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A ^{19}F-ATCUN Peptide NMR Probe for Selective Cu^{2+} or Ni^{2+} Sensing in Complex Biological Media

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We report the design and synthesis of ratiometric ^{19}F-ATCUN (Xxx-Zzz-His) peptide probes for the selective and reversible detection of Ni^{2+} and Cu^{2+} by ^{19}F NMR spectroscopy. In contrast to fluorescence-based approaches, ^{19}F NMR enables background-free detection in biological media, due to the absence of fluorine in native biomolecules. The probes remain stable and functional in complex cell media (DMEM), enabling the detection of Cu^{2+} and Ni^{2+} by ^{19}F NMR with a limit of detection (LOD) of 4 μM and quantification (LOQ) of 10 μM, relevant for studying copper-related pathologies.

1. Introduction

Copper is essential for humans and most living organisms, while nickel, though nonessential for humans, is required by some plants and microorganisms.[1] Above a certain threshold, both elements become toxic. Hence there is interest in detecting and quantifying these metals in a specific manner in different biological or environmental systems, as a tool to better decipher the homeostasis and toxicity of these metal ions. Cu occurs as Cu^{+} and Cu^{2+} in biological systems. Cu^{2+} is the prevalent state in dioxygen-rich and oxidizing environment, such as extracellular biological fluids like blood serum, urine, bile, or cerebrospinal fluid. The total concentration and the different species depend on the system, but in general Cu^{+} or Cu^{2+} are mostly bound to ligands. Both thermodynamic and kinetic factors must be considered when evaluating its bioavailability. While Cu is classically strongly and inertly bound to enzymes, more labile and exchangeable Cu^{2+} is found either strongly bound to blood circulating proteins, mainly serum albumin (HSA, log $K_{d\ (at\ pH\ 7.4)} = 13$),[2] or weakly bound to small ligands like amino acids, organic acids or anions in the urine.[3] Under physiological conditions total copper concentrations are in the ~10–20 μM range, and labile Cu^{2+} often lower, ~3–6 μM or less. Higher concentrations can be reached under Cu dyshomeostasis like in genetic Wilson's disease leading to a copper overload,[4] or by Cu intoxication. Labile Cu^{2+} concentration might also be higher in some site-specific cases. For instance, concentrations of up to 200–250 μM were estimated for Cu release in the synaptic cleft.[5] More broadly, elevated levels of labile Cu^{2+} have also been associated with amyloidosis such as Alzheimer's and Parkinson's diseases.[6,7] Therefore, accurate measurement of labile Cu^{2+} pool provides valuable insights into copper biology as well as diagnosis and therapeutic monitoring of copper-related disease.[8]

The ATCUN motif (Amino Terminal Copper and Nickel binding motif),[9] defined by the sequence H_2N-Xxx-Zzz-His (where His: histidine and Xxx or Zzz are natural amino acids, excluding proline for Zzz) is found at the N-terminus of human serum albumin and represents a prime example of a selective Cu^{2+}- and Ni^{2-+}binding site. This motif forms a highly stable 4N coordination complex with Cu^{2+}, exhibiting large and distinct affinity ranges (log $K_{d\ (at\ pH\ 7.4)} = 12$–15 which remains stable over a broad pH range (pH 5–10)[9]), and with Ni^{2+} (log $K_{d\ (at\ pH\ 7.4)} = 6$–8),[10] depending on the identity of Xxx and Zzz. Such properties make the ATCUN motif well-suited to compete especially with the exchangeable or labile Cu^{2+} pool present in biological fluids.[8] The use of much stronger ligands, for instance ethylenediaminetetraacetic acid (EDTA),[3] might be less appropriate, as they lack selectivity for the labile Cu^{2+} fraction and may extract total copper, including the nonexchangeable pool from essential Cu-enzymes. Consequently, several fluorescent sensors incorporating the ATCUN motif have been reported,[11–14] predominantly employing green-emitting dyes that rely on fluorescence quenching due to the paramagnetic nature of Cu^{2+}. However, in complex biological media (serum, urine …), intrinsic fluorescence from biomolecules can interfere with luminescence-based detection. To circumvent this issue, our group[15] has recently introduced a novel "turn-off ATCUN probe" based on

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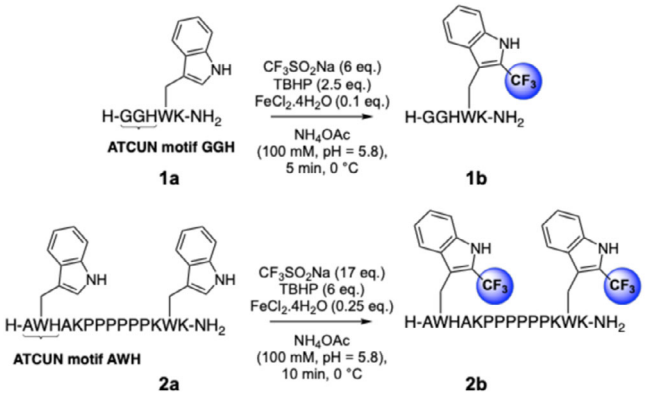
Tb^{3+} luminescence sensitized by tryptophan via an antenna effect. This system exhibits long-lifetime luminescence of Tb^{3+} (2 ms), enabling time-delayed detection (~100 µs time delay) to effectively mitigate background autofluorescence.

Despite these advantages, the inherently weak luminescence of lanthanides can be further attenuated by the inner filter effect of biological media. Interestingly, a complementary strategy involves the use of the ATCUN motif, which forms stable Cu^{2+} complexes serving as paramagnetic probes in ^{1}H NMR. These enable the investigation of long-range interactions within biomolecules, providing valuable insights into protein structure, dynamics, and interactions.[16] By contrast, the absence of fluorine background in biofluids enables ^{19}F nucleus (spin $\frac{1}{2}$) to be selectivity observed in complex mixtures with high sensitivity by NMR or MRI.[17] Furthermore, NMR signal is quantitative and this property has been exploited to design ^{19}F sensor ligands, such as ^{19}F-BATPA,[18] able to form complexes with various diamagnetic cations (Ca^{2+}, Mg^{2+}, K^{+}, Zn^{2+}, Fe^{2+}, and Mn^{2+}) with different affinities. The large chemical shift dispersion of ^{19}F and its high sensitivity to electronic environments allows identifying bound ions through distinct chemical shifts in ^{19}F-NMR and therefore provides insights into their exchange dynamics. However, in the case of Cu^{2+} detection, the ^{19}F-NMR signal is suppressed due to paramagnetic nature of Cu^{2+}, that leads to severe shortening of T1 and T2 relaxation times of ^{19}F resonances located in the vicinity of the metal and subsequently a loss of NMR signal.[19] This quenching of the ^{19}F signal was exploited to design different ^{19}F-labeled probes (ATSM,[20] Cyclam[21]) able to specifically bind Cu^{2+} at relevant concentrations. By altering the redox environment of the ligand, Cu^{2+} is reduced to diamagnetic Cu^{+}, leading to an increase in ^{19}F T_1 and T_2 relaxation times, and consequently to the reappearance of the ^{19}F NMR signal. Importantly, all of these studies demonstrate that ^{19}F-NMR probes cation chelators enable direct ion identification at biologically relevant concentrations and are particularly advantageous for analyzing opaque cell suspensions or complex media, where fluorescence-based measurements are often impractical. Inspired by these findings, we set out to develop a ^{19}F-ATCUN NMR probe as a next-generation strategy to detect exchangeable Cu^{2+} in biological fluids, aiming to overcome the limitations of luminescent ATCUN probes in complex media.

2. Results and Discussion

We selected the peptide **1a** (Scheme 1 & SI Figure S1) derived from the well-characterized ATCUN motif Xxx-Zzz-His with the simplest sequence GGH (log Kd $_{(at\ pH\ 7.4)}$ Cu^{2+} ≈ 12.4; log Kd $_{(at\ pH\ 7)}$ Ni^{2+} ≈ 7).[9,10] A tryptophan (Trp) residue was introduced at the fourth position of peptide **1a** to enable direct selective C2-trifluoromethylation of the indole ring via late-stage modification, thereby avoiding the need to introduce the unnatural amino acid Fmoc-Trp(CF_3)-OH during SPPS. This chemical modification was achieved using optimized Langlois method,[22,23] which exploits the reactivity of trifluoromethyl radicals CF_3• generated in situ from sodium trifluoromethanesulfinate ($NaSO_2CF_3$)



Scheme 1. Synthesis of ^{19}F-ATCUN probes **1b** and **2b** by direct trifluoromethylation of native peptides ATCUN **1a** and **2a**.

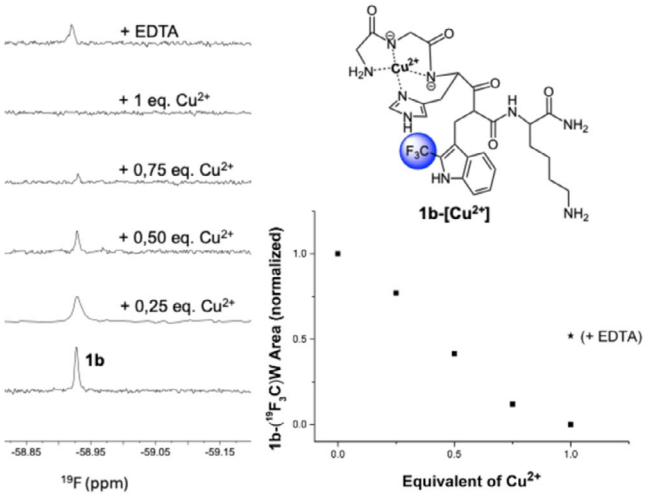


Figure 1. Reversible titration of probe **1b** with Cu^{2+} in HEPES monitored by quenching of the ^{19}F signal from W(CF_3) at -58.92 ppm. Conditions: [probe **1b**] = 100 µM, [EDTA] = 10 mM, HEPES 50 mM, pH 7.4. ^{19}F NMR: 471 MHz, 90% D_2O, 25 °C, 32 scans, 500 MHz spectrometer with cryoprobe (see SI).

in the presence of *tert*-butyl hydroperoxide (TBHP) and a catalytic amount of Fe^{2+} as a radical initiator. The resulting CF_3• radical then adds to the (hetero)aromatic moiety to form a carbon-centered radical, which undergoes single-electron oxidation to a cation, followed by re-aromatization through proton.[23] This reaction was carried out directly on the native peptide **1a** under mild aqueous conditions. To enhance chemoselectivity[23] toward Trp over His, the reaction was performed at low temperature (0 °C) and in acidic buffer ammonium acetate at pH (5.8), where the imidazole side chain of His is protonated, reducing its reactivity. Successful mono-incorporation of the CF_3 group on isolated peptide **1b** was confirmed by LC-MS (SI Figures S3–S4). Furthermore, the selectivity of the Trp modification was validated by ^{19}F NMR spectroscopy (SI Figure S5), which exhibited a characteristic fluorine chemical shift[23] (δ = -58.93 ppm) corresponding to the C_2-trifluoromethylated Trp residue on the indole ring, with no significant signal attributable to any modification of the His imidazole ring (δ = -60.6 ppm).[23]

Cu^{2+} binding to the probe **1b** was next investigated in HEPES buffer at pH 7.4 (Figure 1). As anticipated, a progressive

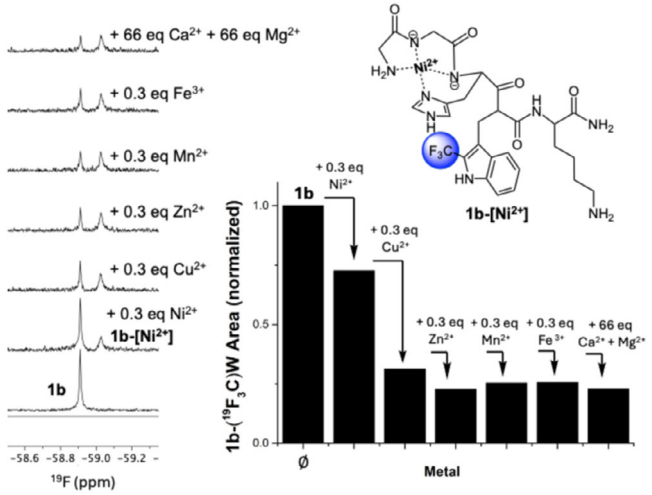


Figure 2. Selectivity of probe **1b** against physiologically relevant metal ions, assessed via quenching of the ^{19}F signal from $W(CF_3)$ at -58.91 ppm. Upon ATCUN(Ni^{2+}) binding, a new ^{19}F NMR signal appeared at −59.02 ppm. Conditions: [probe **1b**] = 60 μM, [Cu^{2+}] = [Zn^{2+}] = [Fe^{3+}] = [Co^{2+}] = [Mn^{2+}] = 20 μM, [Mg^{2+}] = [Ca^{2+}] = 4 mM, HEPES 500 mM, pH 7.4. ^{19}F NMR: 471 MHz, 90% D_2O, 25 °C, 512 scans, 500 MHz spectrometer with cryoprobe (see SI).

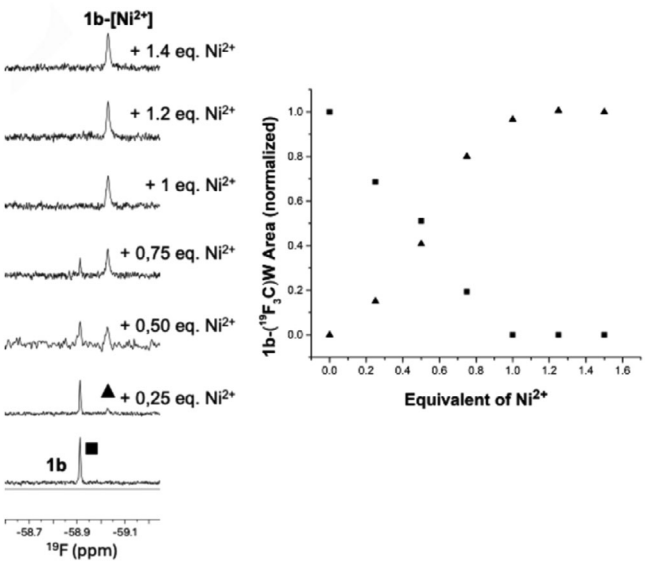


Figure 3. Titration of probe **1b** with Ni^{2+} in DMEM, showing the disappearance of $W(CF_3)$ ^{19}F signal (□) at -58.92 ppm of free probe **1b** and the appearance of $W^{(CF}3)$ ^{19}F signal (▲) at 59.02 ppm of bound **1b**-[Ni^{2+}] probe. Conditions: [probe **1b**] = 50 μM, DMEM 10%, pH 7.4. ^{19}F NMR: 471 MHz, 80% D_2O, 25 °C, 256 scans, 500 MHz spectrometer with cryoprobe (see SI).

broadening of the ^{19}F- $W(CF_3)$ signal (δ = -58.92 ppm) was observed upon Cu^{2+} titration, consistent with paramagnetic relaxation enhancement (PRE) effects. This leads to signal attenuation up to a 1:1 stoichiometric ratio between **1b** and Cu^{2+}, as confirmed by integration of the signal area. Importantly, the addition of an excess of the high-affinity Cu^{2+} chelator ethylenediaminetetraacetic acid (EDTA) (log $K_{d\ (at\ pH\ 7.4)} \approx 15.9$) led to the recovery of the ^{19}F-$W(CF_3)$ signal at its original chemical shift, demonstrating the reversible nature of **1b** probe. This reversibility highlights the potential of probe **1b** in ^{19}F-NMR for dynamic monitoring of Cu^{2+} fluctuations in complex biological environments. The selectivity of probe **1b** was subsequently assessed in the presence of physiologically relevant extracellular metal ions (Figure 2). Notably, the ^{19}F-$W(CF_3)$ signal was only affected by Cu^{2+} and Ni^{2+}, while no significant changes were observed upon addition of Zn^{2+}, Fe^{3+}, Co^{2+}, Mn^{2+} (20 μM), or Mg^{2+} and Ca^{2+} (4 mM), confirming the high selectivity of the ATCUN motif toward Cu^{2+} and Ni^{2+}. Nickel(II) did not induce PRE effects, instead coordination to ATCUN-$W(CF_3)$ led to the appearance of a new ^{19}F-$W(CF_3)$ signal (δ = -59.02 ppm) downfield by approximately 0.1 ppm (Figure 2). This is in line with the reported low-spin and hence diamagnetic Ni^{2+} in ATCUN. The observed shift upon Ni^{2+}binding reflects a change in the local electronic environment upon metal coordination, enabling ratiometric discrimination between the free and Ni^{2+}-bound forms of the probe. Thereafter, a titration of probe **1b** was performed in HEPES buffer in the presence of increasing concentrations of Ni^{2+} (SI Figure S10). Signal integration revealed a linear ratiometric relationship between the free and Ni^{2+}-bound forms of probe **1b** allowing to determine, under these experimental conditions ([**1b**] = 30 μM, 512 scans, 18 min acquisition, 500 MHz spectrometer with cryoprobe) a limit of detection (LOD) ~ 4 μM and a LOQ ~ 10 μM. Furthermore, we demonstrated the potential of this probe for quantitative Ni^{2+} detection (50 μM) in complex biological environments, such as DMEM cell culture medium (Figure 3). The development of a ratiometric probe is particularly advantageous in the case of paramagnetic Cu^{2+}, where the disappearance of the ^{19}F-$W(CF_3)$ signal may not unambiguously indicate metal binding, but could also result from peptide degradation, precipitation, or nonspecific interactions with biomolecules in complex biological environments. To address this limitation, we designed an improved probe architecture[24] **2a** (Scheme 1 & SI Figure S2) by incorporating a second ^{19}F-$W(CF_3)$ residue as an internal reference, spatially separated from the metal-binding ATCUN motif. This was achieved by inserting a rigid hexaproline ($PolyPro_6$) spacer between the two Trp residues, exploiting its propensity to adopt a polyproline type II helical conformation, thereby minimizing PRE effects from Cu^{2+} on the distal Trp. We further refined the design of the probe by modifying the ATCUN motif, replacing the GGH sequence with AWH. This variant displays a comparable affinity for Cu^{2+} but is characterized by slower Cu^{2+} exchange kinetics, offering improved stability of the ^{19}F signal in complex biological environments (serum, urine).[25] Additionally, the incorporation of a Trp residue at the second position of the ATCUN sequence **2a**, directly within the metal coordination sphere, was designed to increase the efficiency of PRE effects and thereby fine-tune the quenching of the ^{19}F signal upon Cu^{2+} binding.

Using the same synthetic strategy, the peptide construct **2a** was subjected to selective C_2-trifluoromethylation of Trp via the Langlois method under the previously optimized conditions (SI Figure S6). The successful and selective modification of both tryptophan residues (SI Figures S7,S8), along with the absence of histidine modification, was confirmed by MS/MS analysis (SI Figure S9), yielding the final construct **2b**, thus

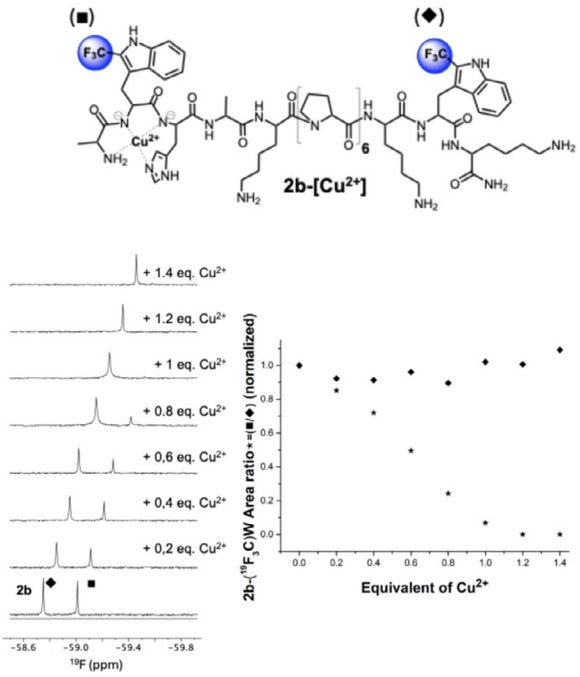


Figure 4. Titration of the ratiometric probe **2b** with Cu^{2+} in DMEM, showing the disappearance of the W(CF_3) ^{19}F signal of the free probe **2b** (□) at -59.02 ppm, while the internal reference W(CF_3) ^{19}F signal (◆) at -58.75 ppm remains unaffected. (⋆) corresponds to the ratio of the two signals (□/◆). Conditions: [probe **2b**] = 50 µM, DMEM 10%, pH 7.4. ^{19}F NMR: 471 MHz, 80% D_2O, 25 °C, 1024 scans, 500 MHz spectrometer with cryoprobe (see SI).

validating the site-selectivity and robustness of this late-stage functionalization. Finally, the ratiometric behavior of the probe **2b** was evaluated in HEPES buffer (SI Figure S11) to confirm its Cu^{2+}-binding properties. Upon titration, a 1:1 stoichiometric ^{19}F-NMR response was observed, with selective quenching of the coordination-site ATCUN-W(CF_3) signal (δ = -59.02 ppm), while the distal internal $(Pro)_6$–W(CF_3) remained unaffected (δ = -58.75 ppm), thus serving as a reliable internal reference. In both cases, for probe **1b** with Ni^{2+} and ratiometric probe **2b**, the observed upfield shift during titration may reflect a peptide conformational change that alters the local fluorine environment, potentially affecting ^{19}F relaxation. Lastly, upon EDTA addition the ATCUN–W(CF_3) signal was recovered, while the distal signal remained unaffected throughout the experiment, confirming the reversible nature of Cu^{2+} binding under these conditions. These results validated the probe's ability, under these experimental conditions ([**2b**] = 50 µM, 1024 scans, 36 min acquisition, 500 MHz spectrometer with cryoprobe) to perform accurate ratiometric quantification of Cu^{2+} levels with a LOD ∼ 4 µM and a LOQ ∼ 10 µM. Encouraged by these results, the ratiometric probe **2b** was further tested in a complex biological cell medium (DMEM) (Figure 4). Remarkably, the probe enabled successful titration of Cu^{2+} in the presence of potential biological interferents and competing ligands. It allowed detection of Cu^{2+} concentrations as low as 50 µM, demonstrating its suitability for monitoring labile copper pools in biologically relevant environments.

3. Conclusion

In this study, we developed and characterized novel ^{19}F-labeled ATCUN-based peptide probes for the selective and reversible detection of Ni^{2+} and Cu^{2+} in biological environments. By incorporating C_2-trifluoromethylated tryptophan residues using the Langlois method, we designed both single- and dual-labeled ratiometric sensors capable of distinguishing between free and metal-bound forms via distinct ^{19}F NMR signals. The use of ^{19}F NMR provides a significant advantage over fluorescence-based approaches by eliminating interference from biological background, as fluorine is absent from native biomolecules. Advantageously, this late-stage derivatization strategy in mild conditions can, in principle, be extended to other peptide scaffolds. Specifically, within the ATCUN motif, replacing the canonical GGH sequence with AWH slowed Cu^{2+} exchange kinetics, improving signal stability in complex media. Finally, the resulting probes exhibited high selectivity for both Cu^{2+} and Ni^{2+} in cell culture conditions enabling Ni^{2+} detection via chemical shift variations and Cu^{2+} detection via signal quenching. Although higher sensitivity would be desirable for most labile Cu^{2+} pools, these findings highlight the potential of ratiometric ^{19}F NMR probes as robust tools for noninvasive and background-free sensing of labile Cu^{2+} in opaque or autofluorescent biological systems.

Supporting Information

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

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: ^{19}F NMR probe · ATCUN motif · biological copper sensor · ratiometric detection · trifluoromethylation

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