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Pseudo-ligandless Click Chemistry for Oligonucleotide Conjugation

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Abstract

Particularly for its use in bioconjugations, the copper-catalyzed (or promoted) azide-alkyne cycloaddition (CuAAC) reaction or ‘click chemistry’, has become an essential component of the modern chemical biologist’s toolbox. Click chemistry has been applied to DNA, and more recently, RNA conjugations, and the protocol presented here can be used for either. The reaction can be carried out in aqueous buffer and uses acetonitrile as a minor co-solvent that serves as a ligand to stabilize the copper. The method also includes details on the analysis of the reaction product.

Keywords

Azide; Alkyne; DNA; RNA; bioconjugation; labeling; nucleic acids

INTRODUCTION

Among bioconjugation reactions, the high efficiency of the copper-catalyzed azide alkyne cycloaddition (CuAAC) reaction has made it near synonymous with the term click chemistry. The use of CuAAC for bioconjugations was initially widespread with saccharides and peptides. In such bioconjugations, where a covalent addition to a biomolecule is performed, copper is typically added in significant excess, superstoichiometric with respect to the biomolecule. These bioconjugation reactions also use an accelerating ligand that coordinates the copper ion. These have been investigated extensively and optimized reliable protocols for bioconjugations are available (Hong et. al.,2009; Preloski et. al.,2011). In seeking to apply these to oligonucleotides, particularly RNA which is especially sensitive to metal mediated hydrolysis, we also investigated other recently described water soluble ligands for copper. In the ensuing studies on RNA click reactions in aqueous buffer, we discovered that acetonitrile as a minor co-solvent obviated the need for a separate copper stabilizing ligand (Paredes and Das, 2011). The use of acetonitrile is in keeping with a prescribed tenet of click chemistry as it allows for simpler purification. The role of ligand in accelerating the copper mediated azide-alkyne cycloaddition reaction is likely negligible as the copper is in excess over the reactants and the role of copper stabilization may be fulfilled by the cyano group of acetonitrile. Further, we found that the click reaction can be favored over competing oxidative damage in DNA and rapid hydrolytic damage in RNA through

control of the copper and acetonitrile concentrations (Paredes and Das, 2012), with faster reactions with acetonitrile. Thus the advantage in reactions using acetonitrile for RNA and DNA is apparent in both the speed of the reaction which abates oligonucleotide degradation and also purification as the acetonitrile is easily removed with solvent.

The procedure detailed here is for the optimized general method for an acetonitrile-containing pseudo-ligandless click reaction to an oligonucleotide. An important step in the method, noted previously by Finn and coworkers (Preloski et. al., 2011), is thorough degassing and the details for this are provided, along with separate alternate protocols for examples and conditions to demonstrate the conjugation of a DNA or RNA to a dye molecule, as well as reactions to conjugate DNA or RNA to other DNA and RNA strands. The specific sequences used in these examples are noted in Table 1. The relative amounts of each of the reagents and reactants used in the various examples remain the same and are noted in Table 2. These reactions are fast and yield near quantitative product. Support protocols for the purification and analysis of the reaction product are also provided.

Rather than a small molecule such as the fluorescent Cyanine 3 dye azide, an oligonucleotide bearing an azide can be clicked instead onto the DNA or RNA of interest. If the oligonucleotides are now connected end to end through alkyne and azide on their termini, the product is a linear strand. However, if the alkyne is in an internal position such as through the 2'-position, the result is a backbone branched DNA (Paredes et. al., 2013). Likewise, connecting an RNA through a 5'-terminal azide to the 2'-position of another RNA strand, yields a backbone branched RNA that has a non-hydrolyzable triazole linkage that mimics 2',5'-branched splicing intermediates. The alternate protocols for DNA and RNA click reactions to other DNA or RNA are for these backbone branched oligonucleotides. Support protocols for analysis or purification by HPLC or by electrophoresis through polyacrylamide gels are also provided. The basic protocol can be extended to use in reactions in which the oligonucleotides are outside the range exemplified in the Alternate Protocols, though we recommend that Alternate Protocol 1 in which a fluorescent dye is conjugated to an oligonucleotide is performed as an initial test, with the support protocol for analysis to verify success of the reaction.

BASIC PROTOCOL 1

General Pseudo-ligandless Click Reaction

The procedure below describes the conjugation of a dye molecule or ligation of a DNA or RNA strand to a DNA or RNA sequence of interest. The DNA or RNA sequence contains an alkyne that may be terminal (3'- or 5'-) or internal (as a 2'-*O*-propargyl group) with the dye or other oligonucleotide containing the reactive azide (see Fig 1). The reactions would work equally well if the oligonucleotide sequence to which the conjugation was to be performed contained the azide and was used as the limiting reagent, or if the modifications were alternately positioned within the oligonucleotide sequence. Rather than a dye molecule used here, other small molecules and even polymers can be used. Rather than THPTA or PMDETA or other ligand to stabilize the copper, acetonitrile is used. All materials or their close analogues are commercially available. The DNA and RNA that included the 5'-azide were synthesized using the method described by Miller and Kool (Miller and Kool, 2005).

Oligonucleotides with azide modifications may be obtained from commercial sources, though other linkers and chemistry may be used in to install the azide. The alternate protocols provide specific guidance for examples where the click-reactive site is at the oligonucleotide terminus (Alternate Protocol 1) or at an internal 2'-position (Alternate Protocol 2). The two alternate protocols use differing concentrations of oligonucleotides that serve to show a range of conditions. However, using the basic protocol below and the relative proportions of reagents in Table 2 concentrations well outside this range (with oligonucleotide upto tens of μM) can be used.

Materials—Solution of alkyne DNA or RNA in ddH₂O

Solution of azide Cy3 dye or DNA or RNA in ddH₂O

20% acetonitrile (ACN) solution in ddH₂O

10 Phosphate Buffered Saline (PBS) (see recipe)

10 mM aqueous copper (II) sulfate (CuSO_4) solution

50 mM aqueous sodium ascorbate solution (prepared fresh)

Gel loading solution (see recipe)

1.7 mL microcentrifuge tube

0.65 mL microcentrifuge tube

Mini-centrifuge

Vortex Genie with foam insert for microtubes

All materials from degassing support protocol

Protocol steps

1. Flush three 0.65 mL microcentrifuge tubes with argon for ten seconds.
2. Add 70 μL of 10 mM CuSO_4 into one of the flushed microcentrifuge tubes. Add 70 μL of 50 mM sodium ascorbate to another flushed tube.

These are mini-stocks that will be degassed and then added into the click reaction mixture. Volume can range from 50 to 100 μL for efficient degassing

3. Table 2 describes the relative proportions of reagents used in the CuAAC reaction. For reactions where the oligonucleotide is being conjugated to a small molecule, the DNA or RNA strand is the limiting reagent; when branched oligonucleotides are being made, the stem is the limiting reagent; i.e., in all cases here the oligonucleotide bearing the alkyne is the limiting reagent. Reaction volumes can range from 50 to 100 μL and concentration of limiting reagent can also range from 50 to 100 μM , giving

a range of 2.5 to 10 nmol for effective conjugation (see Table 2 for relative ratios of reagents used and the Alternate protocols for specific amounts).

4. Add the required volumes of PBS, ACN, alkyne-oligonucleotide, azide-reactant (Cy3 or oligonucleotide) and ddH₂O, to the third argon flushed microcentrifuge tube – this is the reaction mixture tube.
5. Degas the reaction mixture tube with argon three times, as described in the degassing protocol (Support Protocol 1).
6. Degas the two small tubes containing CuSO₄ and sodium ascorbate three times each.
7. Add sodium ascorbate to the reaction mixture, degas the mixture as well as the sodium ascorbate tube after addition.
8. Add the degassed solution of CuSO₄ to the reaction mixture and perform a final degassing.
 - a. Copper addition starts the reaction
9. Spin down the reaction tube on a benchtop mini centrifuge to return all liquid to the bottom, put the reaction on the Vortex shaker at its lowest setting and let react for a total of 2-3 h at ambient room temperature
 - a. Length of reaction depends on conjugating molecule – typically 2 h for reactions at the 5'- (or 3'-) terminus (Alternate Protocol 1) and 3 h for reactions with an internal 2'-*O*-alkyne (Alternate protocol 2).
10. Roughly halfway through the reaction add 1/3 of the starting amount of sodium ascorbate to the solution. Degas the reaction mixture one final time.
11. Stop the reaction by either desalting the reaction mixture using a desalting column according to manufacturer specifications (Glen Pak RNA or Glen Pak DNA when conjugating dye) or by mixing an equal volume of the reaction with gel loading solution and purifying using denaturing (8 M urea) PAGE (for branched oligonucleotides). Following this step, the DNA or RNA click-product may be purified (and analysed) by HPLC or gel electrophoresis (Support Protocols 2 or 3).

Support Protocol 1 [critical protocol]

Degassing of Solutions and Reaction

Degassing of the solutions is critical to the success of the click reaction. The use of copper (II) in the reactions render the oligonucleotides vulnerable to oxidative and radical damage through processes that involve any dissolved oxygen in the solutions. This does not require any specialized equipment and we have found that simply blowing argon through the solutions and reaction mixture, as described below, prior to addition of the copper solution is effective.

Materials

Argon gas

50 mL Erlenmeyer flask

19/22 Rubber Septum

3 mL Syringe

7" balloon

21G 1.5" needle

Rubber bands

Vortex Genie with foam insert for microtubes

1. Assemble the argon balloon apparatus
 - a. Flush a 50 mL Erlenmeyer flask with argon and cap with a rubber septum and set aside. Take a 3 mL plastic syringe, remove the plunger and cut the barrel in half. Attach a 7" balloon to the end of the syringe barrel with a rubber band. Fill the balloon with dry argon. Attach a needle to the other end of the syringe and pierce through septum into Erlenmeyer flask. The balloon with argon can be kept in the flask with the needle through the septum and used as needed.
2. Blanket the solution in the microcentrifuge tube with the argon filled balloon by pointing the syringe over the solution, for 30 s. Close tube and place on the vortexer, with shaker attachment, and let it shake while degassing other tubes. Repeat as many times as indicated in procedure
 - a. Avoid letting the needle come into contact with the solutions to prevent contamination.

See Video Protocol

Support Protocol 2

Purification and analysis of pseudo-ligandless click reaction – HPLC

Introduction—Following the desalting step at the end of the reaction, this protocol is for the purification and analysis of a small scale click reaction.

Materials—0.1 M TEAA (pH 7) (see recipe)

80/20 (v/v) ACN/aqueous 0.1 M TEAA (see recipe)

Waters HPLC – running Breeze software or equivalent system for binary separations

PDA detector

Waters XBridge C₁₈ column 5 μ m (4.6×150 mm)

1. After desalting the reaction mixture, lyophilize the sample and resuspend in ddH₂O. Dilute 1 nmol of the reaction mixture to 100 μ L in 0.1 M TEAA and analyze by HPLC at 25 °C with a flow rate of 1 mL/min with 80/20 (v/v) ACN/ aqueous 0.1M TEAA buffer and a linear gradient from 2.5% to 25% in 10 min and then to 50% in 20 min
2. Collect the fractions that correspond to peaks of the product, which will have absorbance both at 260 nm and 550 nm (for nucleic acid and Cy3, respectively, see Fig. 2). The collected fraction can be concentrated and lyophilized and a small portion reinjected in the HPLC to confirm purity (see Fig. 3) as well as analyzed by mass spectrometry.

SUPPORT PROTOCOL 3 (optional)

Purification and analysis of pseudo-ligandless click reaction – Denaturing (8 M urea) polyacrylamide gel electrophoresis

Materials—Ethanol

20% Gel Mix 8 M Urea 1X TBE (recipe below)

Tetramethylethylenediamine (TEMED)

10% (by volume) aqueous ammonia persulfate (APS)

0.5X TBE buffer

ACN

H₂O

50/50 (v/v) ACN/H₂O

Glass plates for 14 cm wide gel; 17 cm $\times$ 12 cm (width $\times$ height; one plate is 2 cm shorter)

Binder clips

1 mm thick comb with at least 4 teeth of approximately 7mm width for sample loading

1 mm thick spacers

100 mL beaker

Whatman Elutrap Device

SepPak Classic C18 desalting cartridge (Waters)

Lyophilizer

1. Clean two glass plates with ethanol, put 1 mm thick spacers on opposite ends of the plates. Sandwich the spacers between the glass plates with ethanol cleaned sides facing each other. Secure the spacers on the edge of the glass plates by using two binder clips on each side of the spacers.
2. Mix 50 mL of 20% Gel mix with 50 μ L of TEMED and 500 μ L of 10% APS in the 100 mL beaker. Make sure to avoid trapped bubbles in this solution. Pour the gel mixture in between the glass plates. Insert comb and let polymerize for half an hour.
3. Clean out the wells and pre-run the gel at 12 W (10 min) in 0.5X TBE
4. Load the sample into the cast gel that is premixed 1:1 (v:v) with gel loading solution
5. Run in 0.5X TBE buffer at 12 W for 3 hours. Visualize bands by UV shadowing.
6. Excise desired bands from the gel and elute using Whatman Elutrap device with 0.5X TBE buffer (250V, 3 h)
7. Desalt the eluted branched oligonucleotide using a SepPak Classic C18 cartridge (2x 1mL ACN, 2x 1mL H₂O, oligonucleotide, 1x 4mL H₂O, 2x 1mL 50/50 (v/v) ACN/ H₂O) and lyophilize.

ALTERNATE PROTOCOL 1

Pseudo-ligandless click reaction for conjugation of alkyne DNA or RNA to azide Cy3 dye

Introduction—This protocol is for the click conjugation of a fluorescent dye to the 5'-terminus of DNA or RNA. This protocol with reactive dye and the expected output of click-conjugated oligonucleotide along with the support protocol 2 can serve as an initial test to verify success of the click conjugation, before changing the scale of the reaction. The protocol may be followed without change for conjugations to the 3'-terminus of DNA and if moieties other than the fluorescent dye (whether other small molecules, oligonucleotides or polymers) are used.

Materials—Same as in basic protocol 1

1. Follow the general protocol with the amounts of reactants and reagents given below:
 - a. 5 μ L of 10X PBS buffer, final concentration 1X
 - b. 1.5 μ L of 20% ACN, final concentration 0.6%
 - c. 5.4 μ L of 1.4 mM Cy3 azide, final concentration 150 μ M
 - d. DNA- or RNA-alkyne to a final concentration of 50 μ M (9 μ L of 286 μ M DNA3 or
 - e. 24.1 μ L or 23.1 μ L of ddH₂O (for DNA3 or RNA3, respectively)

3. Analyze by gel according to the denaturing gel protocol

A typical result is shown in Figure 4.

REAGENTS AND SOLUTIONS

PBS (10X)

80 g NaCl

2 g KCl

14.4 g Na₂HPO₄

2.4 g KH₂PO₄

Dissolve in 800 mL of ddH₂O, Adjust pH to 7.5 using HCl or NaOH. Adjust final volume to 1000 mL with ddH₂O. Filter with 0.2 µm filter. Store at ambient room temperature for six months.

2 M TEAA

120 mL Glacial Acetic Acid

550 mL ddH₂O

275 mL Triethylamine

Add the glacial acetic acid to the ddH₂O. With vigorous stirring, slowly add the triethylamine in several small portions. When the final solution has cooled adjust the pH to 7.6 with glacial acetic acid or triethylamine as needed. Pass solution through a 0.2 µm filter. Store at 4 °C for up to 6 months.

0.1 M TEAA

100 mL 2 M TEAA

1900 mL ddH₂O

Add the ddH₂O to the 2 M TEAA buffer in a plastic beaker and mix well with a glass rod. Adjust the final pH to 7.0 by dropwise addition of small quantities (50 to 100 µL) of trimethylamine at a time. The final volume should not be affected by the small additions which typically total less than 500 µL. Store at ambient room temperature for six months in an autoclaved or sterile bottle.

80/20 (v/v) ACN/ aqueous 0.1 M TEAA

80 mL ACN (HPLC grade)

50 mL 2 M TEAA

150 mL ddH₂O

In an autoclaved or sterile bottle combine ACN, 2 M TEAA and ddH₂O. Store at ambient room temperature for six months.

10X TBE

108 g Tris Base

55 g Boric Acid

7.5 g EDTA disodium salt

Dissolve the Tris base, boric acid, and EDTA in 800 mL of ddH₂O. Heat at 37°C for 15 minutes to dissolve solids if necessary. Adjust the final volume to 1000 mL with ddH₂O, no pH adjustment is necessary. Store at ambient room temperature for up to six months.

0.5X TBE

1900 mL ddH₂O

100 mL 10X TBE Buffer

Mix two solutions together and store at ambient room temperature for six months.

Gel Loading Solution for Denaturing PAGE

45 mL of Formamide

5 mL of 0.1 M EDTA (pH 8)

Mix the two solutions and store at ambient room temperature for six months.

20% Gel Mix 8 M Urea 1X TBE

480 g Urea

100 mL 10X TBE buffer

500 mL 40% Gel mix (29:1 acrylamide: bisacrylamide)

Weigh out the urea and add the TBE buffer and Gel mix. Mix the solution with a glass rod to dissolve as much urea as possible. Then put the solution in a 65 °C water bath to completely dissolve solids, about 15 minutes. Adjust the final volume to 1 L with ddH₂O if necessary and filter through a 0.45 µm filter. Store at ambient room temperature for up to 4 months away from light.

COMMENTARY

Background Information

Following Sharpless and coworkers' discovery that the azide-alkyne 1,3-dipolar cycloaddition can be rapidly catalyzed by Cu(I) (Kolb et. al. 2001), the use of ascorbate as a mild reductant that allows the use of Cu(II) salts, and various ligands, particularly tris-

(benzyltriazolylmethyl)amine (TBTA), its ddH₂O soluble analogue, THPTA and *N*, gel solution *N*, *N*, *N*, *N'*-pentamethyldiethylenetriamine (PMDETA) among others that significantly accelerate the reaction and stabilize the Cu(I), have led to the widespread use of the reaction (Finn and Fokin, 2010). The copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction has vaulted over others to become near synonymous with the term click reaction or click chemistry. The highly efficient reaction between the reacting azide and alkyne groups that do not cross react with biologically abundant functional groups have made it indispensable in bioconjugations. The literature for click chemistry with proteins, glycans and DNA is already quite extensive (Sletten and Bertozzi, 2009; El-Sagheer and Brown, 2010) and for RNA it is growing (Schulz and Rentmeister, 2014). Finn and coworkers have optimized protocols for the reaction - especially for bioconjugations with excellent details of the process that the reader is advised, indeed urged, to be familiar with (Preloski et. al., 2011). The widespread use of the click reaction has nevertheless generated a vast literature with somewhat inconsistent application of reaction conditions. In the case of oligonucleotides, particularly with RNA that is prone to metal mediated hydrolysis, we were able to narrow down the condition space so as to furnish the desired products with minimal degradation (Paredes and Das, 2013). The use of acetonitrile to replace TBTA or PMDETA in reactions works well for conjugations of oligonucleotides, especially to polymers, easing concerns of ligand adsorption to hydrophobic portions (Averick, et. al. 2010; Averick, et.al. 2013). These pseudo-ligandless click reactions with DNA and RNA work well enough at ambient room temperature to furnish near quantitative product in a few hours, though in some cases we have seen the benefit of conducting the reaction at higher temperatures (up to 42 °C).

Critical Parameters

For success of the reaction with oligonucleotides, we find that attention to the relative amounts of reagents is crucial. Likely the high variability in the concentration and amount of the biomolecules of interest to researchers coupled with the ease and low cost of copper (II) salts and ascorbate reductant have led to the varying conditions found in the literature. While a majority of biomolecules, including DNA, are relatively tolerant to differences, RNA succumbs to degradation much more rapidly once outside the optimal copper and acetonitrile concentrations relative to the oligonucleotide. We have found these concentrations and conditions to work well with a broad range of DNA and RNA sequences, without any other optimization. Most critical to success though, is the recognition that the processes that degrade the oligonucleotides involve oxidative processes. Therefore *proper and adequate degassing of solutions*, which is straightforward, cannot be overemphasized.

Troubleshooting

Following these conditions and protocols, we have had little troubleshooting to do. In cases where we have found inadequate reaction and the limiting oligonucleotide still largely unreacted, elevation of the reaction temperature (to 37 °C or 42 °C) by placing in a water bath has helped – with product yields of at least 85%, if not near quantitative. Although not detailed here, as the purification and analysis rely on different and varying methods, the protocol for the click reaction is also readily applicable without other optimization to conjugations of DNA or RNA with polymers.

In the case of conjugations of oligonucleotides to proteins (Averick, et.al., 2012), the use of acetonitrile, even at the 0.6% v/v concentration may lead to less than complete reaction and some degradation. Therefore for reactions that involve peptides or proteins, we do not recommend the use of the acetonitrile/pseudo-ligandless conditions described here, but rather the use of THPTA as the ligand for copper (Presolski, et.al., 2011), along with guanidinium salt to stabilize the peptide.

Anticipated Results

The click conditions reported here have been used successfully for the conjugation of DNA and RNA to a wide variety of molecules - small and large (including polymers). These reactions are essentially quantitative. For reactions involving just oligonucleotides, such as ligations or branching as described here, where two negatively charged strands are to be conjugated, the reactions may need gentle heating as the charge repulsion and/or other sequence related effects may hinder complete reaction within 2 to 3 h.

Time Considerations

The reactions described here are relatively straightforward and can be performed in three to four hours, including the time for degassing and preparation of the reagent solutions. Purification times will vary depending on the choice of method.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Pseudo-ligandless click chemistry for oligonucleotide conjugations. The oligonucleotide contains an alkyne for reaction with a small molecule (fluorescent Cyanine3) azide or another oligonucleotide. The reactions use 0.6% acetonitrile as a co-solvent to stabilize the Cu(I) cation. The insets show the chemical structures of the different alkyne and azide modifications in the sequences and Cyanine3.

Figure 2.

HPLC Traces of the Oligonucleotide Click Reactions to azide Cy3. The crude reaction products (black traces) compared to unreacted starting material (dashed trace). Panels A and B show the absorbance at 260 nm of the DNA and RNA reactions, respectively. The dashed traces are of the oligonucleotide starting materials. Panels C and D show the absorbance at 550 nm of the DNA and RNA reactions, respectively. In these, the dashed lines are traces from the Cy3 azide starting material.

Figure 3. HPLC Traces of purified click products of DNA or RNA with Cy3 (black traces) compared against unreacted starting material (dashed traces). Panels A and B show the absorbance at 260 nm of the DNA and RNA reactions, respectively. Panels C and D show the 550 nm absorbance of the DNA and RNA reaction, respectively. In these, the dashed traces are of the azide Cy3 starting material.

Figure 4.

Backbone branched oligonucleotide products and starting materials using denaturing polyacrylamide gels. The backbone branched oligonucleotide products are clearly seen as the highest bands in the click reaction lanes, compared to 2'- O- propargyl modified reactant 'stems' and 5'- azide modified 'branches'. Panel A click reaction lane shows the backbone branched DNA product, the excess 15mer branch and some unreacted 11mer stem in order from the top. Panel B shows the backbone branched RNA product in the click reaction lane, followed by slight unreacted 13mer stem and the excess 6mer branch in the order from the top. Markers are xylene cyanol and bromophenol blue.

Table 1

Oligonucleotide Sequences

Name	Sequence (5'-3')	5' Modification	3' Modification
DNA1	GCA GT A(2'- <i>O</i> -propargyl) CAC GC	--	--
DNA2	CCA GTT TGG CCG AGG	N ₃	--
DNA3	GCT AT CCA TCA GAA TTC GCG ACG	Hexynyl	--
RNA1	GUA CUA A(2'- <i>O</i> -propargyl) CAA GUU	--	--
RNA2	GUA UGA	N ₃	--
RNA3	UGG AAC GUU GAG UGU UAA UCG A UGA	Phosphate	<i>O</i> -propargyl

Table 2

Relative proportions of reagents used in pseudo-ligandless click reactions

Reagent	Relative Proportion
Oligonucleotide (alkyne)	1X
Small Molecule azide/ Oligonucleotide azide	2-3X
sCuSO ₄	10X
Sodium Ascorbate	50X
ACN	0.6%
PBS	1X