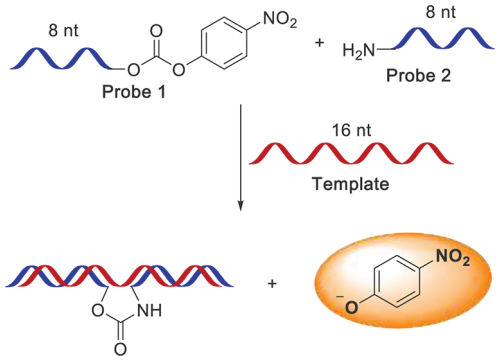
A Simple Reaction for DNA Sensing and Chemical Delivery

Elia Janett, Kim-Long Diep, Katharina M. Fromm, and Christian G. Bochet*

ABSTRACT: Reactions templated by nucleic acids are currently at the heart of applications in biosensing and drug release. The number of chemical reactions selectively occurring only in the presence of the template, in aqueous solutions, and at room temperature and able to release a chemical moiety is still very limited. Here, we report the use of the *p*-nitrophenyl carbonate (NPC) as a new reactive moiety for DNA templated reactions releasing a colored reporter by reaction with a simple amine. The easily synthesized *p*-nitrophenyl carbonate was integrated in an oligonucleotide and showed a very good stability as well as a high reactivity toward amines, without the need for any supplementary reagent, quantitatively releasing the red *p*-nitrophenolate with a half-life of about 1 h.



KEYWORDS: *nucleic acids, sensing, click chemistry, templation, carbonate, release*

Sensing and release events are crucial operations in Nature, in particular in complex organisms. Evolution led to the emergence of highly specific sensing by exploiting the pairing of complementary nucleotides. Coupling this molecular recognition event to a chemical reaction is the key to a highly precise and selective release of chemicals. While traditional bimolecular reactions suffer from low reaction rates if the concentration of the reagents is below 1−10 μM, the use of DNA or RNA strands as templates allows the increase of the effective concentration of the reaction partners, bringing them in proximity with each other and hence accelerating the reaction rate.[1] This was first explored by Orgel[2] and Gilham,[3] while Joyce[4] used DNA strands for the directed ligation of a peptide with an oligonucleotide.

Nowadays, many other reactions can occur selectively in the presence of a nucleic acid template. Their characteristics and scopes are well presented in several reviews.[1,5−9] The native chemical ligation proposed by Seitz[10] and Winssinger[11] or the formation of amide bonds proposed by Abe and Ito,[12] Li,[13] and Seeman and Canary[14] are representative examples. Abe and Ito as well as Kool showed the efficiency of the nucleophilic attack of phosphorothioates or thiols on tosylates[15] or halides,[16−18] as well as on electrophilic phosphorothioesters.[19] A templated aromatic nucleophilic substitution was also developed by Abe and Ito.[20] One of the currently most used reactions is the Staudinger reaction of azides with phosphines[21−24] or ruthenium complexes.[25] Photochemistry has also been exploited by several research groups.[26−29] The tetrazine-based reaction[30] as well as several reactions that form C−C bonds, like the Wittig reaction,[31,32] the aldol-type condensation,[33,34] the Diels−Alder ligation,[35] and the Heck cross-coupling[31] are further examples, together with the formation of boronates.[36]

While the above examples mainly focus on linking moieties together via a template, the number of reactions that lead to the release of a useful chemical entity is however much lower. First examples are the release of a nitrophenolate molecule from the templated reaction of a nitrophenyl ester with an imidazole[37] and the release of a coumarin moiety from a coumarinyl ester.[38]

In times of increasing bacterial resistance, we got interested in this type of chemistry in order to detect bacteria (and other microbes) based on their genetic information released, e.g., by extracellular RNA.[40] This detection should be highly sensitive, fast, and able to release, e.g., a signaling moiety or an active compound. Most of the above reactions have not been exploited for the delivery of an active compound, showing stability or reactivity issues, and cannot be applied to oligonucleotide sensing in physiological conditions. Such a system hence must work in water, at room temperature, at almost neutral pH, and should not require additional reagents or catalysts, which are potentially cytotoxic.[6]

Here, we present the synthesis, the assembly, and the kinetic analysis of two systems based on a DNA probe derived from *Listeria monocytogenes* and containing a *p*-nitrophenyl carbonate (NPC) able to release a colored *p*-nitrophenolate upon attack of a DNA probe modified with a terminal hexylamine in a 3-strand strategy (Charts 1 and 2). The NPC represents a new function

Chart 1. DNA-Templated Reaction of the *p*-Nitrophenyl Carbonate

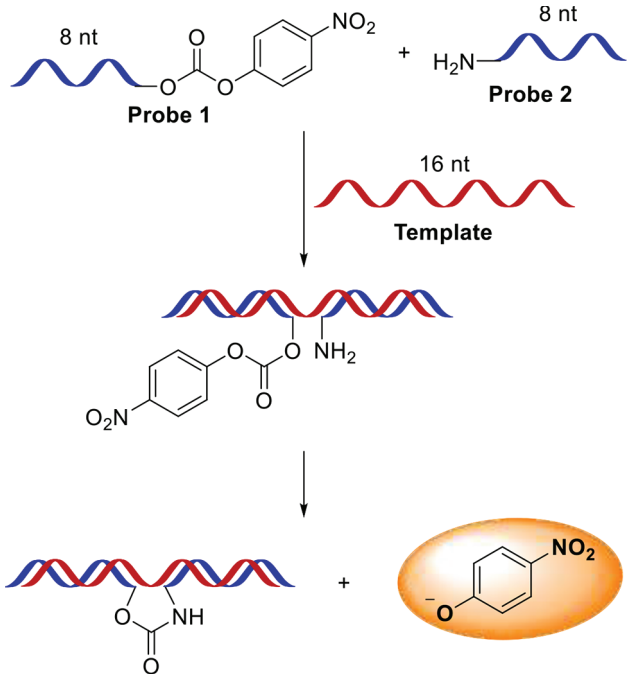
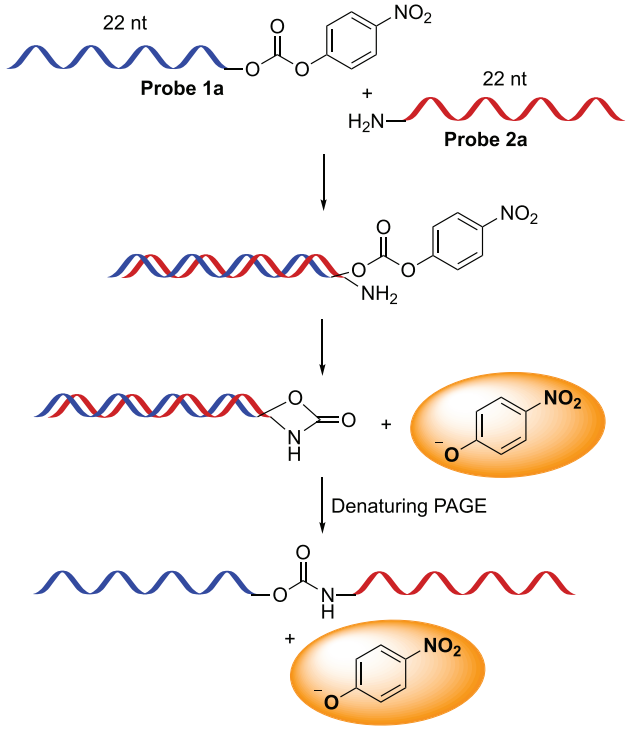


Chart 2. Proof of Principle Using a 2-Strand Model

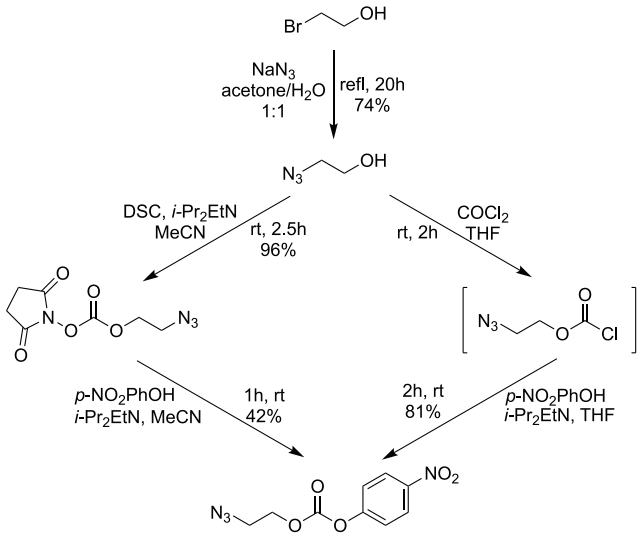


for DNA-templated nucleophilic substitutions. It was easily synthesized and introduced on oligonucleotides, showing good stability and reactivity in physiological conditions. The system works in PBS buffer at pH 7.4 or higher and at room temperature, in the presence of picomoles of template, without any additional reagent, solvent, or base. It therefore broadens the currently limited panel of DNA-templated reactions suitable for the delivery of active molecules, simply replacing the *p*-nitrophenol with an active compound.

NPC SYNTHESIS

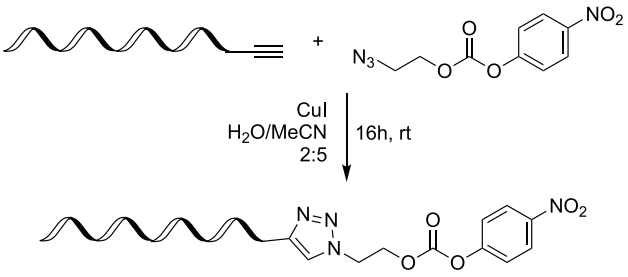
The NPC function was identified after initial stability issues when working with the *p*-nitrophenyl ester described by Taylor.[37] Following a report by Hamachi,[39] who proposed an acyl imidazole function for protein ligation, we envisioned the *p*-nitrophenyl carbonate as a species that shows the advantage of being more stable than the *p*-nitrophenyl ester and more reactive than the acyl imidazole (see SI). An NPC containing an azide terminus, for the connection with the oligonucleotide, was easily synthesized in three steps with good yields using either phosgene or disuccinimidyl carbonate (DSC) (Scheme 1).

Scheme 1. Synthesis of the 2-Azidoethyl 4-Nitrophenyl Carbonate



As a first step, in order to follow the DNA-templated reaction by denaturing polyacrylamide gel electrophoresis (PAGE), we decided to work with only two oligodeoxynucleotides (ODN), having complementary sequences and with two reactive functions at the 3′ and 5′ termini (Chart 2). Since the reaction ligates the two strands, the final product can be easily distinguished from the initial reacting strands. The two probes were designed to be fully complementary with a length of 22 nucleotides in order to have a strong hybridization at room temperature. The *p*-nitrophenyl carbonate ODN (**Probe 1a**) was obtained by Cu(I) catalyzed azide−alkyne cycloaddition of the commercially available 5′-hexynyl-ODN with the synthesized 2-azidoethyl 4-nitrophenyl carbonate (Scheme 2). The amino-modified ODN with a hexyl-amine linker at the 3′ position (**Probe 2a**) is commercially available. In a control

Scheme 2. Synthesis of Probe 1a



experiment, we used a second, noncomplementary strand (**Control Probe**).

hexynyl-5′-ODN:
5-hexynyl-5′-AAT GAT AAA GTG TGG CGC ATG C-3′
Probe 2a:
5′-GCA TGC GCC ACA CTT TAT CAT T-3′-$(CH_2)_6NH_2$
Control Probe:
$H_2N(CH_2)_6$-5′-C GTA CGC GGT GTG AAA TAG TAA-3′

SYNTHESIS OF PROBE 1A

A solution of the 5′-hexynyl-ODN, 50 equiv of the azide and 10 equiv of CuI in a mixture of ddH_2O and acetonitrile was left at room temperature overnight, and then purified by precipitation and analyzed by MALDI-TOF spectrometry (see SI for detailed procedure and analyses).

REACTION WITH PROBE 2A

Mixtures of **Probe 1a** and **Probe 2a** in PBS buffer at pH 7.4 were allowed to react at room temperature for the given time, and then analyzed by denaturing PAGE (Figure 1). An immediate

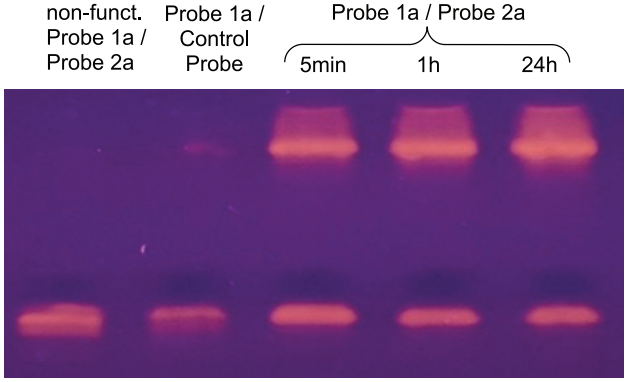


Figure 1. PAGE analysis (10 μM mixtures of **Probe 1** and **Probe 2** in PBS buffer (150 mM NaCl, 4 mM phosphate, 10 mM $MgCl_2$) at pH 7.4 were let react at room temperature for a given time).

hybridization between the two strands was confirmed by the decrease of ~30% of the absorbance at 260 nm. Additional bands, observed after only 5 min of reaction time and corresponding to ODNs of larger size than **Probes 1** and **2**, indicate that the ligation reaction occurred. As a control, mixtures of **Probe 1a** and the noncomplementary **Control Probe** (lane 2) and mixtures of nonfunctionalized **Probe 1a** and **Probe 2a** (lane 1) were also allowed to react together for 24 h, showing no ligation.

The ligation between the two strands was further proven by measuring the MALDI-TOF spectrum of the ligated product. The corresponding lane was cut from the gel, the DNA was extracted by letting the gel piece in an elution buffer overnight, and the salts were removed prior to the analysis using 3k Amicon spin filters. The expected mass was observed.

In order to quantify the release rate of the templated vs the nontemplated reaction, the formation of the *p*-nitrophenolate was followed by UV/vis spectroscopy at 405 nm. The reaction follows a first-order kinetics, and the half-lives were determined between pH 7.4 and 9 (Table 1). The reaction rates were affected by the pH and 3−4 times faster at high rather than neutral pH, presumably due to the protonation state of the amine.[41] In the presence of a mismatched sequence (control probe), the reaction was by a factor of 3−4 slower under similar pH conditions.

Table 1. Half-Lives and First-Order Kinetic Constants

pH	7.4	8.0	8.5	9.0	9.0 PTO
			full match		
$\tau_{1/2}$ (min)	141	94	70	44	146
k ($\times 10^{-3}$ min^{-1})	4.9	7.4	9.8	16	4.8
			mismatch		
$\tau_{1/2}$ (min)	465	284	240	162	190
k ($\times 10^{-3}$ min^{-1})	1.5	2.4	2.9	4.3	3.7

Instead of an amine, the phosphorothioate function (PTO) proposed by Abe, Ito, and Kool[15] was also tested, showing however a reduced reactivity.

After proving the efficiency of the reaction with the 2-strand model and its potential for the detection of a DNA or RNA template, we challenged the 3-strand system of Chart 1. Two new sequences were thus designed, allowing to bring the two reactive functions in proximity with each other in the presence of the template (Chart 1). Four different templating sequences were tested, as well as an RNA template, all derived to match an rRNA of *L. monocytogenes*. **Template 1** is a DNA 16-mer, fully complementary. **Template 2** is a 17-mer, the additional base being introduced to add free space for the reactive functions. **Templates 3** and **4** are DNA 16-mer containing respectively one and two mismatches, and **Template 5** is a fully complementary RNA 16-mer.

hexynyl-5′-ODN:
5-hexynyl-5′- AAT GTG CC -3′
Probe 2b:
5′- GAG ATG AG -3′-$(CH_2)_6NH_2$
Template 1:
5′- GGC ACA TTC TCA TCT C -3′
Template 2:
5′- GGC ACA TT**A** CTC ATC TC -3′
Template 3:
5′- GGC ACA **G**TC TCA TCT C -3′
Template 4:
5′- GG**A** ACA TTC TCA **G**TC C -3′
Template 5:
5′- GGC ACA UUC UCA UCU C -3′

SYNTHESIS OF PROBE 1B

The new probe was synthesized following the same protocol as for the synthesis of 22-mer-NPC-ODN, giving a fully functionalized product in quantitative yield (see SI).

TEMPLATED REACTIONS

The same procedure was followed as before. Mixtures of **Probe 1b**, **Probe 2b**, and **Template 1**, **2**, **3**, **4**, or **5** in PBS buffer at different pH were allowed to react at room temperature for the given time (Chart 1). The absorbance at 405 nm was monitored by UV/vis spectroscopy over time to determine the rate of release of the *p*-nitrophenolate. As a control experiment, we performed the reaction in the absence of a template. Slightly shorter half-lives were observed than in the first experiment (Table 2),the functions reacting thus faster than in the 2-strand system. The pH-dependence and the rate difference between the templated and the control reaction followed the same trend as observed above, although with an improvement of the discrimination. The template length did not affect the reactivity

Table 2. Half-Lives and First-Order Kinetic Constants

pH	7.4	8.0	8.5	9.0	7.4	7.4	7.4	7.4
Template	**T1**	**T1**	**T1**	**T1**	**T2**	**T3**	**T4**	**T5**
				templated				
$\tau_{1/2}$ (min)	89	74	57	34	84	145	293	69
k ($\times 10^{-3}$ min^{-1})	7.8	9.3	12	21	8.3	4.8	2.4	10
				control				
$\tau_{1/2}$ (min)	370	316	201	154	370	247	304	241
k ($\times 10^{-3}$ min^{-1})	1.9	2.2	3.5	4.5	1.9	2.8	2.3	2.9

(column 5) nor the replacement of the DNA template by RNA (column 8). On the other hand, the introduction of a single mismatch is enough to drastically decrease the reactivity (column 6), whereas two mismatches completely inhibit the reaction (column 7). Thus, conditions are met for a reasonably fast (34 min) and selective (k_{cat}/k_{uncat} ca. 5) detection of nucleic acid sequences, at picomolar concentration.

In conclusion, the identification of a new function for the release of a chemical entity by nucleic acid templation broadens the still limited panel of reactions suitable for DNA and RNA sensing and for drug delivery in biologically compatible conditions. The *p*-nitrophenyl carbonate functionalized oligonucleotides show a good stability and are quickly assembled. The aromatic core offers the possibility to be further functionalized by introducing self-immolative linkers to simultaneous release of several chemical compounds. Since the released unit is colored, the 3-strand system is ideally suited for fast colorimetric detection of bacteria, as proven here for *L. monocytogenes*. Moreover, depending on the application, the sensitivity can be further improved by any standard spectrometric technique.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.0c00988.

Experimental details, analytical outputs, and kinetic data (PDF)

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Notes

The authors declare no competing financial interest.

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