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Copper catalysis in living systems and *in situ* drug synthesis

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Abstract: The copper catalyzed azide–alkyne cycloaddition (CuAAC) reaction has proven to be a pivotal advance in chemical ligation strategies with applications ranging from polymer fabrication to bioconjugation. However, application *in vivo* has been limited by the inherent toxicity of the copper catalyst. Here we report the application of heterogeneous copper catalysts in azide–alkyne cycloaddition chemistries in biological systems ranging from cells to zebrafish, with chemistries spanning from fluorophore activation to the first reported *in situ* generation of a triazole-containing anticancer agent from two benign components, opening up many new avenues of exploration of CuAAC chemistry.

Bioorthogonal reactions have emerged as a powerful tool in allowing the study of biological processes in their native environment.^[1–4] A key feature of these reactions is that they must have high selectivity and efficiency, and function within the complex biological environment of a cell, making the development of new bioorthogonal reactions a formidable challenge.^[5] The prototypical “click” reaction is the highly efficient and selective copper catalyzed azide–alkyne 1,3-cycloaddition (CuAAC) reaction that generates a triazole,^[6–8] a scaffold that has been found in antiproliferative, antimicrobial, and anticonvulsant agents.^[9,10] The wide applicability of the CuAAC reaction stems from the robustness of the azide and alkyne functional groups, which remain inert in complex biological systems. In the presence of Cu(I), the reaction proceeds with high rates and efficiency even in challenging biological environments, resulting in the regiospecific 1,4-triazole product, which is also highly robust and inert. A drawback of the CuAAC reaction is the use of Cu(I) ions, which within biological systems leads to the production of reactive oxygen species (such as hydroxyl radicals), the induction of DNA strand breaks, and thiol coordination chemistry, limiting its use *in vivo* where prolonged exposure to the Cu(I) catalyst is required.^[11,12] Cu(I) is prone to oxidation and disproportionation, therefore, current catalyst formulations utilize a Cu(II) salt with the addition of sodium ascorbate as a reductant. In addition, Cu(I)-stabilizing

ligands, such as triazol-based tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA)^[13] and tris(3-hydroxypropyltriazolylmethyl)amine (THPTA),^[14] are added to sequester free Cu(I) ions and to enhance reaction kinetics. Stabilizing ligands with increased water solubility allow for lower concentrations of Cu(II) salts and have been used to label *N*-azidoacetylglactosamines on the surface of Jurkat cells and in the enveloping layer of zebrafish embryos, with reduced cytotoxicity and fast reaction kinetics.^[15–17] Ting^[18] and Taran^[19] used copper-chelating azides in combination with water-soluble ligands to increase the efficiency of the CuAAC reaction, also enabling the use of Cu(I) concentrations in the micromolar range in cell based assays. Recently, a non-toxic metal-organic nanoparticle catalyst was reported, which was efficient in intracellular CuAAC chemistry.^[20] To overcome the toxicity of copper ions, Bertozzi pioneered the development of Cu-free strain-promoted azide–alkyne cycloadditions (SPAAC);^[21,22] however, strained alkynes can react with intracellular nucleophiles.^[23] Although transition metals are traditionally considered detrimental to living systems, biocompatible transition metal chemistry has recently had success in bioorthogonal activation strategies.^[24–28] Chen demonstrated the palladium-mediated cleavage of a propargyloxycarbonyl-caged catalytic lysine in OspF, a bacterial phosphotyrase, switching-on protein function in host cells.^[29,30] Meggers has reported the activation of a prodrug of Doxorubicin *via* in-cell ruthenium catalysis,^[31] while Mascareñas demonstrated the localization of the Ru catalyst to mitochondria and subsequent catalytic activity inside the subcellular compartment.^[32] Palladium-mediated chemistry has been demonstrated by protecting group cleavage and Suzuki-Miyaura cross-coupling for the activation of 5-Fluorouracil or Amsacrine.^[33–35]

Thus, although CuAAC chemistry has been successfully used for many bioconjugations, the *in situ* generation of active compounds by coupling two inert partners, such as prodrugs, remains elusive. Herein, we report the development and application of non-toxic, biocompatible and implantable heterogeneous copper catalysts that were functional both *in vitro* and *in vivo* and were able to catalyze *in situ* the synthesis of an anticancer agent from two component halves.

Heterogeneous, robust, and highly biocompatible entrapped copper nanoparticle catalysts (E-Cu-NP) were synthesized in three steps starting from amino functionalized TentaGel resin (110 μm) by incubation with a solution of Cu(OAc)₂, followed by reduction and entrapment (Supporting Fig. S1). Transmission electron microscopy (TEM) images of cross sections of the resin beads showed the generation of nanoparticles (51.2 $\pm$ 4.2 nm) distributed throughout the bead (160.4 $\pm$ 10.3 μm in water, Supporting Fig. S1), with a Cu content of 0.37 $\pm$ 0.02 μmol Cu/mg resin as determined by ICP-OES (n = 3). Leaching levels were less than 1% after a 72 h treatment in H₂O at 37 $^{\circ}\text{C}$, showing the robustness of the E-Cu-NP catalyst. The catalytic activity of the E-Cu-NPs was

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Supporting information for this article is given via a link at the end of the document.

Figure 1. E-Cu-NP mediated cycloaddition of 3-azido coumarin **1** and TPP-alkyne **2**: (a) Reaction scheme of the CuAAC reaction. (b) Flow cytometry histograms of SKOV-3 cells incubated with **1** (20 μM), **2** (20 μM) and NaAsc (50 μM) for 18 h at 37 $^{\circ}\text{C}$ with the E-Cu-NPs (0.1 μmol Cu) in black and untreated cells in grey ($\lambda_{\text{Ex/Em}}$ 355/450 nm). (c) SKOV-3 cells incubated with **1** and **2** and E-Cu-NPs (top panels) and SKOV-3 cells incubated with **1** and **2** (bottom panels) and stained with MitoTrackerTM Red (mitochondrial stain) and imaged by fluorescence microscopy. Panels show from left to right: synthesized compound **3** (blue), mitochondria (red), and merged image (pink indicates colocalization of **3** to mitochondria).

investigated utilizing the coumarin 'profluorophore' 3-azido-7-hydroxy coumarin **1** ($\lambda_{\text{Ex/Em}}$ 346/455 nm), which exhibits massively decreased fluorescence compared to the unsubstituted coumarin scaffold, due to the quenching effect of the electron-rich α -nitrogen of the azido group.^[36] 3-Azido coumarin **1** was reacted with alkyne **2** and the E-Cu-NPs (Fig. 1a) under a variety of physiological conditions (phosphate buffered saline (PBS), 5 % fetal bovine serum (FBS), and HeLa (cervical adenocarcinoma) cell lysate) with full conversion of **1** into triazole **3** ($\lambda_{\text{Ex/Em}}$ 343/477 nm, Supporting Fig. S2) after 3 h, with no indication of byproducts and a 22-fold increase in fluorescence (Supporting Fig. S3). These results were comparable to the conventional CuSO_4 system with the chelating ligand THPTA and reducing agent sodium ascorbate (NaAsc), which gave full conversion to **3** in 10 min in PBS and reduced conversion in the presence of biomolecules in cell lysate and FBS (Supporting Fig. S4).

The profluorophore "activation" was then investigated in a cell-based assay. SKOV-3 (ovarian adenocarcinoma) cells were

incubated with **1** and **2** and the E-Cu-NPs, which are located in the extracellular space (Supporting Fig. S6), and analyzed by flow cytometry and live cell fluorescence microscopy. The cells showed an 8-fold increase in fluorescence of the entire cell population compared to untreated cells (Fig. 1b), with the fluorescence within the SKOV-3 cells colocalized with the mitochondria stain (MitoTrackerTM Red) (the triphenylphosphonium (TPP) moiety is a well-established mitochondria targeting group^[37]), confirming the synthesis of **3** and localization of **3** into the mitochondria (Fig. 1c). These results were mirrored with HeLa cells (Supporting Fig. S5). The E-Cu-NPs were non-cytotoxic with full cell viability observed under these reaction conditions. The conventional homogeneous catalyst system (CuSO_4 /THPTA), however, showed significant toxicity after 24 h and 48 h, indicating the great advantage of the E-Cu-NPs for biologically compatible and *in vivo* applications (Supporting Fig. S4).

Figure 2. *In situ* synthesis of the cytotoxic agent **6**: (a) The E-Cu-NP mediated cycloaddition of **4** and **5**. (b) Cell viability of SKOV-3 cells treated with compounds **4**, **5**, and **6** for 72 h at 0 μM to 10 μM (MTT assay, $n = 3$). (c) SKOV-3 cells incubated with **4** (10 μM), **5** (10 μM) and NaAsc (50 μM) in the presence of the E-Cu-NPs (0.1 μmol Cu) and cell viability measured after 72 h (MTT assay, $n = 3$). *** $P < 0.001$ by one-way ANOVA with Dunnett post-test, compared to the untreated control group. (d) Flow cytometry analysis of cell proliferation with histograms showing cells in the G0/G1, S, and G2/M phases (Click-iT™ EdU assay): left panel shows untreated control cells; right panel shows cells treated with **4**, **5** and NaAsc in the presence of E-Cu-NPs showing an increase in cells in the G0/G1 phase. (e) Flow cytometry analysis of apoptotic effect of *in situ* synthesis of **6** (Annexin-V/FITC apoptosis assay): Cytograms showing Annexin-V/FITC and PI intensity of untreated cell population (left) and cells treated with **4**, **5** and NaAsc in the presence of E-Cu-NPs showing an increase in apoptotic cells.

Combretastatin A-4 (CA-4) is a tubulin polymerization inhibitor and highly cytotoxic against a variety of cancer cell lines,^[38–40] with the triazole analogue **6** known to inhibit K562 leukemia cancer cell growth with an IC_{50} of 1.3 μM.^[41,42] Thus, the triazole **6** was synthesized from the azide **4** and alkyne **5** in an E-Cu-NP catalyzed cycloaddition (Fig. 2a) under physiological conditions (PBS, 37 °C) with 64 ± 4% conversion to the triazole **6** after 3 h ($n = 3$), as monitored by HPLC (Supporting Fig. S7). Remarkably, this catalyst activity was retained in cell lysate and in 5% FBS (with conversions of 64 ± 5 % and 52 ± 2 %, respectively) (Supporting Fig. S6), indicating the compatibility of the reaction and E-Cu-NPs in the presence of a complex mixture of biomolecules.

The E-Cu-NP catalyzed cycloaddition was investigated in a cell-based assay, allowing the *in situ*, extracellular, synthesis of the cytotoxic agent. The cytotoxicity of precursors **4** and **5**, as well as the triazole product **6** was evaluated on SKOV-3 and HeLa cells with neither **4** and **5** showing any cytotoxicity up to 10 μM. Triazole **6** exhibited cytotoxicity against both SKOV-3 and HeLa cells, with <30% cell viability at 10 μM (Fig. 2b, Supporting Fig. S8). When cells were incubated with **4** and **5** in the presence of the E-Cu-NPs (at 37 °C for 72 h), a 70 % decrease in cell viability was observed (compared to the controls). These results were mirrored in HeLa cells (Supporting Fig. S8). To show that the *in situ* formation of **6** affected cell cycle progression, cell growth was analyzed (*via* a Click-iT™ EdU assay). Only cells incubated with **4** and **5** in the presence of E-Cu-NPs showed significant arrest in cell cycle progression at G0/G1 phase (59 ± 11 % vs 24 ± 3 % in untreated cells, Fig. 2d, Supporting Fig. S10), with the *in situ* formation of **6** inducing apoptosis in SKOV-3 cells, as analyzed by double staining with Annexin-V/FITC and propidium iodide (PI) (43 ± 9 % vs 13 ± 3 % in untreated cells, Fig. 2e, Supporting Fig. S9). Cell lysis, followed by HPLC and HR-MS analysis of the cell contents, identified **6** (as well as precursors **4** and **5**) inside the cells (Supporting Fig. S11).

The biocompatibility of the E-Cu-NPs and application as an implantable device was evaluated by introducing a single bead of the catalyst into the yolk sac of zebrafish embryos 24 hours post fertilization (hpf). The zebrafish with the implanted catalyst showed no signs of toxicity or alteration of phenotype and they developed normally into their larval stage, as monitored by phase contrast microscopy (Fig. 3a and b). To evaluate the catalytic activity of the E-Cu-NPs *in vivo*, the “activation” of **1** with TPP-alkyne **2** was carried out (Fig. 3c and d). A clear fluorescent signal was observed in embryos that had been implanted with the catalyst, with localized increase in fluorescence (indicated by white arrow) and fluorescence distributed throughout the yolk sac, with a 5.7 and 3.2-fold increase, respectively, over background fluorescence of embryos implanted with a non-functionalized bead (Fig. 3d and Supporting Fig. S12).

In conclusion, we have shown the facile synthesis of Cu nanoparticles embedded within a polymeric support and demonstrated their functionality as biocompatible catalysts. These were used to activate a coumarin-profluorophore, and to drive the cycloaddition reaction of two components of a drug to successfully allow the *in situ* synthesis of a cytotoxic anticancer agent that induced apoptosis. The biocompatibility and scope of the E-Cu-NP catalyst was shown by implantation in the widely used and respected zebrafish model, where not only was no toxicity observed, but where an active fluorophore was generated by the covalent conjugation of two “halves” placed within the milieu. The E-Cu-NP functionalized resin beads thus function as an implantable device and could be placed at the desired site of action *in vivo*. This concept opens up a number of research avenues of *in situ* synthesis of bioactive compounds from two fragments by the introduction of bio-inert E-Cu-NPs that function within a cellular and *in vivo* environment.

Figure 3. Biocompatibility studies of the E-Cu-NPs in zebrafish embryos: (a) Brightfield and (b) fluorescence images of zebrafish embryos after insertion of a single resin bead of the E-Cu-NP catalyst, (i) 24 hpf, (ii) 3 days post fertilization (dpf) and (iii) 4 dpf. (c) Zebrafish (2 dpf) with non-functionalized bead and (d) Zebrafish (2 dpf) with E-Cu-NP bead incubated with compound **1** (5 μM) and **2** (5 μM) in the water for 24 h and fluorescence intensity displayed as heat map images (see Supporting Fig. S12 for fluorescence quantification).

Experimental Section

Experimental details are provided in the supplementary material.

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Keywords: Heterogeneous catalysis • Copper • Bioorthogonal chemistry • Prodrugs

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**Copper catalysis in living
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