Fluorescence Spectroscopy

DNA Detection through Signal Amplification by Using NADH:Flavin Oxidoreductase and Oligonucleotide–Flavin Conjugates as Cofactors**

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Sensitive detection of DNA on the basis of hybridization to a complementary DNA probe and complex-specific signal (for example, fluorescence) detection, may be improved by molecular amplification methods.[1] DNA target amplification is one of the most widely used methods and is mainly based on the polymerase chain reaction (PCR). Recently, signal-amplification techniques based on catalytic reactions, which might be useful for PCR-independent detection of label-free DNA sequences, have also been investigated.[2–5] These methods allow each probe-hybridization event to be converted into many signal events because the catalyst (a chemical or an enzyme) turns over many copies of the sensing-reaction substrate. As a consequence, high sensitivity can be attained. For example, the insertion of ferrocene moieties or redox-active intercalators allows hybrids to catalyze electrochemical reactions that can be monitored either amperometrically or chemically.[2,3] Herein we propose an original and simple strategy that involves the cofactor of an enzyme as the catalytic species. In this system, the DNA probe is an oligonucleotide covalently attached to the cofactor, and the enzyme is selected on the basis of its ability to catalyze the cofactor-dependent conversion of a fluorogenic substrate into an optically silent product (Figure 1). If the enzyme is functional only with a single-stranded cofactor–oligonucleotide conjugate, and not when the latter is hybridized to its complementary strand, enzymatic conversion of the substrate, monitored by fluorescence spectroscopy, can serve as a tool to differentiate whether the probe is hybridized or not (Figure 1). Since the enzyme turns over many copies of the fluorogenic substrate, the difference in the fluorescence

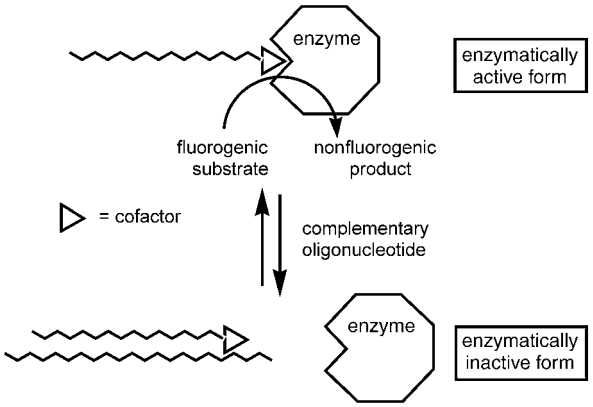

Figure 1. Schematic representation of DNA detection by using an enzyme and its cofactor conjugated to an oligonucleotide probe.

signals obtained with the free and the hybridized probes can be greatly amplified enzymatically.

This new concept is illustrated herein with an enzyme that catalyzes the oxidation of reduced pyridine nucleotides, either nicotinamide adenine dinucleotide phosphate (NADPH) or nicotinamide adenine dinucleotide (NADH), by molecular oxygen in the presence of a riboflavin, either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD), as a cofactor. Such an enzyme is called an NAD(P)H:flavin oxidoreductase or flavin reductase.[6] We selected the flavin reductase from *Escherichia coli*, named Fre, which is soluble, monomeric, and very easy to purify in large amounts.[7,8] Structural, mechanistic, and substrate-specificity studies in our laboratory[9–12] have shown that Fre contains an active site which accomodates both the flavin and the reduced pyridine nucleotide and that the reaction proceeds in two steps (Scheme 1): first, a hydride transfer from NAD(P)H to the

$$\mathrm{NAD(P)H + H^+ + flavin_{ox} \longrightarrow NAD(P)^+ + flavin_{red}}$$

$$\mathrm{flavin_{red} + O_2 \longrightarrow flavin_{ox} + H_2O_2}$$

Scheme 1. The reaction catalyzed by Fre, an NAD(P)H:flavin oxidoreductase.

oxidized flavin and then an oxidation of the reduced flavin by molecular oxygen, thereby regenerating the cofactor for a new cycle. With small amounts of flavin it is thus possible to oxidize large excesses of NAD(P)H, a process that can be easily monitored spectrophotometrically since NAD(P)H absorbs light at 340 nm whereas $NAD(P)^+$ does not. The reaction can also be followed by fluorescence spectroscopy since NAD(P)H is fluorogenic ($\lambda_{excitation} = 340$ nm, $\lambda_{emission} = 460$ nm).

Fre was selected for its ability to interact with the flavin substrate exclusively through the isoalloxazine ring with a minor contribution of the ribityl chain, thus allowing a variety of modifications of the latter with moderate effect on the activity.[9] **Fl1** (Scheme 2), a riboflavin analogue in which the OH groups of the ribityl chain at positions 2–4 have been removed, and its 5′ conjugate to thymidine through a phosphate group, **Fl2**, which have previously been synthe-

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[**] NADH = nicotinamide adenine dinucleotide, reduced form. This work was supported by the CNRS program "Puces à ADN".

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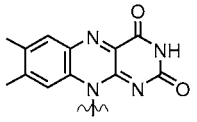
Conjugates:



Fl

Fl—$(CH_2)_6$—OH **Fl1**

Fl—$(CH_2)_6$—O—P(=O)(O$^-$)—5'—d(T)$_n$ **Fl2:** n = 1, **1**: n = 11, **2**: n = 16

Fl—$(CH_2)_6$—O—P(=O)(O$^-$)—5'—d(CCCCCACCACTTCCCCTCTC) 3' **3**

Targets:

4: 5' d(CGTGT**(A)$_{11}$**GGCAGTAC) 3'

5: d(GCCTGAT**GAGAGGGGAAGTGGTGGGGG**AGACATAGCCCACC)

6: d(GCCTGAT**GAGAGGGGAAGTGGTGG**CCCAGACATAGCCCACC)

7: d(GCCTGAT**GAGAGGGGAAGTGG**ATTCCCAGACATAGCCCACC)

8: d(GCCTGAT**GAGAGGGGA**CT**TGGTGGGGG**AGACATAGCCCACC)

Scheme 2. Oligonucleotide–flavin conjugates and the corresponding targets. Fl designates the isoalloxazine ring.

sized,[13] are excellent substrates of Fre, as shown from a comparison of the obtained Michaelis constant (K_M) and maximal rate (V_{max}) values with those determined for the natural FAD cofactor (Table 1). Since the probes represented in Figure 1 are oligonucleotide–flavin conjugates, which should function as cofactors of the flavin reductase, we used conjugates **1**–**3** (Scheme 2) in which the 5′ end of the oligonucleotide is attached to the terminus of the side chain of **Fl1**. These conjugates, previously exploited in other applications, were prepared as previously described.[13,14] The broad specificity of Fre with regard to flavins was confirmed with the demonstration, for the first time, that macromolecular single-stranded oligonucleotide–flavin conjugates can also be used by the enzyme (Table 1). Since the enzymatic reduction of these compounds by NADH follows Michaelis–Menten behavior (not shown), it was possible to determine kinetic V_{max} and K_M parameters (Table 1). The data indicated that the K_M value is indeed only affected moderately by the covalent attachment of the 5′ end of an oligonucleotide to the lateral chain of the flavin. The V_{max} value is also only affected moderately. It is likely that recognition by Fre is possible because the spacing provided by the C_6 linker allows the redox-active isoalloxazine moiety to enter the active site. The three-dimensional structure of Fre in complex with riboflavin indeed shows that the ribityl chain is, to a great extent, exposed to the solvent.[12] Thus, oligonucleotide–flavin conjugates can be readily detected in solution from the oxidation of NADH in the presence of catalytic amounts of Fre. This reaction can be monitored on the basis of the decay of the intensity of either the absorption band at 340 nm or the fluorescence at 460 nm. The large excess of NADH with respect to the flavin (20:1–2000:1), as well as the lack of flavin fluorescence below 470 nm and the limited absorption of the flavin between 290–350 nm, prevents flavin absorption/fluorescence from interfering under the assay conditions used.[15,16] In Figure 2 is shown the effect on NADH oxidation at 25 °C of adding increasing amounts of **5**, a 41-mer oligonucleotide containing a 20-mer sequence complementary to that of **3**, to a 10 μM solution of conjugate **3**. We determined a melting temperature (T_m) value of 62 °C for the **3**:**5** duplex under the conditions of the assay and verified that the presence of Fre has no effect on this value. Whereas the addition of a twofold excess of **5** almost totally inhibited the reaction, the addition of a tenfold excess of a noncomplementary oligonucleotide had no effect on the enzyme activity (data not shown). Thus, **5** behaves as an efficient inhibitor of the reaction as a consequence of its ability to hybridize to **3** and to generate a duplex in which the flavin moiety is not recognized by Fre anymore, probably because of increased

Table 1: Oligonucleotide–flavin conjugates are cofactors of Fre, the NAD(P)H:flavin oxidoreductase from *Escherichia coli*.[a]

Flavin substrate	V_m [nmol min^{-1} mg^{-1}]	K_M [μM]
FAD	16000	1
Fl1	14000	2
Fl2	12000	1
1	8500	4
2	10000	6
3	12000	6

[a] Conditions: The oxidation of 250 μM NADH was monitored spectrophotometrically (λ_{max} = 340 nm; ε = 6220 mM^{-1} cm^{-1}) in the presence of 2 μg mL^{-1} Fre and 1–45 μM flavin substrate at 25 °C. The buffer was 50 mM tris(hydroxymethyl)aminomethane/HCl (Tris-HCl; pH 7.6) with 1 M NaCl (for **1** and **2**) or 10 mM NaCl (for **3**).

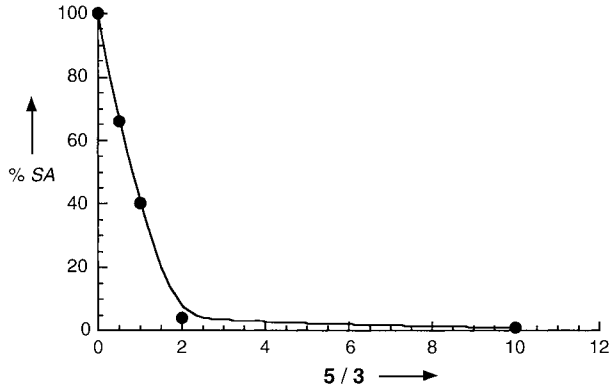


Figure 2. Inhibition of enzymatic oligonucleotide–flavin-dependent NADH oxidation by the complementary strand. The assay mixture contained 10 μM **3**, the flavin reductase (0.36 μg), 200 μM NADH in 50 mM Tris-HCl, and 10 mM NaCl buffer at 25 °C, as well as the complementary oligonucleotide **5** (Scheme 1) in increasing amounts. The activity is assayed on the basis of the oxidation of NADH as monitored spectrophotometrically at 340 nm. **5**/**3** designates the molar excess of oligonucleotide **5** with regard to **3**; %SA designates the percentage specific activity [nmol min^{-1} per mg protein] with 100% corresponding to an assay in the absence of **5**.

steric hindrance. It is also possible that the isoalloxazine moiety is made less available because of specific interactions in the duplex, in agreement with the reported observation that the fluorescence at 520 nm of an oligonucleotide–flavin conjugate is almost totally quenched within a duplex and that the redox properties of the isoalloxazine ring in such a duplex are greatly modified.[17,18]

We propose that enzyme oxidation of NADH is a simple and sensitive method to detect a target DNA by using an oligonucleotide–flavin probe and catalytic amounts of Fre. The enzyme is crucial as it not only greatly accelerates the sensing reaction but it also allows a clear differentiation between the free and hybridized probes, since Fre recognizes only the former as a cofactor. The intensity of the signal (the difference between free and hybridized probes) can be made very large as a result of the possibility of consuming large concentrations of NADH when the probe is free (Figure 2). Mismatches in the complementary sequence of the target DNA could also be detected by this technique. Indeed, various degrees of inhibition of the flavin reductase activity were observed with targets of variable complementarity. The results (Table 2) indicate that Fre-activity measurements

Table 2: Inhibition of **3**-dependent NADH oxidase activity by hybridization.

Target DNA	Specific activity [%][a]
none	100
5	1
6	12
7	30
8	5

[a] 100% corresponds to 6000 nmol min^{-1} per mg protein. The assay was carried out in the presence of 10 equivalents of target DNA (**5**–**8**) with regard to oligonucleotide **3**, 200 μM NADH, and 3 μg mL^{-1} Fre in 50 mM Tris-HCl (0.1 mL, pH 7.6) with 10 mM NaCl.

make it possible to discriminate targets of slightly different sequences. Less inhibition occurs as a result of mutations at the 3′ part of the target facing the 5′ part of the probe **3** (compare **5** to **6** and **7**, Scheme 2 and Table 2) or of mutations in the interior of the DNA target (compare **5** to **8**, Scheme 2 and Table 2). This effect probably results from a combination of increased accessibility of the flavin moiety and decreased amounts of the hybridized probe at equilibrium and might have applications for the detection of mutated sequences in solution.

The experimental results shown in Figure 3 indicate that the enzymatic NADH oxidation reaction can be carried out in a standard 96-well plate containing the oligonucleotide–flavin probe **1** (0.5 μM, 7.5 pmol) and the enzyme in solution (15 μL), and monitored by a simple camera working at an excitation wavelength of 302 nm, which corresponds to radiation that is very little absorbed by the flavin. The reaction was initiated by the addition of 1 mM NADH, and fluorescence images of the plate were obtained after incubation for 15 min at 18°C. As shown in Figure 3, intense fluorescence was detected only in the well containing the complementary oligonucleotide **4** after the reaction (right column, middle well). No fluorescence was detected when the reaction was carried out in the absence of **4** or in the presence of a noncomplementary oligonucleotide (Figure 3). Finally, when the reaction was carried out at temperatures above 25°C, no difference was observed whether the oligonucleotide **4** was present or not, since at this temperature a large amount of **1** is not hybridized to **4** (the T_m value for **1**:**4** duplex is 27°C).

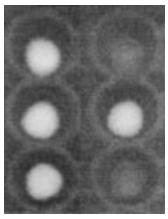


Figure 3. Fluorescence detection of DNA hybridization by using the oligonucleotide–flavin/flavin reductase technique. The image is obtained after incubation (15 min) of 0.5 μM **1** with 1 mM NADH, 100 mM NaCl, and Fre (1.5 μg) in 50 mM Tris-HCl (15 μL, pH 7.5) at 18°C in the presence or absence of the target **4** followed by UV illumination at 302 nm. Left: time zero; right: after 15 min. Top: **1** alone; middle: **1** in the presence of **4** (10 equiv); bottom: **1** in the presence of a noncomplementary 22-mer oligonucleotide (10 equiv).

In this work, no attempt at optimization in terms of sensitivity has been carried out and further investigation is required to reach the highest sensitivities recently reported for comparable systems.[2–4] Theoretically, enhanced sensitivity with this system can be achieved either by prolonging the reaction or by increasing the concentration of the enzyme. For example, the same image as that shown in Figure 3 was also obtained with 50 nM **1** (0.75 pmol in 15 μL) with the same enzyme concentration but with a reaction time of 2 h. In fact, one of the limitations resides in the K_m value for the flavin. Improvement of the system will depend on the development of oligonucleotide–flavin conjugates with smaller K_m values. Previous studies have shown that this goal is realistic.[9] Another limitation is the weak fluorescence of NADH, but this problem can be solved with more-fluorescent analogues, since we have shown that Fre also displays unique binding properties regarding reduced pyridine nucleotides, thereby allowing extensive modifications of the latter.[10]

In conclusion, the concept summarized in Figure 1 for DNA detection has been illustrated experimentally.[19] We have shown that single-stranded oligonucleotide–flavin conjugates are good substrates of an NAD(P)H:flavin oxidoreductase. The fact that hybridization to the complementary strand inhibits the enzyme reaction selectively provides the basis for an enzymatic amplification of nucleic acid sensing. The advantage of the method is the simplicity of the probe and of the sensing reaction.

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