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# Nucleic acid controlled catalysts of carboxylic esters hydrolysis

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ABSTRACT

Two $Cu^{2+}$-binding ligands were covalently attached to termini of short DNAs. The optimal compound of this type forms a catalytically inert complex with $Cu^{2+}$. In the presence of a complementary nucleic acid the complex is decomposed forming products, which may catalyze hydrolysis of carboxylic acid esters. We have demonstrated that this process can be applied for sequence specific detection of nucleic acids.


Detection of nucleic acids is used for identification of microorganisms, in diagnosis of human diseases and in forensics. A number of stoichiometric and catalytic methods have been developed.[1] Stoichiometric assays exhibit excellent sequence specificity, but are not sufficiently sensitive for analysis of rare nucleic acid sequences. Catalytic assays do not have this disadvantage. However, they are not applicable for detection of nucleic acids in cells, since enzymes are typically used as catalysts. Enzymes do not permeate cellular membrane and are not stable in the presence of intracellular proteases.

This stimulates development of new approaches of nucleic acid detection, which rely exclusively on chemical reagents. Majority of such chemical assays are based on stoichiometric and catalytic template reactions.[2] Krämer and co-workers have recently reported an alternative strategy.[3] In particular, they have prepared a cyclic DNA, which releases $Cu(phen)^{2+}$ complex in the presence of an analyte nucleic acid and 1,10-phenanthroline. This complex catalyzes oxidation of 2′,7′-dichlorodihydrofluorescein in air saturated solution containing cysteamine. Concentration of the fluorescent product formed in this reaction correlates with concentration of the analyte DNA. At the conditions of this assay, highly reactive hydroxyl radicals are produced. They may induce decomposition of the catalyst and analyte DNA.[4] As a consequence the analyte DNA cannot be fully recovered after the assay and there is a possibility of false positive results.

We have explored the possibility of replacement of the redox reaction in this assay for the reaction of hydrolysis of the activated esters (Fig. 1).

The latter process occurs at very mild conditions, which affect neither catalyst nor analyte DNA.[5] The optimal **L-DNA-L** will have the following properties. **L-DNA-L** binds 1 equiv $Cu^{2+}$ in dilute solution forming a stable, but catalytically inert complex (–Cu-**L-DNA-L**–). In the presence of a complementary nucleic acid (blue colored in Fig. 1) this complex is converted into the active Cu-**L-DNA-L**. The latter compound catalyzes hydrolysis of substrates present in the solution (Fig. 1). In this case rate of substrate hydrolysis will correlate with concentration of the complementary nucleic acid.

For monitoring nucleic acid dependent hydrolytic activity we have used the substrates developed in our laboratories: **S1**,[6] **S2**[7] (Fig. 2). **S1** is a 2,2′-bipyridine derivative. It was chosen because its hydrolysis can be conveniently monitored by fluorescence spectroscopy. Products of this reaction are its weak inhibitors. Therefore, **S1** hydrolysis is the truly catalytic process with respect to

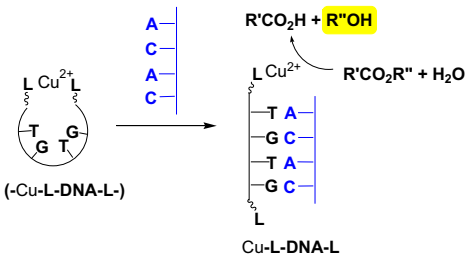


**Figure 1.** A concept of nucleic acid controlled $Cu^{2+}$ based catalysts of carboxylic acid ester hydrolysis; **L** is a $Cu^{2+}$ binding ligand; $R'CO_2R''$ is a $Cu^{2+}$ sensitive carboxylic acid ester (**S1** or **S2**, Fig. 2).

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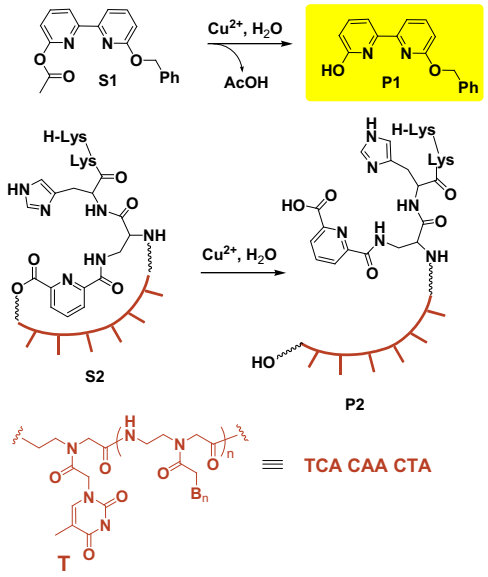
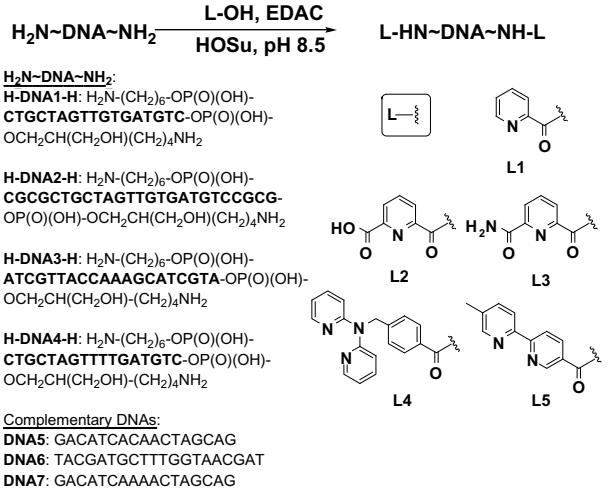



**Figure 2.** Substrates (**S1**, **S2**) used for monitoring nucleic acid induced release of $Cu^{2+}$ ions. $Cu^{2+}$ catalyzed transformation of these substrates results in formation of products **P1** (fluorescent) and **P2** correspondingly.

$Cu^{2+}$.[6] **S2** is a cyclic peptide nucleic acid (PNA), which is transformed into the linear form (**P2**) upon hydrolysis. This process can be monitored by MALDI-TOF mass spectrometry. While **S2** does not bind nucleic acids, **P2** does. Sequence of **P2** was chosen to be complementary to that of **L-DNA1-L**. Since **P2**/**DNA1** duplex is rather stable ($T_m \sim 50$ °C), we could expect that the product of **S2** hydrolysis will induce formation of the hydrolysis catalyst from the (–Cu-**L-DNA-L–**). This would lead to time dependent increase of **S2** hydrolysis rate, which is a feature of an autocatalytic process. Autocatalytic reactions generate large amounts of products per equivalent of a trigger (nucleic acid). This may be applied for analysis of rare nucleic acid sequences.[1]

**L-DNA-L**'s were prepared by coupling of commercially available amino-modified DNAs with carboxylic acids (**L**-OH) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) at pH 8.5 (Scheme 1). The conjugates were purified by HPLC and their composition was confirmed by MALDI-TOF mass spectrometry. Purity of the DNAs prepared was >90%.[8]




**Scheme 1.** Synthesis of terminally modified DNAs and sequences of unmodified DNAs used in this study. EDAC: 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydrochloride.

Structures of the ligands conjugated to the DNAs are shown in Scheme 1. A mixture of $Cu^{2+}$ and **L1-DNA1-L1** hydrolyzes **S2** four times slower than free $Cu^{2+}$ does (Table 1). However, complementary **DNA5** ($T_m$(**DNA5**/**DNA1**) = 51 °C) does not affect the hydrolysis. These data indicate that $Cu^{2+}$ binds to **L1-DNA1-L1** unspecifically rather than in a way illustrated in Figure 1 for (–Cu-**L-DNA-L–**). The possible binding sites are nucleobases and the phosphodiester backbone of the conjugate. In **L2-DNA1-L2**, **L3-DNA1-L3**, **L4-DNA1-L4** and **L5-DNA1-L5** the terminal ligands have higher affinity toward $Cu^{2+}$ than **L1**. These ligands were expected to compete more efficiently for the metal ion with the other $Cu^{2+}$ binding sites within the DNA. The best conjugate in this series turned out to be **L2-DNA1-L2**. In particular, **S2** hydrolysis by $Cu^{2+}$, **L2-DNA1-L2** mixture is slowed down by a factor of ~24 with respect to that by $Cu^{2+}$, **L1-DNA1-L1** mixture (entries 1, 2, Table 1). The hydrolysis is accelerated 15 times upon addition of 1 eq **DNA5**. Comparable DNA-induced acceleration is observed when **S1** is used as a substrate (entry 2, Table 1). The effect is substantially smaller in the case of **L3-DNA1-L3** and **L4-DNA1-L4**. In particular, **S2** hydrolysis by mixtures of $Cu^{2+}$ with the corresponding conjugate is only 3.4- to 5.1-fold slower than that by $Cu^{2+}$, **L1-DNA1-L1** mixture (entries 1, 4, 5, Table 1). The hydrolysis is barely accelerated (1.5–1.9 times) upon addition of **DNA5**. This reflects lower $Cu^{2+}$ affinity of **L3** and **L4** than that of **L2**. Ligand **L5** has the highest $Cu^{2+}$ affinity among the studied metal ion binders. As a consequence, **L5-DNA1-L5** binds $Cu^{2+}$ very strongly and the resulting complex, (–Cu-**L5-DNA1-L5**) does not catalyze hydrolysis of **S2** at all. Unfortunately, the complex is so stable that it is not activated by **DNA5** (entry 6, Table 1). Even an analogue of this compound containing a longer DNA sequence (20-mer, **L5-DNA3-L5**) forms the complex, which is not activated by the complementary nucleic acid, **DNA6** ($T_m$(**DNA3**/ **DNA6**) = 56 °C, entry 7, Table 1).

Next, we have prepared **L2-DNA2-L2**, which contains the DNA sequence able to fold in solution into the hairpin structure with a four basepair stem region. In the hairpin the terminal ligands are pre-organized. This should stabilize the terminal $CuL_2$ complex in (–Cu-**L2-DNA2-L2–**). The expected consequence would be the reduction of the background hydrolysis rate. In practice, activity of (–Cu-**L2-DNA2-L2–**) turned out to be identical to that of (–Cu-**L2-DNA1-L2–**) (entries 2, 3, Table 1). This indicates that the hairpin is unstable at our experimental conditions: 40 °C. Substrate **S2** is hydrolyzed very slowly at <40 °C. Therefore, it is not practical to study this system at lower temperatures. In contrast, **S1** is quickly hydrolyzed at 22 °C. We have found that, at these conditions rate of **S1** hydrolysis in the presence of (–Cu-**L2-DNA2-L2**–) is slower than that in the presence of stemless (–Cu-**L2-DNA1-L2**–) (entries 2, 3, Table 1). Unfortunately, this positive effect is canceled out by the high cycle stability. In the result, only 3.5-fold rate increase is observed upon addition of **DNA5** to (–Cu-**L2-DNA2-L2**–), which is about 20% of the increase exhibited by the **L2-DNA1-L2** containing cycle.

As it is explained above, **S2** hydrolysis by (–Cu-**L2-DNA1-L2**–), **DNA5** mixtures could be an autocatalytic reaction. However, in the presence of 0.1–1 equiv complementary **DNA5** rate of **S2** hydrolysis is not increased at the initial stages of the reaction (Fig. 3, only data for 1 equiv **DNA5** are shown). We have observed that **S2** cleavage in the presence of (–Cu-**L2-DNA4-L2**–), **DNA7** mixture is as fast as it is in the presence of (–Cu-**L2-DNA1-L2**–), **DNA5** mixture (entries 2, 10, Table 1). In the former case **P2** activation is not possible, since sequences of **DNA4** and **P2** are not fully complementary to each other. This indicates that **P2** induced activation of the catalyst does not play an important role in this reaction. Thus, at our experimental conditions **S2** hydrolysis is a catalytic rather than autocatalytic process.

**Table 1**
Parameters of hydrolysis of substrates **S1** and **S2** in the presence of complexes of $Cu^{2+}$ with **L-DNA-L** conjugates

| Compound | **L-DNA-L**[a] | Substrate **S1** $(dF/dt)_0$, $min^{-1}$ [b] | | Substrate **S2** [e] $(d[\mathbf{P2}]/dt)_0$, nM $min^{-1}$ | |
|---|---|---|---|---|---|
| | | + analyte/− analyte [c] | Ratio[d] | + analyte/− analyte | Ratio |
| **1** | L1-DNA1-L1 | — | — | 21(2)/19(2) | 1.1 |
| **2** | L2-DNA1-L2 | >230/33(4) | >7 | 12(2)/0.8(5) | 15 |
| **3** | L2-DNA2-L2 | 44(2)/13(2) | 3.4 | 12(1)/0.8(7) | 15 |
| **4** | L3-DNA1-L3 | — | — | 8.3(7)/5.6(8) | 1.5 |
| **5** | L4-DNA1-L4 | — | — | 7.1(4)/3.7(3) | 1.9 |
| **6** | L5-DNA1-L5 | 1.4(3)/1.1(2) | 1.3 | 0 | 0 |
| **7** | L5-DNA3-L5 | — | — | 0 | 0 |
| **8** | $CuSO_4$ | — | — | −/>87 | — |
| **9** | L2-DNA1-L2 | — | — | 4.7(8)/0.8(5) | 6 |
| **10** | L2-DNA4-L2 | — | — | 9(1)/0.8(5) | 11 |
| **11** | — | — | — | −/0.4(4) | — |

[a] Structures and DNA sequences of **L-DNA-L** conjugates are shown in Scheme 1; [**L-DNA-L**] = 2 μM; [$CuSO_4$] = 1.5 μM; [MOPS] = 10 mM, pH 7.0, [NaCl] = 50 mM.
[b] $\lambda_{ex}$ = 347 nm, $\lambda_{em}$ = 410 nm; [**S1**] = 0.1 μM.
[c] Analyte is either **DNA5** (entries 1–6) or **DNA6** (entry 7) or **DNA7** (entries 9, 10); their sequences are given in Scheme 1; [analyte] = 2 μM.
[d] Ratio = $(dF/dt)_0$ (with analyte)/$(dF/dt)_0$ (without analyte) or $(d[\mathbf{P2}]/dt)_0$ (with analyte) / $(d[\mathbf{P2}]/dt)_0$ (without analyte).
[e] [**S2**] = 1 μM.

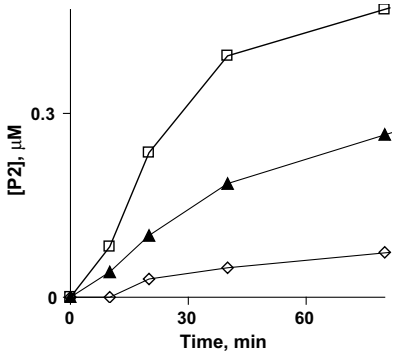



**Figure 3.** Hydrolysis of substrate **S2** (1 μM) in solutions containing MOPS (10 mM, pH 7.0), NaCl (50 mM), $CuSO_4$ (1.5 μM), **L2-DNA1-L2** (2 μM) (open diamonds) and, additionally, either **DNA5** (2 μM, open squares) or **DNA7** (black triangles).

Finally, we have investigated whether the optimized reaction can be used for sequence specific detection of nucleic acids. We have prepared **DNA7**, which contains a single mismatch (C8 → A8 mutation) with respect to **DNA5**. Activation of (–Cu-**L2-DNA1-L2**–) cycle in the presence of both DNAs was compared (Fig. 3). At our experimental conditions **S2** hydrolysis is 2.6 times slower in the presence of the mismatch DNA then in the presence of the complementary DNA (entries 2, 9, Table 1).

In summary, the catalytic reaction studied can be used for sequence specific detection of nucleic acids. Since the reaction is conducted at very mild conditions, the analyte nucleic acid can be easily recovered by using HPLC. Moreover, the catalyst stays unchanged during the reaction, which minimizes the possibility of false positive results. Nucleic acids can be detected by using either fluorescence spectroscopy or MALDI-TOF mass spectrometry. The important disadvantage of the current assay is high rate of background hydrolysis.

## Acknowledgements

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8. Purity of the conjugates was determined by integration of the major product peak (**L-DNA-L**) in analytical HPLC of pure samples. The most prominent side products were conjugates lacking one of the ligands (**L-DNA**) and those ones, which have one additional ligand (**L-DNA-L**∼**L**).HPLC column: Macherey-Nagel Nucleosil 300-5, C4, 250/4.6; solvent A: 0.1 M $(NEt_3H)(OAc)$ in water, pH 7.0; solvent B: $CH_3CN$; gradient I: 0% B for 5 min, in 30 min to 25% B, in 10 min to 90% B, 90% B for 9 min; gradient II: 0% B for 5 min, in 35 min to 40% B, in 5 min to 90% B, 90% B for 9 min.**L1-DNA1-L1**: HPLC, gradient I, $R_t$ 21.2 min. Yield: 67%. MALDI-TOF MS: calcd for $C_{192}H_{248}N_{62}P_{18}O_{114}$ $[M-H]^-$ 5803.1, found 5806.5.**L2-DNA1-L2**: HPLC, gradient I, $R_t$ 26.8 min. Yield: 57%. MALDI-TOF MS: calcd for $C_{194}H_{248}N_{62}P_{18}O_{118}$ $[M-H]^-$ 5891.1, found 5894.4.**L3-DNA1-L3**: HPLC, gradient I, $R_t$ 28.1 min. Yield: 43%. MALDI-TOF MS: calcd for $C_{194}H_{250}N_{64}P_{18}O_{116}$ $[M-H]^-$ 5889.1, found 5890.9.**L4-DNA1-L4**: HPLC, gradient II, $R_t$ 30.7 min. Yield: 23%. MALDI-TOF MS: calcd for $C_{216}H_{268}N_{66}P_{18}O_{114}$ $[M-H]^-$6167.2, found 6169.3.**L5-DNA1-L5**: HPLC, gradient II, $R_t$ 25.1 min. Yield: 35%. MALDI-TOF MS: calcd for $C_{204}H_{258}N_{64}P_{18}O_{114}$ $[M-H]^-$ 5985.1, found 5983.1.**L2-DNA2-L2**: HPLC, gradient I, $R_t$ 29.5 min. Yield: 42%. MALDI-TOF MS: calcd for $C_{270}H_{344}N_{94}P_{26}O_{166}$ $[M-H]^-$ 8363.5, found 8358.1.**L1-DNA3-L1**: HPLC, gradient I, $R_t$ 23.1 min. Yield: 71%. MALDI-TOF MS: calcd for $C_{220}H_{282}N_{79}P_{21}O_{125}$ $[M-H]^-$ 6680.2, found 6684.5.**L5-DNA3-L5**: HPLC, gradient II, $R_t$ 33.2 min. Yield: 19%. MALDI-TOF MS: calcd for $C_{232}H_{292}N_{81}P_{21}O_{125}$ $[M-H]^-$ 6862.3, found 6863.0.**L2-DNA4-L2**: HPLC, gradient I, $R_t$ 28.3 min. Yield: 37%. MALDI-TOF MS: calcd for $C_{194}H_{251}N_{59}P_{18}O_{119}$ $[M-H]^-$ 5868.1, found 5865.0.