Discrete Cu(i) complexes for azide–alkyne annulations of small molecules inside mammalian cells†

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The archetype reaction of "click" chemistry, namely, the copper-promoted azide–alkyne cycloaddition (CuAAC), has found an impressive number of applications in biological chemistry. However, methods for promoting intermolecular annulations of exogenous, small azides and alkynes in the complex interior of mammalian cells, are essentially unknown. Herein we demonstrate that isolated, well-defined copper(I)–tris(triazolyl) complexes featuring designed ligands can readily enter mammalian cells and promote intracellular CuAAC annulations of small, freely diffusible molecules. In addition to simplifying protocols and avoiding the addition of "non-innocent" reductants, the use of these premade copper complexes leads to more efficient processes than with the alternative, in situ made copper species prepared from Cu(II) sources, tris(triazole) ligands and sodium ascorbate. Under the reaction conditions, the well-defined copper complexes exhibit very good cell penetration properties, and do not present significant toxicities.

Introduction

Organometallic catalysis has changed the field of organic synthesis in the last half century, and has found important applications in other areas such as materials, energy or environmental sciences. In spite of such wide impact, the use of transition metal catalysis in biological contexts remains underdeveloped, probably due to the general belief that metal-promoted reactions are incompatible with the air atmospheres and aqueous environments of biological habitats, and that the metal complexes can be highly cytotoxic.†

Only recently, a few examples demonstrating the viability of achieving transition metal promoted transformations in biological contexts, and even in intracellular environments, have been disclosed. Most of these reports deal with palladium or ruthenium-catalyzed uncaging of designed substrates equipped with inactivating handles. More challenging intracellular metal-promoted coupling reactions involving two different abiotic precursors are much scarcer. This is not surprising, as these reactions require the cell entrance and "meeting" of up to three different partners, namely the metal complex and two exogenous reactants (Fig. 1a). Thus, while several groups have demonstrated the viability of achieving Suzuki or Sonogashira couplings on appropriately modified proteins in E. coli, the only two examples described so far in mammalian cells involve the use of palladium nanoparticles, and fixed cells.‡

Fig. 1 (a) Outline of a metal-promoted bimolecular coupling of exogenous molecules in living cells; (b) CuAAC reaction between anthracenyl azide 1 and propargyl alcohol 2.

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Curiously, the well-known copper(i) catalyzed azide–alkyne cycloaddition (CuAAC) reaction,7 has been very scarcely explored inside the complex environment of living mammalian cells. Thus, while early work on the use of this reaction for biological purposes was restricted to bacteria,8 most of the other “in vivo” applications have been limited to the modification of cell surface labelled glycans.9 Probably, the established notion that copper is highly cytotoxic, and the requirement of excess of several additives, including “non-innocent” ascorbate, have precluded further research to implement the reaction in the challenging and crowded atmosphere of mammalian cells.9,10

In recent years, several groups have developed water soluble ligands for Cu(i) that accelerate the reaction and also act as sacrificial scavengers/reductants of the reactive oxygen species (ROS) generated by copper, decreasing its cellular toxicity.11 The accelerating effect can be further improved if the copper chelated ligand is covalently linked to the azide, a tactic that has even been used for the intracellular labelling of proteins. However, with this strategy, the copper complex is likely sequestered by the products, owing to the presence of the triazole moiety.12

Water soluble copper ligands linked to a cell penetrating peptide have been recently used to promote click reactions inside cells, but with low efficiency, and only for alkyne-modified proteins.10b If the modification of intracellular proteins is a highly relevant, and far from trivial, goal, achieving intracellular copper promoted reactions between two “freely diffusing small molecules” is even more challenging. Accessing to this type of reactivity can open new, exciting opportunities for biological or metabolic intervention, and for a metal-dependent generation of active drugs or optical signals. To the best of our knowledge, the only example of such type of intracellular CuAAC reaction relies on the use of cross-linked copper containing polymers termed metalorganic nanoparticles (MONPs), and requires high concentration of sodium ascorbate.13 Another class of copper nanostructures that can also promote the reaction in water has been recently reported, however their activity is confined to the extracellular milieu.14

Herein we report the first examples of an intracellular CuAAC transformation involving two exogenous, freely spreading substrates (small molecule azide and alkyne), promoted by discrete Cu(i) complexes (Fig. 1b). We also present data on the compared reactivity, redox stability, cell uptake and toxicity of in situ made copper species versus Cu(i) predefined complexes. These studies allow for the discovery of an independently isolated, well-defined Cu(i) complex equipped with the BTTE ligand (3-4-){bis[1-(1,1-dimethylethyl)-1H-1,2,3-triazol-4-y]methyl}amino)methyl]-1H-1,2,3-triazole-1-ethanol, L3), which performs much better than the in situ mixture obtained from the ligand, a Cu(ii) source and sodium ascorbate.

**Results and discussion**

Our work was conceived on the hypothesis that designed, well-defined Cu(i) complexes might cross cell membranes and keep their oxidation +1 state under the reductive atmosphere of the cell. Thus we proposed to study the intracellular reactivity of tris-triazolyl–Cu(i) complexes generated in situ by reduction of Cu(ii) precursors with ascorbate, as well as of isolated, well-defined Cu(i) complexes (Chart 1).15 As substrates we chose fluorogenic azides that undergo an increase in fluorescent emission upon annulation with the corresponding alkynes.16 The most habitual azide substrate for these purposes is 3-azido-7-hydroxycoumarin, however, in our hands, preliminary control tests with HeLa cells indicated that this azide presents a substantial background signal. We therefore moved to the 9-(azidomethyl)anthracene (1, Fig. 1b) which is almost non-fluorescent, but undergoes a ca. 150-fold increase in fluorescence upon its annulation with alkynes (Fig. S2†). This increase can be explained in terms of suppression of the internal PET (photoinduced electron transfer) quenching on moving from the azide to the triazole structure.16,37

As tris(triazolyl)methylamine ligands we selected BTAA (3-4-){bis[1-(1,1-dimethylethyl)-1H-1,2,3-triazol-4-yl]methyl}amino)methyl]-1H-1,2,3-triazole-acetic acid, L1), which has been shown to be rather effective in CuAAC in aqueous media, and even in E. coli.16 We also prepared the analogues BBTE (L2) and BTE (L3), which feature a hydroxyl group susceptible of conjugation to different units. Indeed, we synthesized the derivative L4 which contains a triphenylphosphonio moiety designed to favor cellular internalizations and, eventually, mitochondrial localizations (Chart 1a). As predefined Cu(i) catalysts, we initially aimed to explore several previously characterized species such as pyrazolyl, NHC (N-heterocyclic carbene) phosphe or phosphinite copper complexes (C1–C4, Chart 1b). It is surprising that the catalytic activity of this type of well-defined Cu(i) complexes had never been explored in bio-relevant settings.

Before moving to cellular environments, we investigated the performance of the above complexes in aqueous media. With ligands L1–L4, the catalytic reactions were carried out using 75 mol% of copper, by mixing CuSO4 with 2 equiv. of the ligand in water (with 2% DMSO) at room temperature for 10 min, and adding the solution to either water or PBS (phosphate buffered saline)
solution) mixtures of anthracenyl azide 1 (100 μM) and propargyl alcohol 2 (200 μM), followed by sodium ascorbate (NaAsc, over 30 equiv.).

For comparison purposes, we analyzed the conversion after 10 and 20 min, by using calibration curves (see Fig. 2 and Section S4 in the ESI†).

In the absence of ligands, i.e., when only CuSO₄ and sodium ascorbate are employed, the reaction proceeds with poor yields (<10% even after 24 h, Fig. 2a, dark blue bars). However, with ligand L₃ the product was obtained in 32% yield in water and 22% in PBS, after 10 min, while L₂ was less effective. Notably, using the phosphonium containing ligand L₄ we observed 50% of the triazole after 10 min, in both water and PBS (phosphate-buffered saline, Fig. 2a, purple bars), and a very good 70% yield after 20 min. As expected, if we skip the pre-treatment of the Cu(n) complexes with sodium ascorbate, there is no reaction. UV-Vis and ¹H-NMR analysis confirmed that mixing CuSO₄ and the ligand and sodium ascorbate generates a tris(triazole) Cu(i) species (Fig. S9 and S10†).

The performance of the predefined, isolated Cu(i) complexes C₁–C₄ (Chart 1b) was also assessed in the absence or presence of ascorbate, at 37 °C (20 min, Fig. 2b). The carbene complex C₃ is almost inactive, and the phosphite and phosphinite complexes C₂ and C₄ also led to very poor conversions (less than 2% of the product). With the complex [Cu(NCMe)(Tpa⁺)] [PF₆] (C₁) the reaction was slightly more efficient (13% in water and 5% in PBS). Importantly, addition of sodium ascorbate allowed much better conversions, especially, with C₂ and C₃. These results suggest that under the reaction conditions (open air flask), the Cu(i) species are readily oxidized, something that was further confirmed by EPR. Therefore, while C₁ and C₂ are stable in solid state, in DMSO they are very rapidly oxidized under air to give paramagnetic Cu(n) species (Fig. S11 and S12†).

Overall, the best conversions were achieved with the in situ made copper complexes in presence of ligand L₄. Indeed, using this ligand it was possible to obtain the product in a satisfactory 46% yield, after 20 min, using just 25 μM of the copper source (Fig. 2c).†

With the above information in hand, we moved to living mammalian cells using two different cell lines: HeLa and A549 (living human cervical cancer cells and adenocarcinomic human alveolar basal epithelial cells, respectively). In the experiments with sodium ascorbate, the copper containing mixture added to the cells was prepared by mixing CuSO₄ and the ligand (L) in a 1 : 2 ratio in water for 1 h, followed by treatment with an aqueous solution of sodium ascorbate (6 equiv.) for 30 min.† With the defined, discrete Cu(i) species C₁–C₄, cells were directly incubated with a freshly made DMSO solution of the complexes. The experiments were carried out by mixing cultured cells with the copper solutions (75 μM for in situ made complexes and 50 μM for discrete Cu(i) species) for 30 min in fresh DMEM (Dulbecco's modified Eagle's medium), followed by two washing steps with DMEM prior to the addition of the reactants. The resulting cells were incubated with the azide 1 (100 μM) and the alkyne 2 (200 μM) in fresh DMEM for 60 min and washed twice with DMEM, before observation under the fluorescence microscope. It is important to note that we do not use cell fixation techniques, which allows for the preservation of the native living environment, and avoids artefacts or over-interpretations.

In the experiments with in situ made copper species, in absence of ligands or with L₂, we did not detect any intracellular fluorescence, while with L₁ and L₃ the fluorescent intensity was weak (Fig. S18†). However, we were glad to observe that when using L₄ as ligand, there was a clear blue intracellular fluorescence across the cytoplasm and in vesicles, with the cells showing an unaltered morphology (Fig. 3, panel C, D and E). Control experiments in absence of the copper species (Fig. 3, panel A and B), using the same threshold observation parameters, confirm that the signal must necessarily come from the expected reaction.²⁰,²¹

Remarkably, despite their low in vitro activity, the predefined Cu(i) complex C₁, the phosphite complex C₂ and the phosphinite complex C₄ were able to raise some intracellular fluorescence in experiments carried out in the absence of ascorbate, while C₃ failed to elicit any fluorescence (Fig. S19†).

Using MTT cytotoxicity assays we observed that more than 90% of the cells survived after 2 h of treatment with the standard Cu(n)/L₄/ascorbate mixture, using 75 μM of the copper

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**Fig. 2** (a) Yields of the CuAAC with in situ preformed copper complexes. Reaction conditions: CuSO₄ (75 μM) was mixed with 2 equiv. of the ligands L₁–L₄ in H₂O and the mixture was added to another solution containing anthracenyl azide 1 (100 μM) and propargyl alcohol 2 (200 μM) in either H₂O or PBS. Then, NaAsc was added (2.5 mM) and the reaction was maintained at 25 °C; (b) yields of the CuAAC with the preformed copper complexes C₁–C₄ (75 μM), using the above concentrations of reactants, with/without sodium ascorbate (2.5 mM), at 37 °C, 20 min; (c) conversion profiles of the CuAAC with different Cu(i) complexes in PBS, in reactions carried out with 25 μM of the copper species. The reaction yields were calculated using a fluorescence calibration curve that was obtained with increasing concentrations of the triazole 3, from 0 to 100 μM.
source (80% survival after 12 h). With C1 the cell survival was slightly lower, reaching values of approx. 80% after 2 h (Fig. S2f).

The reactivity observed with the tris(pyrazolyl) copper species C1, prompted us to pursue the specific preparation of a well-defined Cu(i) complex equipped with a tris(triazole) ligand. Thus, we focused on the isolation of a complex similar to C1 but containing the ligand L3 or L4. While with L4 we have not yet been successful, we could isolate a Cu(i) complex (C5) by mixing [Cu(NCMe)₃][PF₆] with equimolar amounts of L3 in methanol, and subsequent precipitation (Section S2†).

EPR monitoring of fresh DMSO solutions of this complex (C5) demonstrated a higher redox stability than C1. Therefore, while in the case of C1, 80% of Cu(i) is oxidized to Cu(II) after 20 min, under the same conditions, less than 30% of C5 was oxidized (Fig. S12 and S13†). The in vitro performance of complex C5 was quite similar to that of tris(pyrazolylmethane)-containing complex C1, however we were pleased to observe that this complex presents an excellent performance in native cellular settings, in the absence of sodium ascorbate (Fig. 4a, panel C and F); much better than that observed when the cells are incubated with the standard pre-made mixture containing Cu(i)/L3 and ascorbate (Fig. 4a, panel B and E).

To better appreciate the differences in efficiency, we have established a protocol to calculate reaction yields of the intracellular transformations, based on fluorescence measurements using a microplate reader (see Section S13 in the ESI†). The data were normalized with respect to the amount of anthracenyl azide (1, limiting reactant) uptaken by cells. Gratifyingly, when complex C5 was used, the product was obtained in approx. 18% yield, which is over 7 times greater than that obtained using the in situ prepared complex with ligand L3.

The intracellular reactivity was also analyzed by flow cytometry, which confirmed that cells treated with C5 presented higher levels of fluorescence when compared with that resulting from the in situ made L3/copper complex. Indeed, C5 performed the best among all the copper species so far studied (Fig. 4b). The use of an extensive washing protocol to remove extracellular copper should assure that the reactions are taking place inside the cells. However, we further confirmed this by observing a total lack of reactivity in control experiments using extracellular media. Furthermore, we also observed that adding copper chelators like EDTA to the extracellular solution, in experiments carried out with living cells, has no effect on the results (see Fig. S20†).

Interestingly, there is a clear correlation between the copper uptake and the observed activity. Therefore, the phosphonium containing ligand L4 promoted a relatively high intracellular accumulation of copper. The ICP-MS analysis also indicates that the well-defined Cu(i) complexes C1 and C2 are very well internalized, which explains why we do observe some intracellular reactivity despite their poor in vitro activity. More important, the copper complex C5 is also very well internalized, leading to almost three times more internal copper than that from the corresponding in situ made copper complex with the ligand L3.

**Fig. 3** Fluorescence micrographies in experiments carried out in HeLa cells using the in situ made copper(i) complexes: 75 μM CuSO₄, 2 equiv. L4, 6 equiv. NaAsc. (A, B) Cells incubated with azide 1 (100 μM) and alkyne 2 (200 μM) for 1 h, followed by double washing with DMEM (2 × 5 min). (C, D) Cells after incubation with the copper containing mixture (30 min), DMEM washings (2 × 5 min), and treatment with 1 (100 μM) and 2 (200 μM) for 1 h, followed by double washing with DMEM (2 × 5 min). (E) Zoom of panel D. Basal levels of fluorescence were normalized by LUT equalization. Scale bar, 12.5 mm. (A and C and brightfield).

**Fig. 4** (a) Fluorescence micrographies resulting from the CuAAC reactions with the in situ made Cu(i)/L3 species and with C5, using HeLa cells. (A, D) Cells incubated only with azide 1 (100 μM) and alkyne 2 (200 μM) for 1 h, followed by double washing with DMEM (2 × 5 min). (B, E) Cells after incubation with in situ made copper species with L3, using standard ascorbate reducing conditions (75 μM, 30 min incubation), DMEM washings (2 × 5 min), and treatment with 1 (100 μM) and 2 (200 μM) for 1 h, followed by double washing with DMEM (2 × 5 min). (C, F) Results using complex C5. A 10 mM solution of C5 in DMSO was freshly prepared in an open flask, and used immediately with no further precautions. Cells after incubation with C5 (50 μM, 30 min incubation), DMEM washings (2 × 5 min), and treatment with 1 (100 μM) and 2 (200 μM) for 1 h, followed by double washing with DMEM (2 × 5 min). Basal levels of fluorescence were normalized by LUT equalization. Scale bar, 12.5 mm; (b) flow cytometry analysis for the quantification of fluorescent cells after the reactions promoted by copper complexes. The results with L1–L3 refer to the copper-promoted reactions using these ligands and NaAsc (standard conditions): (A, B and C are brightfield).
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featuring designed ligands can readily enter mammalian cells
possible to tune the cell uptake and reactivity of Cu(I)
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usible molecules.

There are no conflicts to declare.

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18 It is important to note that in DMEM or cell lysates, the CuAAC reactivity is seriously compromised, probably because of the presence of a high proportion of thiol.

19 An excess of ligand is used to favour the formation of the copper complex and avoid free copper ions.

20 No fluorescence was observed when cells were treated with azide (1) and Cu(i) complexes in absence of alkyne (2).

21 Similar results were obtained with A549 cells (Fig. S19†).