Inorganic Chemistry
Communication

Inorg. Chem. **2009**, *48*, 9593–9595 **9593**
DOI: 10.1021/ic9006795

A Fluorogenic, Nucleic Acid Directed "Click" Reaction

Elmar Jentzsch and Andriy Mokhir*

Anorganisch-Chemisches Institut, Ruprecht-Karls-Universität Heidelberg, Im Neuenheimer Feld 270, 69120 Heidelberg, Germany

Received April 7, 2009

A fluorogenic, nucleic acid directed, Cu^{+}-catalyzed chemical reaction was developed that allows sequence-specific detection of nucleic acids at concentrations as low as 20 nM through monitoring of the increase of the fluorescence intensity. A single nucleotide mismatch in the template strand leads to the complete inhibition of this reaction. We observed that a Cu^{+} ion stabilized with a water-soluble ligand is a significantly more efficient catalyst than free Cu^{+}.

Nucleic acid directed (templated) chemical transformations have been applied for the sequence-specific detection of nucleic acids,[1] in the preparation of nanosized structures,[2] in the multistep synthesis of complex organic compounds, and in the discovery of new chemical reactions.[3] Only a few known reactions of this type are catalyzed by metal-containing compounds. In particular, it has been found that coordinatively unsaturated Cu^{2+} complexes are efficient catalysts of the hydrolysis of carboxylic esters,[4] stable Cu^{+} complexes catalyze the ligation of modified oligodeoxyribonucleotides (ODNs) in a "click" reaction,[5] and Mn^{2+} and Ni^{2+} ions catalyze ligations of ODNs by forming metallosalen complexes.[2a,6]

Fluorescence spectroscopy is a highly sensitive, noninvasive, and quick method of analysis of chemical and biological compounds. It has been used for monitoring of organic nucleic acid templated reactions both in vitro and in cells.[1] In contrast, analogous metal-complex-catalyzed reactions have not been studied by this method yet. One of the reasons for this is that metal ions as well as their coordinatively unsaturated complexes are efficient quenchers of typical fluorophores.[7] The possibility of monitoring such reactions by fluorescence spectroscopy would facilitate their optimization and would advance the understanding of their mechanism. In particular, the mechanism of the metal-catalyzed reactions could be studied in detail on a single-molecule level.[8] Moreover, the metal-catalyzed fluorogenic reactions could be applied for the sequence-specific detection of nucleic acids in biological samples.

Herein we report on an efficient nucleic acid directed, Cu^{+}-catalyzed coupling of two nonfluorescent conjugates (dye*∼ODN1 and ODN2∼azide) with the formation of a fluorescent product ODN2∼dye∼ODN1 (Figure 1). Such transformations are called "click" reactions.[9] They have already been applied in the synthesis of chemical compounds, labeling and conjugation of biomolecules,[9] and analysis of Cu.[10] Inspired by the work of Wong and co-workers,[11] we selected nonfluorescent 4-ethynyl-1,8-naphthaleneimide (dye*) as a 5′ modifier of the ODN1 strand. In the template reaction, the dye* was supposed to be transformed into fluorescent 4-(1,2,3-triazol-4-yl)-1,8-naphthaleneimide (dye).[11] To conjugate the dye* to an ODN, a new building block was prepared starting from anhydride **1** (Scheme 1). In particular, the anhydride was first condensed with $BocNHCH_2CH_2NH_2$. Then the Br atom in the product (**2**) was substituted for the $Me_3SiC{\equiv}C$ fragment. Finally, the protecting groups were cleaved to obtain the required building block (**4**). We investigated the coupling of **4** (100 μM) to azide **5** (1 equiv) in a water−dimethyl sulfoxide (5%) solution (pH 7.5) in the presence of a Cu^{+}-TBTA complex (1 equiv).

*To whom correspondence should be addressed. E-mail: Andriy.Mokhir@urz.uni-heidelberg.de.

(1) (a) Silverman, A. P.; Kool, E. T. *Chem. Rev.* **2006**, *106*, 3775–3789. (b) Jacobsen, M. F.; Cló, E.; Mokhir, A.; Gothelf, K. V. *ChemMedChem* **2007**, *2*(6), 793–799. (c) Grossmann, T. N.; Strohbach, A.; Seitz, O. *ChemBioChem* **2008**, *9*, 2185–2192.

(2) (a) Gothelf, K. V.; Brown, R. S. *Chem.—Eur. J.* **2005**, *11*, 1062−1069 and references cited therein.(b) Andersen, C. S.; Yan, H.; Gothelf, K. V. *Angew. Chem., Int. Ed.* **2008**, *47*, 5569–5572.

(3) (a) Tse, B. N.; Snyder, T. M.; Shen, Y.; Liu, D. R. *J. Am. Chem. Soc.* **2008**, *130*, 15611−15626 and references cited therein;(b) Kanan, M. W.; Rozenman, M. M.; Sakurai, K.; Snyder, T. M.; Liu, D. R. *Nature* **2004**, *431*, 545–549. (c) Rozenman, M. M.; McNaughton, B. R.; Liu, D. R. *Curr. Opin. Chem. Biol.* **2007**, *11*, 259–268. (c) Rozenman, M. M.; Kanan, M. W.; Liu, D. R. *J. Am. Chem. Soc.* **2007**, *129*, 14933–14938.

(4) (a) Boll, I.; Kraemer, R.; Brunner, J.; Mokhir, A. *J. Am. Chem. Soc.* **2005**, *127*, 7849–7856. (b) Boll, I.; Jentzsch, E.; Kraemer, R.; Mokhir, A. *Chem. Commun.* **2006**, 3447–3449.

(5) (a) Kumar, R.; El-Sagheer, A.; Tumpane, J.; Lincoln, P.; Wilhelmsson, L. M.; Brown, T. *J. Am. Chem. Soc.* **2007**, *129*, 6859–6864. (b) Xu, Y.; Suzuki, Y.; Komiyama, M. *Angew. Chem., Int. Ed.* **2009**, published online under DOI: 10.1002/anie.200806306.

(6) Czlapinski, J. L.; Sheppard, T. L. *J. Am. Chem. Soc.* **2001**, *123*, 8618–8619.

(7) Mokhir, A.; Kiel, A.; Herten, D. P.; Kraemer, R. *Inorg. Chem.* **2005**, *44*, 5661−5666 and references cited therein.

(8) Kiel, A.; Kovacs, J.; Mokhir, A.; Kraemer, R.; Herten, D.-P. *Angew. Chem., Int. Ed.* **2007**, *46*, 3363–3366.

(9) Meldal, M.; Tornøe, C. W. *Chem. Rev.* **2008**, *108*, 2952–3015.

(10) Wolfbeis, O. S. *Angew. Chem., Int. Ed.* **2007**, *46*, 2980–2982.

(11) Sawa, M.; Hsu, T.-L.; Itoh, T.; Sugiyama, M.; Hanson, S. R.; Vogt, P. K.; Wong, C.-H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 12371–12376.

Published on Web 09/21/2009

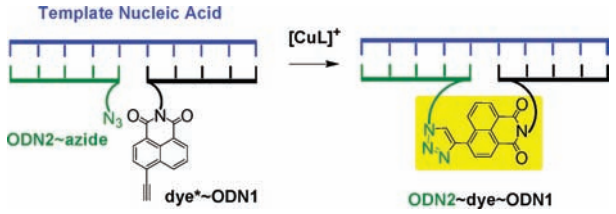
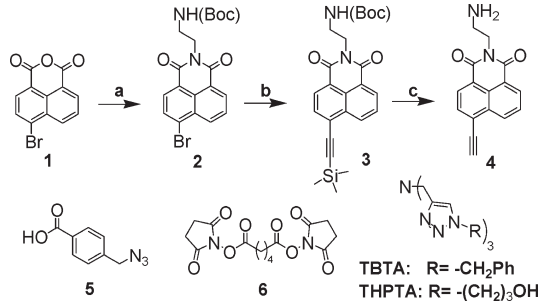


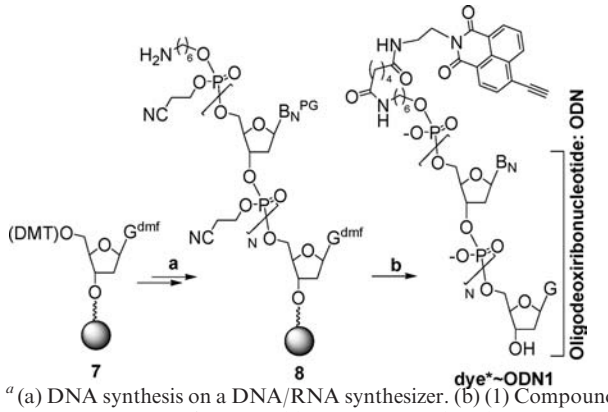
Figure 1. Fluorogenic, Cu^+ complex ($[CuL]^+$) catalyzed, nucleic acid directed chemical reaction. Substrates (dye*~ODN1 and ODN2~azide) are coupled to each other on the template nucleic acid (blue); the dye* fragment in one of the substrates is not fluorescent; it is converted to a fluorescent dye (yellow) in this reaction.

Scheme 1. Synthesis of Building Block **4**[a]



[a] (a) $BocNHCH_2CH_2NH_2$; (b) $Me_3SiC{\equiv}CH$, $Pd(PPh_3)_2Cl_2$, CuI, NEt_3, THF; (c) (1) HCl, THF, (2) NEt_3, H_2O. Known reagents and ligands used in this study: compound **5**,[12] compound **6**,[13] ligands TBTA[14] and THPTA.[15]

Scheme 2. Synthesis of the Dye*~ODN1 Conjugate[a]

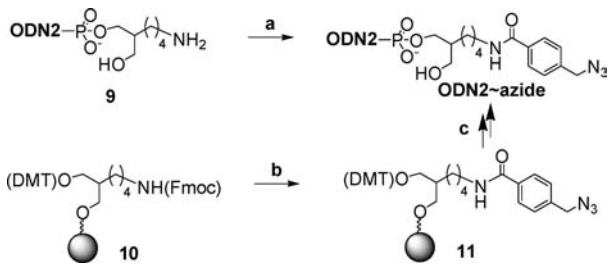


[a] (a) DNA synthesis on a DNA/RNA synthesizer. (b) (1) Compound **4**, HBTU, HOBT, DIEA, DMF; (2) aqueous NH_3 (25%).

During at least 15 min, the reaction rate was constant and the fluorescent product (λ_{em} = 460 nm and λ_{ex} = 340 nm) was formed: $(dF_{460\ nm}/dt)_0 = 36.4$. In contrast to the complex, free Cu^+ was inactive as a catalyst at these conditions. However, in the presence of 50-fold excess Cu^+, the reaction took place.

The dye* was conjugated to ODN1 using solid-phase synthesis (Scheme 2). In particular, we first prepared 5′-amino-group-terminated ODN **8** from commercially available solid support **7**, standard phosphoramidites, and 5′-amino-modifier C6 phosphoramidite. Then, the amino group of ODN **8** was converted to the activated carboxylic acid fragment by using bifunctional linker **6**,[13] followed by the addition of **4**. The resulting crude conjugate (dye*~ODN1) was cleaved from the solid support and deprotected by concentrated ammonia (55 °C, 2 h). The product was purified by high-performance liquid chromatography (HPLC), and fractions containing >90% of the conjugate were combined, lyophilized, redissolved in water, and used in all further experiments.

Scheme 3. Synthesis of the ODN2~Azide Conjugate[a]



[a] (a) Compound **5**, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, *N*-hydroxysuccinimide. (b) (1) DBU, DMF; (2) compound **5**, HBTU, HOBT, DIEA, DMF. (c) DNA synthesis on a DNA/RNA synthesizer.

Scheme 4. Sequences of ODNs Used in This Study

Probes (5'-->3'):
dye*~ODN1: dye*~GTT CAT CAC G
ODN2a~azide: CGC TCC CAC~azide
ODN2b~azide: ATA CGC TCA TAC~azide

Templates (5'-->3'):

ODN3a: CGT GAT GAA CGT GGG AGC G	match
ODN3b: CGT GAT GAA CGT ATG AGC GTA T	match
ODN3c: CGT GAC GAA CGT ATG AGC GTA T	mismatch

ODN2~azide was prepared by two methods (Scheme 3). The solution-phase synthesis starting from commercially available amino-ODN **9** was inefficient because both the starting material **9** and the product ODN2~azide should be purified by HPLC. This leads to the reduction of the overall yield of the reaction. In an alternative procedure, the Fmoc group of **10** was deprotected with a 1,8-diazabicyclo-[5.4.0]undec-7-ene (DBU)/*N*,*N*-dimethylformamide (DMF) mixture. Compound **5**[12] was then coupled in the presence of O-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluoro-phosphate (HBTU), *N*-hydroxybenzotriazole (HOBT), and diisopropylethylamine (DIEA), and, finally, the protected ODN2~azide conjugate was assembled on a DNA/RNA synthesizer. Its deprotection, cleavage from the support, and HPLC purification led to the pure conjugate. The yield was 22% with respect to the amount of DMT groups on the initial solid support. Sequences of the probes and templates used in this study are given in Scheme 4. UV melting points (T_m) of the duplexes formed from these probes and templates in an aqueous buffer are well above 25 °C, which indicates that the duplexes are stable at our experimental conditions (see the Supporting Information).

The reaction between dye*~ODN1 (2 μM) and ODN2a~azide (1 equiv) in the presence of a 50-fold excess of Cu^+ is facilitated by the template ODN3a (Figure 2). In particular, the parameter $(dF_{460\ nm}/dt)_0$ for the template-directed reaction is 9.3 times larger than that for the background reaction. The ligation product ODN2a~dye~ODN1 was identified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry: m/z 6539.9. Calcd for $C_{224}H_{258}N_{72}O_{125}P_{18}$ $[M - H^+]^-$ m/z 6543.6. At a lower concentration (200 nM) of the substrates, the template-directed, Cu^+-catalyzed reaction is dramatically slowed because Cu^+ becomes less stable. A further increase of the Cu^+ concentration leads to the undesired oxidative cleavage of ODNs and the destabilization of the ODN duplex.

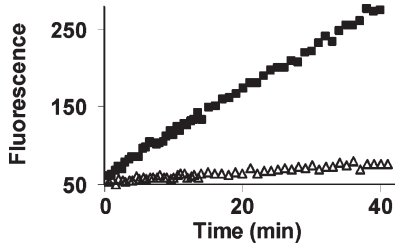


Figure 2. Monitoring of the nucleic acid directed click reaction by fluorescence spectroscopy. Substrates: dye*∼ODN1 (2 μM) and ODN2a∼azide (2 μM). Catalyst: Cu^+ (formed in situ from 100 μM $CuSO_4$ and 1 mM sodium ascorbate). The reaction was conducted without the template (open triangles) and with 2 μM of the template ODN3a (filled squares). Buffer: 10 mM MOPS, pH 7.5, 150 mM NaCl, 22 °C; λ_{em} = 460 nm and λ_{ex} = 340 nm. The fluorescence intensity is given in arbitrary units.

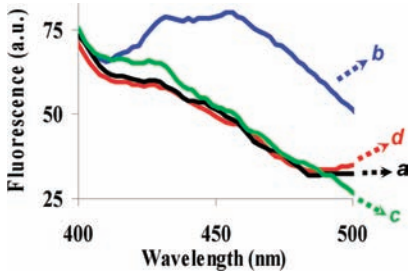


Figure 3. Fluorescence spectra (λ_{ex} = 340 nm) acquired 17 min after the addition of dye*∼ODN1 (200 nM) to mixtures A−C (25 °C). mixture A: ODN2b∼azide (1 equiv), 20 μM $CuSO_4$, 100 μM sodium ascorbate, 60 μM THPTA, 0.003% Triton-X (black trace a). Mixture B: same as A plus template ODN3b (1 equiv; blue trace b). Mixture C: same as A plus a mismatched template ODN3c (1 equiv; green trace c). The spectrum shown as red trace d was measured immediately after the addition of dye*∼ODN1 (200 nM) to mixture A. The same spectra were obtained with mixtures B and C. Buffer: 10 mM MOPS, pH 7.5, 150 mM NaCl. The spectra were acquired at 85 °C; the fluorescence intensity is given in arbitrary units.

Next, we substituted Cu^+ for a more stable Cu^+ complex to find that the template reaction takes place at lower concentrations of the substrates and template. The Cu^+-TBTA complex[14] turned out to be unsuitable because its water solubility is low. In contrast, the solubility of its analogue Cu^+-THPTA[15] (Scheme 1) is high. The latter catalyst was used earlier in the template-directed click reaction.[6] We observed that the coupling of alkyne **4** and azide **5** in the presence of the Cu^+-THPTA complex results in a strongly fluorescent product. Surprisingly, the reaction of similar substrates bound to ODNs (dye*∼ODN1 and ODN2a∼azide, both 2 μM) in the presence of the template ODN3a (1 equiv) and the Cu^+-THPTA complex (1 equiv) did not generate the fluorescence signal: $(dF_{460\ nm}/dt)_0 \sim 0$. In contrast, the fluorescence intensity was slightly increased when this reaction was conducted in the absence of the template: $(dF_{460\ nm}/dt)_0 = 1.5$. The analogous effect was also observed at the reduced concentration of the substrates: 200 nM, $(dF_{460\ nm}/dt)_0 = 0.3$ with the template (1 equiv); $(dF_{460\ nm}/dt)_0 = 0.5$ without the template. Replacing ODN2a for ODN2b and changing the template sequence correspondingly did not induce the fluorescence increase in the template reaction. We have hypothesized that these data indicated either that (a) the metal complex failed to catalyze the nucleic acid directed azide−alkyne coupling due to, e.g., the steric hindrance or that (b) the ligation product was formed and its fluorescence was quenched in the product. The possibility (a) was rejected because we could detect the ligation product in the complex-catalyzed reaction by both HPLC (Figure S10 in the Supporting Information) and MALDI-TOF mass spectrometry. In contrast to the Cu^+-THPTA-catalyzed template reaction, the one catalyzed by free Cu^+ resulted in the fluorescent product (Figure 2). On the basis of the latter fact, we concluded that the quenching was linked to the Cu^+-THPTA complex. We suggest that the associate of the ODN2∼dye∼ODN1/ODN3a duplex and the Cu^+-THPTA complex is formed. In this associate, the dye is positioned in close proximity to the Cu^+-THPTA complex, which results in the efficient quenching of its fluorescence. Other experimental facts confirm our hypothesis. In particular, we observed that heating leads to the dramatic increase of the fluorescence intensity of solutions supposedly containing the ODN2∼dye∼ODN1/ODN3a/Cu^+-THPTA associate. No such temperature-dependent change was observed in the solution, which did not contain the ODN3a template. Moreover, decomposition of Cu^+-THPTA in the presence of S^{2-} ions leads to the fluorescence increase (Figure S11 in the Supporting Information).

Because Cu^+-THPTA is more active and stable than free Cu^+, the complex-catalyzed template reaction could be conducted at lower concentrations of the probes and templates. In particular, 20−200 nM of the template was readily detectable either after heating of the reaction mixtures to 85 °C (traces a−c, Figure 3 and Figure S12 in the Supporting Information) or after their treatment with Na_2S (Figure S11 in the Supporting Information). The ligation is strongly inhibited in the presence of a single mismatch in the template (trace d in Figure 3 and Figure S12 in the Supporting Information), which indicates that this reaction can potentially be used in single-nucleotide-polymorphism detection. Known assays based on the best organic chemical reactions provide somewhat better sensitivity: 1 nM DNA.[1]

In summary, we developed a fluorogenic, nucleic acid directed, Cu^+-catalyzed reaction and observed that in this transformation free Cu^+ is a less active catalyst than the Cu^+-THPTA complex. The disadvantage of the complex is that it quenches the fluorescence of the ligation product. This problem could be solved by the heating of solutions obtained after the template reaction to 85 °C or by their treatment with Na_2S. We observed that the complex-catalyzed template reaction occurs at low substrate concentration (20 nM) and is sensitive to single mismatches in the template strand.

Acknowledgment. We thank the University of Heidelberg, German Research Council, and Konrad-Adenauer-Stiftung for financial support and C. Sämann and G. Rudolf for conducting some experiments.

Supporting Information Available: Synthesis of building blocks and chemically modified ODNs. This material is available free of charge via the Internet at http://pubs.acs.org.

(12) Zhou, Z.; Fahrni, C. J. *J. Am. Chem. Soc.* **2004**, *126*, 8862–8863.
(13) Hill, M.; Bechet, J.-J.; D'Albis, A. *FEBS Lett.* **1979**, *102*, 282–286.
(14) Wang, Q.; Chan, T. R.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2002**, *125*, 3192–3193.
(15) Chan, T. R.; Hilgraf, R.; Sharpless, K. B.; Fokin, V. V. *Org. Lett.* **2004**, *6*, 2853–2855.