Metallomics

A ratiometric fluorescent sensor for the mitochondrial copper pool

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COMMUNICATION

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Copper plays a key role in the modulation of cellular function, defence, and growth. Here we present InCCu1, a ratiometric fluorescent sensor for mitochondrial copper, which changes from red to blue emission in the presence of Cu(I). Employing this probe in microscopy and flow cytometry, we show that cisplatin-treated cells have an impaired ability to accumulate copper in the mitochondria.

Copper plays an essential role in moderating cellular function, defence, and growth.[1] Proteins that employ copper for its structural geometry or redox activity are ubiquitous and rely on a bioavailable pool of copper to draw from when needed. This requires cells to rigorously maintain their copper levels both to provide copper where needed, and to avoid uncontrolled production of reactive oxygen species. This is achieved with a series of intracellular ligands[2] and network of chaperones[3-5] to buffer and traffic copper to its appropriate subcellular destinations.

There is much current interest in understanding the effects of perturbations in copper homeostasis on the genesis and progression of disease.[6-8] Copper and oxidative stress are tightly linked, because of the metal's redox activity in the biological redox window.[9] The distribution and levels of copper are not only markers for cellular health, but also inflammation and potentially the progression of cancer.[10,11] There is a small but growing body of evidence that perturbed copper homeostasis may be crucial for cancer treatment,[12] from the seminal finding that the prognosis of platinum-based chemotherapy correlates with tumour expression levels of the copper transporter CTR1,[13-15] to recent investigations of copper starvation as a cancer therapy.[16,17] The latter is suggested on the basis of observations that the depletion of copper has been found to inhibit angiogenesis and trigger apoptosis.[18,19] Thiosemicarbazone complexes of copper have also been explored as antitumour agents through the induction of reactive oxygen species.[20] The possible redistribution of copper and alteration of redox homeostasis has been associated with therapeutic effects on tumours.[21] Copper has since been found to play both signalling and critical proteomic roles in cell migration, tissue invasion and metastasis,[22] with disruption of labile copper stores affecting neural activity.[23]

Despite this compelling evidence for the importance of copper in the treatment of cancer, the primary area of research in this field has focussed on the interaction of cisplatin, the highly successful platinum-based anticancer drug,[24] with CTR1.[3,25] Many reports have suggested that this interaction is a major mode of cisplatin transport into cells,[26] and that down-regulation of CTR1 is therefore a mode by which tumours can acquire resistance,[15,27-29] but little is known about the subsequent effects on copper homeostasis. Furthermore, while the soft nature of both Cu(I) and Pt(II) is cited as explanation for this interaction, only recent studies have investigated how cisplatin interacts with other proteins involved in copper trafficking, such as copper chaperones Cox17[30] and Atox1,[31] and the copper efflux protein ATP7a.[32] The relationship between platinum and copper proteins is complicated and may involve yet unknown copper chaperones, whose interactions need to be investigated. The involvement of cisplatin and its metabolites in copper homeostasis is likely to reach beyond its import and impact copper's intracellular and vesicular trafficking, as well as organelle-level distribution and uptake.

Given the important role of copper in biology, there is a growing need to visualise copper at a cellular level, and therefore investigate the subtle consequences of cellular stress, cancer and therapeutics on copper homeostasis. To investigate the effects of cisplatin on copper at a subcellular level, we chose to use fluorescence imaging, an essential technique that has the potential to sensitively and selectively visualise metal ions and other chemical species.[33] Fluorescent Cu(I) probes are sensitive to the labile copper pool, which comprises weakly-bound metal ions that are readily available

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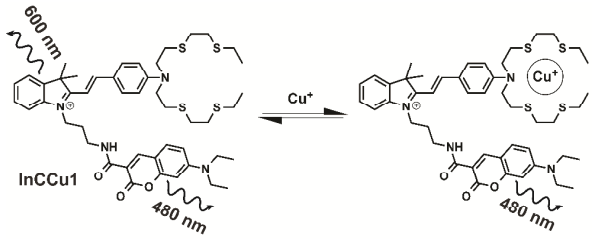
to the metalloproteome.[34] It is this pool that fluorescent probes for Cu(I) generally target, rather than the total copper pool.

Designing Cu(I) fluorescent sensors is challenging because of the tendency of the ion to quench fluorescence emission, which is reflected in the relatively few reports of copper sensors compared to those for other metals.[35-39] Intensity-based fluorescent sensors, in which the presence of Cu(I) is signalled by a change in intensity at a single wavelength, are simplest to develop, but are influenced by local probe concentrations and fluorescent artefacts. This can be minimised with ratiometric imaging, where the emission ratio from two different colour channels is collected, thereby standardising the probe's response to copper, and removing any probe's concentration effects. To this end, we report here the synthesis of a novel Cu(I) probe: indolinium-coumarin copper sensor 1 (**InCCu1**) (Scheme 1), and demonstrate its ratiometric response to Cu(I) both *in vitro* and *in cellulo.* **InCCu1** is selective and sensitive to Cu(I), and was found to localise in mitochondria, making it an ideal platform to specifically investigate the mitochondrial copper pool during cancer therapy.

InCCu1 consists of two fluorophores, an indolinium-based hemicyanine component, and a coumarin, linked *via* an alkyl linker such that there is no conjugation between the two fluorophores. The indolinium group is attached to a bis(2-((2-(ethylthio)ethyl)-thio)ethyl)amine (BETA)-receptor, a thiol-rich copper chelator used in reported Cu(I) probes, and its fluorescence is therefore sensitive to the presence of Cu(I). The coumarin group was incorporated to provide a non-responsive fluorescence peak as an internal standard.

InCCu1 was synthesised in a modular fashion. Coumarin carboxylic acid was prepared according to established procedures[40] and activated by the formation of its *N*-hydroxysuccinimide ester. Following functionalisation with 3-bromopropylamine hydrobromide to attach the propyl linker, the brominated product was reacted in a neat mixture with 2,3,3-trimethylindolenine. Trimethylindolenine is a useful starting material as it contains both a place for substitution at the tertiary nitrogen, and starting point for extending conjugation due to the acidic methyl group, as achieved in similar indolinium metal sensors.[41] This gave the indolinium-coumarin conjugate, which could be decorated with the BETA-receptor. The BETA receptor, coupled subsequently to a benzaldehyde linking group, was prepared by methodology adapted from reports by the Fahrni and Chang groups, both of whom have reported similar Cu(I) receptor structures.[42-45]



Scheme 1. Structure of indolinium-coumarin-copper sensor 1 (**InCCu1**) in equilibrium with Cu(I).

First, *N*-phenyldiethanolamine was chlorinated and formylated in one step with phosphoryl chloride, followed by substitution with 3-thiapentan-1-thiol at both chloride groups to give BETA-benzaldehyde. Finally, the fluorophores and receptor component were conjugated by a condensation reaction to give **InCCu1** in moderate yield.

The fluorescence properties of **InCCu1** were then measured in HEPES buffer (20 mM, pH 7.4). The probe was found to exhibit two distinct fluorescent emission maxima at 480 nm and 600 nm (Figure 1a). The probe had strong absorbance peaks at 430 nm and 550 nm, and the indolinium peak showed appreciable excitation at 430 nm (Figure S1). When increasing amounts of copper were added, the coumarin fluorescence at 480 nm was unchanged, while fluorescence of the indolinium group at 600 nm decreased, but with little change in the absorbance spectrum (Figures 1a, S1). The ratio of coumarin to indolinium intensity (I_{600}/I_{480}) increased linearly between 0 and 1.0 equivalents of Cu(I) (Figure S2), and the response could be reversed upon subsequent removal of Cu(I) (Figure S3). The affinity of **InCCu1** for Cu(I) was measured in a competitive thiourea buffer,[42] giving an observed K_d value of 4.38 x 10^{-12} M (Figure 1b). Furthermore, the ratio of **InCCu1** was unaffected by other biologically relevant metals (Figure 1c): the ratio increase was observed only upon the addition of Cu(I), and this response was retained in the presence of other metal ions. **InCCu1** did not show any response to Ag(I), Hg(II) or Pt(II) salts or to cisplatin (Figure S4). Finally, the fluorescence ratio of **InCCu1** did not vary across the physiological pH range 5-8 (Figure S5).

To test the uptake of **InCCu1** in cells, DLD-1 cells were treated

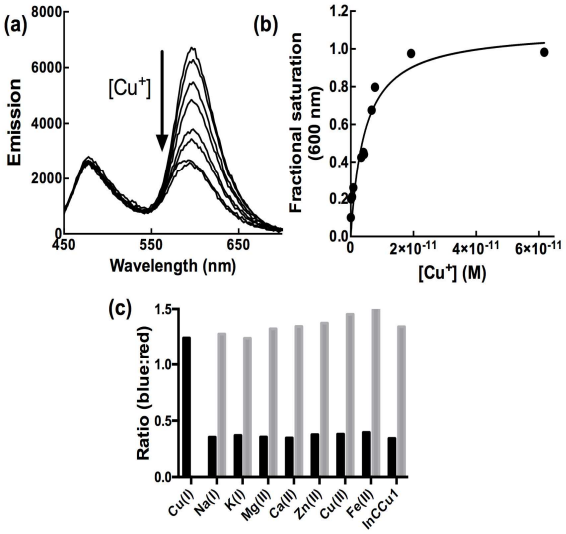


Figure 1 Photophysical properties of **InCCu1**. (a) Fluorescence response of 100 μM **InCCu1** to Cu(I) in HEPES buffer (20 mM, pH 7.4) with increasing equivalents of copper. Cu(I) concentrations are 0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 and 2.0 μM delivered from acetonitrile stock solutions. Excitation provided at 430 nm and collected from 450-700 nm. (b) Normalised fluorescence response of 100 μM **InCCu1** to thiourea buffered Cu(I) solutions to determine K_d in HEPES buffer (20 mM, pH 7.4). Excitation provided at 430 nm, and emission collected at 600 nm. Observed K_d = 4.4 (±1.2) x 10^{-12} M. (c) Fluorescence responses of **InCCu1** (100 μM) to essential metal ions. Bars represent the emission ratio determined by dividing integrated emission from 450-515 nm with 550-650 nm, with excitation at 430 nm. Black bars represent the addition of 1 equivalent of the appropriate metal ion. Grey bars represent the subsequent addition of 100 μM Cu^+ to the solution.

with the probe and then washed with PBS before imaging. Bright fluorescence could be observed in both the blue (425-525 nm) and red (570-670 nm) channels following excitation at 405 nm. Furthermore, a spectral scan of the cells was collected that showed the two characteristic emission peaks observed *in vitro* (Figure S6). The distinct staining pattern of the probe suggested mitochondrial co-localisation, which was confirmed in co-localisation experiments with MitoTracker DeepRed (Figure S7). The Pearson's correlation coefficient of 0.96 indicates good co-localisation of the probe with MitoTracker.

In order to verify the response of **InCCu1** to cellular copper, DLD-1 cells were pre-treated with copper sulfate followed by the probe. The cells were imaged by confocal microscopy, which revealed a statistically significant increase in the emission ratio in copper-treated cells (Figure 2). This difference could also be observed by multiphoton microscopy, with a two-photon excitation wavelength of 820 nm, confirming that the probe can also be imaged by this modality (Figure S8). To confirm that this change could be attributed to receptor-copper binding, we prepared a control probe, **InC**, which lacked the sulfur-rich receptor (Scheme 2), and which was therefore insensitive to the addition of copper *in vitro* (Figure S9). Cells pre-treated with copper sulfate, and then interrogated with **InC** showed no significant change in emission ratio compared to cells treated with vehicle control (Figure 2).

We then investigated the effects of cisplatin treatment on mitochondrial copper uptake. Having determined a dosage of cisplatin (10 μM, 16 h) that was not cytotoxic (>90% viability) and did not affect the mitochondrial membrane potential or the mitochondrial localisation of **InCCu1** (Figure S10), we pre-treated DLD-1 cells with cisplatin overnight, followed by a 2 h copper treatment. The fluorescence was measured with flow cytometry, which allows the collection of the fluorescence ratio from a large population of individual cells. Cells treated with copper alone followed by **InCCu1** showed a lower red/blue ratio than cells treated with vehicle control, consistent with the mitochondrial accumulation of copper observed in confocal microscopy (Figures 3, S11). Interestingly, cisplatin pre-treated cells did not show a significant ratio change with copper loading. This suggests that cisplatin is interfering with copper accumulation in the mitochondria,

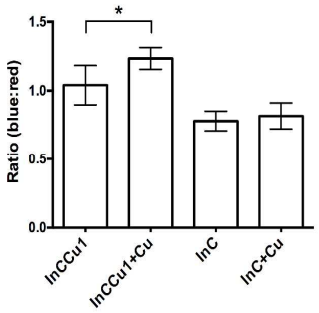
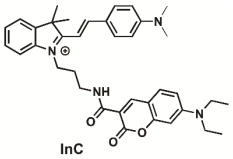


Figure 2. InCCu1 reveals a significant change in copper levels while InC does not. Cells were treated either with copper sulfate overnight (100 μM) or nothing, followed by **InCCu1** or InC (0.1 μM, 15 min) prior to imaging on a confocal microscope. Mean fluorescence ratio was calculated from ratios of intensities of blue channel (λ_{em} = 425-525 nm) over red channel (λ_{em} = 570-670 nm). Error bars represent standard error of mean for quintuplicate measurements.



Scheme 2. Structure of the control probe, indolinium-coumarin (**InC**).

perhaps by interfering with the chaperones involved in copper delivery to this organelle.

The ready supply of copper to the mitochondria is crucial, particularly due to the essential role of the copper protein cytochrome C oxidase (CCO) in the electron transport chain. It has been previously shown that in cases of global copper deficiency, the cell prioritises mitochondrial copper over cytoplasmic copper.[46] The results presented here suggest that cisplatin may be perturbing this pathway even at sub-toxic doses. An interaction between cisplatin and Cox17, the CCO copper chaperone, has been implicated in *in vitro* studies,[47] while Cox17 expression levels have been shown to correlate

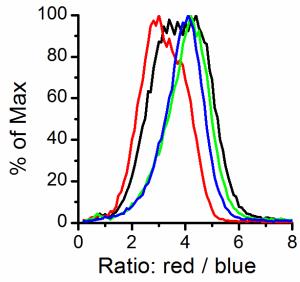


Figure 3: Flow cytometry with **InCCu1** reveals that cisplatin treatment compromises mitochondrial copper uptake. Histograms show ratio of red emission 585(15) / blue emission 450(50), excitation at 405 nm for DLD1 cells treated with **InCCu1** (0.2 μM, 15 min) following treatment with: PBS vehicle control - black, and copper sulfate (100 μM, 2 h) - red, or cisplatin treatment (10 μM, 16 h) followed by PBS vehicle (2 h) - green, cisplatin (10 μM, 16 h) followed by copper sulfate (100 μM, 2 h) – blue.

with mitochondrial platinum levels in cisplatin-treated cells.[30] While studies such as these have focussed on the potential role of copper transport proteins in trafficking cisplatin into and within the cell,[26] perhaps the effect of cisplatin on copper proteins, and subsequently on copper homeostasis, is more significant. It could also be that cisplatin's reported interaction with CTR1 has effects on mitochondrial copper levels, whether through perturbation of cytoplasmic copper, or through interaction with other copper handling proteins. Despite the common mantra that cisplatin's main mode of action lies in its interaction with DNA, there is certainly much evidence that its effect on mitochondrial function plays a role in apoptosis.[48,49] These initial results highlight that one aspect of this interaction could be through perturbation of mitochondrial copper homeostasis, therefore indicating that further study of these processes in the future will be valuable.

Conclusions

InCCu1 is a new mitochondrial copper sensor that can provide information about intracellular copper levels. Importantly, its

ratiometric output allows the standardised investigation of copper levels, unaffected by probe concentration and background fluorescence artefacts. We have shown here how **InCCu1** can be applied in biological investigations of the effect of cisplatin on copper homeostasis. In the future, the information that can be gained from a mitochondrial probe such as **InCCu1** will be greatly supplemented by the development of similar sensors that are targeted to other organelles, enabling the subcellularly resolved mapping of the changes in labile copper pools.

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Significance to metallomics

The effect of therapeutic metal ions on essential metal homeostasis is crucial in understanding the modes of actions and limitations of metal-based therapies. In particular, the putative interactions of cisplatin with copper proteins suggest that platinum-containing drugs should affect copper homeostasis. By developing a ratiometric fluorescent sensor for the mitochondrial copper pool, we show that cisplatin does perturb copper accumulation in the mitochondria.