Bioorthogonal Chemistry Hot Paper

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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. Herein, we report the application of heterogeneous copper catalysts in azide–alkyne cycloaddition processes in biological systems ranging from cells to zebrafish, with reactions 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 for CuAAC chemistry.*

Bioorthogonal reactions have emerged as a powerful approach 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. Furthermore, Cu^{I}-stabilizing ligands, such as triazole-based tris[(1-benzyl-1*H*-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*-azidoacetylgalactosamines on the surface of Jurkat cells and in the enveloping layer of zebrafish embryos, with reduced cytotoxicity and fast reaction kinetics.[15–17] The groups of 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 that was efficient in intracellular CuAAC chemistry.[20] To overcome the toxicity of copper ions, Bertozzi and co-workers pioneered the development of copper-free strain-promoted azide–alkyne cycloaddition (SPAAC) reactions;[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 and co-workers demonstrated the palladium-mediated cleavage of a propargyloxycarbonyl-caged catalytic lysine in OspF, a bacterial phospholyase, switching on protein function in host cells.[29,30] Meggers and co-workers have reported the activation of a prodrug of doxorubicin through in-cell ruthenium catalysis,[31] whereas Mascareñas et al. 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-couplings for the activation of 5-fluorouracil and amsacrine.[33–35]

Thus, although CuAAC chemistry has been successfully used for many bioconjugation processes, 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-NPs) were synthesized in three steps starting from amino-functionalized TentaGel resin by incubation with a solution of $Cu(OAc)_2$,

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followed by reduction and entrapment (see the Supporting Information, Figure S1). Transmission electron microscopy (TEM) images of cross-sections of the resin beads showed the generation of nanoparticles (51.2 ± 4.2 nm) distributed throughout the bead (160.4 ± 10.3 μm in water; Figure S1), with a Cu content of 0.37 ± 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 H_2O at 37 °C, showing the robustness of the E-Cu-NP catalyst. The catalytic activity of the E-Cu-NPs was investigated utilizing the coumarin "pro-fluorophore" 3-azido-7-hydroxycoumarin **1** ($\lambda_{ex/em} = 346/455$ nm), which exhibits strongly quenched fluorescence compared to the unsubstituted coumarin scaffold owing to the quenching effect exerted by the electron-rich α-nitrogen atom of the azido group.[36] 3-Azidocoumarin **1** was reacted with alkyne **2** and the E-Cu-NPs (Figure 1 A) under a variety of physiological conditions, namely phosphate buffered saline (PBS), 5% fetal bovine serum (FBS), and HeLa (cervical adenocarcinoma) cell lysate, with full conversion of **1** into triazole **3** ($\lambda_{ex/em} = 343/477$ nm; Figure S2) after 3 h, with no indication of side product formation and a 22-fold increase in fluorescence in the PBS experiment (Figure S3). These results were comparable to those achieved with the conventional $CuSO_4$ system with the chelating ligand THPTA and the reducing agent sodium ascorbate (NaAsc), which gave full conversion into **3** in 10 min in PBS and reduced conversion in the presence of biomolecules in cell lysate and FBS (Figure 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 (Figure S6), and analyzed by flow cytometry and live-cell fluorescence microscopy. The cells showed an eightfold increase in fluorescence of the entire cell population compared to untreated cells (Figure 1 B), with the fluorescence within the SKOV-3 cells colocalized with the mitochondria stain (MitoTracker™ Red; the triphenylphosphonium (TPP) moiety is a well-established mitochondria-targeting group),[37] confirming the synthesis of **3** and localization of **3** in the mitochondria (Figure 1 C). These results were mirrored with HeLa cells (Figure 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 biolog-

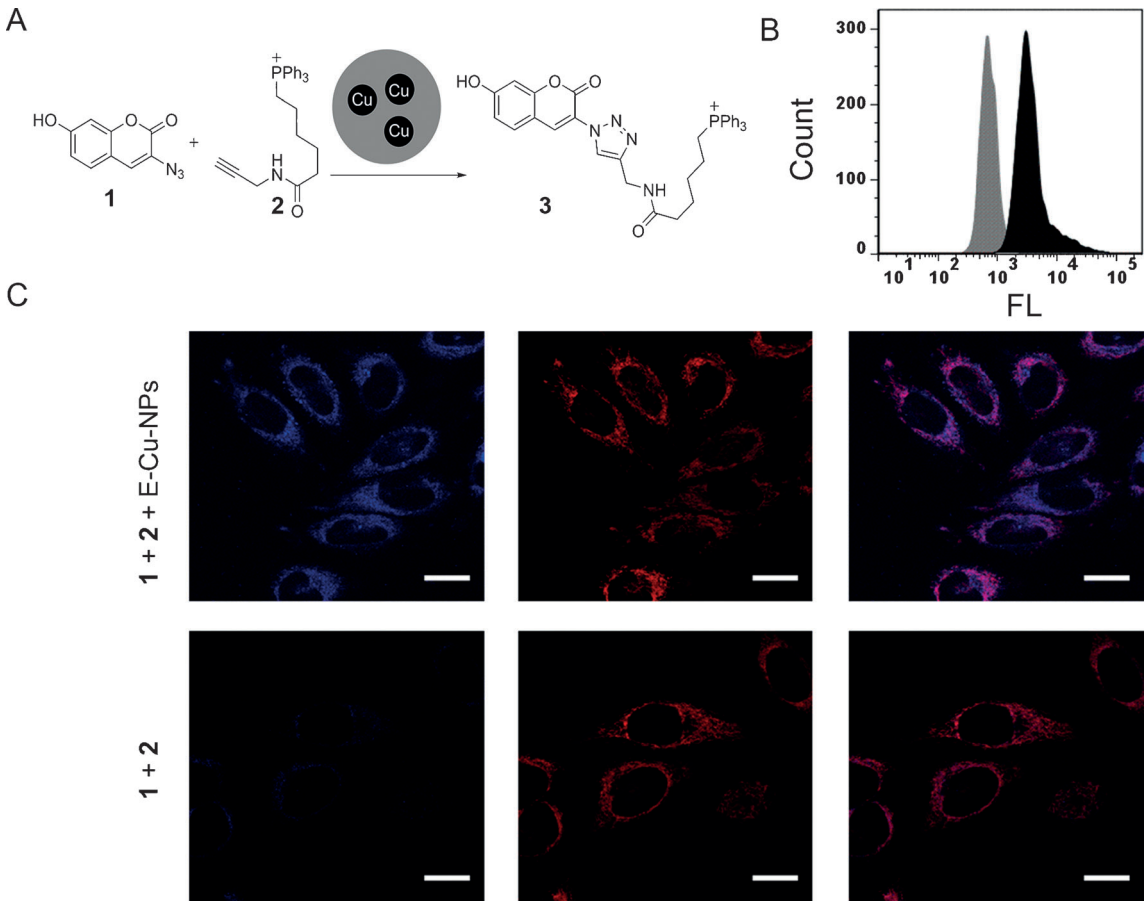

Figure 1. E-Cu-NP mediated cycloaddition of 3-azidocoumarin **1** and TPP alkyne **2**. A) The CuAAC reaction. B) Flow cytometry histograms of SKOV-3 cells incubated with **1** (20 μM), **2** (20 μM), and NaAsc (50 μM) for 18 h at 37 °C with the E-Cu-NPs (0.1 μmol Cu) in black and untreated cells in gray ($\lambda_{ex/em} = 355/450$ 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 MitoTracker™ 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** and the mitochondria).

ically compatible, and possibly in vivo, applications (Figure S4).

Combretastatin A4 (CA4) 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} value of 1.3 μM.[41,42] Thus triazole **6** was synthesized from azide **4** and alkyne **5** in an E-Cu-NP catalyzed cycloaddition (Figure 2A) under physiological conditions (PBS, 37 °C) with 64 ± 4% conversion into triazole **6** after 3 h ($n = 3$), as monitored by HPLC analysis (Figure S7). Remarkably, this catalyst activity was retained in cell lysate and in 5% FBS (with conversions of 64 ± 5% and 52 ± 2%, respectively; Figure S7).

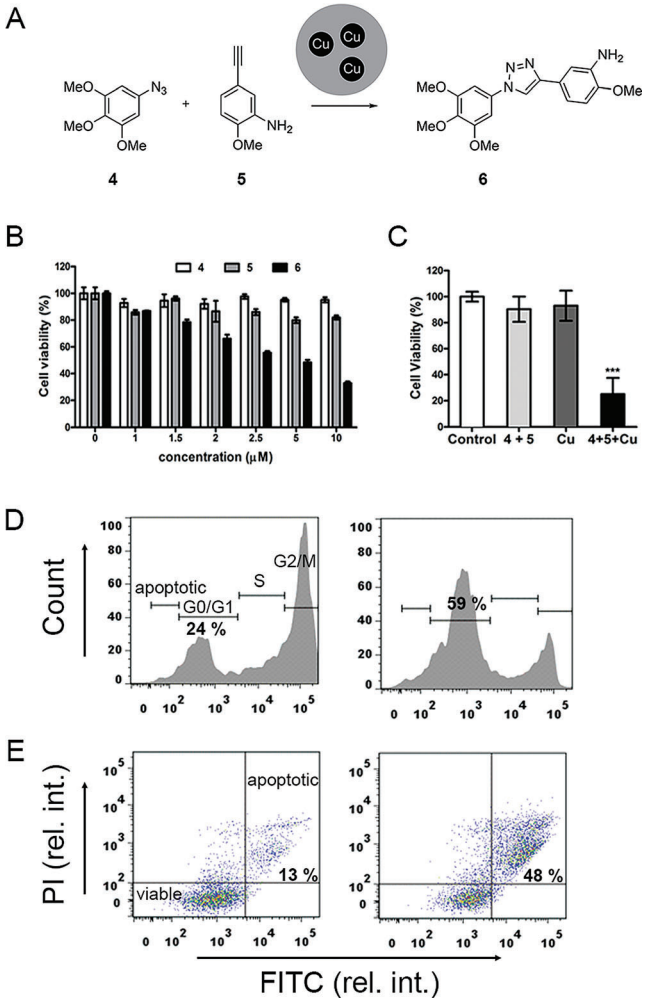

Figure 2. In situ synthesis of the cytotoxic agent **6**. A) The E-Cu-NP mediated cycloaddition of **4** and **5**. B) Cell viability of SKOV-3 cells treated with compounds **4**, **5**, and **6** (purified material) for 72 h at 0–10 μM (MTT assay, $n = 3$). C) SKOV-3 cells incubated with **4** (10 μM), **5** (10 μM), and NaAsc (50 μM) in the presence of the E-Cu-NPs (0.1 μmol Cu); 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). The left panel shows untreated control cells, and the 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 the apoptotic effects of the in situ synthesis of **6** (Annexin-V/FITC apoptosis assay): Cytograms showing the Annexin-V/FITC and PI intensity of untreated cell populations (left) and cells treated with **4**, **5**, and NaAsc in the presence of E-Cu-NPs, showing an increase in apoptotic cells for the treated cells.

The E-Cu-NPs 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 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 (Figure 2B and Figure 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; Figure 2C). These results were mirrored in HeLa cells (Figure S8). To show that the in situ formation of **6** affected cell cycle progression, cell growth was analyzed (in 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 the G0/G1 phase (59 ± 11% vs. 24 ± 3% in untreated cells; Figure 2D and Figure 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; Figure 2E and Figure 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 (Figure S11).

The biocompatibility of the E-Cu-NPs, and their 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 catalyst implanted into zebrafish showed no signs of toxicity, no phenotype alteration was observed, and the embryos developed normally into their larval stage, as monitored by phase contrast microscopy (Figure 3A,B). To evaluate the catalytic activity of the E-Cu-NPs in vivo, the "activation" of **1** with TPP alkyne **2** was carried out (Figure 3C,D). A clear fluorescence signal was observed in embryos that had been implanted with the catalyst, with a 5.7-fold localized increase in fluorescence (indicated by the white arrow) and a 3.2-fold increase in fluorescence distributed throughout the yolk sac, compared to the background fluorescence of embryos implanted with a non-functionalized bead (Figure 3d and Figure S12).

In conclusion, we have described the facile synthesis of copper nanoparticles embedded within a polymeric support and demonstrated their functionality as biocompatible catalysts. They were used to activate a coumarin profluorophore and to drive the cycloaddition reaction of two components of a drug to successfully enable the in situ synthesis of a cytotoxic anticancer agent that induces apoptosis. The biocompatibility and scope of the E-Cu-NP catalyst were shown by in vivo 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 the 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

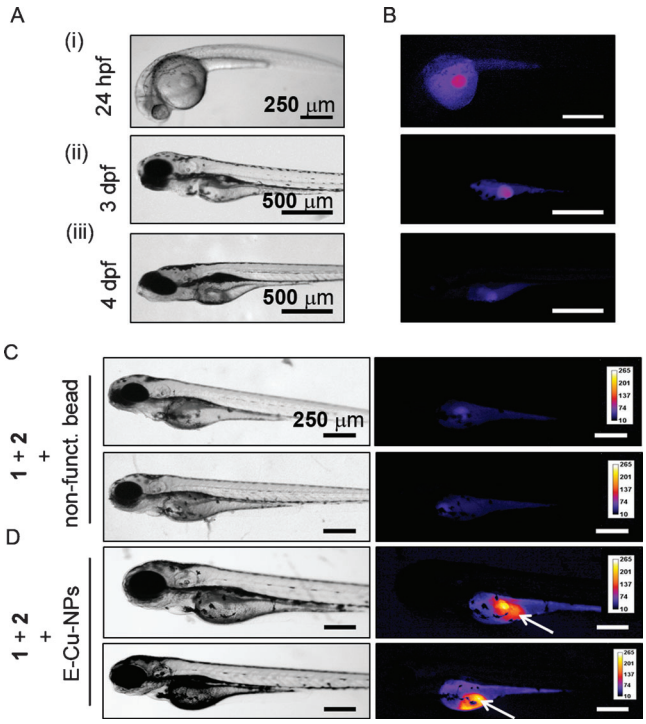

Figure 3. Biocompatibility studies with the E-Cu-NPs in zebrafish embryos. A) Bright-field and B) fluorescence images of zebrafish embryos after insertion of a single resin bead of the E-Cu-NP catalyst i) 24 hpf, ii) 3 days post fertilization (dpf), and iii) 4 dpf. C) Zebrafish (2 dpf) with a non-functionalized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

(the site of the disease known from CT or MRI scans, facilitating implantation), allowing the generation of an active drug from inactive prodrugs as a means to eliminate toxic side effects resulting from non-localized treatment with cytotoxic drugs. This concept opens up a number of research avenues for the in situ synthesis of bioactive compounds from two fragments by the introduction of bio-inert E-Cu-NPs that function within cellular and in vivo environments.

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Keywords: bioorthogonal chemistry · copper · heterogeneous catalysis · prodrugs

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Bioorthogonal Chemistry

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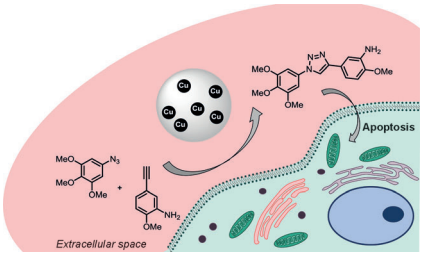
Copper Catalysis in Living Systems and In Situ Drug Synthesis



Localized drug synthesis: Biocompatible copper nanoparticle catalysts were synthesized and employed in the activation of a profluorophore in living cells and in zebrafish embryos. Furthermore, an anticancer drug was synthesized in situ from two benign components, leading to apoptosis in ovarian cancer cells.

