DNA-enhanced CuAAC ligand enables live-cell detection of intracellular biomolecules

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Abstract

Of the various conjugation strategies for cellular biomolecules, Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is the preferred click chemistry approach due to its fast reaction rate and the commercial availability of a wide range of conjugates. While extracellular labeling of biomolecules using CuAAC has been widely adopted, intracellular labeling in live cells has been challenging; the high copper concentration required for CuAAC reaction can be toxic to biological systems. As a critical first step towards achieving intracellular labeling with CuAAC, an ultrasensitive CuAAC ligand is needed to reduce the required copper concentration while maintaining fast reaction kinetics. Here, we develop a new DNA oligomer-conjugated CuAAC ligand for click reaction biomolecular labeling. The DNA oligo attachment serves several purposes: 1. Increases availability of local copper atoms in proximity to the ligand, which drives up reaction rates, 2. Enables the ligation of azide tags with up to 10-fold lower copper concentrations as compared to commercially available CuAAC ligands, 3. Allows nucleic acid template-driven proximity ligation through the choice of the attached DNA sequence and 4. Allows the CuAAC ligand and copper to traverse the cell and nuclear membrane. We demonstrate that this ligand enhances the intracellular 5-ethynyl uridine labeling of nascent RNAs using fluorogenic dyes. We also show that our DNA-enhanced CuAAC ligand enables the ligation of fluorogenic dyes to label both sialylated glycans on the surface on live cells as well as the live-cell intracellular labeling of nascent RNAs. This new ligand advances our efforts toward the final goal of applying CuAAC for live-cell applications.

Introduction

Bioorthogonal chemistry is a broadly applied conjugation strategy to study biological processes with molecular details[1–4]. These rapid reactions are highly selective without side-reactions towards native functional groups[5]. One of the first bioorthogonal reactions reported was Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) whereby a dipolar cycloaddition between a chemically inert terminal alkyne and an azide is facilitated[6–9]. The CuAAC reaction is compatible with aqueous conditions[10], is usually free of by-products[1] and benefits from the commercial availability of a wide range of conjugates. However, the high copper catalyst concentration required in combination with ascorbate and oxygen can result in reactive oxygen species (ROS) that can be detrimental to nucleic acids[11] and toxic to biological systems[12], thus limiting the CuAAC reaction inside of live cells.

Biocompatible ligands have been developed to reduce the toxicity of the copper catalyst and enhance the reaction kinetics[8,9] and copper-chelating azides have reduced the total copper requirement for the reaction[13]. However, even with these developments, only a few studies have achieved intracellular labeling using CuAAC. This is due to copper toxicity at the necessary concentration range, low uptake of reagents, and competing intracellular ligands capable of sequestering the copper. For example, a copper-chelating azide was used to enhance CuAAC reactivity[14] for the detection of an alkyne labeled drug however this strategy precludes the use of fluorogenic azides. In another example, the CuAAC accelerating ligand was conjugated to a cell penetrating peptide and demonstrated to detect l-homopropargylglycine using a fluorogenic dye[15]. However, the cell viability was diminished compared to commercial ligands due to the copper toxicity and yields for cytosolic proteins were significantly decreased. For these reasons, CuAAC in live cells is typically used for the labeling of cell-surface biomolecules such as glycans[8] and lipids[16]. Alternatively, the incorporation of azide and alkyne reporters is frequently

achieved in live cells, however, cell fixation is performed prior to CuAAC driven ligation of detection probes[17,18].

Copper-free, strain-promoted azide-alkyne cycloaddition (SPAAC) may also be used for covalent tagging of biomolecules[19]. This method is highly selective[20,21], and may be used for intracellular labeling of biomolecules[22]. However, SPAAC is limited by commercial availability of cyclooctynes[23] providing an alkyne functional group for the reaction, reagent instability, and nucleophilic addition with cellular nucleophiles[20] . Thus, the development of a CuAAC reaction that may be used for intracellular, live-cell labeling is desirable.

We have developed a new, DNA oligomer-conjugated CuAAC accelerating ligand $BTT_{(1,2)}$-DNA that can reduce the required copper concentration while enhancing reaction kinetics. We show that the DNA oligo attachment can increase availability of local copper atoms in proximity to the ligand, thus enabling the ligation of azide tags with much lower copper concentrations as compared to commercially available CuAAC ligands. The DNA oligo attachment also enables a nucleic acid template-driven proximity ligation through the choice of the attached DNA sequence. We show that $BTT_{(1,2)}$-DNA enables the ligation of azide with up to 10-fold lower ligand concentrations in the fluorogenic plate-reader assay with a variety of fluorogenic azide probes[24]. We demonstrate that increasing the number of binding sites of the nucleic acid template can increase the CuAAC reaction yield accelerated by $BTT_{(1,2)}$-DNA. We also show that $BTT_{(1,2)}$-DNA enhances the intracellular 5-ethynyl uridine detection of nascent RNAs with a 12-fold lower ligand concentration in fixed cells. Importantly, we show that $BTT_{(1,2)}$-DNA enables the ligation of fluorogenic dyes on the surface of live cells to metabolically labeled, sialylated glycans with a 4-fold lower ligand concentration. Additionally, we are able to achieve intracellular, live-cell labeling and specific detection of nascent RNA molecules in the nuclear compartment of cells. In short, $BTT_{(1,2)}$-DNA enables sensitive detection of biomolecules in fixed and live cells, and advances our efforts toward the final goal of applying CuAAC for intracellular, live-cell applications.

Results

Synthesis and characterization of $BTT_{(1,2)}$-DNA CuAAC accelerating ligand

The commercially available BTTAA ligand was used to stabilize the Cu(I) oxidation state and maintain the fast kinetics for CuAAC in aqueous solution. To enhance sensitivity and reduce the total copper concentration required for CuAAC, we adopted the strategy used to develop BTTAA [9], using the precursor molecule (S1) that contains two *tert*-butyl groups with an alkyne, to synthesize a CuAAC accelerating ligand, $BTT_{(1,2)}$-DNA (**Fig. 1a**) with a DNA scaffold. We ligated S1 to a single strand DNA oligonucleotide probe bearing a single or double 3' azide, added using TdT and azido-UTP, via CuAAC, to produce $BTT_{(1,2)}$-DNA (**Fig. 1a**). We confirmed the synthesis of this probe using a TBE-Urea gel and the ratio of the single/double-CuAAC accelerating ligand is 47.8%/52.2% (**Fig. 1b**) based on the incorporation of a single or double azido-UTP via TdT enzyme. We performed HPLC and dialysis to purify both populations of $BTT_{(1,2)}$-DNA ligand and tested the efficacy of each version to catalyze the formation of a ligation product via CuAAC. We found that the fold change of the $BTT_{(1)}$-DNA ligand was 11.10 and that of $BTT_{(2)}$-DNA ligand was 11.28, indicating that they performed similarly (**Fig. 1c**).Therefore, we opted to mix the two populations of $BTT_{(1,2)}$-DNA ligand. To lower the sample loss due to HPLC and accelerate the purification process, we opted to purify directly with dialysis for subsequent experiments after confirming the similar efficacy of the mixed population of $BTT_{(1,2)}$-DNA ligand purified by HPLC and dialysis, and dialysis alone.

Pilot CuAAC studies using the $BTT_{(1,2)}$-DNA ligand suggested the formation of ligation product in the absence of free copper (data not shown), so we performed inductively coupled plasma mass

spectrometry (ICP-MS) to assess the presence of copper in the purified ligand. We were surprised to find that the ratio of Cu:BTT$_{(1,2)}$-DNA is 7.5:1, even after dialysis (**Fig. 1d**).

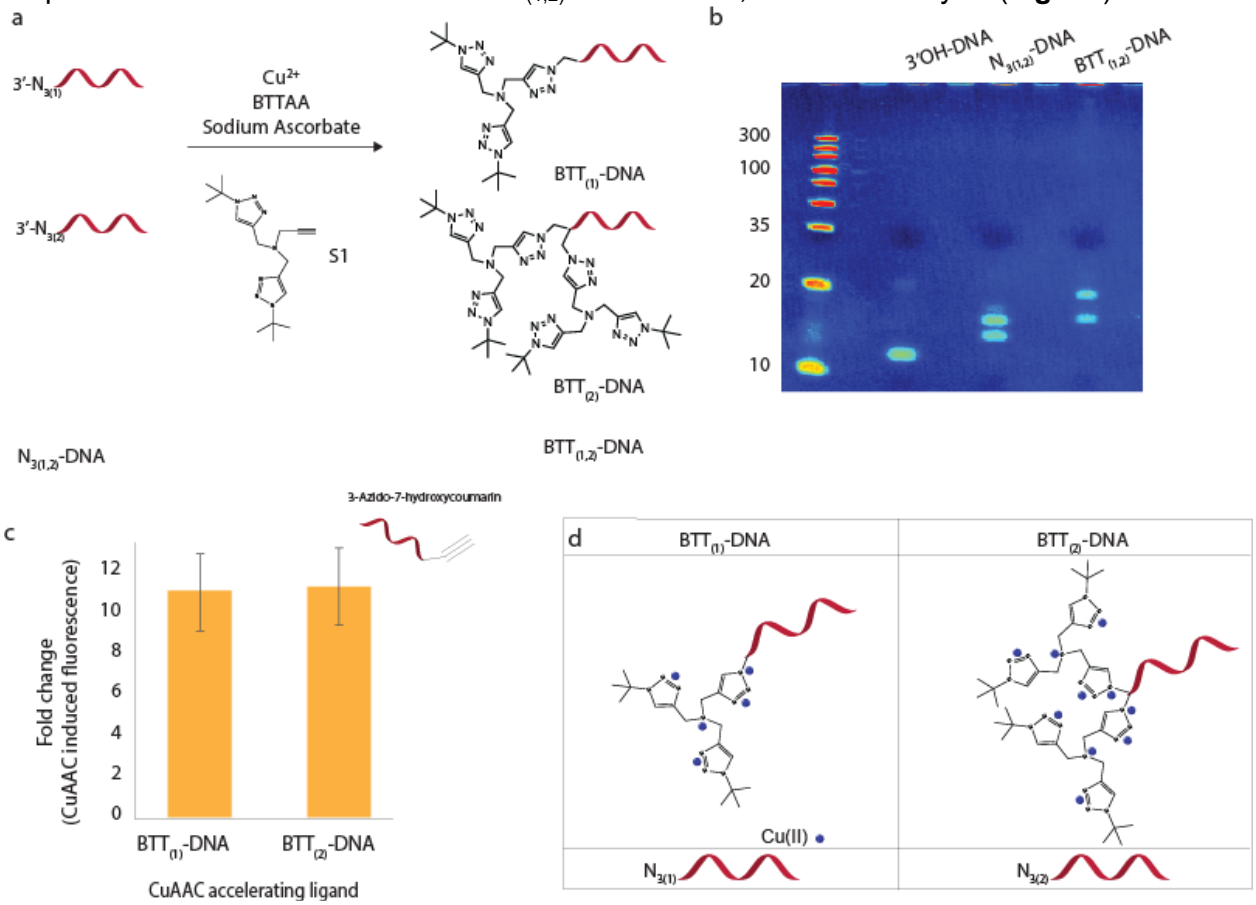

Fig 1. Synthesis and characterization of BTT$_{(1,2)}$-DNA ligand. a) Strategy to synthesize BTT$_{(1,2)}$-DNA ligand via CuAAC using (S1). b) 15%TBE-Urea gel electrophoresis for BTT$_{(1,2)}$-DNA ligand. c) Relative efficacy of BTT$_{(1)}$-DNA and BTT$_{(2)}$-DNA ligand in accelerating CuAAC. An alkyne labeled DNA oligonucleotide is ligated to 3-Azido-7-hydroxycoumarin in the presence of BTT$_{(1)}$-DNA and BTT$_{(2)}$-DNA ligand and sodium ascorbate. Error bars represent the standard deviation of three biological replicates. d) Structure of BTT$_{(1,2)}$-DNA ligand based on ICP-MS.

Reaction kinetics of BTT$_{(1,2)}$-DNA ligand

The relative reactivity of Cu(I) catalysts in the form of BTTAA-Cu(I) and the new Cu(I) accelerating ligand BTT$_{(1,2)}$-DNA ligand complexed with an average of 7.5 copper molecules was determined in the fluorogenic plate-reader assay by reacting a 15 mer DNA oligomer bearing a 5' alkyne moiety (henceforth referred to as 5' alkyne DNA) with a series of azido fluorogenic dyes[24] using CuAAC (**Fig. 2a)**. To assess the reactivity of the Cu(I) catalyst in complex with BTTAA and the BTT$_{(1,2)}$-DNA ligand, the reaction was optimized (**Supplementary Fig.1**) and the 5' alkyne DNA was ligated to a series of fluorogenic dyes using CuAAC with the fluorescence measured over 2 hours (**Fig. 2b-f)**.

BTT$_{(1,2)}$-DNA consistently showed higher activity in accelerating the CuAAC reaction than BTTAA for all dyes. For the CuAAC reactions that were assisted by 100 nM BTT$_{(1,2)}$-DNA ligand, we observed an increase in fluorescence intensity from 59.46 to 87.68 RFUs over a 2 hour reaction. For the reactions that were assisted by 1 μM BTT$_{(1,2)}$-DNA ligand, fluorescence intensity increased from 79.64 to 355.82 RFUs over 2 hours. At 100 nM ligand concentration, the BTT$_{(1,2)}$-DNA ligand outperformed the commercial ligand 1.56 fold, demonstrating that BTT$_{(1,2)}$-DNA is an effective ligand in the nanomolar range and in the absence of free Cu(I). The

difference was even more striking at 1 μM $BTT_{(1,2)}$-DNA at which the fluorescence produced was 2.33 fold higher than commercial ligand at 2 hours (**Fig. 2b**). Upon click reaction in the presence of $BTT_{(1,2)}$-DNA ligand, the fluorescence of the CalFlour 647 azide produced at 1 μM $BTT_{(1,2)}$-DNA was 1.56 fold higher than BTTAA ligand at 2 hours and the $BTT_{(1,2)}$-DNA ligand also outperformed the BTTAA ligand 1.63 fold at the concentration of 100 nM (**Fig. 2c)**. The CalFlour 488 azide showed a significant difference at 1μM $BTT_{(1,2)}$-DNA ligand concentration and the fluorescence produced was 1.77 fold higher than commercial ligand at 2 hour , while the difference between the $BTT_{(1,2)}$-DNA and BTTAA ligand at the concentration of 100 nM was negligible (**Fig. 2d**). To our surprise, CalFlour 555 azide showed a significant difference between the $BTT_{(1,2)}$-DNA and BTTAA ligand was observed at 100nM of the ligand and 1.96-fold increase was achieved (**Fig. 2e**). While CalFlour 580 had the lowest reaction yield in the 100nM to 1μM concentration range of ligand and the pattern looked different from other 3 CalFlour azide dyes, we observed a 1.26-fold increase and 1.32-fold increase in fluorescent signal produced from the $BTT_{(1,2)}$-DNA ligand accelerated reaction as compared to the commercial ligand at 100 nM and 1μM of ligand, respectively (**Fig. 2f**).

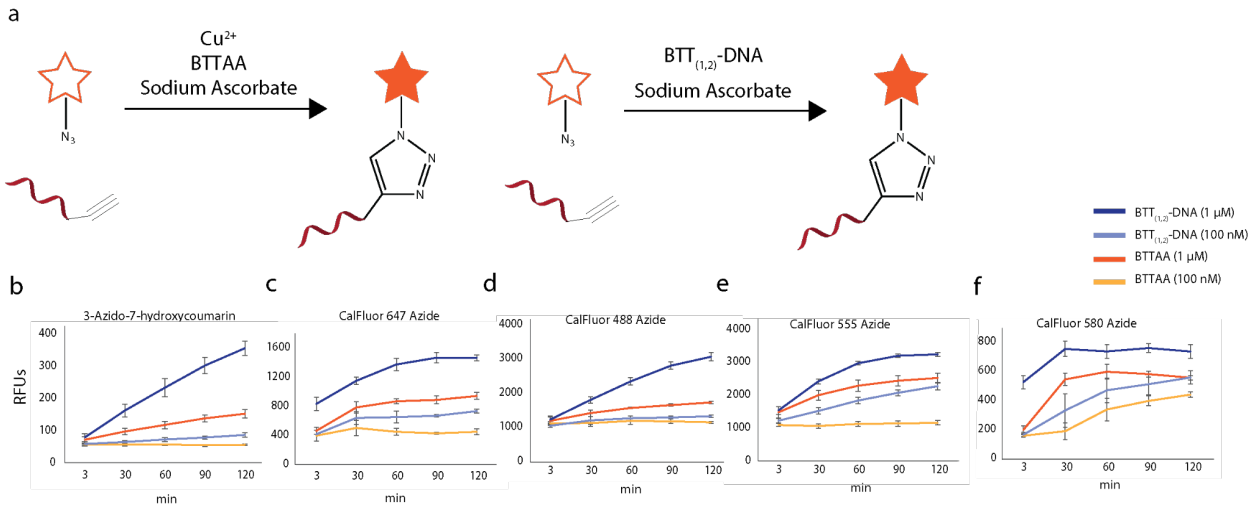

Fig 2. Reaction kinetics of CuAAC accelerated by the $BTT_{(1,2)}$-DNA ligand with different azido fluorogenic dyes. a) (left) schematic showing an alkyne labeled DNA oligonucleotide is ligated to a fluorogenic dye in the presence of Cu2+, BTTAA and sodium ascorbate. (right) an alkyne labeled DNA oligonucleotide is ligated to a fluorogenic dye in the presence of $BTT_{(1,2)}$-DNA ligand and sodium ascorbate. CuAAC reaction kinetics of reaction shown in (a) using b) 3-Azido-7-hydroxycoumarin c) CalFlour 647 azide d) CalFlour 488 azide e) CalFlour 555 azide , and f) CalFlour 580 azide. Error bars represent the standard deviation of a minimum of three biological replicates.

DNA and RNA template-driven proximity ligation with $BTT_{(1,2)}$-DNA ligand

To increase the local concentration of the reaction components, we used a DNA splint that was complementary to both the 5' alkyne DNA and 3' $BTT_{(1,2)}$-DNA ligand (**Fig. 3a**).We supplemented the reaction with spermine, a polyamine bearing multiple amino groups, to improve hybridization (**Supplementary Fig. 2**). We observed that the addition of both DNA and RNA splints enhanced the ligation of the fluorogenic azide with 5' alkyne DNA (2.13-fold and 2.21-fold respectively), facilitated by the $BTT_{(1,2)}$-DNA probe (**Supplementary Fig. 2**, **Fig. 3a**). To assess the appropriate distance to position the 5' alkyne on the DNA probe, to maximize ligation to the fluorogenic azide, we performed the fluorogenic plate-reader assay for 8 different 5' alkyne DNA probes and selected the DNA alkyne probe that has a 2 nucleotides space from the $BTT_{(1,2)}$-DNA probe (**Supplementary Fig. 2).** We also evaluated the splint concentration range which could drive the proximity ligation and found that a splint concentration as low as 100 nM can drive the reaction (**Supplementary Fig. 2**).

To enhance the reaction kinetics, we designed DNA splints harboring 2 and 3 repeats of the binding sequence, which can double and triple the ligation, respectively (**Fig. 3a**). We assessed the absorbance after a 1-hour incubation time in fluorogenic plate-reader assay for each condition. We found that the total fluorescence produced from a single DNA splint-enhanced reaction is 236.71±12.16 RFUs, which is 2.33-fold higher than the fluorescence produced from random collisions (**Fig. 3b**). We also observed that splints harboring more binding sites had higher fluorescence intensity. The absorbance increased from 236.71±12 RFUs to 310.98±7.11 RFUs and 344.10±9.82 RFUs when the binding sites double and triple, respectively (**Fig. 3b**, **Supplementary Fig. 2)**.

5-ethynyl uridine (EU) labeling of nascent RNAs followed by fixed cell detection with $BTT_{(1,2)}$-DNA ligand

EU is a alkyne derivatized uridine analog that may be metabolically incorporated into nascent RNAs in live cells. Following metabolic incorporation, cells were fixed and permeabilized, then reacted with CalFluor 647 Azide using CuAAC, comparing the performance of $BTT_{(1,2)}$-DNA ligand to commercially available BTTAA (**Supplementary Fig 3**). The total fluorescent signal produced in the nucleus from EU labeling, and in the presence of the $BTT_{(1,2)}$-DNA ligand was 3.36-fold higher than the BTTAA accelerated sample (**Supplementary Fig 3, Fig. 3c**). The $BTT_{(1,2)}$-DNA enhanced CuAAC with a 12-fold lower ligand concentration (**Fig. 3c**). The $BTT_{(1,2)}$-DNA ligand produced a 3.1 signal to noise ratio while the commercial ligand produced 1.94 (**Supplementary Fig 3, Fig. 3c**).

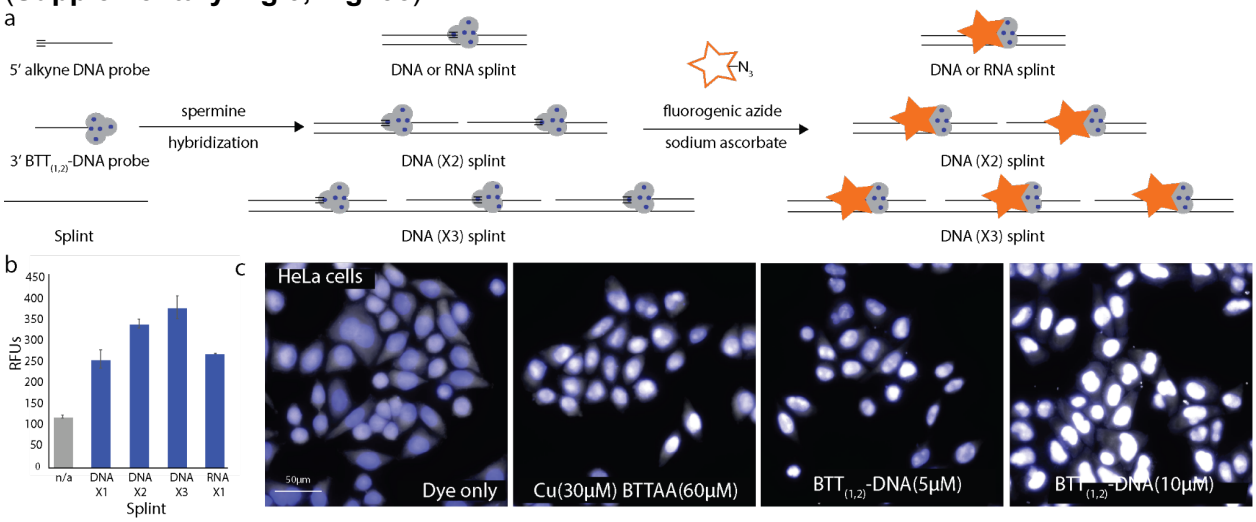

Fig 3.DNA and RNA template-driven proximity ligation with $BTT_{(1,2)}$-DNA ligand. a) Schematic of nucleic acid template-driven proximity ligation. b) Fluorescence measurement of nucleic acid template-driven proximity ligation with DNA splints harboring different repeats of binding sequence. Error bars represent the standard deviation of three biological replicates. c) Fluorescence microscopy of the intracellular 5-ethynyl uridine labeling of nascent RNAs via CuAAC in the presence of $BTT_{(1,2)}$-DNA ligand and BTTAA ligand in fixed cells. (from left to right) Fixed cells are treated with CuAAC in the presence of 1) CalFlour 647 azide and sodium ascorbate, 2) CalFlour 647 azide, Cu2+, BTTAA and sodium ascorbate, 3-4) CalFlour 647 azide, $BTT_{(1,2)}$-DNA ligand (5-10 μM) and sodium ascorbate. (white) CalFlour 647 azide labeling of nascent RNAs incorporated by 5-ethynyl uridine, (blue) DAPI staining of nuclei.

Copper induced cellular toxicity in the presence of CuAAC ligands

To evaluate the cytotoxicity of copper with the new Cu(I) accelerating ligand $BTT_{(1,2)}$-DNA, we compared Jurkat cells that were treated with premixed BTTAA-$CuSO_4$ complex, at the recommended copper concentration (20-30 μM), to Jurkat cells treated with $BTT_{(1,2)}$-DNA ligand in the optimal concentration range (5-10 μM) ligand (each molecule of ligand is complexed to approximately 7.5 molecules of copper; **Fig. 1**) and sodium ascorbate. These samples were compared to untreated cells and cells reacted with 20-30 μM $CuSO_4$ and sodium ascorbate in

the absence of ligand. Viable cells were counted with trypan blue assay. The new accelerating ligand $BTT_{(1,2)}$-DNA ligand at 10 μM outperformed the commercial BTTAA ligand for at least 12 hours (**Fig. 4a).** Interestingly, when we lowered the concentration to 5 μM, $BTT_{(1,2)}$-DNA ligand still outperformed the commercial BTTAA ligand, but not as significantly as $BTT_{(1,2)}$-DNA ligand at 10 μM after 6 hour-incubation(**Fig. 4a)**.

$BTT_{(1,2)}$-DNA ligand enables the detection of cell-surface sialic with lower ligand concentration

To test the activity of $BTT_{(1,2)}$-DNA in driving the CuAAC reaction for live cells, we first metabolically labeled HeLa cells with N -(4-pentynoyl)mannosamine (Ac_4ManNAl) to introduce an alkyne tag to cell surface sialic acids[25], clicked a fluorophore to the alkynyl sugar and imaged using fluorescence microscopy. Following metabolic labeling, we performed CuAAC on ice as well as at 37°C. For the CuAAC on ice, the $BTT_{(1,2)}$-DNA accelerated CuAAC produced equal fluorescent signal as compared to the commercial ligand but with a 4-fold lower ligand concentration (**Fig. 4b, Supplementary Fig.4**). At 37°C, the fluorescent signal on the membrane for the commercial ligand was equivalent to the signal observed on ice. Interestingly, for the CuAAC facilitated by the $BTT_{(1,2)}$-DNA ligand, we found many bright cells which were showing fluorescent signals localized in the nucleus while the fluorescent signal on the membrane was 50% of the signal produced from the commercial ligand (**Fig. 4b**).

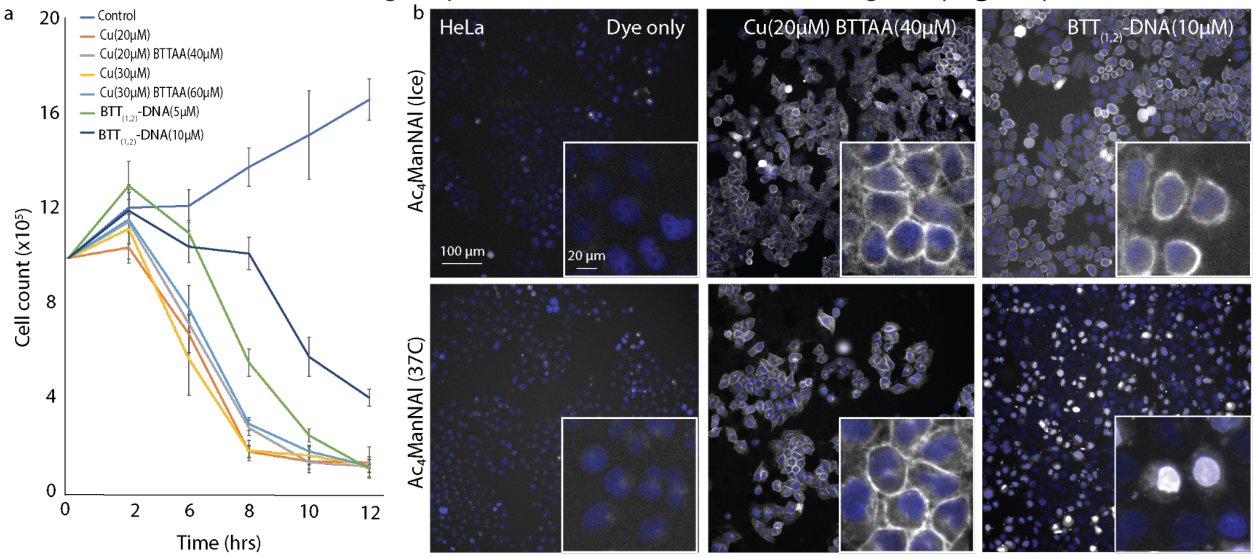

Fig.4 Biocompatibility and performance of the $BTT_{(1,2)}$-DNA ligand assisted CuAAC on the surface of living cells. a) Cell growth assay comparing $BTT_{(1,2)}$-DNA ligand to commercial ligand on Jurkat cells. Error bars represent the standard deviation of four biological replicates. b) Metabolic labeling and detection of cell-surface sialic acids with $BTT_{(1,2)}$-DNA and commercial BTTAA ligand. (white) CalFlour 647 azide labeling of sialic acids, (blue) Hoechst 33342 staining of nuclei.

Detection of EU labeled nascent RNAs in living cells with $BTT_{(1,2)}$-DNA ligand

To evaluate the efficacy of the $BTT_{(1,2)}$-DNA in driving CuAAC in the detection of intracellular biomolecules, we metabolically labeled nascent RNAs in HeLa cells using 5-EU (**Fig. 5a**). Briefly, we cultured the HeLa cells with EU for 3 hours, then added the CuAAC component directly to the live cells with a fluorogenic dye.

Using the $BTT_{(1,2)}$-DNA ligand to click the fluorogenic dye, we observed positive signal in 20% of the cell population (**Fig. 5b-c, Supplementary Fig.5**). When we lowered the $BTT_{(1,2)}$-DNA ligand concentration to 5 μM, significant difference was still observed however to a lesser extent.

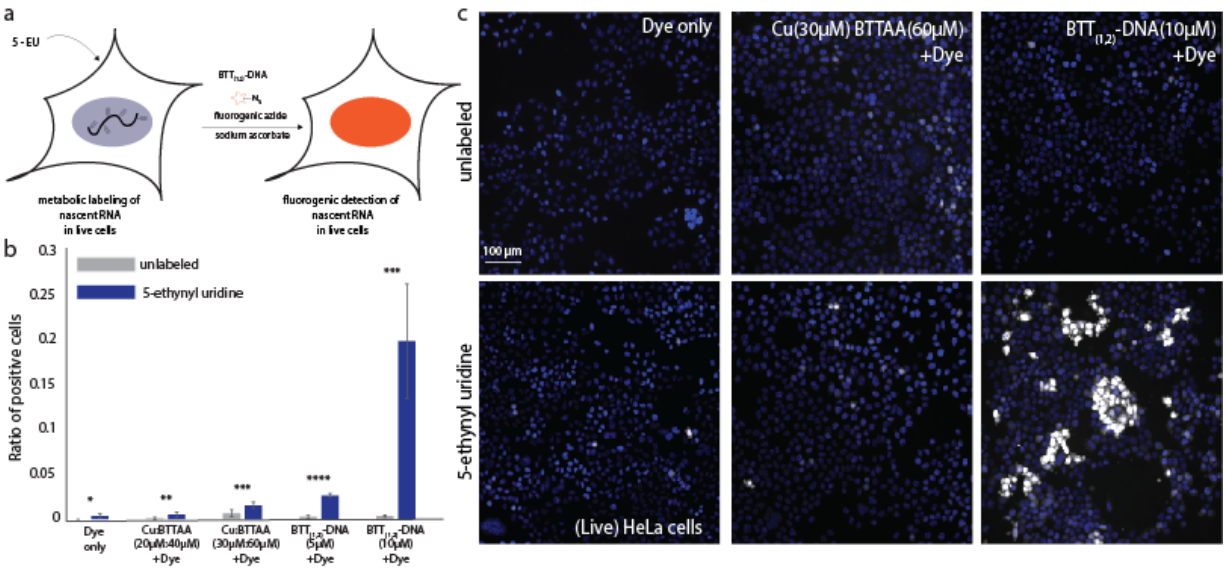

Fig.5 Intracellular labeling and detection of nascent RNAs in living cells. a) Schematic of metabolic 5-ethynyl uridine labeling of nascent RNAs and detection using a fluorogenic dye via CuAAC in the presence of $BTT_{(1,2)}$-DNA ligand in living cells. b) Analysis of the ratio of positive cells. Error bars represent the standard deviation of two biological replicates with a total six images. c) Representative fluorescence micrographs of live, 5-ethynyl uridine labeled cells subjected to CuAAC with a fluorogenic dye in the presence of $BTT_{(1,2)}$-DNA ligand and BTTAA ligand. (white) CalFlour 647 azide labeling of nascent RNAs incorporated by 5-ethynyl uridine, (blue) Hoechst 33342 staining of nuclei.

Discussion

Here, we develop a DNA conjugated CuAAC accelerating ligand, $BTT_{(1,2)}$-DNA, that reduces the required copper concentration while maintaining fast reaction kinetics. With this new CuAAC accelerating ligand, the total ligand concentration required for the CuAAC *in vitro* may be decreased to the nanomolar range while remaining complexed to an average of 7.5 copper molecules per ligand with different fluorogenic azido dyes, thus eliminating the need for additional copper in the reaction. This new ligand also enables the CuAAC reagents to traverse the cell and nuclear membrane, showing stronger intracellular labeling in fixed cells and achieving live cell detection of biomolecules in the nucleus.

Previous studies have shown DNA splint-enhanced CuAAC[26–28] by bringing together the azide and the alkyne probes; however, these systems preclude the use of fluorogenic dyes that are ideal for live-cell applications. We show that the $BTT_{(1,2)}$-DNA ligand may further drive the CuAAC reaction kinetics when hybridized to a DNA or RNA splint in close proximity to an alkyne-modified probe and clicked to a fluorogenic azide. This demonstrates that a completely non-fluorescent system may be used to drive the CuAAC reaction kinetics of a fluorogenic dye. We find that the reaction is enhanced with the addition of spermine to the mixture before CuAAC. We suspect that the spermine contributes to the stability of the DNA-DNA duplex and DNA-RNA duplex[29], which is important for the subsequent CuAAC reaction. Additionally, spermine can help mask the negative charge of oligonucleotides[30] that may be sequestering the copper away from the desired ligation site. We also show that DNA splints with multiple binding sites can amplify the total fluorescence produced.

Our experiments demonstrate that the $BTT_{(1,2)}$-DNA ligand can be used for standard CuAAC ligation assays such as 5-ethynyl uridine labeling and detection of nascent RNAs in fixed cells. We observed that the $BTT_{(1,2)}$-DNA ligand outperformed the commercial reagent[9] with a 12-fold lower ligand concentration in fixed cells. Additionally, the fluorescence produced in the nucleus was much higher for the reaction in the presence of 10 μM $BTT_{(1,2)}$-DNA ligand, indicating that the $BTT_{(1,2)}$-DNA ligand can also outperform the commercial ligand in biological environments with lower concentration and higher yield.

We demonstrate that $BTT_{(1,2)}$-DNA ligand lowers the cytotoxicity induced by copper for at least 12 hours. Interestingly, we observed that the 5 μM $BTT_{(1,2)}$-DNA ligand concentration offered the greatest protection from copper toxicity until 6 hours, after which the 10 μM $BTT_{(1,2)}$-DNA ligand concentration offered the greatest protection. This observation was made across multiple biological replicates. The prolonged protection may be because the copper molecules are not covalently bound to the ligand, and therefore will dissociate as they achieve equilibrium with the environment. The equilibrium takes longer for the $BTT_{(1,2)}$-DNA ligand at high concentration, leading to longer-term protection at the 10 μM $BTT_{(1,2)}$-DNA ligand concentration.

The $BTT_{(1,2)}$-DNA ligand displayed some unique properties when used for live cell labeling. We observed the ligation of fluorogenic dyes on the surface of live cells to metabolically labeled sialylated glycans with a 4-fold lower ligand concentration than with the commercial ligand at low temperature. Interestingly, when we increased the CuAAC reaction temperature from 0°C to 37°C, the fluorescent signal produced on the cell membrane diminishes for reaction using the $BTT_{(1,2)}$-DNA ligand, and several cells show high fluorescent signal in the nucleus, which does not appear in the commercial BTTAA ligand. Because this reaction was performed with a fluorogenic dye, this result suggested to us that 1. The $BTT_{(1,2)}$-DNA ligand was freely entering the cells, and 2. We may be visualizing the Ac_4ManNAl as it is getting metabolized into CMP-SiaNAl in the nucleus[22]. The primary reason why DNA cannot freely diffuse into cells is that the negative charge of the DNA is repelled by the negative charge on the surface of mammalian cells. It is possible that the pre-complexed copper molecules are sufficiently masking the charge of the DNA, thus allowing it to diffuse freely.

With the knowledge that our ligand can drive an intercellular CuAAC reaction, we demonstrate that the $BTT_{(1,2)}$-DNA ligand may be used for intercellular detection of EU labeled nascent RNAs in live cells. This reaction is typically performed by metabolically labeling live cells with EU, fixing and permeabilizing the cells and then detecting the EU labeled RNA using CuAAC. Our live-cell labeling and detection could enable future applications such as tracking the dynamics of global cellular transcription over time. Overall, the newly developed CuAAC accelerating ligand enables sensitive detection of biomolecules in fixed and live cells and holds great promise for further application of CuAAC for intracellular, live-cell detection.

Materials and Methods

Enzymatic production of N_3-labeled oligo probe.

A 15mer ssDNA oligo (5'-GGTGCTCTTCGTCCA-3') was obtained from IDT and reconstituted to 100 μM with nuclease-free H_2O. We mixed 250 μl of 1mM of Azide-PEG4-aminoallyl-dUTP (Jena Bioscience), 100 μl of 10X transferase buffer, 100 μl of 100μM 15mer ssDNA oligo, 30 μl of TdT enzyme (20,000U/ml), 100 μl of 10X $CoCl_2$, 420 μl of NF-H_2O in a total reaction volume of 1000 μl. The reaction was vortexed for several seconds then incubated at 37°C for 90 minutes. The reaction was stopped by increasing the temperature to 70°C for 10 minutes on a heat block. The product was purified by ethanol precipitation and the pellet was resuspended

with 50 µl NF-H_2O, resulting in a pure 3' N_3-labeled 15mer ssDNA oligo. The concentration of N_3-labeled 15mer ssDNA oligo was measured using the Qubit ssDNA Assay Kit (Fisher scientific).

Synthesis and purification of $BTT_{(1,2)}$-DNA ligand.
A 100-fold molar excess of *N,N*-bis((1-*tert*-butyl-1*H*-1,2,3- triazol-4-yl)methyl)prop-2-yn-1-amine (S1; purchased from the AECOM chemical biology core facility) was reacted with 3' N_3-labeled 15 mer ssDNA oligo using BTTAA ligand-assisted CuAAC ([BTTAA]:[$CuSO_4$]=2:1) (See **Supplementary methods).** The reaction mixture was shaken at 600 rpm for 1 hour at 37°C, resulting in a crude $BTT_{(1,2)}$-DNA ligand.The $BTT_{(1,2)}$-DNA ligand was purified using 3.5kD MWCO dialysis tubing for 24 hours in 3.5 L of water at room temperature (Repligen). Invitrogen Qubit ssDNA Assay Kit was used to measure the concentration of the purified $BTT_{(1,2)}$-DNA ligand.

Inductively Coupled Plasma Mass Spectrometry (ICP-MS).
To assess the copper that is complexed with the $BTT_{(1,2)}$-DNA ligand, we prepared the ligand for ICP-MS. The sample was dissolved (0.1 ml) in 0.9 ml of concentrated HNO_3. The sample was heated for an hour at 90°C. Then the temperature was increased to 150°C at which the sample was boiled. During the boiling period, 0.5 ml of concentrated HNO_3 was added to the sample twice. When the sample was completely dried, we added 0.5 ml of concentrated HNO_3 and diluted it with NF-H_2O to bring the final volume to 10 ml. To confirm the accuracy of our ICP-MS data, we submitted two replicates. Samples were submitted to Robertson Microlit Laboratories at Ledgewood, New Jersey for ICP-MS.

Tissue culture conditions
HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Jurkat cells were grown in RPMI 1640 medium containing 10% FBS. All cells were incubated in a 5% CO2, water saturated incubator at 37°C.

Click labeling of sialylated glycans on live cells.
Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for three days then harvested and resuspended in fresh media. 2.5 µl of Ac_4ManNAl (5mM; Click Chemistry Tools) was added to each well of the 18-well chamber (cellvis) and dried for 20 min. Cells were seeded into the Ac_4ManNAl-treated 18-well chamber and the total volume per well is 150 µl and incubated for 24h. The cells were washed 2X with PBS/1%FBS (pH 7.4) and treated with 100 µl of Invitrogen™ NucBlue™ Live ReadyProbes™ reagent for 30 minutes at 37°C to stain the nucleus of the live cells.

Cells were washed 3X with PBS/1%FBS (pH 7.4) followed by treatment with 10 µM CalFluor 647 Azide (Click Chemistry Tools), premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$]=2:1, [$CuSO_4$]= 0.5-30 µM) or $BTT_{(1,2)}$-DNA ligand ([$BTT_{(1,2)}$-DNA]= 1-10 µM) and 2.5 mM freshly prepared sodium ascorbate for 30 minutes in a 5% CO_2 incubator at 37°C. The cells were washed 3X with 200 µl of PBS/1%FBS (pH 7.4) to remove the unreacted CalFluor 647 Azide dye. Then 100 µl of PBS/1%FBS (pH 7.4) was added to each well to prepare for imaging.

Epifluorescent microscopy of cultured cells
For HeLa cells, microscopy was performed using a Nikon inverted research microscope eclipse Ti2-E/Ti2-E/B using a Plan Apo λ 20X/0.75 objective or Plan Apo λ 60X/1.40 oil objective.The Epi-fi LED illuminator linked to the microscope assured illumination and controlled respective brightness of four types of LEDs of different wavelengths. Images were acquired using the Neutral Density (ND16) filter for labeling with CalFluor 647 Azide. Images were acquired and

processed using ImageJ, and were shown as a single z-plane. Images acquired using the Neutral Density (ND16) filter are false-colored gray.

5-ethynyl uridine (EU) labeling of nascent RNAs in fixed cells
Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for three days and harvested by centrifugation (300Xg,3 min) and resuspended in fresh media and seeded into the 18-well chamber and the total volume per well is 150 µl. After one day incubation at 37°C, 1.5 µl 5-ethynyl uridine (EU) was added to each well to the 1mM final concentration and put back to the incubator for 3 hour incubation. Cells were rinsed with 1X PBS, and fixed with 4% formaldehyde for 10 minutes, then permeabilized with 70% EtOH in NF-H_2O at 4°C overnight. Cells were washed with 2X SSC/10% formamide for 30 minutes at 37°C, followed by treatment with 10 µM CalFluor 647 Azide, premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$]=2:1,[$CuSO_4$]= 0.5-30 µM) or $BTT_{(1,2)}$-DNA ligand ([$BTT_{(1,2)}$-DNA]= 1-10µM) and 2.5 mM freshly prepared sodium ascorbate for 30 minutes at 37°C. After click reaction, the cells were washed again with 150 µl wash buffer for 30 minutes at 37°C and counterstain with DAPI stain diluted with 2X SSC/10% formamide for 20 minutes at 37°C. Then 2X SSC was added to each well to prepare for imaging.

5-ethynyl uridine (EU) labeling of nascent RNAs in live cells
Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS for three days then harvested and resuspended in fresh media and seeded into the 18-well chamber and the total volume per well is 150 µl. After one day incubation at 37°C, 1.5 µl 5-ethynyl uridine (EU) was added to each well to the 1mM final concentration and put back to the incubator for 3 hour incubation.The negative control is the untreated cells. The cells were washed 2X with PBS/1%FBS (pH 7.4) and treated with 100 µl of Invitrogen™ NucBlue™ Live ReadyProbes™ reagent for 30 minutes at 37°C to stain the nucleus of the live cells. Cells were washed 3X with PBS/1%FBS (pH 7.4) followed by treatment with 10 µM CalFluor 647 Azide (Click Chemistry Tools), premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$]=2:1, [$CuSO_4$]= 10-30 µM) or $BTT_{(1,2)}$-DNA ligand ([$BTT_{(1,2)}$-DNA]= 5-10 µM) and 2.5 mM freshly prepared sodium ascorbate for 30 minutes in a 5% CO_2 incubator at 37°C. The cells were washed 3X with 200 µl of PBS/1%FBS (pH 7.4) to remove the unreacted CalFluor 647 Azide dye. Then 100 µl of PBS/1%FBS (pH 7.4) was added to each well to prepare for imaging.

Cell growth assay
Jurkat cells were grown in RPMI 1640 medium supplemented with 10% FBS. After 3 days the cells were harvested by centrifugation (300Xg,3 min) and seeded into 48-well plates at a concentration of 1 million viable cells per well. The total volume of each well is 500 L. Cells were treated with premixed BTTAA-$CuSO_4$ complex ([BTTAA]:[$CuSO_4$]=2:1,[$CuSO_4$]=20-30 µM) or $BTT_{(1,2)}$-DNA ligand ([$BTT_{(1,2)}$-DNA]= 5-10 µM) and 2.5 mM freshly prepared sodium ascorbate. We used the untreated cells as a negative control and cells reacted with 20-30 µM $CuSO_4$ and 2.5 mM sodium ascorbate in the absence of BTTAA as the positive apoptosis control. Cells were incubated at 37°C and viable cells were counted at 2,6,8,10,12 hours using the Trypan blue dye exclusion method.

Author Contributions
K.N. and S.H.R. designed the project, analyzed the data, and wrote the manuscript with input from all authors. K.N. performed all the experiments for this project. L.B helped with the synthesis of the $BTT_{(1,2)}$-DNA ligand and Y.L. helped with the 5-ethynyl uridine (EU) labeling of nascent RNAs in fixed cells using commercial ligand.

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