Electronic Supplementary Information

Optimizing the amino terminal Cu(II)- and Ni(II)-binding (ATCUN) motif for Cu(II) complexes with improved inertness

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EXPERIMENTAL SECTION

Materials

All solvents, chemicals, and reagents were purchased from commercial sources and used without further purification. Fluorenylmethoxycarbonyl(Fmoc)-α-L-amino acids, coupling reagents, *N,N*-dimethylformamide (DMF), piperidine, triisopropylsilane (TIPS), trifluoroacetic acid (TFA) as well as other chemicals necessary for solid-phase peptide synthesis (SPPS) were purchased from Sigma-Aldrich (NovaBiochem). Fmoc-Rink amide aminomethyl-polystyrene resin (Fmoc-Rink amide AM Resin) was purchased from Iris Biotech. $CuCl_2 \cdot 2H_2O$, ethylenediaminetetraacetic acid in solution of 0.1 M (EDTA) and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) were purchased from Sigma-Aldrich.
HPLC was performed using gradients of mQ water (0.1 % TFA) and MeCN/$H_2$0 9:1 (0.1 % TFA).

Peptide synthesis

Peptides H_2N-Xxx_1-Xxx_2-His-Xxx_4-Gly-Lys-Gln-NH_2 and H_2N-Asp-Ala-His-Lys-NH_2 were synthesized manually following standard Fmoc/*t*Bu-SPPS protocols. The synthesis was performed on a 0.1 mmol scale on Rink amide AM (0.74 mmol/g loading, 100-200 mesh) resins. Amino acid (4 equiv) coupling was performed at room temperature for about 45 min, using 3-[bis(dimethylamino)methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate (HBTU, 3.9 equiv) as coupling agent, *N,N*-diisopropylethylamine (DIEA, 8 equiv) as base and DMF as the solvent. After coupling, the presence of unreacted *N*-terminal free amine was checked with TNBS (2,4,6-Trinitrobenzenesulfonic acid) reagent, and the coupling was repeated in case of a positive test. Capping of the unreacted free amine group was carried out using 5% acetic anhydride and 10% DIEA in DMF for 5 min. *N*-terminal Fmoc deprotection was performed using 20% piperidine in DMF. Peptides were cleaved from the resin and acid-labile side-chain deprotected by treatment with TFA/TIPS/H_2O (95:2.5:2.5) for 2 hours. The peptide crude was precipitated with cold ether and purified by reverse-phase HPLC (High Performance Liquid Chromatography) on a C18 column (XBridge Peptide BEH C18 OBD Prep Column from Waters, 19 mm × 150 mm, pore size 130 Å, particle size 5 μm) using a LaPrep Sigma (VWR International) instrument with UV–vis detection at 214 nm and 280 nm. The purity of fractions was evaluated by reverse-phase analytical HPLC (Hitachi Primaide equipped with a C18 column (XBridge C18 BEH 300 Å, 5 μm, 4,6 × 150 mm, 37 °C) (Figure S3). The method used a gradient of buffers A (1 % TFA in mQ water) and B (0.1 % TFA in MeCN/$H_2$0 9:1): after 2 minutes at 5 %, the percentage of B ramped up to 50 % in 15min, and then 100 % of B was applied for 5 min (purge), before equilibrating at 5%.

The mass of each peptide was measured on a LC-MS Agilent 1100 series equipped with a C18 column (XBridge C18 BEH 300 Å, 5 µm, 4,6 × 150 mm, 37 °C) and coupled to an ESI mass detector (Agilent 6120) (Figure S4). Pure peptide fractions were lyophilized.

The peptide Tyr-Tyr-His-Trp-Gly-Gly-Gly-Lys-NH_2, that has another solubility tag and that was used for the ^{64}Cu radiolabeled experiments, was synthesized, purified and analyzed in an analogous manner, with the exception of the coupling step, which was doubled here and performed with 5 eq. Fmoc-protected amino acid, 5 eq. HBTU and 10 eq. DIEA.

Preparation of stock solutions

All stock solutions were prepared in ultrapure water (ρ = 18.2 MΩ·cm^{-1}). The concentration was calculated based on the molecular weight of the peptide and the counter ions (considering 1 TFA anion per basic residue (His, Lys) and terminal amine), and we confirmed the concentrations of the peptides by absorption at 280 nm using ε^{280nm}(Trp) = 5690 mol·L^{-1}·cm^{-1} and ε^{280nm}(Tyr) = 1280 mol·L^{-1}·cm^{-1}.[1] The concentration of these solutions was also corrected by copper titration. The concentration of $CuCl_2 \cdot 2H_2O$ stock solution was verified by UV–vis, directly with the absorbance of the aqueous copper (ε^{780nm} = 12 M^{-1} cm^{-1}),[2] and also by titrating it with a commercial 0.1 M standard solution of EDTA in HEPES buffer pH 7.4. During such titration, our experimental value of the absorbance coefficient of Cu-EDTA at 730 nm was in agreement with that of literature (ε^{730nm} = 85 M^{-1} cm^{-1}).[2]

Kinetic assays by UV-vis spectroscopy

UV–vis spectra were recorded on a Cary 60 spectrophotometer fitted with a Peltier temperature controller using a 1 cm path quartz cuvette (100 µL volume). Kinetics of Cu(II) transchelation from the peptide to EDTA was monitored at 25 °C by following the bands at 515 nm and 730 nm corresponding to the copper-peptide complex and copper-EDTA complex, respectively. Cu(II)-peptide complexes were pre-formed at 550 µM concentration of peptide and 500 µM of Cu(II) in 50 mM HEPES buffer, pH 7.4. EDTA (10 mM final concentration) was added to the resulted solution giving the starting time point of the transchelation reaction. 200 µL of paraffine oil were slowly added to avoid evaporation over time. Spectra were collected every 30 min and were background corrected with the signal of the buffer and the cuvette. Data points were fitted with the formula $A = (A_0 - A_{plateau}) \times \exp(-k_{transfer} \times t) + A_{plateau}$ (Figure S1).

Competition assay by fluorescence spectroscopy

Tyrosine fluorescence was monitored using a FluoroMax Plus (Horiba) fluorimeter equipped with a water-circulating temperature controller. The experiments were performed in a 2x10 mm quartz cuvette, in 500 µL of sample total volume, with the final concentrations: 10 µM YYHGGKQ-NH_2, 10 µM DAHK-NH_2 (ie 1 eq.), 8 µM $CuCl_2$ (ie 0.8 eq.) and 10 mM HEPES buffer pH 7.4. Two order of addition were used, in order to start the competition from the two opposite "ends". First ("Path A"), the YYHGGKQ-NH_2 peptide was added to the buffer, then $CuCl_2$ to form the complex, and the DAHK-NH_2 was added at last. Second ("Path B"), DAHK-NH_2 was added to the buffer, then $CuCl_2$ to form the complex, and the YYHGGKQ-NH_2 peptide was added at last. To monitor the competition, emission fluorescence spectra were recorded from 290 to 500 nm, with an excitation at 275 nm. At the plateau (i.e. when system reached equilibrium) final concentration of each entity was deduced, allowing the calculation of equilibrium constant K = ([CuDAHK][YYHGGKQ]) / ([CuYYHGGKQ][DAHK]), which corresponds also to the ratio K_{app}(DAHK) / K_{app}(YYHGGKQ). Because K_{app}(DAHK) is known, we can finally deduce K_{app}(YYHGGKQ).

Competition assay by UV-vis spectroscopy

Competition between ATCUN peptides and EGTA was monitored at 37 °C by following the bands at 515 nm and 680 nm corresponding to the copper-peptide complex and copper-EGTA complex, respectively. Cu(II)-peptide complexes were pre-formed at 500 µM concentration of peptide and 450 µM of Cu(II) in 50 mM HEPES buffer, pH 7.4. Equimolar concentration of EGTA (500 µM) was added to the resulted solution giving the starting time point of reaction. 200 µL of paraffin oil were slowly added to avoid evaporation over time. Spectra were collected every 30 min, were background corrected with the signal of the buffer and the cuvette and collection was stopped after equilibrium was reached (Figure S2). The opposite reaction, in which Cu(II)-EGTA complex was pre-formed and equimolar concentration of ATCUN peptide was added, was also monitored using the same conditions. Final concentration of each entity was deduced, allowing the calculation of equilibrium constant K, which corresponds also to the ratio K_{app}(EGTA) / K_{app}(peptide). Because K_{app}(EGTA) is known (see below), we can finally deduce K_{app}(peptide).

Calculation of the apparent affinity constants of EDTA and EGTA

A ligand L (EGTA or EDTA) have several protonation states, associated to different pKa. When L is bound to Cu^{2+}, it is fully deprotonated, a form noted L^{4-}.

The affinity of a L ligand for a given metal ion is expressed as an association constant considering the ligand is its L^{4-} state only:

$$K_{ML} = \frac{[ML]^{(4-n)-}}{[M^{n+}] \times [L^{4-}]}$$

For Cu(II) complexes of EDTA and EGTA, the log K_{ML} values are 18.7 and 17.6, respectively.[3,4]

However, at the pH of our experiments (pH 7.4), different protonated states are in equilibrium (L^{4-}, HL^{3-}, H_2L^{2-}, etc…). Hence the affinity value found in the textbooks (i.e. K_{ML}) have to be corrected at a given pH by considering the proportion of the L^{4-} species. It results in an "apparent affinity" (K_{app}), that also called "conditional affinity".

$K_{app} = K_{ML}$ x α

With α = $[L^{4-}] / [L]_{Tot}$ where $[L]_{Tot}$ is the total concentration of L, i.e. the sum of all its forms. The ratio $[L^{4-}] / [L]_{Tot}$ is the fraction of L that is fully deprotonated (L^{4-} form).

$$\alpha = \frac{[L^{4-}]}{[H_4L] + [H_3L^-] + [H_2L^{2-}] + [HL^{3-}] + [L^{4-}]}$$

α can be determined properly by considering all the deprotonations steps:

1st deprotonation $H_4L \leftrightarrow H_3L^{1-} + H^+$, to which $K_a(1)$ corresponds

2nd deprotonation $H_3L^{1-} \leftrightarrow H_2L^{2-} + H^+$, to which $K_a(2)$ corresponds

3rd deprotonation $H_2L^{2-} \leftrightarrow HL^{3-} + H^+$, to which $K_a(3)$ corresponds

4th deprotonation $HL^{3-} \leftrightarrow L^{4-} + H^+$, to which $K_a(4)$ corresponds

With $$\frac{1}{\alpha} = \frac{[L^{4-}]}{[L^{4-}]} + \frac{[HL^{3-}]}{[L^{4-}]} + \frac{[H_2L^{2-}]}{[L^{4-}]} + \frac{[H_3L^-]}{[L^{4-}]} + \frac{[H_4L]}{[L^{4-}]}$$

Hence $$\frac{1}{\alpha} = 1 + \frac{[H_3O^+]}{K_a(4)} + \frac{[H_3O^+]^2}{K_a(3)K_a(4)} + \frac{[H_3O^+]^3}{K_a(2)K_a(3)K_a(4)} + \frac{[H_3O^+]^4}{K_a(1)K_a(2)K_a(3)K_a(4)}$$

However, a good approximate of α can be obtained simply by considering only the ratio between $[L^{4-}]$ and the species of L. At a given pH in which one species largely predominates (i.e. represent more than 99 %), one can approximate that the concentration of that one species is equal to the sum of concentrations of all L species.

In the case of EDTA, we have $pK_a(3)$ = 6,11 and $pK_a(4)$ = 10.17.[4] Then, at pH 7.4, we have $pK_a(3)<(pH-1)$ and $(pH+1)<pK_a(4)$, which means that $[HL^{3-}] \approx [L]_{Tot}$, hence we consider α to be:

$$\alpha = \frac{[L^{4-}]}{[H_4L] + [H_3L^-] + [H_2L^{2-}] + [HL^{3-}] + [L^{4-}]} \approx \frac{[L^{4-}]}{[HL^{3-}]} = \frac{K_a(4)}{[H_3O^+]} = \frac{10^{-pKa(4)}}{10^{-pH}}$$

Hence, we calculate K_{app} of EDTA at pH 7.4 as:

$\log K_{app}(EDTA)_{pH\ 7.4} = \log (K_{ML} \text{ x } \alpha) = \log (K_{ML}) - pK_a(4) + pH = 18.7 - 10.17 + 7.4 = 15.93$

This means that EDTA at pH 7.4 is a stronger ligand for Cu^{2+}, by more than 1 order of magnitude, than the most stable ATCUN ligands reported so far.[5,6]

In the case of EGTA, at 20 °C we have $pK_a(2) = 2.66$, $pK_a(3) = 8.85$ and $pK_a(4) = 9.47$.[4] Then, at pH 7.4, we have pKa(2)<(pH-1) and (pH+1)<pKa(3), which means that $[H_2L^{2-}] \approx [L]_{Tot}$, hence we consider α to be:

$$\alpha = \frac{[L^{4-}]}{[H_4L] + [H_3L^-] + [H_2L^{2-}] + [HL^{3-}] + [L^{4-}]} \approx \frac{[L^{4-}]}{[H_2L^{2-}]} = \frac{K_a(3)K_a(}{[H_3O^+]}$$

Hence, we calculate K_{app} of EGTA at pH 7.4 as:

$$\log K_{app}(\text{EGTA})_{pH\ 7.4} = \log (K_{ML} \times \alpha) = \log (K_{ML}) - pK_a(3) - pK_a(4) + 2\ pH$$

$$= 17.6 - 8.85 - 9.47 + 2 \times 7.4 = 14.08$$

Production of ^{64}Cu and Radiochemistry

^{64}Cu was produced using a modified TBP/TK201 column method, as previously described.[7]

Production was carried out at IPHC (Strasbourg, France) using a TR 24 ACSI cyclotron via the ^{64}Ni(p,n)^{64}Cu nuclear reaction, with a proton energy of 12 MeV. The resulting ^{64}Cu was synthesized as [^{64}Cu]$CuCl_2$. The solution was evaporated to dryness and subsequently reconstituted in deionized water.

All radioactivity measurements were performed using a Capintec® CRC-55tR dose calibrator. Radio thin-layer chromatography (radio-TLC) was conducted on aluminum-backed silica gel 60 F_{254} plates (Sigma-Aldrich), and analysis was performed using a TLC scanner (miniGita; Raytest, Straubenhardt, Germany).

Radiolabeling with ATCUN and EGTA

For this experiment and the *in vitro* stability assay in serum (see below), the ATCUN peptide used was YYHWGGGK-NH_2. Its solubility tag differs from the other peptides by being one residue longer (Gly) and having a Gly instead of the Gln. These minor changes are expected not to have any impact on the coordination properties of the peptide as the coordinating motif of Cu(II) remains identical.

[^{64}Cu]ATCUN was prepared by mixing [^{64}Cu]$CuCl_2$ (3 MBq in 25 µL of water), HEPES buffer (8 µL, 50 mM, pH 7), and ATCUN (17 µL, 150 µM in HEPES buffer 50mM, pH 7). The reaction mixture was incubated at 37 °C for 15 minutes.

[^{64}Cu]EGTA was prepared by mixing [^{64}Cu]$CuCl_2$ (1.08 MBq in 10 µL of water), HEPES buffer (3 µL, 50 mM, pH 7), and EGTA (7 µL, 150 µM in HEPES buffer 50mM, pH 7). The reaction mixture was incubated at 37 °C for 15 minutes.

Radiolabeling efficiency was assessed by radio thin-layer chromatography (radio-TLC) using a mobile phase composed of HEPES buffer (50 mM, pH 7) and acetonitrile in a 6:4 (v/v) ratio. A 2–5 µL aliquot of the reaction mixture was applied directly to the TLC plate.

The RadioTLC of [^{64}Cu]EGTA (Figure S6) was included as a control to validate the radiolabeling and chromatographic method used for [^{64}Cu]ATCUN. Both [^{64}Cu]ATCUN and [^{64}Cu]EGTA migrate with characteristic Rf values (~0.8) distinct from unchelated ^{64}Cu, thus confirming complete complexation (i.e. radiolabeling) and validating this chromatographic method to monitor later the serum stability of this complex.

In Vitro Stability in serum

The *in vitro* serum stability of [^{64}Cu]ATCUN was evaluated by incubating 50 µL of the radiolabeled compound in 200 µL of mouse serum at 37 °C (Invitrogen). Samples were collected at 1, 3, 18, and 24 hours (Figure S7). Stability was monitored using instant radio-TLC as described above.

SUPPORTING DATA

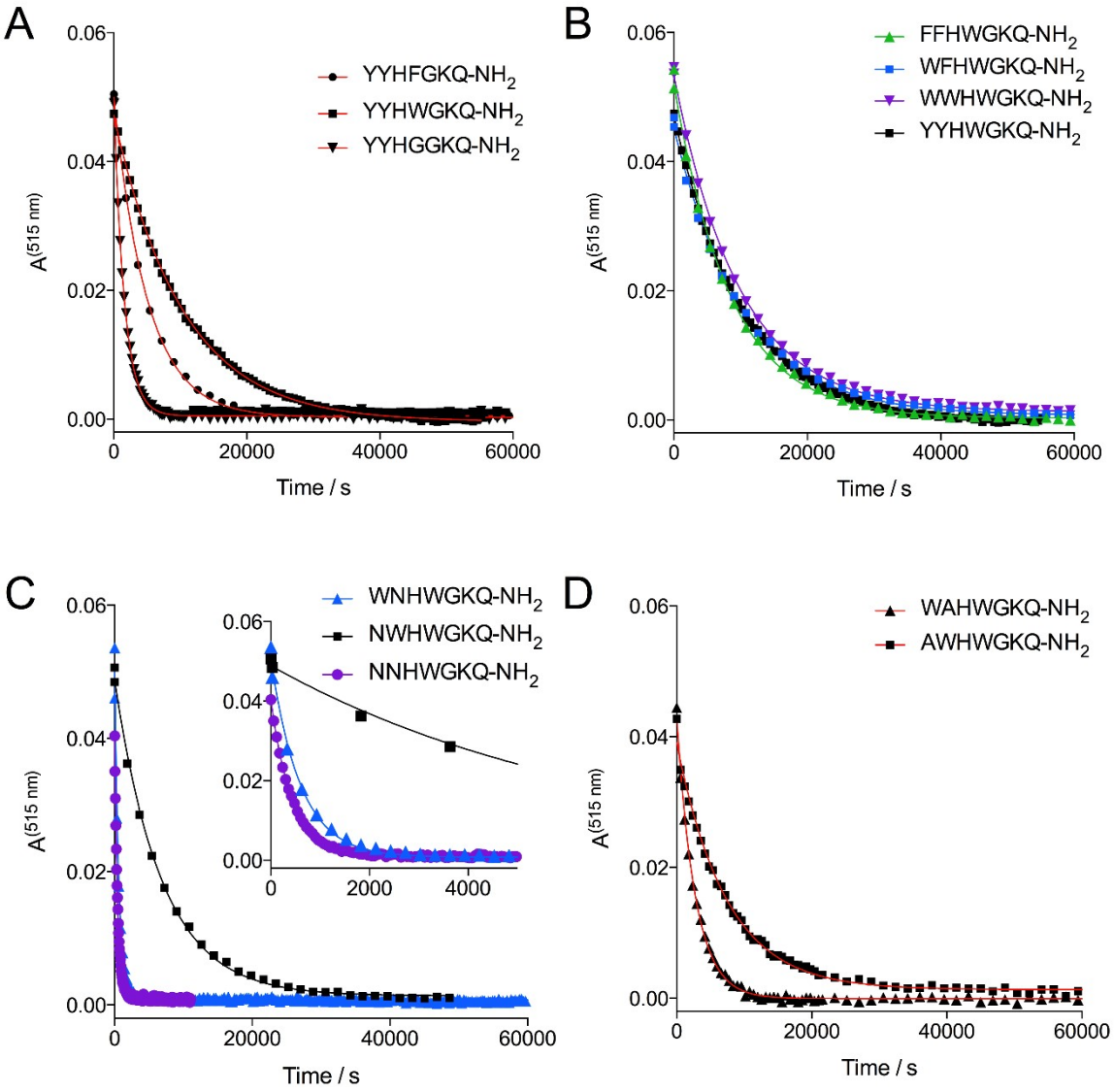


Figure S1. Kinetics of transchelation of Cu(II), from the ATCUN peptides to the competitor (EDTA). (A) Effect of the residue at the fourth position. (B) The nature of the aromatic residues at the two first positions have little influence. (C,D) A Trp residue has a different effect on the inertness of the copper-peptide complex, weather it is at positions 1 or 2. A significant decrease of the inertness properties was observed when residue at position two was not a Trp nor an aromatic. Conditions: 550 µM peptide, 0.9 equiv. $CuCl_2$, 20 equiv. EDTA, HEPES buffer 50 mM, pH 7.4, 25 °C.

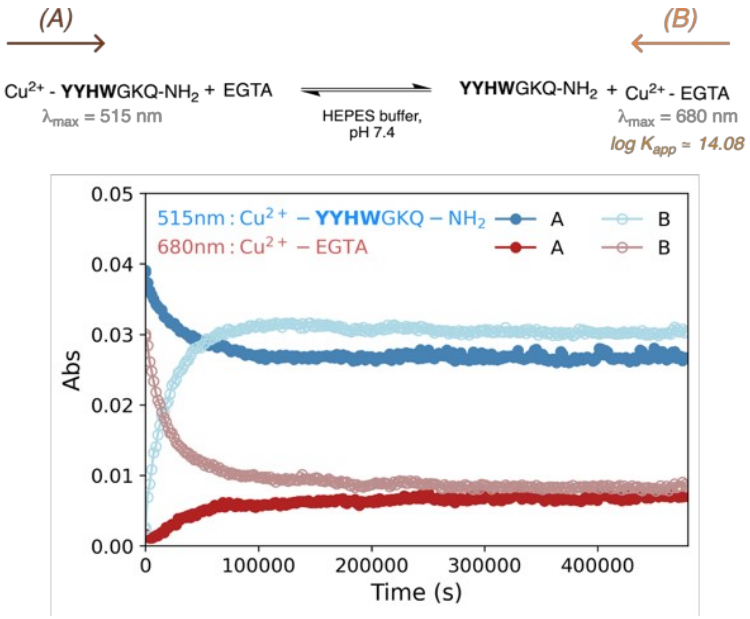
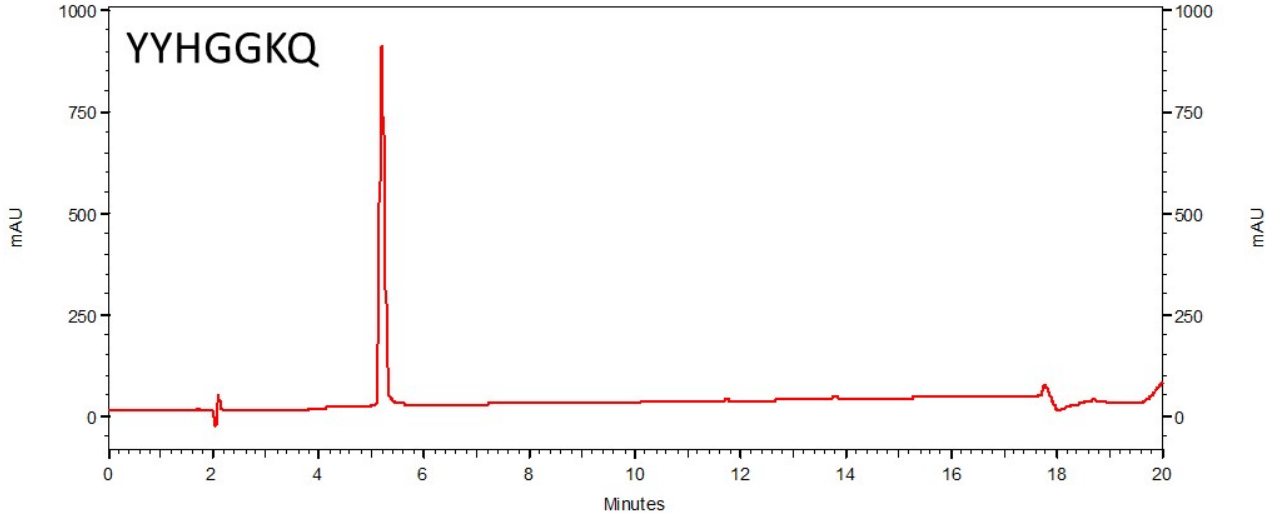


Figure S2. Evaluation of the thermodynamic stability of the Cu(II)-YYHWGKQ-NH_2 complex. Equilibrium reaction was monitored by UV-spectroscopy with equimolar concentration of EGTA as competitor. In the experiment "A", the copper in first coordinated into the peptide and then the competitor is added, while in the experiment "B", the copper in first coordinated into EGTA and then the peptide is added. Conditions: 500 µM peptide, 1 equiv. EGTA, 0.9 equiv. $CuCl_2$, 50 mM HEPES buffer, pH 7.4.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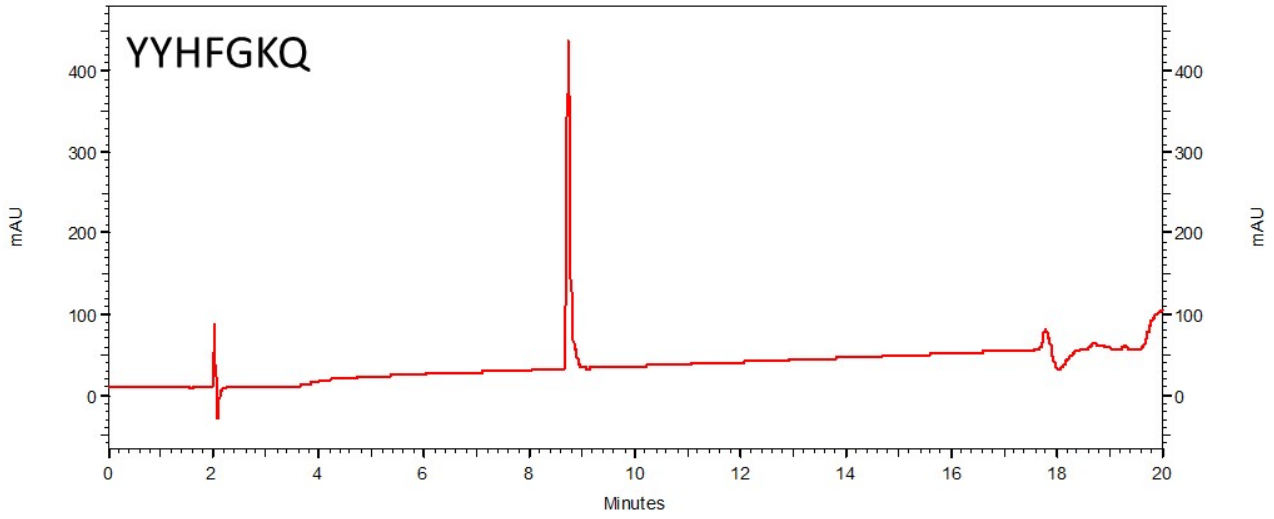
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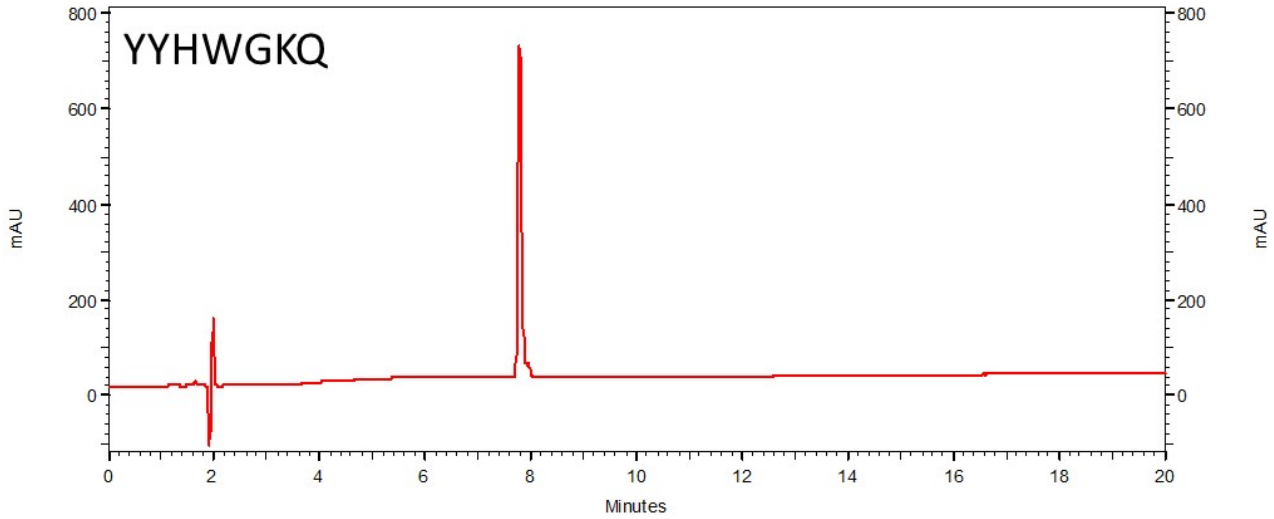
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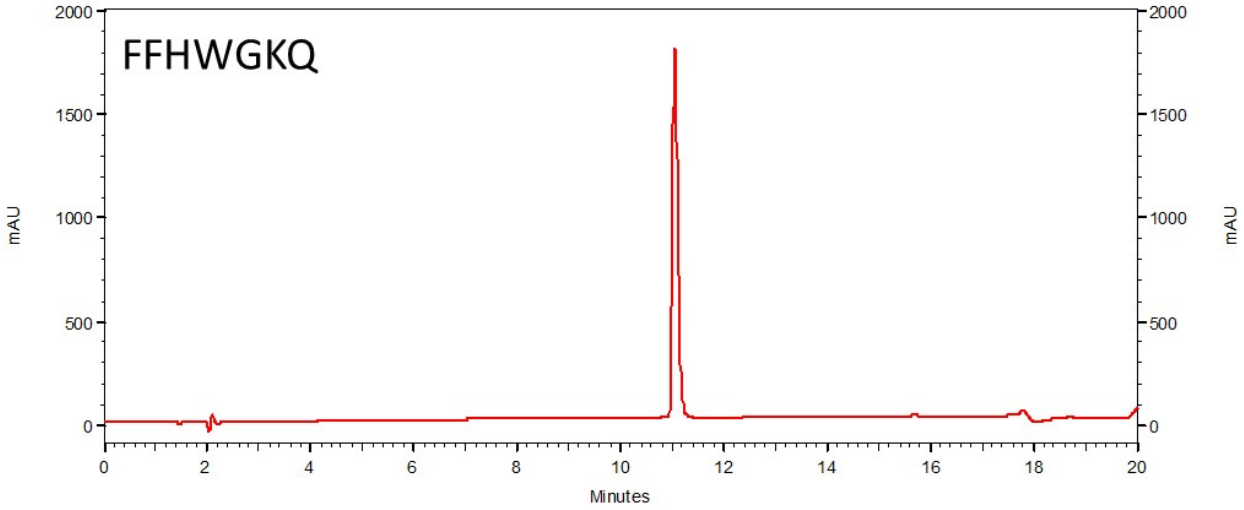
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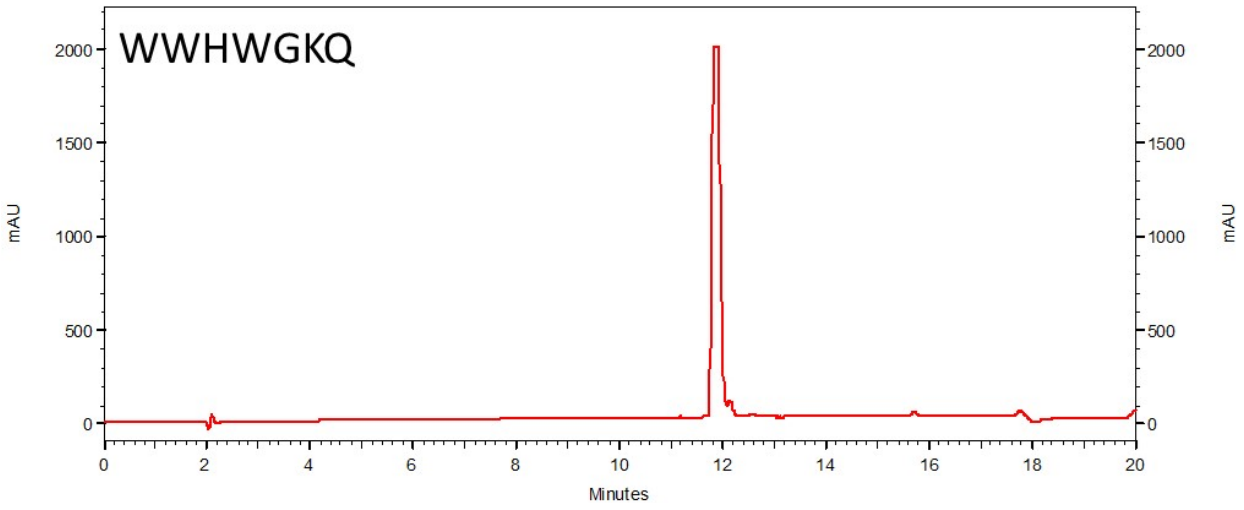
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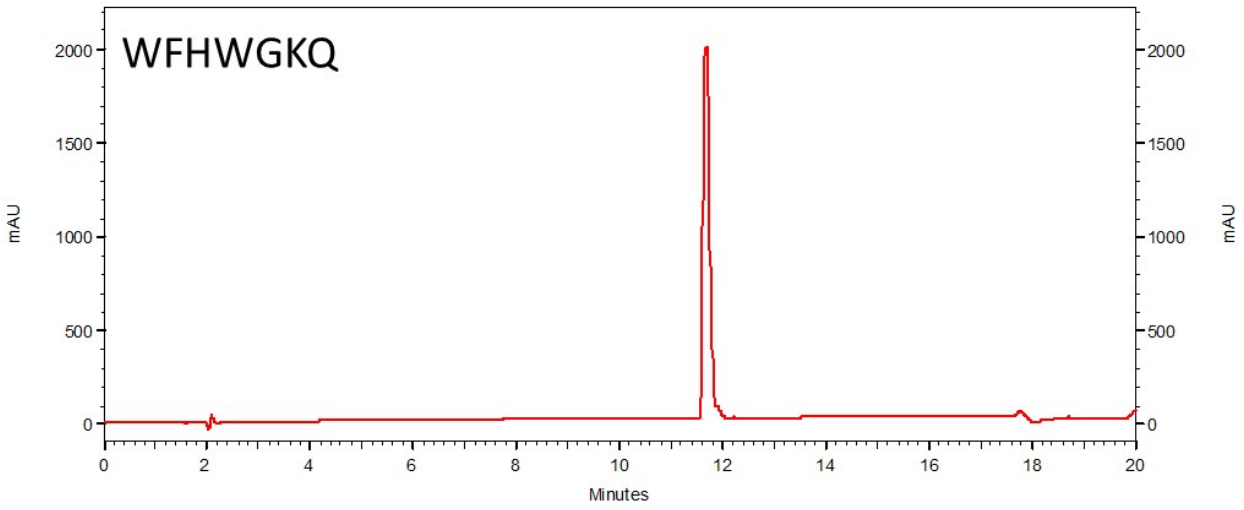
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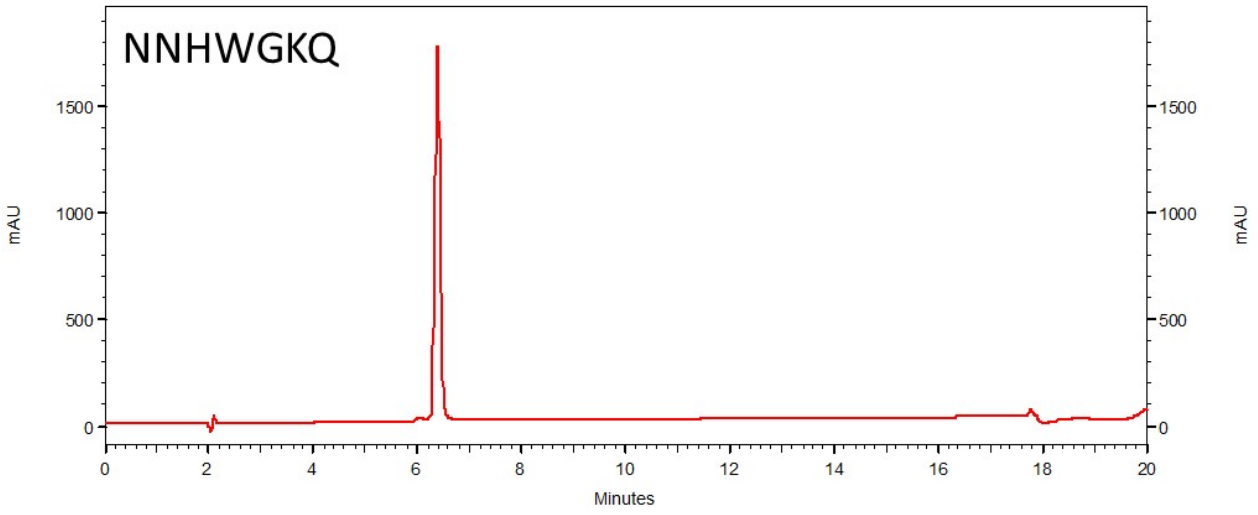
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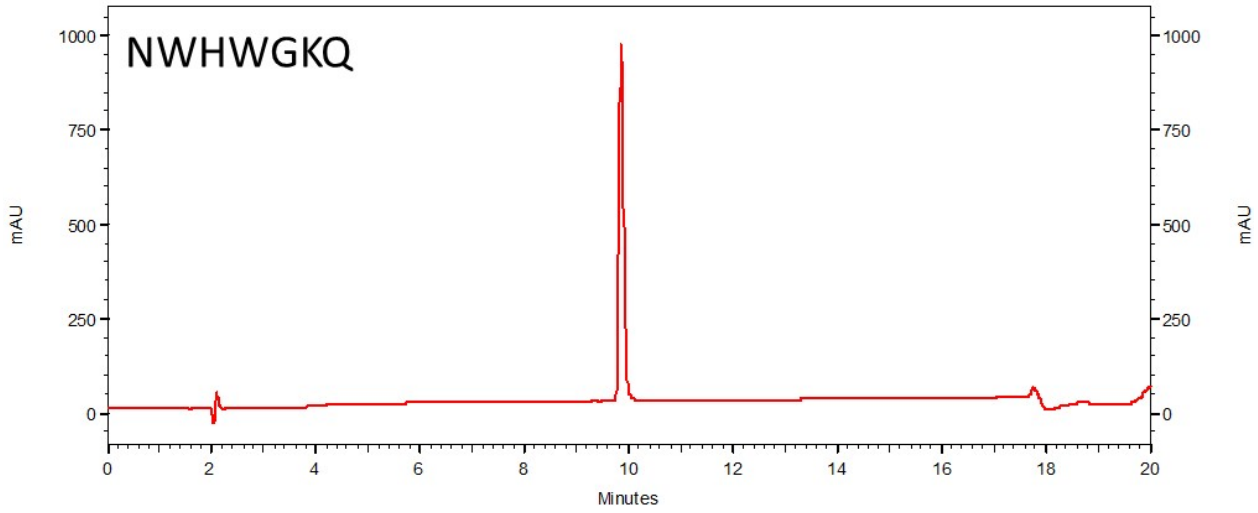
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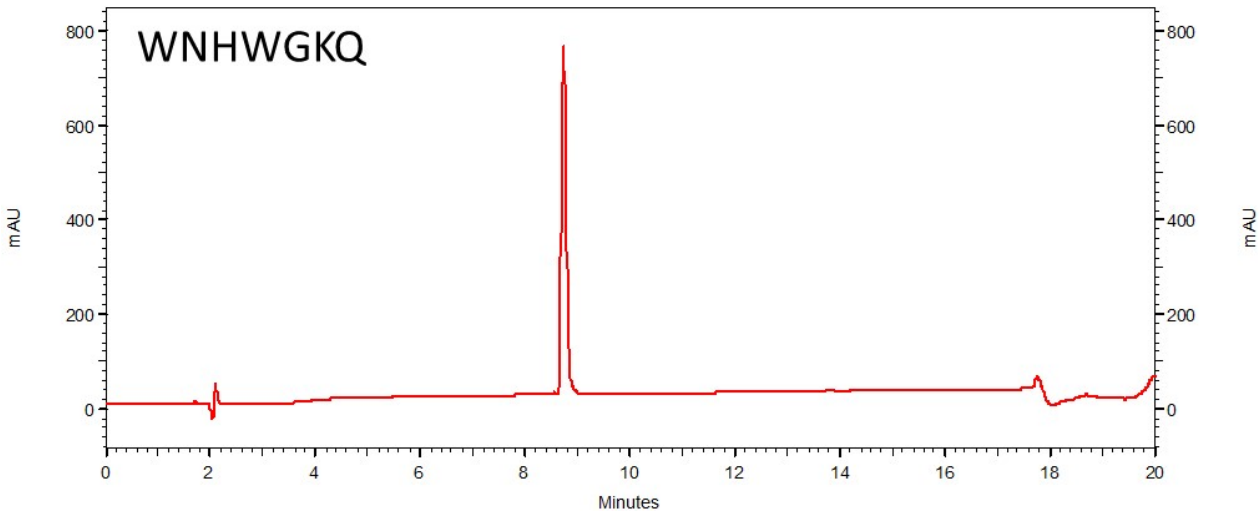
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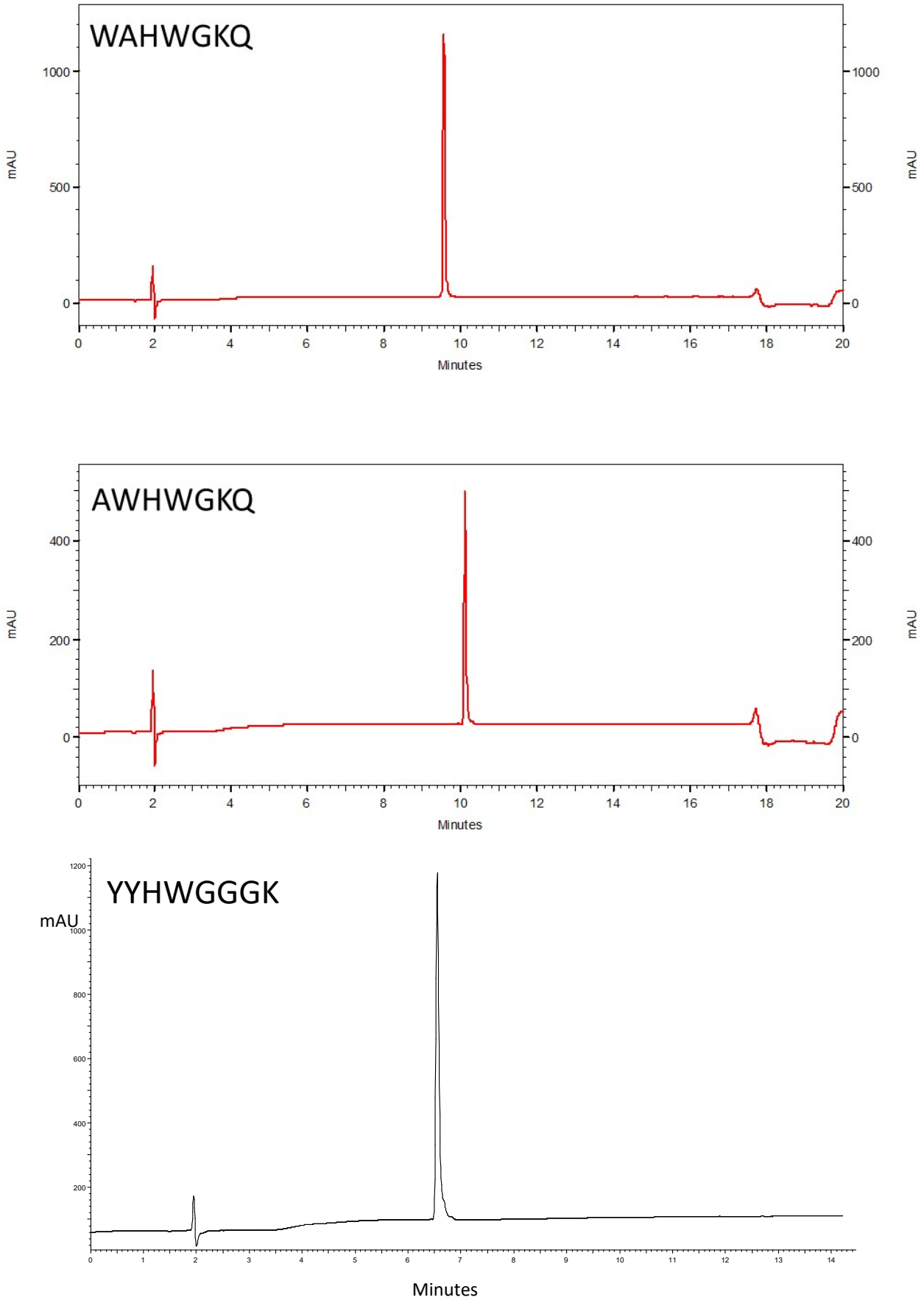

Figure S3. Analytical chromatograms at 214 nm of the purified peptides.

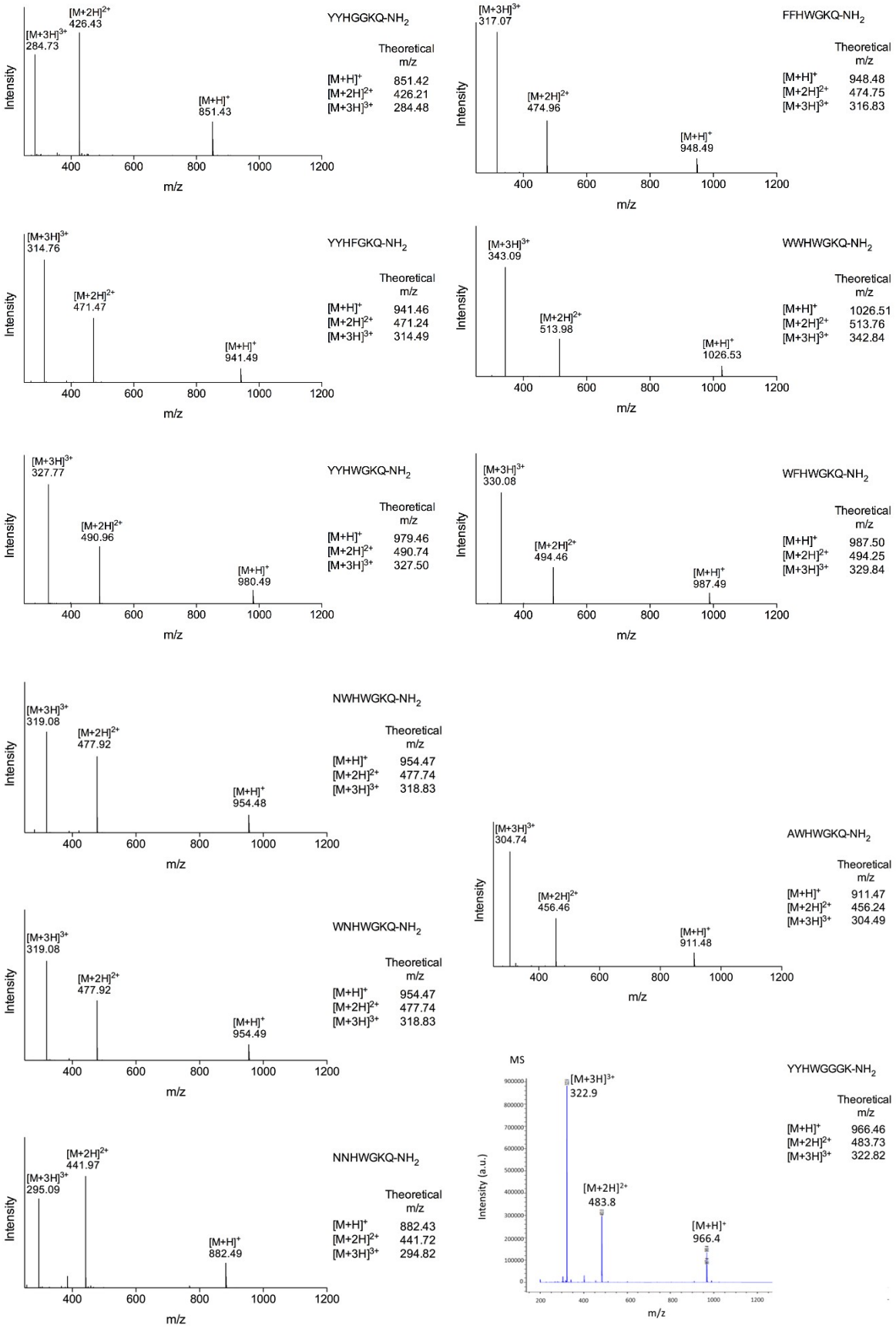

Figure S4. Mass spectra for each peptide, extracted from the LC-MS analysis.

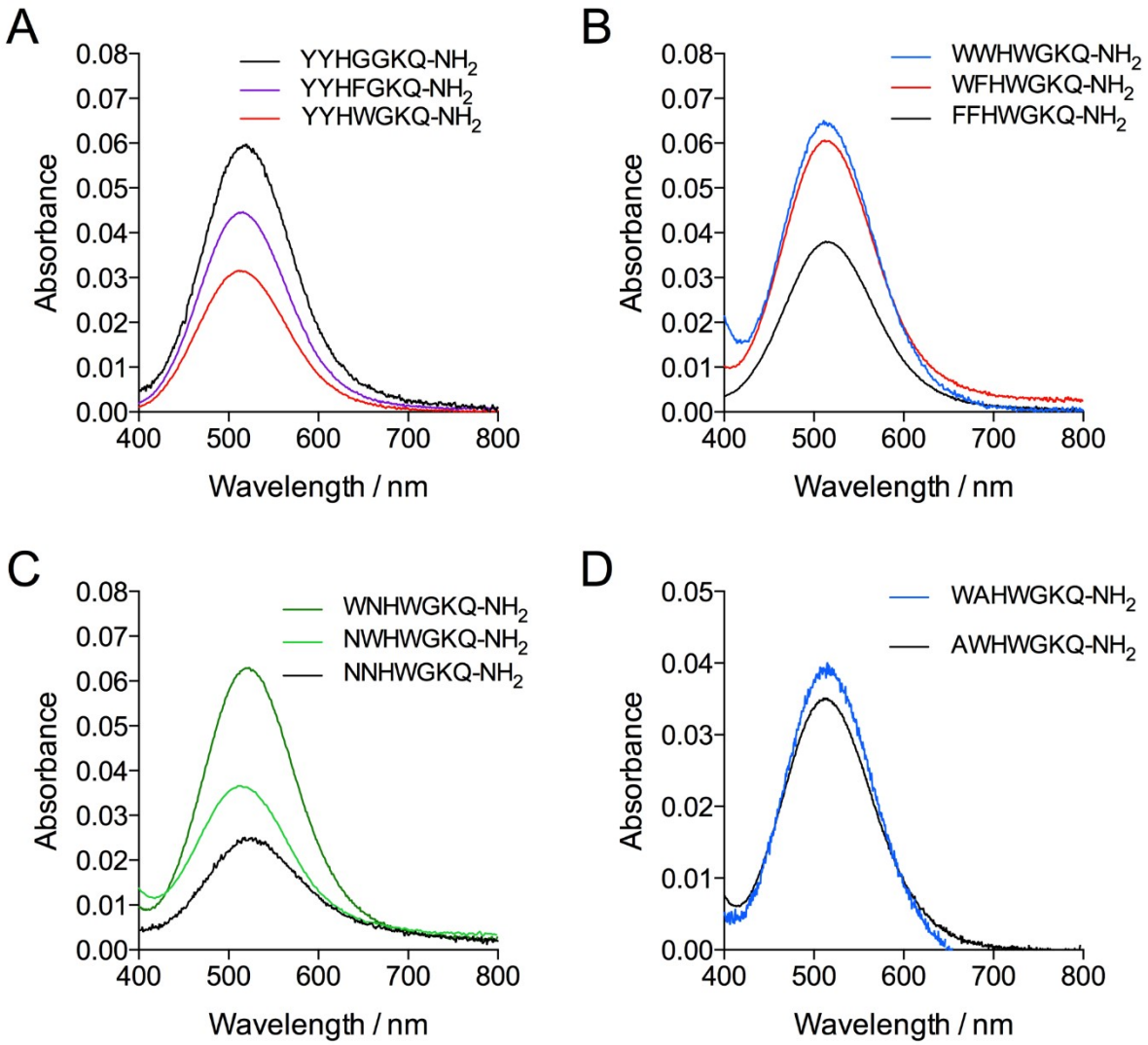

Figure S5. Electronic absorption (Visible window) spectra of the different Cu^{2+}-ACTUN complexes studied in this report. These spectra were recorded at different concentrations: **A**) YYHGGKQ-NH_2 (Black, 560 µM), YYHFGKQ-NH_2 (Blue, 420 µM), YYHWGKQ-NH_2 (Red, 310 µM). **B**) FFHWGKQ-NH_2 (Black, 370 µM), WFHWGKQ-NH_2 (Blue, 560 µM), WWHWGKQ-NH_2 (Red, 560 µM). **C**) NNHWGKQ-NH_2 (Black, 260 µM), NWHWGKQ-NH_2 (Ligth green, 330 µM), WNHWGKQ-NH_2 (Dark green, 560 µM). **D**). AWHWGKQ-NH_2 (Black, 400 µM), WAHWGKQ-NH_2 (Blue, 450 µM).

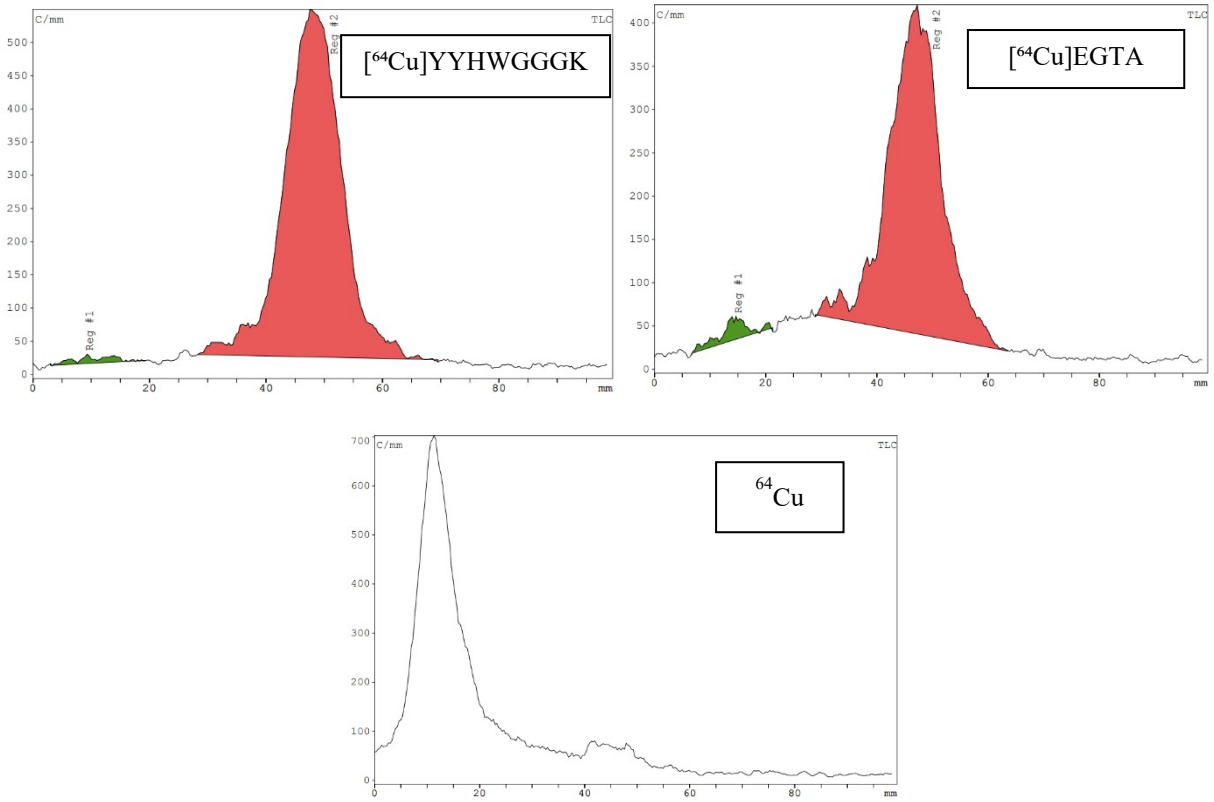

Figure S6. RadioTLC of radiolabeled [^{64}Cu]YYHWGGGK-NH_2 and [^{64}Cu]EGTA complexes, and free ^{64}Cu. Green area = uncomplexed ^{64}Cu ; red area = ^{64}Cu complexed with YYHWGGGK-NH_2 or EGTA.

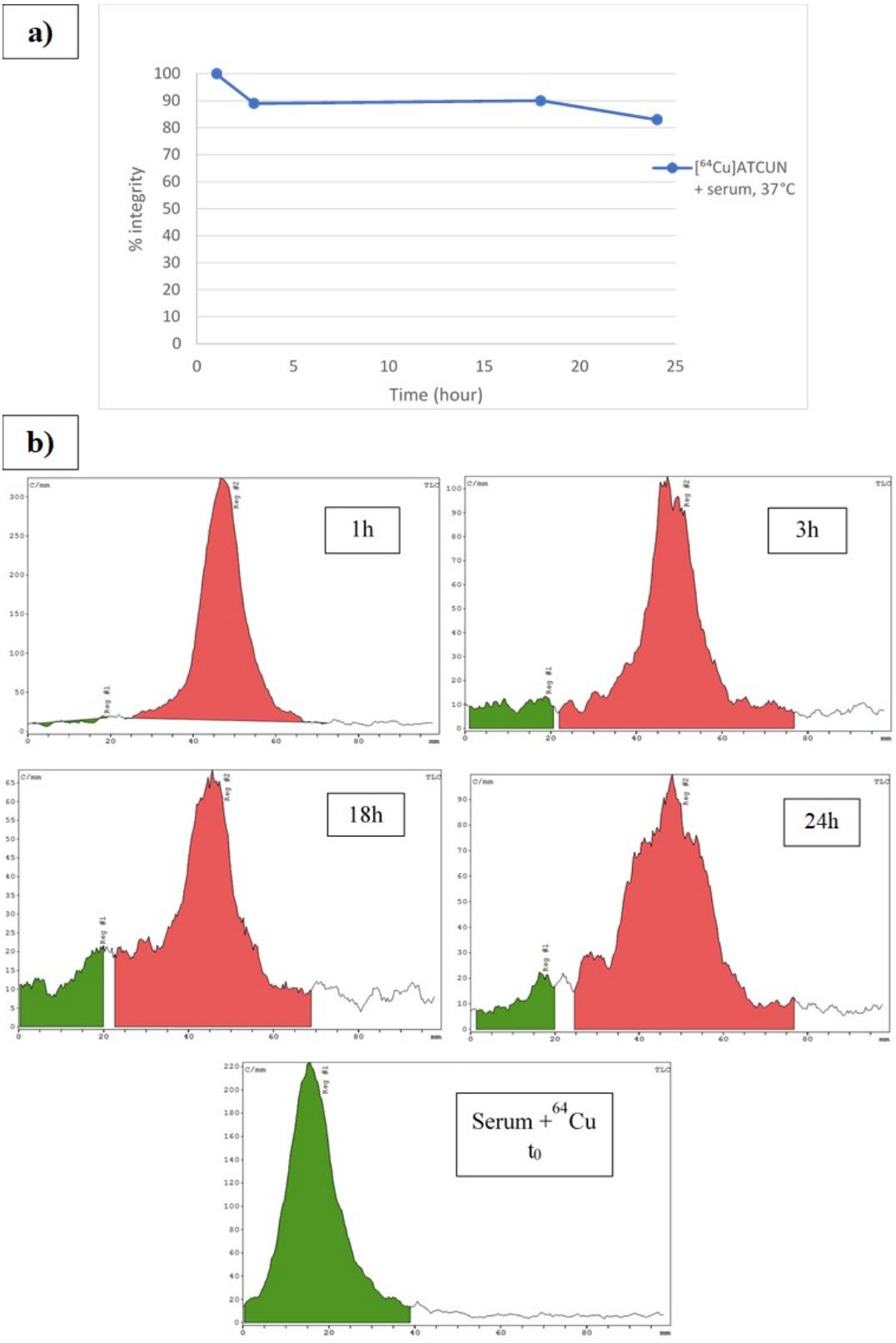

Figure S7. Stability of the radiolabeled [^{64}Cu]YYHWGGGK-NH_2 complex in mouse serum for 24h and the control serum with ^{64}Cu. a) % of integrity of [^{64}Cu]YYHWGGGK-NH_2 as function of time in serum at 37°C. b) radioTLC. Green area = decomplexed ^{64}Cu ; red area = [^{64}Cu]YYHWGGGK-NH_2 complex. The control (ie serum + ^{64}Cu) was analyzed at the time of ^{64}Cu addition at t_0.

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