Influence of Amino Acid Sequence in a Peptidic Cu^+-Responsive Luminescent Probe Inspired by the Copper Chaperone CusF

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Supporting Information

Content

Abbreviations

PyBOP: (Benzotriazol-1-yloxy)tripyrrolidino-phosphonium-hexafluorophosphate; COMU: (1-Cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophos-phate; DOTA: 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; $Pd(PPh_3)_4$: *tetrakis*(triphenylphosphine)-palladium(0); TIS: triisopropylsilane; DIEA: diisopropylethylamine; TFA: trifluoroacetic acid; MeCN: acetonitrile; DCM: dicholoromethane; Et_2O: diethylether; DMF: *N*,*N*-dimethylformamide; *t*Bu: *tert*-butyl; Trt: trityl; Fmoc: 9-fluorenylmethoxycarbonyl; Boc: *tert*-butyloxycarbobyl; Alloc: allyloxycarbonyl; HPLC: high performance liquid chromatography; ESI: electrospray ionization; MS: mass spectrometry; UV-Vis: ultraviolet-visible; CD: circular dichroism.

Materials and methods

Reagents and solvents: DOTA-*tris*(*t*Bu)ester was purchased from Chematech. N-α-Fmoc-protected amino acids for peptide synthesis, PyBOP and COMU coupling reagents and resins were obtained from Novabiochem or Iris Biotech. Other reagents for peptide synthesis, solvents, buffers and metal salts were purchased from Sigma-Aldrich.

All buffer or metal solutions for spectroscopic measurements were prepared with MilliQ water (Millipore) or pure deuterium oxide (D_2O, 99.9% Eurisotop) in a glovebox. Buffer solutions were treated with Chelex 100 resin (Biorad) to remove metal traces. The pH of buffer solutions was adjusted using NaOH and $HClO_4$ to ensure that no chloride is present during Ag^+ complexation studies.

Analyses and purifications: HPLC analyses and purifications were performed on an Agilent Infinity 1200 system or on a VWR LaPrep system. Analytical and preparative HPLC separations were performed at 2 and 14 mL/min, respectively, using Chromolith (Merck) RP-18e 10-4.6mm and XBridge Prep (Waters) C18 5 µm 150-19mm columns, respectively. Mobile phase consisted in a gradient of solvent A (0.1% TFA in H_2O) and B (0.1% TFA in MeCN/H_2O 9:1). For analytical separations, Method A consisted in 5% B during 1 min followed by a 5 to 50 % B gradient in 14 min at 2 mL/min. The eluent was monitored by electronic absorption at 214 nm and 280 nm. ESI-MS analyses were performed on a Thermo LXQ spectrometer.

Spectroscopy: UV-Vis spectra were recorded on a Perkin-Elmer Lambda 35 spectrophotometer or on a Varian Cary 50 spectrophotometer. CD spectra were recorded on an Applied Photophysics Chirascan spectropolarimeter. Luminescence spectra were recorded on a Cary Eclipse spectrometer. Phosphorescence lifetimes were measured in time-resolved mode on a Cary Eclipse spectrometer. UV-Vis, CD and Cary Eclipse luminescence spectrometers are equipped with a thermo-regulated cell holder and spectra were recorded at 298 K.

Time-resolved emission studies were performed using degassed solutions at 20 °C using a frequency-tripled Nd:YAG amplified laser system (30 ps, 30 mJ @1064 nm, 20 Hz, Ekspla model PL 2143) whose output was used to pump an optical parametric generator (Ekspla model PG 401) producing tunable excitation pulses in the range 410-2300 nm. All light signals were analysed by a spectrograph (Princeton Instruments Acton model~SP2300) coupled with a high dynamic range streak camera (Hamamatsu Photonics C7700, 1 ns – 1 ms). Data were analysed using an in-house written software developed in LabVIEW 2014 system-design platform and development environment. The trust-region dogleg algorithm[1] (supported by LabVIEW 2014) was applied to determine the set of parameters that best fit the set of input data. The trust-region dogleg algorithm was used instead of Levenberg-Marquardt algorithm, the latter being less stable in most cases during optimization process, because trust region methods are robust, and can be applied to ill-conditioned problems.

Peptide synthesis

Linear precursor: Peptide synthesis was performed by solid-phase peptide synthesis (SPPS) on NovaPEG HMPB resin (substitution 0.52 mmol/g, 250 mg, 0.13 mmol) using Fmoc chemistry. The coupling of the first amino acid (Gly) was performed manually as followed. Fmoc-Gly-OH (1.4 mmol, 416 mg) and DIPCDI (0.7 mmol, 0.1 mL) were dissolved in anhydrous DCM (8 mL) and DMF (4 mL) under argon. After stirring for 20 min, DCM was evaporated under reduced pressure. The solution was poured into the reactor containing the resin and DMAP (0.04 mmol, 5 mg) was added. After stirring for 30 min, the resin was washed with DMF and DCM. Then, the linear precursor was assembled on a CEM Corporation Liberty1 peptide synthesizer. Fmoc removal was performed by two successive treatments with 20 % piperidine in DMF (10 and 5 min) at room temperature. Coupling reactions were performed with N-α-Fmoc-protected amino acids (0.2 M in DMF, 5 eq.), PyBOP (0.5 M in DMF, 5 eq.) and DIEA (2 M in NMP, 10 eq.) for 30 min at room temperature, except for ^{D}Pro (double coupling, 1h each), Aib (activation with COMU instead of PyBOP, double coupling, 2h each) and the first Met (double coupling, 1h each). Each coupling was followed by a capping treatment performed with 2 % Ac_2O in DMF and DIEA (2 M in NMP, 10 eq.) for 5 min at room temperature. Resin cleavage was performed using 1% TFA in DCM. The solution containing the protected peptide was evaporated under reduced pressure. The residue was dissolved in a minimum of DCM and Et_2O was added to promote precipitation. The supernatant was removed and the solid was washed twice with Et_2O and dried. It was used without further purification in the next step.

Cyclization: The powder corresponding to the linear precursor was dissolved in degassed DMF (1 mg / 1 mL) and PyBOP (0.26 mmol, 2 eq., 137 mg) and DIEA (6.5 mmol, 60 eq., 1.1 mL) were added. The solution was stirred overnight at room temperature. The solvent was evaporated under reduced pressure and Et_2O was added. The supernatant was removed and the solid was washed twice with Et_2O and dried. It was used without further purification in the next step.

Alloc removal: The cyclic peptide was reacted with $Pd(PPh_3)_4$ (0.017 mmol, 0.1 eq., 20 mg) and phenylsilane (4.3 mmol, 25 eq., 0.5 mL) in degassed anhydrous DCM (20 mL) for 1h in the dark. The solution was evaporated under reduced pressure. The residue was dissolved in a minimum of DCM/MeOH (1:2) mixture before precipitation using Et_2O. The supernatant was removed and the solid was washed twice with Et_2O and dried. The product was used without further purification in the next step.

Coupling of DOTA-*tris*(tBu)ester: The powder corresponding to the alloc-free cyclic peptide was dissolved in degassed DMF (8 mL). DOTA-*tris*(*t*Bu)ester, (0.26 mmol, 0.15 mg), PyBOP (0.26 mmol, 135 mg) and DIEA (0.78 mmol, 0.5 mL) were added. The reaction mixture was stirred overnight at room temperature and the solvent was evaporated under reduced pressure. The residue was dissolved in a minimum of DCM/MeOH (1:2, v/v) mixture before precipitation using Et_2O. The supernatant was removed and the solid was washed twice with Et_2O and dried. It was used without further purification in the next step.

Removal of side-chain protecting groups: The DOTA-substituted cyclic peptide was stirred for 4 h in a TFA/TIS/H_2O/DTT/thioanisole/octylsulfide (18 mL / 0.6 mL / 0.6 mL / 650 mg / 300 mL / 300 mL) mixture. The solution was evaporated to yield a yellow oil, which was precipitated on adding cold Et_2O. The crude peptide was purified by preparative RP-HPLC (gradient: 5 to 20% B in 3 min followed by 20 to 40% B in 15 min) to yield the free peptide as a white powder after freeze-drying.

LCC0(^{D}P-P): t_R(analytical) = 10.5 min (method A); ESI-MS average *m/z* = 1193.9 (2+), 796.6 (3+), 597.7 (4+) (calculated av. *m/z* = 1194.43 $[M+2H]^{2+}$, 796.62 $[M+3H]^{3+}$, 597.72 $[M+4H]^{4+}$ for M = $C_{109}H_{172}N_{28}O_{28}S_2$).

LCC0(Aib-^{D}P): t_R(analytical) = 10.1 min (method A); ESI-MS average *m/z* = 1188.3 (2+), 792.5 (3+), 594.7 (4+) (calculated av. *m/z* = 1188.42 $[M+2H]^{2+}$, 792.62 $[M+3H]^{3+}$, 594.71 $[M+4H]^{4+}$ for M = $C_{108}H_{172}N_{28}O_{28}S_2$).

LCC0(P-G): t_R(analytical) = 9.9 min (method A); ESI-MS average *m/z* = 1173.9 (2+), 783.3 (3+), 587.8 (4+) (calculated av. *m/z* = 1174.40 $[M+2H]^{2+}$, 783.27 $[M+3H]^{3+}$, 587.70 $[M+4H]^{4+}$ for M = $C_{106}H_{168}N_{28}O_{28}S_2$).

LCC1(Aib): t_R(analytical) = 9.8 min (method A); ESI-MS average *m/z* = 1181.3 (2+), 787.9 (3+), 591.2 (4+) (calculated av. *m/z* = 1181.39 $[M+2H]^{2+}$, 787.93 $[M+3H]^{3+}$, 591.20 $[M+4H]^{4+}$ for M = $C_{106}H_{166}N_{28}O_{29}S_2$).
LCC1(G): t_R(analytical) = 10.0 min (method A); ESI-MS average *m/z* = 1166.9 (2+), 778.4 (3+), 584.2 (4+) (calculated av. *m/z* = 1167.36 $[M+2H]^{2+}$, 778.58 $[M+3H]^{3+}$, 584.18 $[M+4H]^{4+}$ for M = $C_{104}H_{162}N_{28}O_{29}S_2$).
LCC2: t_R(analytical) = 9.3 min (method A); ESI-MS average *m/z* = 1174.3 (2+), 783.2 (3+), 587.6 (4+) (calculated av. *m/z* = 1174.37 $[M+2H]^{2+}$, 783.25 $[M+3H]^{3+}$, 587.69 $[M+4H]^{4+}$ for M = $C_{105}H_{164}N_{28}O_{29}S_2$).
LCC3: t_R(analytical) = 9.0 min (method A); ESI-MS average *m/z* = 1189.6 (2+), 793.5 (3+), 595.4 (4+) (calculated av. *m/z* = 1189.88 $[M+2H]^{2+}$, 793.59 $[M+3H]^{3+}$, 595.44 $[M+4H]^{4+}$ for M = $C_{105}H_{165}N_{29}O_{30}S_2$).

Formation of Ln^{3+} complexes: The free peptide (0.004 mmol, 10 mg) was dissolved in H_2O and the pH was adjusted to 6.2 using NaOH. Then $TbCl_3$ or $LaCl_3$ (0.019 mmol, 5 mg) was added. The solution was stirred overnight (after 1h, the pH was controlled and adjusted to 6.2 if needed) and then it was purified by preparative RP-HPLC (gradient: 5 to 20% B in 3 min followed by 20 to 40% B in 15 min) to yield the Ln^{3+}-loaded peptide as a white powder after freeze-drying.
LCC0(^{D}P-P)^{Tb}: t_R(analytical) = 10.3 min (method A); ESI-MS average *m/z* = 1271.8 (2+), 848.5 (3+), 636.7 (4+) (calculated av. *m/z* = 1272.38 $[M+2H]^{2+}$, 848.59 $[M+3H]^{3+}$, 636.69 $[M+4H]^{4+}$ for M = $C_{109}H_{169}N_{28}O_{28}S_2Tb$).
LCC0(Aib-^{D}P)^{Tb}: t_R(analytical) = 10.0 min (method A); ESI-MS average *m/z* = 1265.9 (2+), 844.5 (3+), 633.7 (4+) (calculated av. *m/z* = 1266.37 $[M+2H]^{2+}$, 844.58 $[M+3H]^{3+}$, 633.69 $[M+4H]^{4+}$ for M = $C_{108}H_{169}N_{28}O_{28}S_2Tb$).
LCC0(Aib-^{D}P)^{La}: t_R(analytical) = 10.0 min (method A); ESI-MS average *m/z* = 1255.9 (2+), 837.9 (3+) (calculated av. *m/z* = 1256.36 $[M+2H]^{2+}$, 837.91 $[M+3H]^{3+}$ for M = $C_{108}H_{169}N_{28}O_{28}S_2La$).
LCC0(P-G)^{Tb}: t_R(analytical) = 9.7 min (method A); ESI-MS average *m/z* = 1252.3 (2+), 835.2 (3+), 626.7 (4+) (calculated av. *m/z* = 1252.35 $[M+2H]^{2+}$, 835.23 $[M+3H]^{3+}$, 626.68 $[M+4H]^{4+}$ for M = $C_{106}H_{165}N_{28}O_{28}S_2Tb$).
LCC1(Aib)^{Tb}: t_R(analytical) = 10.3 min (method A); ESI-MS average *m/z* = 1259.1 (2+), 839.8 (3+) (calculated av. *m/z* = 1259.34 $[M+2H]^{2+}$, 839.89 $[M+3H]^{3+}$ for M = $C_{106}H_{163}N_{28}O_{29}S_2Tb$).
LCC1(G)^{Tb}: t_R(analytical) = 10.4 min (method A); ESI-MS average *m/z* = 1245.0 (2+), 830.4 (3+) (calculated av. *m/z* = 1245.31 $[M+2H]^{2+}$, 830.54 $[M+3H]^{3+}$ for M = $C_{104}H_{159}N_{28}O_{29}S_2Tb$).
LCC2^{Tb}: t_R(analytical) = 9.5 min (method A); ESI-MS average *m/z* = 1252.2 (2+), 835.1 (3+), 626.5 (4+) (calculated av. *m/z* = 1252.32 $[M+2H]^{2+}$, 835.22 $[M+3H]^{3+}$, 626.67 $[M+4H]^{4+}$ for M = $C_{105}H_{161}N_{28}O_{29}S_2Tb$).
LCC3^{Tb}: t_R(analytical) = 9.2 min (method A); ESI-MS average *m/z* = 1267.5 (2+), 845.4 (3+), 634.4 (4+) (calculated av. *m/z* = 1267.83 $[M+2H]^{2+}$, 845.56 $[M+3H]^{3+}$, 634.42 $[M+4H]^{4+}$ for M = $C_{105}H_{165}N_{29}O_{30}S_2Tb$).

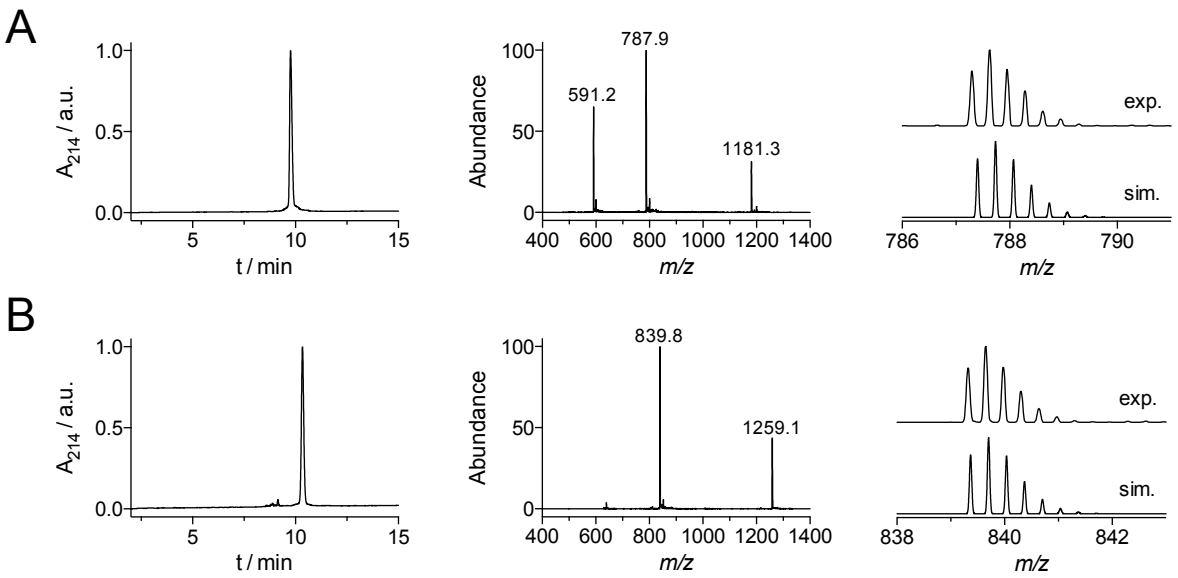

Figure S1. Examples of HPLC chromatograms (*left*) and ESI-MS spectra (*middle*: full MS spectrum; *right*: experimental and simulated isotopic pattern of the $[M+3H]^{3+}$ peak) obtained for (A) LCC1(Aib) and (B) LCC1(Aib)^{Tb}.

Luminescence spectroscopy

Preparation of samples for luminescence monitoring of Cu^+ and Ag^+ binding: For Cu^+, peptide solutions (5-20 µM) in HEPES buffer (10 mM, pH 7.5) containing NH_2OH (2 mM) were prepared in a glove box from stock solutions of peptide (50-200 µM), HEPES buffer (100 mM, pH 7.5) and NH_2OH (100 mM, pH 7.5). The concentration of the peptide was determined using the extinction coefficients at 280 nm (ε = 5 690 M^{-1} cm^{-1}) of tryptophan. Rubber-sealed cuvettes were used. Cu^+ was generated *in situ* by reduction of Cu^{2+} added as a $CuSO_4$ (2 mM) solution in the glove box. For Ag^+, peptide solutions (5-20 µM) in HEPES buffer (10 mM, pH 7.5) were prepared in the glove box or aerobically and a stock solution of $AgClO_4$ (1-5 mM) in H_2O was used for metal additions.

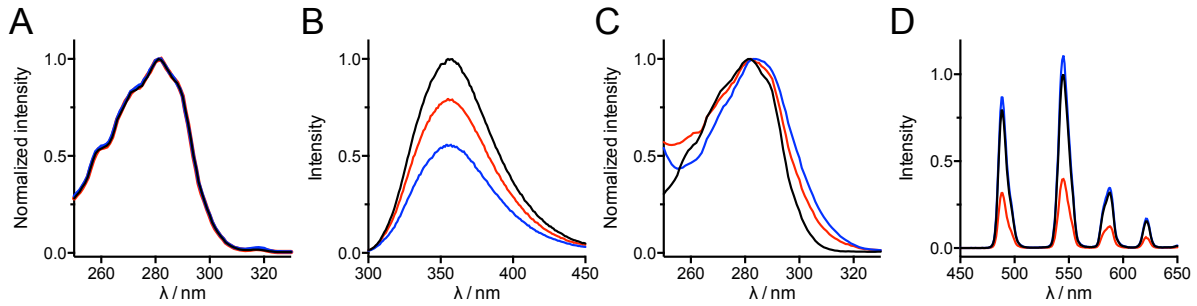

Figure S2. Comparative emission and excitation spectra of LCC0(^{D}P-P)^{Tb} in its metal-free (black), Ag^+-loaded (blue) and Cu^+-loaded (red) forms (degassed solutions). (A) Tryptophan fluorescence excitation spectra (λ_{em} = 357 nm). (B) Tryptophan fluorescence emission spectra (λ_{ex} = 280 nm). (C) Tb^{3+} time-gated luminescence excitation spectra (delay time = 100 µs, λ_{em} = 545 nm). (D) Tb^{3+} time-gated luminescence emission spectra (delay time = 100 µs, λ_{ex} = 280 nm).

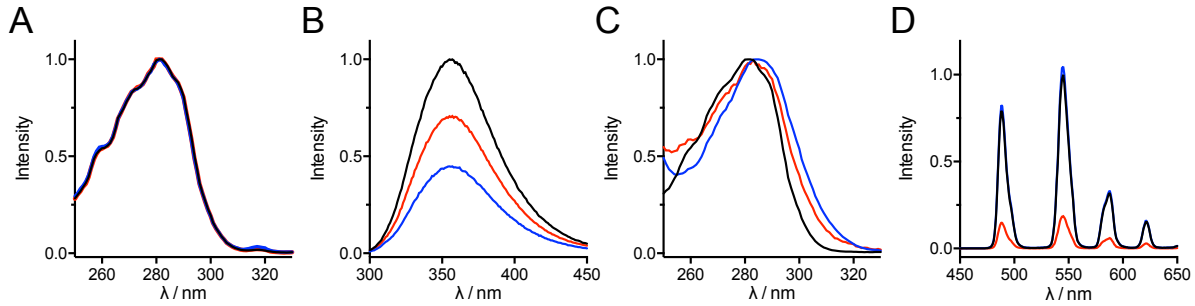

Figure S3. Comparative emission and excitation spectra of LCC0(Aib-^{D}P)^{Tb} in its metal-free (black), Ag^{+}-loaded (blue) and Cu^{+}-loaded (red) forms (degassed solutions). (A) Tryptophan fluorescence excitation spectra (λ_{em} = 357 nm). (B) Tryptophan fluorescence emission spectra (λ_{ex} = 280 nm). (C) Tb^{3+} time-gated luminescence excitation spectra (delay time = 100 µs, λ_{em} = 545 nm). (D) Tb^{3+} time-gated luminescence emission spectra (delay time = 100 µs, λ_{ex} = 280 nm).

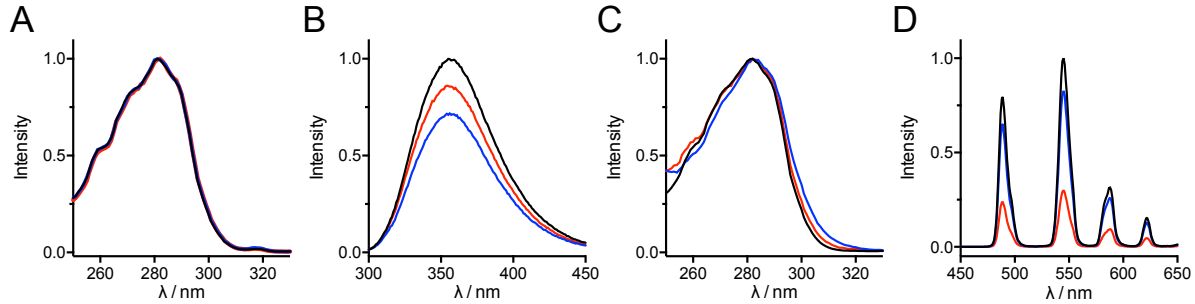

Figure S4. Comparative emission and excitation spectra of LCC0(P-G)^{Tb} in its metal-free (black), Ag^{+}-loaded (blue) and Cu^{+}-loaded (red) forms (degassed solutions). (A) Tryptophan fluorescence excitation spectra (λ_{em} = 357 nm). (B) Tryptophan fluorescence emission spectra (λ_{ex} = 280 nm). (C) Tb^{3+} time-gated luminescence excitation spectra (delay time = 100 µs, λ_{em} = 545 nm). (D) Tb^{3+} time-gated luminescence emission spectra (delay time = 100 µs, λ_{ex} = 280 nm).

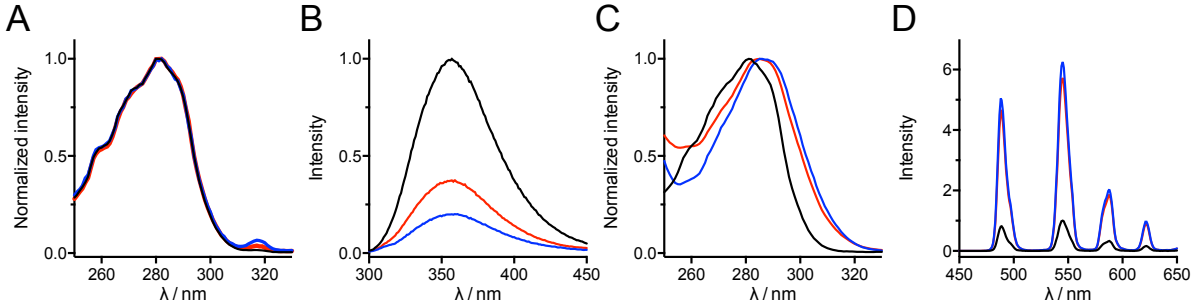

Figure S5. Comparative emission and excitation spectra of LCC1(G)^{Tb} in its metal-free (black), Ag^{+}-loaded (blue) and Cu^{+}-loaded (red) forms (degassed solutions). (A) Tryptophan fluorescence excitation spectra (λ_{em} = 357 nm). (B) Tryptophan fluorescence emission spectra (λ_{ex} = 280 nm). (C) Tb^{3+} time-gated luminescence excitation spectra (delay time = 100 µs, λ_{em} = 545 nm). (D) Tb^{3+} time-gated luminescence emission spectra (delay time = 100 µs, λ_{ex} = 280 nm).

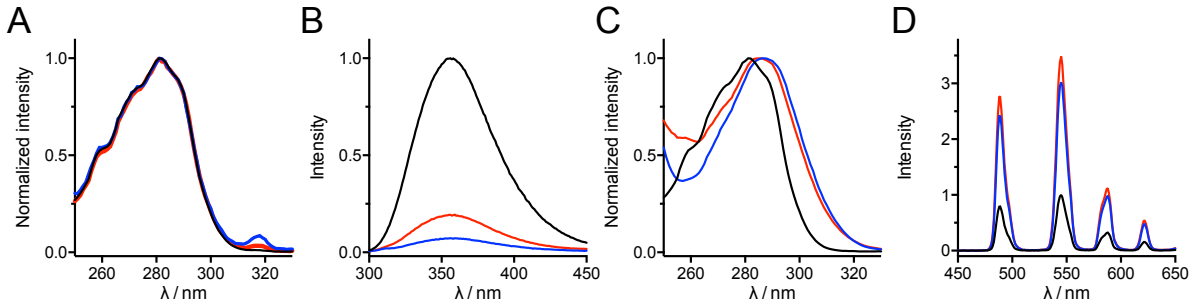

Figure S6. Comparative emission and excitation spectra of LCC1(Aib)^{Tb} in its metal-free (black), Ag^+-loaded (blue) and Cu^+-loaded (red) forms (degassed solutions). (A) Tryptophan fluorescence excitation spectra (λ_{em} = 357 nm). (B) Tryptophan fluorescence emission spectra (λ_{ex} = 280 nm). (C) Tb^{3+} time-gated luminescence excitation spectra (delay time = 100 µs, λ_{em} = 545 nm). (D) Tb^{3+} time-gated luminescence emission spectra (delay time = 100 µs, λ_{ex} = 280 nm).

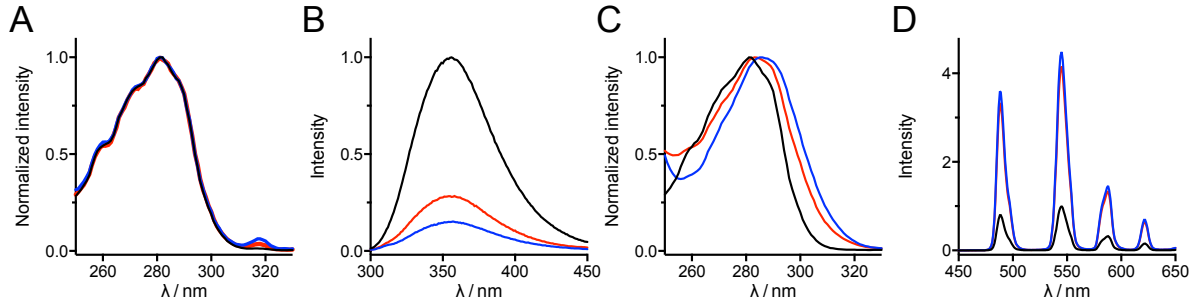

Figure S7. Comparative emission and excitation spectra of LCC2^{Tb} in its metal-free (black), Ag^+-loaded (blue) and Cu^+-loaded (red) forms (degassed solutions). (A) Tryptophan fluorescence excitation spectra (λ_{em} = 357 nm). (B) Tryptophan fluorescence emission spectra (λ_{ex} = 280 nm). (C) Tb^{3+} time-gated luminescence excitation spectra (delay time = 100 µs, λ_{em} = 545 nm). (D) Tb^{3+} time-gated luminescence emissson spectra (delay time = 100 µs, λ_{ex} = 280 nm).

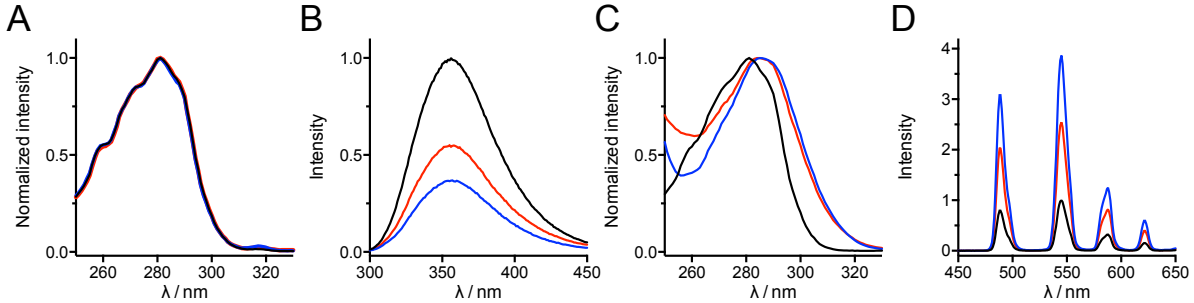

Figure S8. Comparative emission and excitation spectra of LCC3^{Tb} in its metal-free (black), Ag^+-loaded (blue) and Cu^+-loaded (red) forms (degassed solutions). (A) Tryptophan fluorescence excitation spectra (λ_{em} = 357 nm). (B) Tryptophan fluorescence emission spectra (λ_{ex} = 280 nm). (C) Tb^{3+} time-gated luminescence excitation spectra (delay time = 100 µs, λ_{em} = 545 nm). (D) Tb^{3+} time-gated luminescence emission spectra (delay time = 100 µs, λ_{ex} = 280 nm).

Determination of Cu^+ and Ag^+ binding constants

The Cu^+ and Ag^+ binding constants of the various LCC peptides, K_M = [M·LCC]/([M]×[LCC]), were determined as previously described for LCC1^{Tb} by titration experiments using imidazole as a competitor.[2] Titrations were performed in HEPES buffer (10 mM, pH 7.5) containing the peptide (5-20 µM) and imidazole (1-10 mM). For

Ag^+, a solution of $AgClO_4$ was used. For Cu^+, buffer contained NH_2OH (2 mM) and Cu^+ was generated *in situ* by reduction of the added $CuSO_4$ by NH_2OH. Tb^{3+} emission spectra (λ_{ex} = 280 nm) were recorded after each addition of metal (equilibration is reached within a few seconds). The titrations (Figure S9) were fitted using the program SPECFIT[3] with the formation constants of the $[Cu(imidazole)]^+$, $[Cu(imidazole)_2]^+$, $[Ag(imidazole)]^+$, $[Ag(imidazole)_2]^+$ complexes taken from the literature,[4,5] which are reported in Table S1. The competitive titration between $LCC1(Aib)^{Tb}$ (11.0 μM) and imidazole (1.0 mM) is shown in Figure S9 as an example.

Table S1. Formation constants $\beta_{1n} = [M(imidazole)_n]/([M][imidazole]^n)$ for the 1:1 and 1:2 complexes formed between Cu^+ or Ag^+ and imidazole in water.

Metal ion	log β_{11}	log β_{12}	Reference
Cu^+	6.83	10.73	[4]
Ag^+	2.96	6.71	[5]

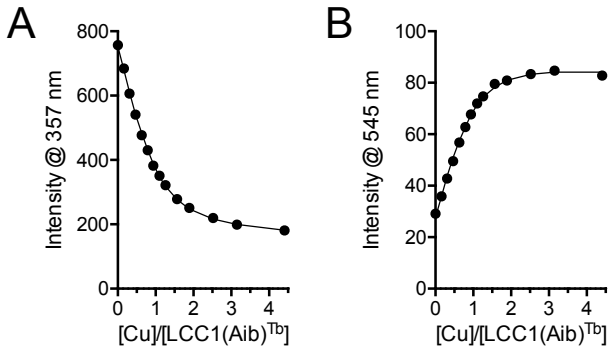

Figure S9. Evolution of (A) fluorescence emission at 357 nm and (B) time-gated (delay time = 100 μs) Tb^{3+} emission at 545 nm (λ_{ex} = 280 nm) upon addition of Cu^+ in solutions of $LCC1(Aib)^{Tb}$ in HEPES buffer (10 mM, pH 7.5) containing imidazole as a competitor. Cu^+ was generated *in situ* by reduction of $CuSO_4$ by NH_2OH. $[LCC1(Aib)^{Tb}]$ = 11 μM, [imidazole] = 1 mM, $[NH_2OH]$ = 2 mM. The solid lines correspond to the fits obtained using SPECFIT[3], which yielded log K_{Cu} = 10.3 (2).

Circular dichroism

Preparation of samples for CD monitoring of Cu^+ and Ag^+ binding: Solutions were prepared as described above for luminescence monitoring but using a phosphate buffer (10 mM, pH 7.0) instead of HEPES.

References

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