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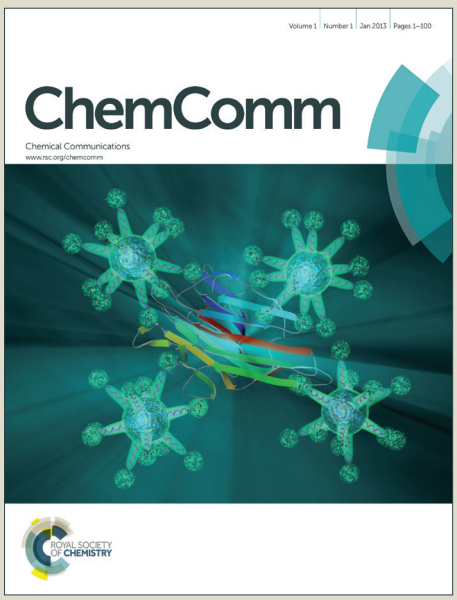

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Highly-Efficient and Versatile Fluorous-Tagged Cu(I)-Catalyzed Azide-Alkyne Cycloaddition Ligand for Preparing Bioconjugates

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A novel ligand (FBTTBE) for Cu(I)-Catalyzed Azide-Alkyne Cycloaddition (CuAAC) has been developed, which demonstrates not only superior catalytic efficiency but also the ease of removing toxic copper species. FBTTBE has also been successfully applied in the synthesis of radiometal-labeled peptide and antibody without observable transchelation with the non-radioactive Cu(I) catalyst.

Click chemistry, particularly the copper(I)-catalyzed azide alkyne cycloaddition (CuAAC)[1], has found applications in a wide range of modern chemistry-related areas, including organic chemistry, drug discovery, drug delivery and chemical biology.[2-6] However, the toxicity of copper(I) from the generation of reactive nitrogen and oxygen species[7] limits its application in living systems.[8,9] For example, upon treatment of 1 mM $CuSO_4$, 1.5 mM sodium ascorbate, and 0.1 mM TBTA (**Figure 1**), Zebrafish embryos do not survive beyond 15 min.[10] Therefore, the removal of copper species is typically required in order to avoid cytotoxicity caused by residual copper ions in biological applications, adding another layer of complexity to the application of CuAAC in living systems. To overcome the cumbersome copper removal problem, major efforts have been made to minimize the risk caused by this metal catalyst. New methodologies and techniques have been developed, including copper-free variants of azide-alkyne click chemistry (e.g., strain-promoted azide–alkyne cycloaddition (SPPAC) and resin-supported catalyst systems).[11-14] However, these strategies cannot fulfill all the requirements due to their inherent deficiencies, including relatively sluggish kinetics in SPAAC and copper leaching problems observed in the resin-supported catalyst systems.[15] Therefore, a more efficient approach is highly desired.

Here we report the development of a novel fluorous tagged tris(triazolylmethyl)amine-based Cu(I) stabilizing ligand (FBTTBE; **Figure 1**). This ligand has great promise towards facilitating the removal of toxic catalytic species while maintaining high catalytic efficiency. The use of a fluorous tag enables the easy separation of the toxic catalyst from the product (non-fluorous species) via the Fluorous Solid-Phase Extraction (F-SPE) approach[16], whereby the separation is accomplished by simply passing the reaction mixture through fluorous silica gel. The bis(tert-butyltriazolyl) methyl amine based catalytic core shows significantly improved kinetics compared with two commercially available Cu(I) ligands, TBTA and THPTA (**Figure 1**).[17] This new design of the catalytic ligand integrates homogenous solution phase reaction conditions with a phase-tag separation, while maintaining high reactivity as well as strong capacity to fully complex the copper ions. It is believed that the synergy of the fluorous-tag and the catalytic core in the designed FBTTBE ligand will result in much broader applications of CuAAC. The linker between the fluorous tag and catalytic core provides the necessary distance to reduce possible steric effects, and in the future it can be replaced by a PEGylated linker to counter the loss of hydrophilic groups (i.e. the hydroxyl in THPTA) for improved aqueous solubility.

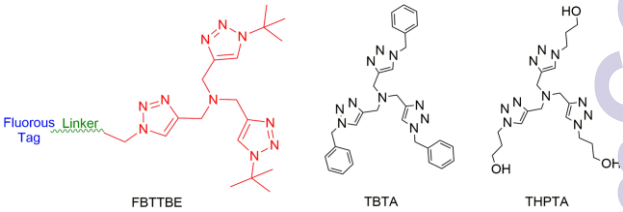

Figure 1. FBTTB, TBTA and THPTA.

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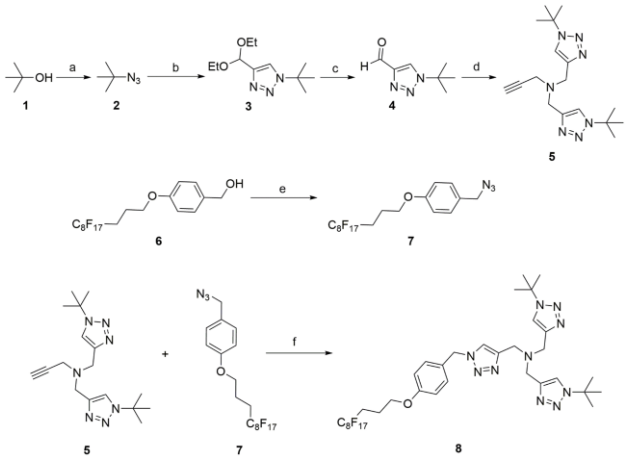
In our study, a model FBTTBE ligand was synthesized *via* multiple steps (**Scheme 1**). Alcohol **1** was treated with sodium azide to generate azide **2**. Subsequently, **2** was reacted with 3,3-diethoxy-1-propyne through a copper catalyzed click reaction to give the corresponding triazole **3**, which was then converted to the. triazolylcarbaldehyde **4** *via* TFA (trifluoroacetic acid) treatment. Facilitated by the reduction reagent $NaBH(OAc)_3$, intermediate **5** was then prepared through the reaction between **4** and propargyl amine.[18] Intermediate **7** was synthesized by treating the alcohol **6** first with thionyl chloride, followed by azidation using sodium azide. In the final step, the FBTTBE ligand **8** was obtained through the click reaction between **5** and **7**.

As discussed above, the fluorous-tag containing FBTTBE ligand features a rapid F-SPE removal capability. Utilizing radioactive $^{64}Cu^{2+}$, the trapping efficiency of the fluorous silica gel was determined. In this experiment, $^{64}Cu^{2+}$ (100 µCi) was added to a non-radioactive Cu^{2+} solution, and the resulting carrier-added $^{64}Cu^{2+}$ (200 µM) was then mixed with 1.5 equiv. of FBTTBE followed by 1.0 eq. of NaAA; the mixture was passed through the fluorous silica gel after a 5 min incubation. Over 99% of the radioactivity remained on silica gel, demonstrating that FBTTBE-Cu(I) can be efficiently trapped. Therefore, it is anticipated that the removal of toxic copper species after CuAAC can be greatly simplified to a one-step filtration using FBTTBE as the catalytic ligand.

In order to investigate its catalytic efficiency, the reactivity of the synthesized FBTTBE ligand was then compared with two widely used ligands TBTA and THPTA. Specifically, we compared the relative reactivity of the canonical Cu(I) catalysts in the form of TBTA–Cu(I), THPTA–Cu(I) and FBTTBE–Cu(I) *via* a reported fluorogenic assay[17] based on the reaction between propargyl amine and 3-azido-7-hydroxycoumarin (**Scheme S1**).



Scheme 1. Synthesis of the FBTTBE ligand. Reagents and conditions: (a) NaN_3, H_2SO_4: H_2O = 1:1 (w/w); (b) 3,3-diethoxy-1-propyne, $NaHCO_3$, $CuSO_4$, sodium ascorbate (NaAA), t-BuOH : H_2O = 1:1 (v/v); (c) TFA, DCM : H_2O = 2:1 (v/v); (d) propargyl amine, $NaBH(OAc)_3$, Dichloroethane; (e) 1). $SOCl_2$, DMF, 2). NaN_3, DMF: THF = 1:1 (v/v); (f) $CuSO_4$, NaAA, t-BuOH : H_2O = 1:1 (v/v).

Upon formation of the triazole ring, strong fluorescence at 47 nm can be quantitatively measured to determine the extent of the reaction. FBTTBE showed the greatest ability to accelerate CuAAC, followed by THPTA, with TBTA having the lowest reactivity (**Figure 2**). The reaction catalyzed by Cu(I)-FBTTBE

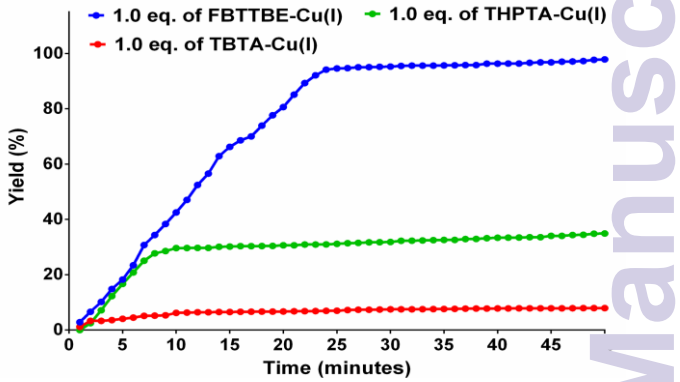


Figure 2. Comparison of Cu(I) stabilizing ligands in the CuA... between propargyl amine and 3-azido-7-hydroxycoumarin ([azidocoumarin] = 10 µM; [propargyl amine] = 15 µM, ligand : Cu(I) = 1.5 : 1).

completed in around 20 min at ambient temperature using 1... µM of Cu(I) and 1.5 eq. (15 µM) of FBTTBE. In contrast, n... more than 40% yield was achieved after 12 h with the TBT... and THPTA ligands. This implies that FBTTBE renders a much higher catalytic capacity in CuAAC compared with TBTA and THPTA. The catalytic efficiency of the non-fluorous tagged analogue BTTBE was found to be higher than that of FBTTBE (**Figure S1**), indicating the fluorous tag will affect the reactivity of the catalytic core. This decreasing of catalytic efficiency could be reversed by adding excess amount of FBTTBE which could be conveniently removed by the fluorous silica gel after the reaction.

The superior catalytic efficiency as well as the ease of separating toxic copper species when using FBTTBE drove us to further investigate its application in preparing agents that could be used in living systems, such as a fluorescent dye attached peptide for fluorescent microscopy staining. Specifically, CuAAC between Cy3-azide and acetylene-AE105 (a urokinase-type plasminogen activator receptor, uPAR-targeted peptidic ligand) catalyzed by various Cu(I)-ligands was compared (**Figure S2**). The Cu(I)-FBTTBE catalyzed CuAAC was complete in 5 min, whereas neither Cu(I)-TBTA nor Cu(I)-THPTA could achieve over 50% yield, even after incubating for 8 h. More importantly, both the Cu(I)-FBTTBE catalyst and excess FBTTBE ligand were rapidly removed by F-SPE. The resulting Cy3-AE105 conjugate was then used to stain human U87MG glioblastoma cells that overexpress uPAR (**Figure 3**).

In addition to the rapid and complete removal of toxic copper species and excellent catalytic efficiency, the FBTTBE catalyst also demonstrated the capability of minimizing transchelation between non-radioactive copper and radiometals. In 2006, Marik and Sutcliffe reported the first use of this method in preparing [^{18}F]fluoropeptides,[19] and since then a large body of work employing the CuAAC has been

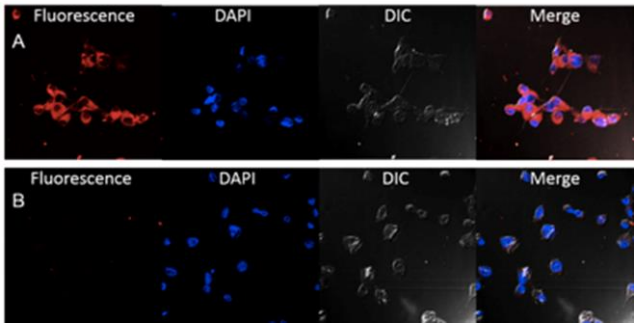


Figure 3. Microscopy staining of U87MG cells with A) Cy3-AE105; and B) Cy3-AE105 with blocking (be more specific).

presented in the preparation of ^{18}F-based PET radiotracers.[20] However, its application in radiometal-based probes still remains noticeably underdeveloped, possibly attributed to the transchelation between non-radioactive copper ions and those radiometals.[21-25] Even with an extremely low concentration of radiometal-containing reactants (e.g., 10-100 nM) and rapid decay of these radionuclides, a relatively large amount of the catalyst is required to ensure that the pmol-reactant reaction can complete within a relatively short time. This leads to not only difficulty in catalyst removal but also significant radiometal transchelation, resulting in significant decreases in specific activity of the corresponding radiopharmaceuticals.[17]

Here, a click reaction used for preparing the ^{64}Cu-labeled peptide was employed to evaluate the performance of FBTTBE in addressing both removal of toxic copper and the radiometal transchelation problems. Specifically, azide modified CB-TE1K1P chelator (N_3-CB-TE1K1P)[26] was incubated with ^{64}Cu for 5 min at 90 °C, and the resulting N_3-(^{64}Cu)CB-TE1K1P chelate was then conjugated to acetylene-AE105 peptide via CuAAC catalyzed by different ligands (**Scheme S2**). FBTTBE exhibited much higher catalytic efficacy than either TBTA or THPTA (**Figure S3**). The rapid F-SPE removal capability was then investigated by comparing the UV peaks in the two HPLC traces of the above CuAAC mixtures before and after F-SPE purification. The retention time of the FBTTBE/FBTTBE-Cu(I) appeared to be 22.3 min according to the HPLC trace of the CuAAC mixture before F-SPE purification (**Figure 4A**). After the F-SPE purification, it was found that the UV peak representing the FBTTBE/FBTTBE-Cu(I) completely disappeared (**Figure 4B**), indicating that the catalyst was completely removed by the fluorous silica gel. In addition, the reaction mixture was also analyzed by the radio-HPLC to monitor potential transchelation. It was found that the radio-purity of the final product (retention time = 20 min, **Figure 4A**) was >98%, while there was no radioactive peak at either 1.5 or 22.3 min for Cu ions and FBTTBE-Cu(I), respectively. Therefore, FBTTBE can be regarded as an excellent ligand for the preparation of radiometal-based radiopharmaceuticals. The resulting (^{64}Cu)CB-TE1K1P-AE105 was subsequently used for PET/CT imaging of nude mice bearing subcutaneous U87MG xenografts that overexpress uPAR **(Figure 5)**.

The FBTTBE ligand has also been successfully applied in the rapid radiometal labeling of antibodies. Proteins are typically

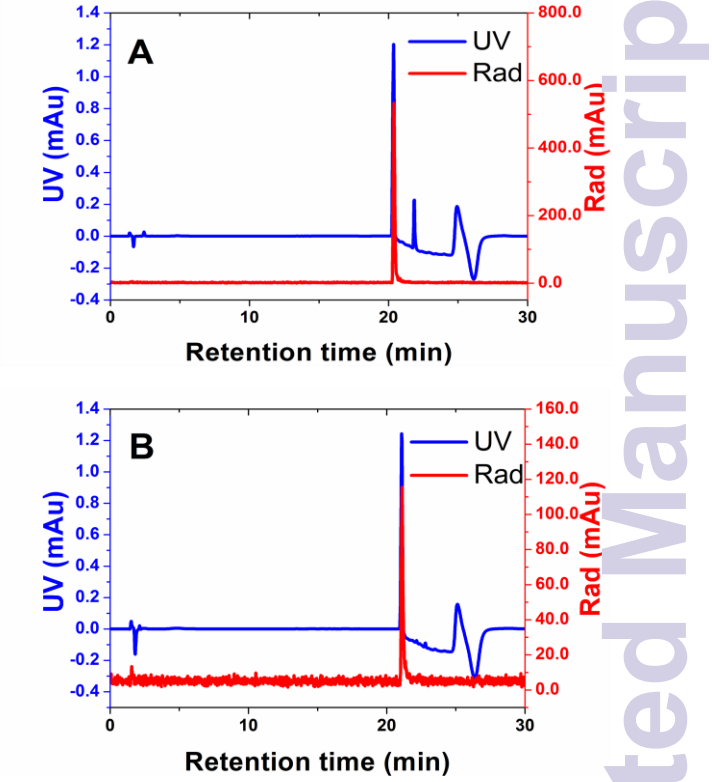


Figure 4. HPLC results: A) N_3-CB-TE1K1P(^{64}Cu) click reaction with acetylene-AE105 catalyzed by 1.0 eq. of FBTTBE; B) Spectrum after the fluorous-silica gel purification of the reaction mixture.

more sensitive to radiolabeling conditions such as pH, temperature and incubation time than small molecules and peptides. As a proof-of concept study, a monoclonal anti-EGFR antibody (cetuximab) was functionalized with an acetylene group using a slightly modified procedure from what was previously reported.[27] The resulting acetylene-cetuximab was conjugated with N_3-(^{64}Cu)CB-TE1K1P *via* the Cu(I)-FBTTBE catalyzed CuAAC under mild conditions (37 °C for 30 min) that would not denature the protein. The results were encouraging in that 50% of the (^{64}Cu)CB-TE1K1P was attached to cetuximab. The specific activity of the resulting ^{64}Cu-labeled cetuximab was 10 µCi/µg after purification by a Zeba desalting column. Together, these data demonstrated the broad applicability of the Cu(I)-FBTTBE catalyst for radiometal labeling of peptides and antibodies.

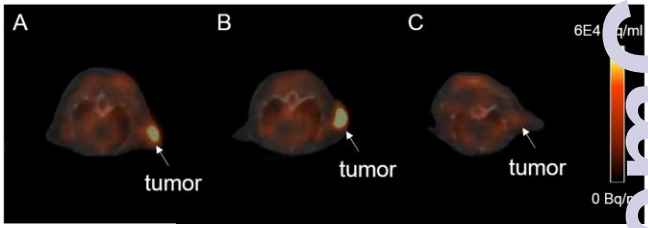


Figure 5. PET/CT imaging of mice bearing U87MG xenografts: A) 1 h p.i; B) 4 h p.i.; c) with blockade, 4 h p.i.

In summary, a novel CuAAC ligand FBTTBE has been successfully developed that not only demonstrated higher catalytic efficiency than two commercial available ligands (TBTA & THPTA), but also simplified the removal of toxic copper species after reactions, rendering it an ideal ligand for CuAAC. In addition, transchelation was avoided when applying FBTTBE in preparing radiometal-based pharmaceuticals, broadening the application of CuAAC beyond ^{18}F-chemistry. Although reactions presented here are primarily geared toward PET and molecular imaging, FBTTBE can be applied in almost any CuAAC where the high reaction rate as well as the complete removal of copper species are desired.

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