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A Terbium(III) luminescent ATCUN-based peptide sensor for selective and reversible detection of Copper(II) in biological media.

Enrico Falcone,^a Paulina Gonzalez,^a Lucie Lorusso,^a Olivier Sénéque,^b Peter Faller,^{*a} and Laurent Raibaut^{*a}

The measurement of exchangeable Cu²⁺ levels in biological samples is gaining interest in the context of copper-related pathologies. Here, we report a Tb³⁺ luminescent turn-off sensor for Cu²⁺ based on the specific and suitable-affinity *Xxx-Zzz-His* (ATCUN) peptide motif, enabling Cu²⁺ detection in the presence of a biological fluorescent background.

Copper is a catalytic co-factor essential for most organisms to accomplish several biochemical tasks, such as cellular respiration, radicals scavenging and neurotransmitter synthesis.¹ Notwithstanding, excess of loosely bound copper can be toxic as its redox cycling between Cu²⁺ and Cu⁺ generates Reactive Oxygen Species (ROS). Therefore, copper is tightly coordinated, mainly as Cu²⁺, by a number of *extracellular* carriers (ceruloplasmin, serum albumin, α_2 -macroglobulin, small ligands), and mainly as Cu⁺ by membrane transporters (Ctr1/2, ATP7A/B) and *intracellular* metallo-chaperones (Atox1, CCS, Cox17), which ensure proper copper homeostasis.^{2,3} However, this tight control is lost in genetic disorders such as Menkes' and Wilson's diseases and in amyloid diseases such as Alzheimer's and Parkinson's diseases.^{4,5} Interestingly, increased levels of serum and urine copper, especially those of the exchangeable (i.e. kinetically labile) copper pool, are gaining interest as potential new markers of these pathologies.⁶⁻⁹ Whilst the measurement of total copper levels by means of classical techniques (AAS, ICP-MS, ICP-AES) is well established, probes for the measurement of exchangeable Cu levels are still under development.¹⁰⁻¹³ In order to be competitive with Cu physiological partners, such a probe should have an affinity of the same order of magnitude. In particular, if the affinity of the probe for Cu is too high, it is always loaded and hence it cannot respond to fluctuations. Likewise, if the affinity is too low, the probe is always empty.

The Amino Terminal Cu²⁺- and Ni²⁺-binding (ATCUN) *Xxx-Zzz-His* motif,¹⁴ naturally found in proteins and peptides such as serum albumin,¹⁵ hepcidin¹⁶ and Neuromedin C (NMC),¹⁷ has been exploited to develop Cu²⁺-selective sensors by its conjugation with common green-emitting dyes.¹⁸⁻²³ The selectivity of such 4N (NH₂, 2×N⁻, N_{im}) binding motif for Cu²⁺ stems from the strong Lewis acid character and the preference for square-planar coordination geometry. Interestingly, the suitable affinity of the ATCUN motif ($12 < \log {}^cK_{7,4} < 15$, depending on the identity of Xxx, Zzz and the sequence after His)¹⁴ ensures competitiveness against the endogenous Cu²⁺ ligands. Indeed, Human Serum Albumin (HSA, $\log {}^cK_{7,4} \approx 13$)²⁴ is only partially loaded with Cu under normal physiological conditions, indicating that it has the right affinity to detect fluctuations. The above-mentioned dye-ATCUN sensors are reversible and based on fluorescence turn-off, as paramagnetic Cu²⁺ quenches fluorescence.²⁵ Conversely, turn-on Cu²⁺-sensors reported are commonly not reversible, as they are based on a chemical reaction that yields a fluorescent product.^{26,27} Indeed, we recently proved that at least one of

several alleged turn-on binding-based probes works through irreversible Cu²⁺-induced sensor oxidation.²⁸

On the other hand, fluorescent sensing in biological media is challenged by the intrinsic fluorescence background of biomolecules such as proteins and cofactors. This issue can be overcome, for instance, employing long-lifetime luminophores, which allow getting rid of the biological fluorescence background via time-delayed detection. In this respect, luminescent trivalent lanthanide ions (Ln³⁺) have long-lifetime emission (in the millisecond range) and show very interesting photophysical properties, such as ligand-independent atom-like narrow emission bands, large Stokes shift and low tendency to photobleach.²⁹ However, the lanthanide-based probes for Cu²⁺ that have hitherto been reported are based on ligands that do not benefit from the advantageous properties of the ATCUN motif, i.e. the high selectivity for Cu²⁺, the appropriate affinity and the reversibility.³⁰⁻³⁶

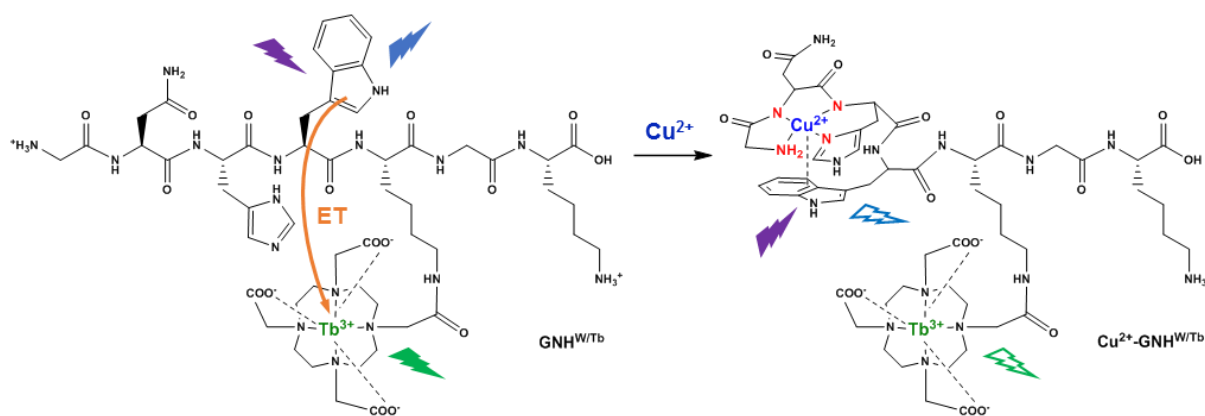


Figure 1. Structure and schematic mechanism of Cu^{2+} sensing by $\text{GNH}^{\text{W/Tb}}$: in absence of Cu^{2+} , Trp excitation (violet flash) gives rise to Trp (blue flash) emission and also Tb^{3+} (green flash) emission via energy transfer (ET, orange arrow); upon Cu^{2+} -binding to the peptide ATCUN motif, both Trp and Tb^{3+} luminescence is quenched.

In this article, we present the design of a turn-off luminescent peptide for Cu^{2+} , combining the selectivity and suitable affinity of the ATCUN motif with the long-lifetime emission of the lanthanide Tb^{3+} , and we prove its suitability for Cu^{2+} sensing in biological-like media via time-resolved luminescence detection. As the low extinction coefficients of the Laporte-forbidden 4f-4f transitions impair the efficient excitation of Ln^{3+} ions, it is necessary to resort to the so-called “antenna effect”, i.e. the energy transfer from a suitable excited chromophore to the accepting excited electronic levels of Ln^{3+} .³⁷ Typically, the amino acid tryptophan (Trp) can be chosen as an antenna due to its recognised ability to sensitize Tb^{3+} ions and its straightforward inclusion into a peptide scaffold.^{38–41}

Thus, we designed a Tb^{3+} luminescent ATCUN peptide, $\text{GNH}^{\text{W/Tb}}$ ($\text{GNHWK}^{\text{DOTA}(\text{Tb})}\text{GK-NH}_2$), containing the first four N-terminal amino acids (GNHW) of the peptide NMC, which includes the ATCUN motif and a Tb^{3+} -DOTA luminescent complex grafted on a lysine residue next to the Trp antenna (see Figure 1). $\text{GNH}^{\text{W/Tb}}$ was obtained using classical peptide synthesis methodology (see Figure S1 and S2).

First, the luminescent properties of $\text{GNH}^{\text{W/Tb}}$ were investigated by steady-state luminescence spectroscopy in HEPES buffer (pH 7.4). In the absence of Cu^{2+} , excitation of Trp at 280 nm promotes its emission at 348 nm, and by the antenna effect the characteristic emission of Tb^{3+} at 487, 544, 585 and 623 nm due to $^5\text{D}_4 \rightarrow ^7\text{F}_j$ ($j = 6, 5, 4, 3$) transitions. Indeed, time-delayed excitation spectrum (at $\lambda_{\text{em}} = 544$ nm), also confirmed that Trp acts as a sensitizing antenna for Tb^{3+} luminescence (Figure S3). Next, the effect of Cu^{2+} binding on the peptide luminescence was investigated by a Cu^{2+} titration (Figure 2A). Cu^{2+} addition caused the gradual quenching of both Trp and Tb^{3+} emission until one equivalent of Cu^{2+} , confirming 1:1 $\text{Cu}^{2+}:\text{GNH}^{\text{W/Tb}}$ stoichiometric binding, as also shown by UV-vis titration via the increase of the d-d band at $\lambda_{\text{max}} = 525$ nm (Figure S4). Conversely, Tb^{3+} luminescence lifetime remained constant upon Cu^{2+} addition and equal to ≈ 1.97 ms (Figure S5) indicating that Cu^{2+} does not quench the Tb^{3+} excited state but quenches the Trp antenna S_1 excited state, resulting in lower efficiency of the antenna effect. Besides, the selectivity of the probe in the

presence of a physiological concentration of other extracellular relevant di- and trivalent cations (Zn^{2+} , Fe^{3+} , Co^{2+} , Mn^{2+} 10 μM , Mg^{2+} and Ca^{2+} 2 mM) was evaluated. These cations did not alter significantly the intensity of Trp (Figure 2B) and Tb^{3+} emission bands (Figure S6), while Cu^{2+} did, even in the presence of all the other cations. Thus, $\text{GNH}^{\text{W/Tb}}$ was able to detect specifically Cu^{2+} among these essential metals for humans, expected to be the main competitors in the extracellular space. Since the ATCUN motif also binds Ni^{2+} ions effectively, we investigated the effect of Ni^{2+} binding on $\text{GNH}^{\text{W/Tb}}$ luminescence, which was efficiently quenched upon Ni^{2+} addition (Figure S7). Nevertheless, the sensor can be considered selective for Cu^{2+} since (i) Ni^{2+} is not a physiological ion for mammals, (ii) the affinity of the ATCUN motif for Ni^{2+} is about 5÷6 orders of magnitude lower than for Cu^{2+} (for NMC, $\log cK^{\text{Ni}}_{7,4} = 7.3$ and $\log cK^{\text{Cu}}_{7,4} = 13.6$), and (iii) Ni^{2+} -binding is much slower than Cu^{2+} -binding.^{42,43}

Next, we tested the ability of $\text{GNH}^{\text{W/Tb}}$ in detecting Cu^{2+} in the presence of a more biologically relevant fluorescent background. In particular, we studied (i) the Cu^{2+} transfer from Pig Serum Albumin (PSA), which contain many fluorescent aromatic amino acids residues, to $\text{GNH}^{\text{W/Tb}}$, and (ii) the Cu^{2+} sensing in a complex mixture such as Luria-Bertani (LB), which is rich in peptides and vitamins, including possible competing Cu^{2+} -binding sites. In both cases, no significant change of the fluorescence emission intensity upon Cu^{2+} addition can be observed (Figures 3A and S8A), as PSA or LB medium signal

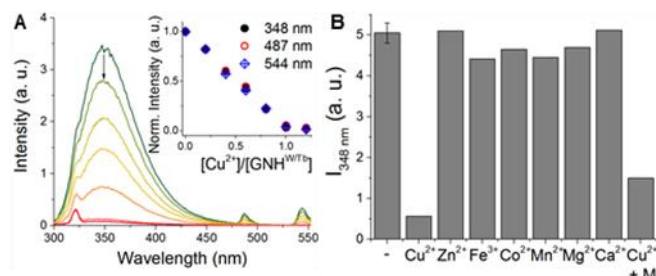


Figure 2. Selective Cu^{2+} sensing by $\text{GNH}^{\text{W/Tb}}$ via Trp and Tb^{3+} luminescence quenching. (A) Cu^{2+} titration of the sensor. Conditions: $[\text{GNH}^{\text{W/Tb}}] = 10 \mu\text{M}$, HEPES 100 mM pH 7.4. (B) Effect of other physiologically relevant metal ions on Trp emission ($M = \text{mix}$ of all the cations). Conditions: $[\text{GNH}^{\text{W/Tb}}] = 10 \mu\text{M}$, $[\text{Cu}^{2+}] = [\text{Zn}^{2+}] = [\text{Fe}^{3+}] = [\text{Co}^{2+}] = [\text{Mn}^{2+}] = 10 \mu\text{M}$, $[\text{Mg}^{2+}] = [\text{Ca}^{2+}] = 2 \text{ mM}$, HEPES 100 mM, pH 7.4.

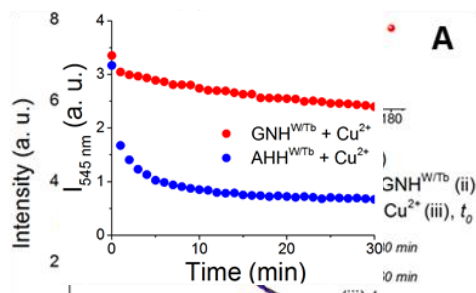


Figure 4. Kinetics of Cu^{2+} binding to XZH^{WTb} in LB medium. Conditions: $[\text{Cu}^{2+}] = 9 \mu\text{M}$, $[\text{XZH}^{\text{WTb}}] = 10 \mu\text{M}$, LB medium 10%.

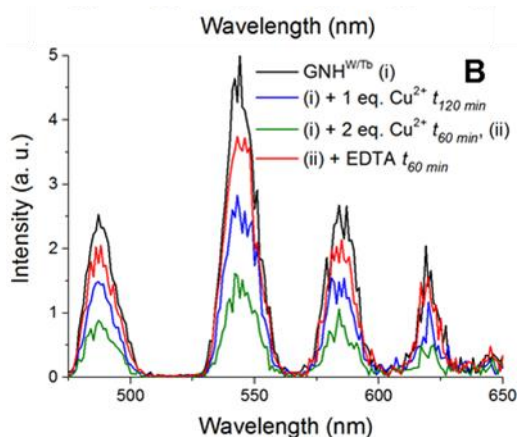


Figure 3. Reversible Cu^{2+} sensing by GNH^{WTb} in the presence of LB medium monitored by steady-state (A) and time-delayed (B) luminescence. Conditions: $[\text{GNH}^{\text{WTb}}] = 10 \mu\text{M}$, $[\text{EDTA}] = 100 \mu\text{M}$, LB 10%. Inset: kinetics profile of Cu^{2+} binding.

override that of the probe. Moreover, under steady-state fluorescence condition, the fluorescent background at higher wavelength was so intense that Tb^{3+} emission bands were undetectable in the presence of PSA or LB medium. Advantageously, long luminescence lifetime of Tb^{3+} allows to perform time-resolved detection and hence to get rid of the autofluorescence of the biological background. Under this condition, with a $100 \mu\text{s}$ time delay before the recording of the emission, only the emission bands of Tb^{3+} were observed (Figures 3B and S8B). As Cu^{2+} was slowly transferred from PSA to GNH^{WTb} , the intensity of these bands progressively decreased (Figure S8B). To assess the reversibility of the sensor, an excess of the stronger Cu^{2+} chelator EDTA was added. The latter took Cu^{2+} slowly out from the sensor, restoring the initial signal. Time-delayed detection also allowed the measurement of Tb^{3+} emission in LB medium (Figure 3B). Specifically, the sensor showed a slow response and the quenching by Cu^{2+} was not complete, even upon the addition of an excess. Finally, the signal was slowly recovered by the addition of excess EDTA. This behaviour suggests that the sensor is likely in kinetic competition with Cu^{2+} -binding medium components. Indeed, Cu^{2+} -binding to ATCUN motif is known to be slower than the binding to non-amide ligands, as amide deprotonation is relatively slow.^{44,45} Therefore, Cu^{2+} could bind first to kinetically-favoured ligands in the medium and then move to the thermodynamically competitive ATCUN motif in the sensor. Overall, the observed behaviour shows that the designed sensor is sensitive to fluctuations of exchangeable Cu^{2+} levels in the presence of fluorescent background and possible competitors. In order to enhance the kinetic competitiveness of the sensor,

we designed a sequence variant, AHH^{WTb} ($\text{AHHWK}^{\text{DOTA}(\text{Tb})}\text{GK-NH}_2$), bearing the ATCUN-like motif *Xxx-His-His*, which occurs for instance in rice albumin (DHH)⁴⁶ and $\text{A}\beta_{12-x}$ fragment peptides (VHH). The AHH motif has been recently investigated thoroughly.^{48,49} At pH 7.4 Cu^{2+} binds predominantly in the ATCUN 4N coordination mode, but a small portion is bound in a 3N (NH_2 , N^- , N_{im}) mode with an external ligand (Figure S9). Cu^{2+} exchanged much more rapidly in AHH than AAH, which was attributed to the possible formation of ternary complexes of the 3N form. In addition, AHH motif also shows higher affinity ($\log K_{7.4} = 14.3$) than GNH. Hence, we first investigated Cu^{2+} - AHH^{WTb} interaction by UV-vis and luminescence titrations (Figures S10), which showed similar behaviour to GNH^{WTb} . Then, we tested the kinetic properties of the probes, first comparing the rate of Cu^{2+} transfer from the simple dipeptide AH to the two sensors (Figure S11). Indeed, the AHH^{WTb} revealed to be faster than the canonical GNH^{WTb} .

Thus, we further compared the Cu^{2+} -binding kinetics of the two sensors in the complex LB medium. The peptide AHH^{WTb} showed again faster Cu^{2+} -binding than the GNH^{WTb} , proving to be more readily responsive to Cu^{2+} levels fluctuation (Figure 4). In conclusion, in this work, we report for the first time a Tb^{3+} luminescent sensor for Cu^{2+} based on a naturally occurring ATCUN motif, proving its suitability for competing with endogenous ligand and for applications in biological media. This represents a proof of principle study showing the favourable application of Ln^{3+} -based ATCUN-bearing peptide probes to study Cu^{2+} handling. Advantageously, the versatility of this peptide scaffold opens the way for improving and tuning sensitivity, affinity and kinetics of this prototype by modulating the amino acid sequence as well as the nature of the antenna- Ln^{3+} couple. Such optimised sensors could not only be applied to monitor Cu^{2+} transfer reactions *in vitro* but have also the potential to be used in cell cultures and biological fluids. We acknowledge Dr Gilles Ulrich (UMR 7515, Strasbourg) for help with lifetime measurements, Dr Loïc Charbonnière and Dr Clémence Cheignon (UMR 7178, Strasbourg) for help with time-delayed luminescence measurements, and Dr Elise Glattard (UMR 7177, Strasbourg) for LB medium preparation. We acknowledge financial support from the French National Research Agency (ANR) through the Programme d'Investissement d'Avenir under contract 17-EURE-0016.

Conflicts of interest

There are no conflicts to declare.

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