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Solid-phase DNA binding detection by EPR spectroscopy

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Abstract—We show here that spin probe labeled oligonucleotides coupled with EPR detection can be used to detect oligonucleotide binding, to optimize conditions used for annealing, and provides a highly selective method for oligonucleotide hybridization detection under conditions that model DNA biochips.

The detection of DNA sequences by DNA biochips is a rapidly developing technology.[1] In its present form, DNA sequences are attached to a chip, the chip is then exposed to solutions containing DNA and, if a complementary sequence is present, it anneals to the chip. The chip is then treated with a fluorescent probe that detects double stranded (ds) DNA. The technique is very sensitive, mainly due to the method of detection. However, there are some aspects of the method that are problematic. For example, hybridization may be incomplete, it may be difficult to optimize hybridization conditions, and detection of hybridization may be non-selective.[2] Thus, it is desirable to develop alternative methods of detection that can also provide insight into oligonucleotide interactions on the chip.

An alternative method of detection we are exploring is based on electron paramagnetic resonance (EPR) using nitroxide spin labels. Conceptually, this method would use spin labeling of either sequences attached to the chip or of the target DNA (RNA or protein) in solution. The label could be attached in either a non-site specific or site-specific manner by any of several methods. Spin-labeling of biological molecules in solution, such as DNA by enzymatic ligation with a spin-labeled nucleoside,[3,4] would require additional manipulation of the samples and attachment of the spin label to the DNA biochip is preferred. We are engaged in developing DNA chips to serve as biosensors and are exploring EPR based methods of detection. Questions addressed in this work include determining whether this method can be used to differentiate DNAs that have annealed to a solid-phase bound oligonucleotide versus DNAs that are in solution, whether the spin label should be attached directly to the chip or to the DNA in solution, the optimal conditions under which the annealing process should be conducted, and the synthesis of new, effective, spin labels for DNA Biochips.

The spin labels used here were the probes **1** or **2** (Fig. 1). Spin label **1** has been previously described[5] and **2** is a similar label but synthetically more simple to prepare.[6] These spin labels were prepared as their phosphoramidites for automated DNA synthesis on controlled pore glass (CPG) using standard procedures. The sequences prepared were T_7–T_{sp} or T_7–T_{sp}–T_7 (T_{sp} = **1** (T_{5sp}) or **2** (T_{6sp})) such that the spin label was located at the terminus or in the middle of the oligonucleotide, respectively. The complementary strands required (A_8 or A_{15}) were also prepared by automated

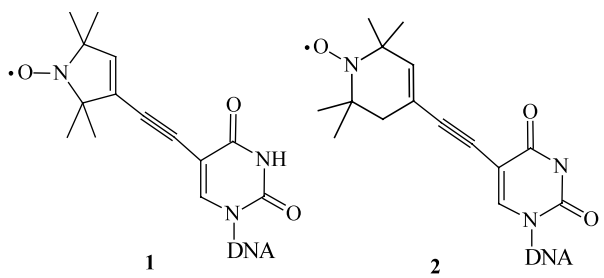

Figure 1. Spin-labeled phosphoramidites **1** (T_{5sp}) and **2** (T_{6sp}) used to prepare spin-labeled oligonucleotides.

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DNA synthesis on either CPG or on polystyrene (OAS-PS, Glen Research). Those prepared on CPG were cleaved from the resin, protecting groups removed, and purified by HPLC. T_7–T_{sp}–T_7 (T_{sp}=T_{5sp} or T_{6sp}) were purified by SAX chromatography (Buffer A: 10 mM NaOH, pH 11.8; Buffer B: 10 mM NaOH, 1 M NaCl, pH 11.8, 30–45% B over 90 min). Likewise, A_{15} was purified on SAX (with 26% B). A_{15}, prepared on OAS-PS, was deprotected but is not cleaved from the resin under the conditions used for deprotection.

We first examined the approach where the spin labels were attached to oligonucleotides that were in solution and the complementary strand was attached to a solid support. As noted, the A_{15} was prepared on OAS-PS (OAS-PS-A_{15}) and not on CPG. This was necessary because removal of the protecting groups used during the automated DNA synthesis of A_{15} without cleavage of the oligonucleotide from a CPG support is not possible. OAS-PS-A_{15} was prepared on an ABI 391 DNA synthesizer in accordance with the supplier's instructions.

In Fig. 2a is the EPR spectrum of T_7–T_{5sp}–T_7 and is typical of a spin-labeled single-stranded DNA. The spectra were simulated with the program NLSL.[7] The

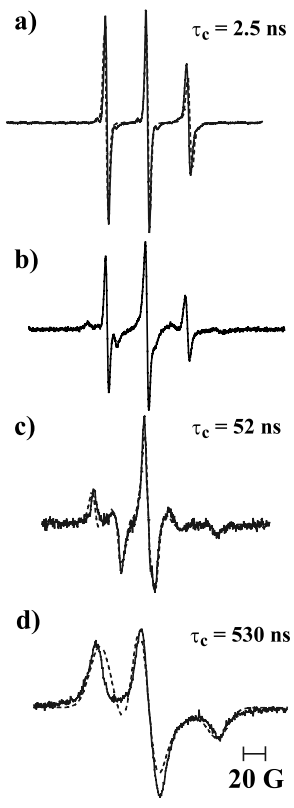

Figure 2. Room temperature, X-band (9.4 GHz EPR spectra of (a) T_7–T_{5sp}–T_7 in 10 mM phosphate buffer, 100 mM NaCl, pH 7.4; (b) OAS-PS-A_{15} after annealing to T_7–T_{5sp}–T_7 in 10 mM phosphate buffer, 100 mM NaCl, pH 7.4; (c) as in (b) except 1 M NaCl, and (d) T_7–T_{5sp}–T_7 in the solid state. Dashed lines are the best fit simulation spectrum to the experimental spectrum.

fitting procedure provides the correlation times (τ_c) of the spin label, a measure of its mobility. The spectrum in Fig. 2a was fit assuming only one species present with τ_c=2.5 ns. When OAS-PS-A_{15} was treated with T_7–T_{5sp}–T_7 under standard annealing conditions (10 mM phosphate buffer, 100 mM NaCl, pH 7.4, 30 min), and the beads then washed with annealing buffer, the spectrum shown in Fig. 2b was obtained. This spectrum is mainly comprised of free T_7–T_{5sp}–T_7 though a second species is clearly present. Repetition of this experiment with the NaCl concentration increased to 1 M gave the spectrum shown in Fig. 2c. In this spectrum, the T_7–T_{5sp}–T_7 appears to be completely annealed to OAS-PS-A_{15} and is easily distinguished from that of T_7–T_{5sp}–T_7, free in solution (Fig. 2a).

Attempts to fit the spectrum shown in Fig. 2c were made assuming one, two, or three species were present. These three species could correspond to the unbound spin-labeled DNA (Fig. 2a), annealed (OAS-PS-A_{15}):(T_7–T_{5sp}–T_7) and immobilized (solid T_7–T_{5sp}–T_7) (Fig. 2d). The best fit was obtained when one species was assumed to be present and the value of τ_c which resulted was 52 ns. Note that simple washing of the sample with 100 mM NaCl (10 mM phosphate buffer pH 7.4) returns the spectrum to that shown in Fig. 2b. Thus, to ensure complete annealing of the oligonucleotides requires high salt concentrations.

The second approach we examined was when the spin label was attached to the solid support and the unlabeled complementary strand was in solution. The EPR spectra (X-band, 9.4 GHz) of CPG-T_7–T_{5sp}–T_7, before and after exposure to the complementary sequence (A_{15}, 10 mM phosphate buffer, 100 mM NaCl, pH 7.4) were not significantly different from one another and would not likely be useful for distinguishing between unbound, single-stranded spin-labeled DNA from the corresponding double stranded species.

We then examined the effect of moving the spin label to the terminus of the bound oligonucleotide. The resulting EPR spectra of the CPG-T_7–T_{5sp} and CPG-T_7–T_{5sp}:A_{15} leads to a different set of EPR spectra than were observed for CPG-T_7–T_{5sp}–T_7 and CPG-T_7–$T_{5sp}T_7$:A_{15}, as shown in Fig. 3. In Fig. 3a is the EPR spectrum of CPG-T_7–T_{5sp} and in Fig. 3b is the spectrum of CPG-T_7–T_{5sp}:A_{15}, after annealing to A_{15} and using the same conditions as were used for OAS-PS-A_{15}:T_7–T_{5sp}–T_7. The spectrum of the unannealed CPG-T_7–T_{5sp} is similar to the free nitroxide (Fig. 2a) while the annealed spectrum of CPG-T_7–T_{5sp}:A_{15}, shown in Fig. 3b, is similar to the annealed spectrum of OAS-PS-A_{15}:T_7–T_{5sp}–T_7 shown in Fig. 2c. Although the two spin-labeled CPG-bound oligonucleotides differ in length, it is likely that the key difference between CPG-T_7–T_{5sp}–T_7 and CPG-T_7–T_{5sp} is the location of the spin label, being at the terminus of the oligonucleotide strand in the latter and in the middle of the strand in the former. This difference is key as the spectra shown in Fig. 3a and b would be useful for distinguishing between unbound and bound oligonucleotides on a DNA biochip.

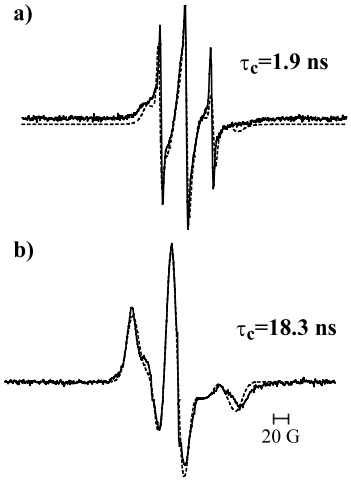

Figure 3. Room temperature, X-band (9.4 GHz EPR spectra of (a) CPG-T_7–T_{5sp} (washed with 10 mM phosphate buffer, 1 M NaCl, pH 7.4) and (b) after annealing with A_{15} (10 mM phosphate buffer, 1 M NaCl, pH 7.4). Dashed lines are the best fit simulation spectrum to the experimental spectrum.

The reason for the differences observed between CPG-T_7–T_{5sp}–T_7:A_{15} and CPG-T_7–T_{5sp}:A_{15} may be due to the difference in the accessibility of it to water. CPG-T_7–T_{5sp}–T_7:A_{15} locates the probe in such a way that it is constrained by the oligonucleotide chain and the chain may prevent or inhibit access to it by water. In contrast, the spin label in CPG-T_7–T_{5sp} is at the terminus of the oligonucleotide and is therefore less constrained by the oligonucleotide and has greater accessibility to water.

Here we have shown that spin-labeled DNAs may prove to be a valuable tool in the development of DNA biochips. Currently, spin labels can not match the sensitivity available from fluorescent probes, though they do offer other characteristics that may make them useful. First, since they are bonded to an oligonucleotide and display unequivocal changes in their EPR spectrum when they bind, they are highly sequence-selective. Second, spin-labeled DNAs should be useful for optimizing annealing conditions such as temperature, time, and annealing buffer. Third, since spin labels are uniquely sensitive to their environment, they may be a useful tool for investigating the binding of oligonucleotides to DNA biochip surfaces. Finally, we stress that there is considerable potential for enhancing the specificity, absolute sensitivity and resolution of the EPR technique by utilizing higher fields and frequencies, as has been documented in the literature.[8,9]

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