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Fluorescent Deazaflavin – Oligonucleotide Probes for Selective Detection of DNA

Cécile Dueymes, Jean Luc Décout,* Philippe Peltié, and Marc Fontecave*

Arrays of oligonucleotide probes (DNA chips) immobilized on glass or silicon surfaces have emerged as powerful new tools for the analysis of DNA and RNA.[1] These specific DNA sensors operate through hybridization reactions which are based on the mutual recognition of two complementary nucleic acid strands that establish hydrogen bonds between their nucleic bases. Hybridization is most commonly assayed by fluorescence, and hence requires that fluorophore labels are covalently attached to the DNA target fragments to be analyzed.

This technology, however, is still fraught with a number of drawbacks and requires new developments. For example, it is still difficult to assess the quality of the oligonucleotides attached to the surface. The homogeneity and the reproducibility of the procedures for preparing the DNA-functionalized surfaces, and consequently the DNA surface density, are difficult to control. Furthermore the polymerase chain reaction (PCR) step, which serves to amplify the DNA targets, and the subsequent chemical step, for the attachment of the fluorophore to the target DNA, are responsible for significant modifications in the relative proportions of the different populations of the nucleic acids to be analyzed.

We propose that some of these problems might be simply solved if the detection label (here a fluorophore) is incorporated on the array capture DNA strand and not on the target to be detected. This strategy might have many advantages. First it greatly reduces the number of manipulations of the targets. Second, it provides a way to control the quality of the DNA array (by using standard fluorescence scanners) in the absence of the targets. Such an array requires that: 1) the fluorescence is not quenched by interaction of the fluorophore with the surface; 2) the label on the probe does not affect its affinity for its complementary oligonucleotide; and 3) the fluorescence is significantly changed as a specific consequence of hybridization of the functionalized DNA

[*] Prof. J. L. Décout
Département de Pharmacologie Moléculaire
UMR Université J. Fourier, CNRS no. 5063
Domaine de la Merci, 38706 La Tronche Cedex (France)
Fax: (+33) 4-76-51-86-67
E-mail: jean-luc.decout@ujf-grenoble.fr
Prof. M. Fontecave, C. Dueymes
DBMS-CB
UMR Université J. Fourier, CNRS, CEA no. 5047
CEA Grenoble
17 Avenue des Martyrs, 38054 Grenoble Cedex 9 (France)
Fax: (+33) 4-38-78-91-24
E-mail: mfontecave@cea.fr
Dr. P. Peltié
LETI, CEA Grenoble
17 Avenue des Martyrs
38054 Grenoble Cedex 9 (France)

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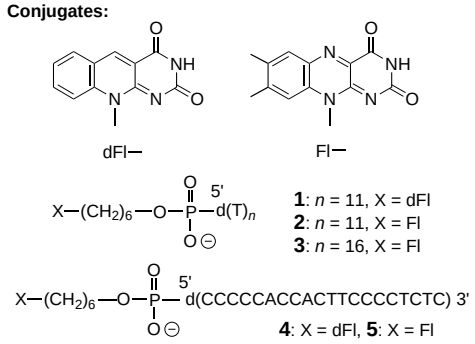
probe to its complementary target, so that DNA recognition can be detected.

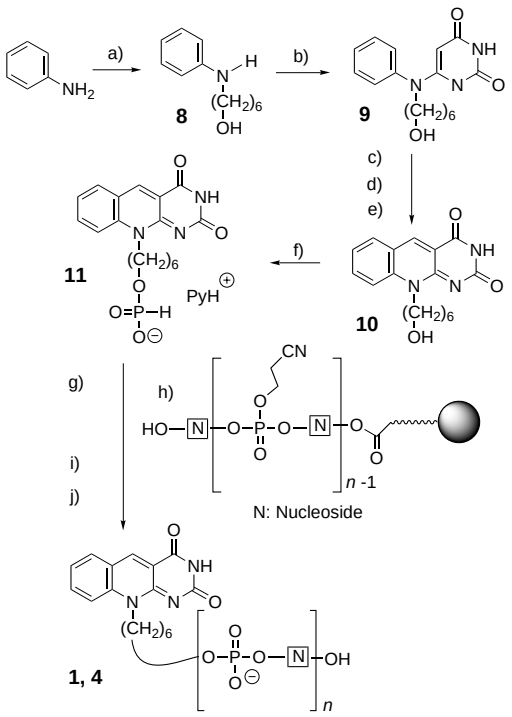
Here we report that flavins and deazaflavins provide the first examples of such a label. Flavins and deazaflavins exhibit a bright blue or green fluorescence at 520–540 and 450–460 nm, respectively, in aqueous solutions, with deazaflavins giving larger fluorescence intensities. We have recently reported an efficient method for the synthesis of stable flavin–oligonucleotide conjugates that is also used here for the preparation of new deazaflavin–oligonucleotide conjugates.[2] Furthermore, these conjugates are stable to UV irradiation.[3] Preliminary results demonstrate here that these molecules are able to hybridize to a complementary oligonucleotide target both in solution and on solid support, and that the hybridization can be detected by a dramatic quenching of the probe fluorescence.

The deazaflavin–oligonucleotide conjugates **1** and **4** (Scheme 1 and 2) were synthesized from the corresponding oligo-2′-deoxynucleotides prepared by the phosphoramidite automated method with standard building phosphoramidites



Scheme 1. Oligonucleotide conjugates of 5-deazaflavin (dFl) and flavin (Fl), as well as their corresponding targets.

(see Supporting Information). The 5′-hydroxyl group of the oligonucleotide attached to the solid support was coupled to the 5-deazaflavin H-phosphonate derivative **11** (synthesized from aniline and 6-chlorouracil) after activation with adamantanecarboxylic acid chloride (Scheme 2).[4] Oxidation of the conjugate on the solid support with iodine and then cleavage and deprotection with sodium hydroxide afforded the conjugates **1** and **4** in high yields (60 and 53%, respectively). The conjugates **1** and **4** were characterized by ^{1}H and ^{31}P NMR spectrometry, mass spectrometry (matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI)), and absorption spectrophotometry.



Scheme 2. Synthesis of the 5-deazaflavin–oligonucleotide conjugates: a) $HO(CH_2)_6Cl$, 120 °C, 74%; b) 6-chlorouracil/H_2O/EtOH, reflux, 65%; c) Ac_2O/Py, RT; d) DMF/$POCl_3$, 100 °C; e) KOH/EtOH, RT, 70% for the three (c–e) steps; f) H_3PO_3/$TPSO_2Cl$/Py, 85%; g) adamantanecarboxylic acid chloride/3′-acetylthymidine/Py; h) nucleoside, coupling step; i) I_2/THF/Et_3N; j) NaOH/H_2O/CH_3OH. Py = pyridine, TP = 2,4,6-triisopropylbenzene.

The fluorescence emission spectrum of the deazaflavin moiety of the conjugates was comparable to that of the free deazaflavin, with a peak maximum at 450 nm (excitation at 396 nm) and a high quantum yield of 0.26, which is comparable to or even better than that of standard fluorophores such as Alexa and Cyanine dyes. Furthermore, the observation that the duplex has the same melting temperature ($T_m = 62$ °C, NaCl 10 mM) whether the probe contained the tag or not[5] indicates that the deazaflavin tag had no effect on the stability of the duplex formed between the oligonucleotide probe and a 42-mer oligonucleotide target, **ICAM20**, which contains the 20-mer complementary sequence (Scheme 1).

Figure 1 shows a representative experiment in which the addition of increasing amounts of the target oligonucleotide **ICAM20** resulted in a decay of the fluorescence intensity of compound **4** at 450 nm to less than 5% of the initial value after the addition of one equivalent of **ICAM20**. Control experiments, carried out either by addition of **ICAM20** in the presence of one equivalent of the free deazaflavin to an oligonucleotide identical to **4** but lacking the deazaflavin group or by addition of increasing amounts of a 45-mer oligonucleotide target with no complementary sequence to a solution of conjugate **4**, resulted in no variation in the fluorescence of the deazaflavin unit. In the case of conjugate **1**, quenching also occured upon hybridization with target **6** ($T_m = 43$ °C, NaCl 1M), and the fluorescence was shown to be fully restored upon heating to a temperature that allowed dissociation of the complex. Several cycles of hybridization/

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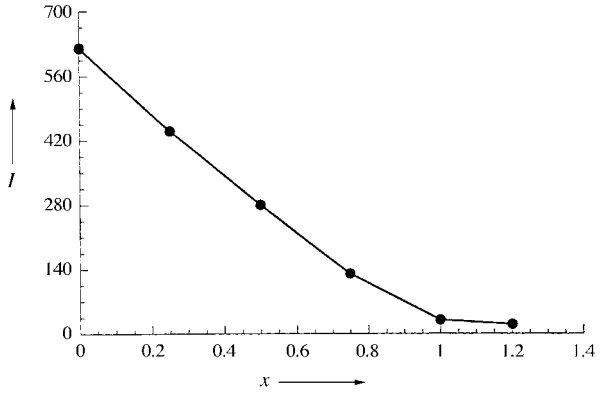

Figure 1. Effect of the addition of increasing amounts of **ICAM20** on the fluorescence intensity (I) of conjugate **4** (2 μM in 50 mM tris(hydroxymethyl)aminomethane-HCl (Tris-HCl), pH 7.5, 10 mM NaCl, 25 °C) at 450 nm (excitation at 396 nm). x represents the target/**4** ratio.

melting, as monitored by fluorescence spectroscopy, were successfully performed. All these highly reproducible results showed that the fluorescence quenching observed here was a specific property of a deazaflavin moiety attached to an oligonucleotide on hybridization to the complementary strand.

Similar results were obtained with the flavin–oligonucleotide conjugates **2**, **3**, and **5** (synthesized as described previously[2]) and using the oligonucleotides **6**, **7**, and **ICAM20** as the complementary strands, respectively. In the case of probe **3**, sequence variations in the proximity of the flavin moiety (GC, CG, or CC in place of the GG doublet adjacent to the polyA strand) in the target **7** had no effect on the quenching efficiency. The results taken together indicate that the extent of fluorescence quenching, with both deazaflavin and flavin as markers, does not depend on particular target lengths and sequences. This observation seems to rule out specific interactions, such as hydrogen bonding between the probe fluorophore and bases of the target, as the origin of the quenching.

An interesting observation is shown in the Stern–Volmer representation $I_o/I(x)$ plotted as a function of increasing amounts x of various targets (Figure 2). The results indicate that fluorescence quenching makes it possible, with a given

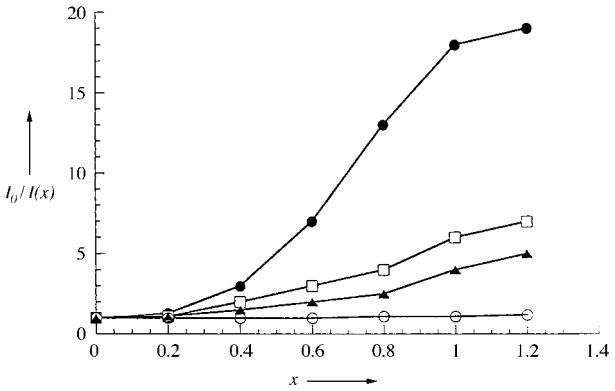

Figure 2. Effect of the addition of increasing amounts of different targets (● **ICAM2O**, □ **ICAM18**, ▲ **ICAM17**, ○ **ICAM14**) on the fluorescence of conjugate **4** under the same conditions as for Figure 1. I_o represents the initial fluorescence intensity of **4** and $I(x)$ its intensity after the addition of x equivalents of the target oligonucleotide.

probe, to discriminate targets of variable complementarity. Significantly decreased quenching occurs because of decreased base pairing, which occurs as a result of mutations at the 3′ part of the sequence facing the 5′ part of conjugate **4** to which the deazaflavin moiety is attached (compare **ICAM20** to **ICAM17** and **ICAM14**) or because of mutations in the interior of the DNA targets (compare **ICAM20** to **ICAM18**). This effect probably results from a combination of an increased distance between the deazaflavin and the target and decreased amounts of the hybridized probe at equilibrium, and hence might have applications for the detection of mutations in solution.

Since deazaflavin–oligonucleotide conjugates might serve as fluorescent probes in the new concept of DNA microarrays proposed here, we finally checked whether the same duplex-specific quenching effect occurred on a solid surface. In this experiment 20 μL of a 10 μM solution of conjugate **4** were deposited at several places on a glass slide and then dried. By shining a mercury 100 W lamp of an epi-fluorescence microscope through an appropriate dichroic mirror for excitation at 396 nm, fluorescence emission could be detected with a high sensitivity spectrophotometer through an optical fiber mounted on the microscope in place of the camera.[6] This arrangement allowed the detection of a well-resolved signal at 500 nm (intensity: 1100 arbitrary units) corresponding to the fluorescence of the deazaflavin moiety of conjugate **4** on the glass surface. Duplex formation was allowed for 10 min on several spots after the addition of five equivalents (20 μL in 0.1M NaCl) of the complementary oligonucleotide **ICAM20**. An assay of the fluorescence of each spot after drying the surface revealed a reproducible large quenching (intensity: 100 arbitrary units). No quenching of fluorescence could be detected when oligonucleotides with no complementary sequence were instead added on fluorescent spots under the same conditions and then dried. This result demonstrates that quenching was specific for the hybridized probe and not the result of some unspecific effect of the added oligonucleotide.

In conclusion, we propose that flavins or deazaflavins might be suitable fluorescent markers for oligonucleotide probes to be used in DNA microarrays. First, these markers do not affect the affinity for the complementary strand. Second, their fluorescence is also detectable on a solid surface by fluorescence scanners, which thus provides a means of controlling the quality of the array. Third, their fluorescence is specifically quenched upon hybridization to a complementary sequence, thus allowing DNA detection. Fourth, the hybridization/fluorescence quenching is reversible. Even though, in general, a generation of a signal is preferred to a decay, the concept of labeled DNA arrays proposed here is also likely to be applicable and to provide significant advantages.

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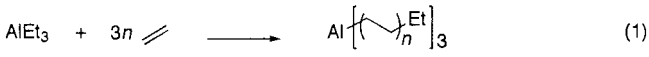
[6] The high-sensitivity spectrophotometer with a grating optimized for visible light (360 – 850 nm) was purchased from Ocean Optics Europe Company.

Iron-Catalyzed Polyethylene Chain Growth on Zinc: Linear α-Olefins with a Poisson Distribution**

George J. P. Britovsek, Steven A. Cohen, Vernon C. Gibson,* Peter J. Maddox, and Martin van Meurs

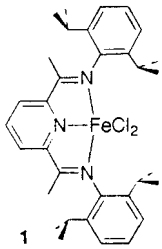
Fifty years ago Ziegler and co-workers reported the *Aufbaureaktion* for alkylaluminum compounds.[1] This reaction involves a stepwise insertion of ethylene into the Al–C bonds of, for example, triethylaluminum to give long-chain trialkylaluminum compounds [Eq. (1)]. Today, this chain-growth reaction is commercially exploited for the synthesis of linear α-olefins and primary alcohols from ethylene.[2]



(1)

Subsequently, Ziegler and co-workers discovered the effect of transition metals such as colloidal nickel on this reaction, which suppressed chain growth to give only butene.[3] The elucidation of this "nickel effect", and the subsequent screening of the periodic table for the effects of other metals led to the discovery of the first transition metal catalyzed ethylene polymerization.[4]

In their early reports,[4] Ziegler and co-workers generally described the aluminum compound as the polymerization catalyst, with the transition metal compound functioning as the cocatalyst, and therefore considered the whole chain-growth process to occur at the aluminum centers or, in other words, a transition metal catalyzed "Aufbaureaktion". Later it was realized that in these polymerization systems, the polymer chain actually grows on the transition metal and not on the aluminum center. But, to complicate matters, in recent years it has been observed that, for certain early as well as late transition metal polymerization catalysts, under certain conditions, the polymer chain is transferred to the aluminum centers during the polymerization reaction, a process that is referred to as *chain transfer to aluminum*. This reaction is sometimes the only transfer process,[5] but more often it occurs alongside the more common β-H transfer process.[6–9] If chain transfer to aluminum constitutes the sole transfer mechanism, and the exchange of the growing polymer chains between the transition metal and the aluminum centers is very fast *and* reversible, the polymer chains will appear to be growing on the aluminum centers. This can then reasonably be described as a transition metal catalyzed chain growth reaction on aluminum or, using Ziegler's terminology, a transition metal catalyzed "Aufbaureaktion". An attractive manifestation of this type of chain-growth reaction (catalyzed or uncatalyzed) is a Poisson distribution of main group alkyl products, as opposed to the Schulz – Flory distribution of olefinic products that arises when β-H transfer accompanies propagation.[10]



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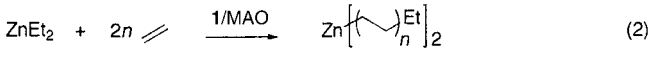
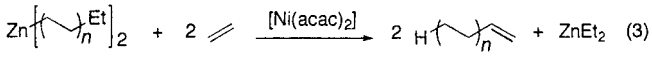
Systems where these criteria are fulfilled, or are close to being fulfilled, have been described for transition metal and rare-earth metallocenes (on Al and Mg)[11–14] and recently for half-sandwich complexes of chromium (on Al).[16] Here we report the first highly active chain-growth process on zinc, catalyzed by bis(imino)pyridineiron catalyst **1** [Eq. (2)]. We also describe a nickel-catalyzed



(2)

displacement of the grown alkyl chain, in the presence of ethylene, to regenerate diethylzinc and to give a Poisson distribution of α-olefins [Eq. (3); acac = acetylacetonate].



(3)

In the absence of $ZnEt_2$, a polymerization system containing **1** (5 μmol) and 100 equivalents of methylalumoxane (MAO) generates high molecular weight polyethylene with a broad molecular weight distribution (Table 1, Figure 1). A small amount of a low molecular weight fraction is also obtained, due to chain transfer to the $AlMe_3$ present in MAO. An increased proportion of low molecular weight material is obtained upon addition of 100 equivalents of $ZnEt_2$ (run 2, Table 1). At 500 equivalents of $ZnEt_2$ (2.5 mmol) a very narrow molecular weight distribution product results (M_n = 700, M_w = 800, M_w/M_n = 1.1). The polymer yield after hydro-

[*] Prof. V. C. Gibson, Dr. G. J. P. Britovsek, M. van Meurs
Department of Chemistry
Imperial College of Science, Technology and Medicine
Exhibition Road, London SW7 2AY (UK)
Fax: (+44) 20-7594-5810
E-mail: V.Gibson@ic.ac.uk
Dr. S. A. Cohen
BP America Inc., Naperville Complex
150 W. Warrenville Road, Naperville, IL, 60563 (USA)
Dr. P. J. Maddox
BP plc, Sunbury Research Center
Chertsey Road, Sunbury on Thames, Middlesex TW16 7LN (UK)
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