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An immobilized and reusable Cu(I) catalyst for metal ion-free conjugation of ligands to fully deprotected oligonucleotides through click reaction†

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Chelation of Cu(I) ions to an immobilized hydrophilic tris(triazolylmethyl)amine chelator on a solid support allowed synthesis of RNA oligonucleotide conjugates from completely deprotected alkyne-oligonucleotides. No oligonucleotide strand degradation or metal ion contamination was observed. Furthermore, use of the immobilized copper(I) ion overcame regioselectivity issues associated with strain-promoted copper-free azide–alkyne cycloaddition.

Both copper-assisted and copper-free strain-promoted click reactions have been used for bioconjugation.[1] We recently reported use of both approaches for conjugation of various ligands to oligonucleotide strands of small interfering RNA (siRNA) duplexes with the goal of improving cellular uptake of these therapeutic agents.[2,3] The drawbacks of copper-assisted azide–alkyne cycloaddition of a ligand to a therapeutic siRNA are the difficulties associated with complete removal of copper ions from the product and strand degradation during conjugation.[2,4] These limitations create chemistry, manufacturing, and control (CMC) issues in drug development. The disadvantages of the copper-free strain promoted method are that synthesis of the activated alkyne requires multiple steps and, more importantly, results in non-regioselective bicyclic triazole (both 1,4- and 1,5-) adducts.[3]

We therefore explored alternative strategies of copper-assisted regioselective 1,4-cycloaddition of an azide and a simple alkyne for oligonucleotide conjugation. Heterogeneous and immobilized copper-assisted click reactions effectively minimize heavy metal contamination of the product,[5] and the support can be reused.[5e,6] Chan *et al.* reported stabilization of Cu(I) ions by chelation to tris(benzyltriazolylmethyl)amine (TBTA); chelation protects the metal ion from oxidation and disproportionation while enhancing its catalytic activity.[7] Immobilization of TBTA onto TantaGel resin allows parallel synthesis and direct screening of cycloaddition products.[6] We rationalized that immobilizing a hydrophilic analog of TBTA, such as tris(triazolylmethyl)amine, chelated with Cu(I) ions onto a solid support would allow use of unstrained alkynes and azides in click conjugation of highly hydrophilic oligonucleotides to ligands without copper ion contamination of the product.

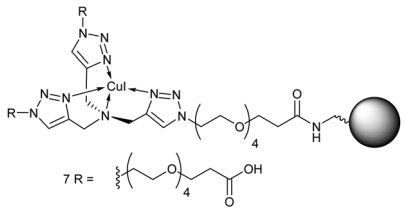

Fig. 1 Immobilized Cu(I) ion on a solid support.

Here we demonstrate the chelation of Cu(I) ions to an immobilized hydrophilic tris(triazolylmethyl)amine on a solid support (Fig. 1). The coupling of a carboxylate moiety of the hydrophilic tris(triazolylmethyl)amine to an amino-functionalized solid support followed by chelation with Cu(I) ions using tetrakis-(acetonitrile)copper(I) hexafluorophosphate $[Cu(CH_3CN)_4PF_6]$ afforded the desired support (**7**). The utility of the immobilized Cu(I) catalyst for quantitative azide–alkyne cycloaddition of completely deprotected alkyne-oligonucleotides to azides was evaluated under azide–alkyne cycloaddition conditions reported previously.[8]

The Cu (I) ion-chelated solid support **7** was synthesized from commercially available 3-[2-[2-[2-(2-azidoethoxy)ethoxy]-ethoxy]-ethoxy]-propanoic acid (**1**) as shown in Scheme 1. Azido carboxylic acid **1** was refluxed with benzyl bromide in acetone in the presence of solid potassium carbonate to afford benzyl ester **2** as colourless oil in quantitative yield. The click reaction of tripropargylamine **3** with 3 molar equivalents of the azide **2** in the presence of sodium ascorbate (10 mol%) and $CuSO_4 \cdot 5H_2O$ (1 mol%) in a 1 : 1 mixture of *tert*-BuOH and H_2O (v/v) yielded the desired tris(triazolylmethyl)amine derivative **4**.

The reaction was performed both at ambient temperature and under microwave-assisted conditions. The ambient temperature reaction was complete after overnight stirring, whereas the microwave-assisted reaction at 80 °C was complete

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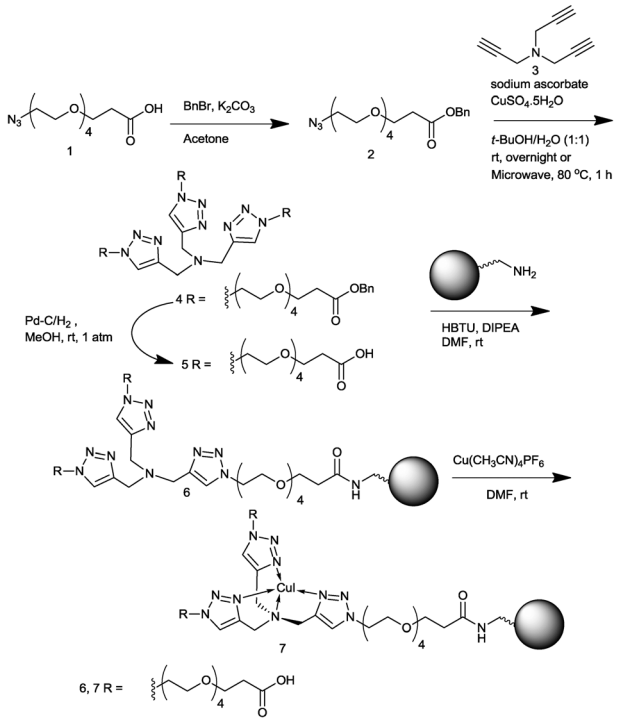
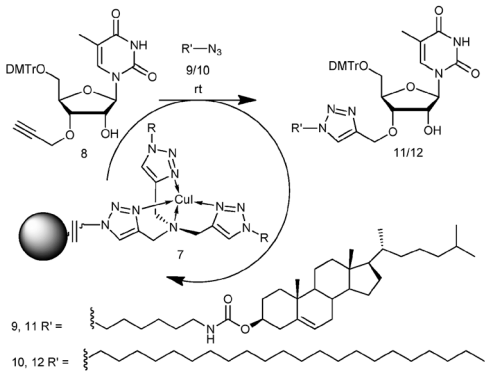


Scheme 1 Synthesis of Cu(I) ion chelated polystyrene resin **7**.



Azide	Solvent	Reaction Time	Product	Yield %
9	Acetone	16 h	**11**	99
9	DMF	6 h	**11**	98
9	DCM/DMF (1:2)	2 h	**11**	99
10	Acetone	16 h	**12**	96
10	DMF	6 h	**12**	97
10	DCM/DMF (1:2)	2 h	**12**	99

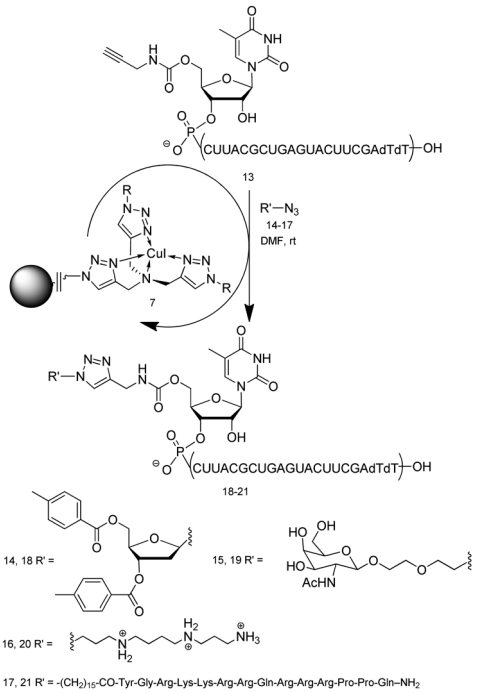
Scheme 2 Immobilized Cu(I) ion-mediated azide–alkyne cycloaddition.

after 1 h in almost quantitative yield. The product after purification was pale green in colour, presumably due to partial chelation of copper ions (from $CuSO_4 \cdot 5H_2O$) to the product. Catalytic hydrogenation of **4** over 10% Pd–C at atmospheric pressure in methanol afforded the tricarboxylic acid **5** as an oil in quantitative yield. The tricarboxylic acid **5** was activated with 0.5 molar equivalents of 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluoro-phosphate (HBTU) in the presence of diisopropylethylamine in DMF and charged with an amine functionalized polystyrene resin (amine content 230 μmol g^{-1}). After gentle agitation at ambient temperature overnight, the chelator-functionalized resin **6** was suspended in DMF and mixed with $Cu(CH_3CN)_4PF_6$ followed by gentle shaking to obtain Cu(I)-chelated resin **7**. The Cu(I) ion content of the resin was estimated by inductively coupled plasma optical emission spectrometry (ICP-OES); the observed loading of the metal ion was between 160 and 220 μmol g^{-1} with variation from batch to batch. Coupling of the chelator to the resin was roughly equimolar with amine present on the support. Loading of compound **5** under similar conditions to long-chain amino alkyl controlled pore glass (lcaa CPG) afforded the chelator-immobilized CPG. The Cu(I) ion content of this resin was proportionate to the amine content (loading capacity) of the resin used and the number of molar equivalents of compound **5** added.

We first evaluated the heterogeneous copper-assisted azide–alkyne 1,4-cycloaddition (CuAAC) reaction in different solvent systems including DCM, DMF, DCM : DMF (1 : 2), THF, toluene, and acetone using 5′-*O*-[bis(4-methoxyphenyl)phenylmethyl]-5-methyl-3′-*O*-2-propyn-1-yl-uridine **8** and alkyl azides[2] **9** and **10** as reactants (Scheme 2). Of the solvents evaluated, acetone, DCM : DMF (1 : 2), and DMF were optimal based on reaction time and yield at ambient temperature. The mixture of DCM and DMF was selected as it effectively solubilizes hydrophobic azides like **9** and **10**. Use of 10 mol% of the immobilized Cu(I) ion was sufficient to drive the reaction to completion. With a 1 : 1 molar ratio of the azide to alkyne the desired product was obtained in excellent purity as determined by TLC and NMR. No detritylation of the nucleoside was observed with the heterogeneous immobilized CuAAC reaction, whereas dimethoxytrityl (DMTr) deprotection is an issue with the conventional CuAAC reaction due to the acidic nature of the reagents employed.

Recyclability of the catalyst was investigated using the alkyne **8** and azides **9** and **10**. The immobilized catalyst **7** was filtered, washed, and reused repeatedly to determine the catalytic efficiency of the chelated Cu(I) ion. The performance of the immobilized catalyst remained the same after five cycles, and the recyclability of the catalyst was solvent independent. There was no detectable leaching of the copper ion from the chelator to the reaction media under any reaction conditions tested. We also evaluated the stability of the catalyst at ambient temperature and confirmed similar activity over a period of several months.

We next evaluated the usefulness of the approach for azide cycloaddition to fully deprotected oligonucleotides containing an alkyne.[2] Due to the highly anionic nature of fully deprotected oligonucleotides, copper ion contamination in the product and RNA strand degradation are limitations of the copper-assisted click conjugation approach with fully deprotected oligonucleotides.[2,4] The chelated copper complex **7** was used to catalyse click reactions between a fully deprotected alkyne-modified oligonucleotide **13** and azido functionalized ligands **14**,[9] **15**,[2] and **16**[2] as shown in Scheme 3. DMF was chosen as the solvent to overcome solubility limitations of azides and unprotected oligonucleotides. As observed in the reactions of small molecules, 10 mol% of the immobilized catalyst **7** was sufficient to drive the reaction to completion overnight at ambient temperature. The catalyst was recovered by filtration and the integrity of the conjugates (**18–20**) was confirmed by LC-MS



Scheme 3 Chelated Cu(I)-assisted azide–alkyne cycloaddition of fully deprotected alkyne oligonucleotides to azides.

analysis. Importantly, LC-MS did not show detectable levels of copper ion in the product or strand cleavage. In our earlier work using the conventional click reaction, strand cleavage was observed and copper ion was detected in the product even after stringent purification.[2]

We then investigated conjugation of oligonucleotide **13** to a lipopeptide. The azido lipopeptide **17** is based on the HIV Tat 48–60 cell permeation peptide (CPP)[10] and was synthesized under standard Fmoc solid-phase peptide synthesis conditions. After coupling of the last amino acid to the solid support, 16-azidohexadecanoic acid[11] was coupled to the *N*-terminus to obtain the desired peptide. The lipophilic alkyl chain functioned as a tether and is considered as an additional pharmacokinetic modulator of the siRNA.[12] The reaction between the alkyne-oligonucleotide **13** and the azido peptide **17** was quantitative with 2 molar equivalents of the azide to the alkyne, and there was no copper ion contamination in the product as confirmed by LC-MS analysis. The catalyst retained activity after repeated use. There was no distinguishable difference in the catalytic activity of the immobilized Cu(I) ion on polystyrene compared to the CPG solid support (data not shown).

In summary, we have demonstrated efficient chelation of Cu(I) ions to derivatized hydrophilic tris(triazolylmethyl)amine on a solid support. The immobilized reagent effectively catalysed the CuAAC reaction under heterogeneous conditions and resulted in quantitative and regioselective conjugation of an azido ligand to a completely deprotected alkyne oligonucleotide with no detectable levels of copper ions in the product or strand degradation. Use of the immobilized and chelated Cu(I) ion overcame regioselectivity issues associated with strain-promoted copper-free azide–alkyne cycloaddition and the metal ion contamination observed with the conventional CuAAC reaction of fully deprotected alkyne-oligonucleotides with azides. Moreover, the recyclability of the catalyst makes this an environmentally friendly and economical strategy[5a,5e,6] for ligand conjugation to oligonucleotides. To expand the scope of this reagent, we are evaluating the utility of this catalyst in synthesizing other click chemistry products including targeted nanoparticles for delivery of siRNAs.

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