residue in probe strand with adenine residue in target sequence. Panel B. Sequence motif for dA-Ap cross-linking reaction. Panel C. Sequence-specific covalent capture of the mutant *BRAF* gene sequence by an Ap-containing probe strand.

Figure 2.

Ap-containing probes selectively cross-link with the 1799 T→A mutant *BRAF* kinase gene sequence. Panel A: Gel electrophoretic analysis of cross-link formation in a 21 bp duplexes containing the first-generation Ap-containing probe and either the mutant (lane 1) or wild-type (lane 5) *BRAF* sequence. Panel B: Cross-linking by the second-generation Ap-probe containing an adenine residue on the 3'-side of the Ap site is completely selective for the mutant *BRAF* sequence. Complete probe and target sequences are shown in Figure S1.

Figure 3.

Cross-link yield as a function of the amount of mutant *BRAF* sequence present in mixtures of mutant and wild-type duplexes. Samples run in triplicate containing various proportions of mutant and wild-type *BRAF* duplexes (21 bp) were denatured by warming at 50–70 °C in the presence of the second-generation probe, cooled, incubated at 37 °C, and the yield of interstrand cross-link assessed by gel electrophoretic analysis.

Figure 4.

Cross-linked DNA generated from the mutant BRAF-probe duplex E can be readily detected by its unique current signature in the α -HL nanopore. The mixtures of species generated by incubation of the third-generation probe with either mutant or wild-type BRAF gene sequence were analyzed using a single α -HL ion channel embedded in a lipid bilayer. Current traces were recorded at +120 mV in Tris (10 mM, pH 7.4) containing KCl (1 M) at 22 °C. Panel A: Analysis of the mixture generated by hybridization of the mutant BRAF sequence with the Ap-containing probe strand. The current block was recorded for 1 min, then voltage polarity was reversed to translocate the cross-linked duplex back to the *cis* solution. Current trace showing persistence of the current block by cross-linked duplex E for 30 min is provided in Figure S6. Panel B: The wild-type BRAF sequence does not generate cross-linked DNA when hybridized with the Ap-containing probe strand. Short current

blocks are consistent with translocation of single-stranded DNA and uncross-linked duplex
F. The illustration depicts the three-step unzipping/translocation process for duplex DNA.

Figure 5. Incremental current decreases induced by sequential, irreversible blocking of individual α -HL pores by cross-linked duplexes in an experiment with multiple channels embedded in the lipid bilayer. The mixture contained cross-linked duplex, uncross-linked duplex, and single strands. The analysis was carried out at 120 mV in Tris buffer (10 mM, pH 7.4) containing KCl (1 M) at 22 °C. The trace shown was low-pass filtered at 1 kHz.