Ultrasensitive and Selective Copper(II) Detection: Introducing a Bioinspired and Robust Sensor

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**Figure S1** | RP-HPLC and inserted ESI-MS of purified peptide 1. HPLC gradient: 5 – 40% of eluent B in 20 min detection at 214 nm

**Figure S2** | RP-HPLC and inserted ESI-MS of purified peptide 2. HPLC gradient: 0% for 4 minutes followed by a gradient of 0 – 30% eluent B in 16 minutes, detection at 214 nm
Figure S3 | Effect of ZnSO₄ concentration on the spectra of peptide 1 and 2 in MES buffer at pH 6.5. a, Fluorescence spectra (NiSO₄ (excitation at λ_ex 494 nm, emission at λ_em 518, 25°C) of peptide 1 (1 µM) at different concentrations of ZnSO₄ (0 – 3 mM, in water). b, UV-Vis spectra of peptide 2 (1 mM) at different concentrations of ZnSO₄ (0 – 2 mM, in water).
Figure S4 | ESI spectra of complex formation between peptide 1 and CuSO₄ in PBS buffer at pH 7.4. ESI spectrum shows formation of 1:1 complex between peptide 1 and Cu(II). Insert shows simulated and measured isotopic pattern.
Figure S5 | ESI spectra of complex formation between peptide 2 and CuSO₄ in PBS buffer at pH 7.4. ESI spectrum shows formation of 1:1 complex between peptide 2 and Cu(II). Insert shows simulated and measured isotopic patterns.
Figure S6 | ESI spectra of complex formation between peptide 1 and NiSO₄ in PBS buffer at pH 7.4. ESI spectrum shows formation of 1:1 complex between peptide 1 and Ni(II). Insert shows simulated and measured isotopic patterns.
Figure S7 | ESI spectra of complex formation between peptide 2 and NiSO₄ in PBS buffer at pH 7.4. ESI spectrum shows formation of 1:1 complex between peptide 2 and Ni(II). Insert shows simulated and measured isotopic patterns.
**Figure S8** | UV-Vis titration of peptide 2 with CuSO₄ at pH 8.0. **a**, UV-Vis titration of peptide 2 (1 mM, TRIS pH 8.0, 25°C) at different concentrations of CuSO₄ (0 – 1.3 mM, water). **b**, absorption coefficient of peptide 2-metal complex as a function of the molar ratio of Cu(II) to peptide 2.

**Figure S9** | UV-Vis titration of peptide 2 with NiSO₄ at pH 10.5. **a**, UV-Vis titration of peptide 2 (1 mM, Tris buffer at pH 10.5, 25°C) at different concentrations of NiSO₄ (0 – 1.8 mM, water). **b**, change in fluorescence intensity as a function of the molar ratio of Ni(II) to peptide 2.

**Figure S10** | UV-Vis titration of peptide 2 with CuSO₄ at pH 6.5. **a**, UV-Vis titration of peptide 2 (0.68 mM, MES buffer pH 6.5, 25°C) at different concentrations of CuSO₄ (0 – 0.7 mM, in water). **b**, absorption coefficient of peptide 2-metal complex as a function of the molar ratio of Cu(II) to peptide 2.
Figure S11 | UV-Vis titration of peptide 2 with NiSO₄ at pH 6.5. a, UV-Vis titration of peptide 2 (0.68 mM, MES buffer pH 6.5, 25°C) with NiSO₄ (0 – 16 mM, in water) monitored at 551 nm. b, change of absorption as a function of the molar ratio of Ni(II) to peptide 2.
Figure S12 | Fluorescence titration of peptide 1 with CuSO₄ at pH 8.0. a, Fluorescence titration of peptide 1 (0.1 µM, phosphate buffer at pH 8.0, 25°C) at different concentrations of CuSO₄ (0 – 0.15 µM, water). b, Change in fluorescence intensity as a function of the molar ratio of Cu(II) to peptide 1 (fluorescence excitation at λₘₐₓ 494 nm, emission at λₘₐₓ 518).

Figure S13 | Fluorescence titration of peptide 1 with NiSO₄ at pH 10.5. a, Fluorescence titration of peptide 1 (0.025 µM, CAPS buffer at pH 10.5, 25°C) at different concentrations of NiSO₄ (0 – 0.038 µM, water). b, Change in fluorescence intensity as a function of the molar ratio of Ni(II) to peptide 1 (fluorescence excitation at λₘₐₓ 494 nm, emission at λₘₐₓ 518).
Figure S14 | Determination of Cu(II) and Ni(II) binding constants to peptide 2 in MES buffer at pH 6.5. a, Effect of increasing concentration of CuSO₄ (0 – 0.7 mM) on peptide 2 at concentration 1mM represented as Benesi-Hildebrand plot of correlation between 1/conc.(Cu(II)/Ni(II)) vs 1/(A₅₅₁ - A₅₅₁.₀). b, Effect of increasing concentration of NiSO₄ (0 – 10 mM) on peptide 2 at concentration 1mM represented as Benesi-Hildebrand plot of correlation between 1/conc.(Cu(II)/Ni(II)) versus 1/(A₅₅₁ - A₅₅₁.₀).

Figure S15 | Determination of Cu(II) and Ni(II) binding constants to peptide 1 at pH 6.5 in MES buffer. a, Double logarithmic plot of the quenching for peptide 1 fluorescence by CuSO₄ in aqueous medium. b, Double logarithmic plot of the quenching for peptide 1 fluorescence by NiSO₄ in aqueous medium. F is measured fluorescence of a solution containing the peptide 1 and the Cu(II) or Ni(II) at different concentrations, F₀ is the fluorescence of a solution of peptide 1 alone (fluorescence excitation at λₑ₅₄₉₄ nm, emission at λₑ₅₅₁₈ nm).

Determination of binding constants from fluorescence quenching data:
The Stern Volmer constant, $K_{sv}$, was determined using the Stern Volmer equation:
$F_0/F = 1 + K_{sv}[Q]$  
**Equation S1**

$Q =$ concentration of quencher (Cu²⁺)
$F_0 =$ fluorescence intensity when no quencher is present
$F =$ fluorescence intensity when Q is added

To calculate the bimolecular quenching rate constant $K_q$, the following equation is used:
$K_q = K_{sv}/\tau_0$  
**Equation S2**

$\tau_0 =$ fluorescence lifetime s⁻¹
$\tau = 10^{-8}$ s⁻¹

The quenching rate constant of diffusion collision for diverse quenchers is defined to be under $2 \times 10^{10}$ M⁻¹ s⁻¹ which means that a value above indicates a static quenching process.

To calculate the binding constant $K_b$ the equation which is used for static quenching process was deployed:
$\log([F_0 - F]/F) = \log K_b + n\log(Q)$  
**Equation S3**

When plotting log([F₀ – F]/F) vs log(Q) the binding constant $K_b$ is obtained.

[1]
Table S1. Summary of Cu(II) and Ni(II) binding constants

| Fluorescence | pH | \( K \) (mol/L) | \( \log K \) | \( \lambda_{\text{max, buffer}} \) |
|--------------|----|-----------------|--------------|----------------------------------|
| peptide 1    | Cu(II) | 6.5 | \((6.3 \pm 0.1) \times 10^6\) | 6.80 |
|              | Ni(II)  | 6.5 | \((1.8 \pm 0.1) \times 10^3\) | 3.26 |
| peptide 1    | Cu(II) | 8.0 | \((2.1 \pm 0.2) \times 10^3\) | 9.3  |
|              | Ni(II)  | 10.5| \((6.1 \pm 0.3) \times 10^{11}\) | 11.79 |
| peptide 2    | Cu(II) | 6.5 | \((3.8 \pm 0.2) \times 10^6\) | 6.57 | 551 |
|              | Ni(II)  | 6.5 | \((5.8 \pm 0.1) \times 10^2\) | 2.76 | 393 |
| peptide 2    | Cu(II) | 8.0 | \((1.0 \pm 0.1) \times 10^8\) | 6.01 | 561 |
|              | Ni(II)  | 10.5| \((2.5 \pm 0.2) \times 10^7\) | 7.39 | 458 |
Figure S16 | Determination of limit of detection in solution (LOD). a, correlation plot of \( F_0 - F \) vs. concentration of copper (II) for determination of limit of detection of peptide 1 (1 \( \mu \text{M} \), 100 \( \text{mM} \) MES buffer pH 6.5, 25°C) upon addition of Cu(II)SO\(_4\) (0 – 60 nM, in water).

The LOD is calculated using the following equation:

\[
\text{LOD} = \frac{(3.3 \times \sigma)}{\text{slope}}
\]

\( \sigma \) = standard deviation of blank solution

1 \( \mu \text{l} \) of a blank solution (100 \( \text{mM} \) MES buffer pH 6.5, 25°C) was pipetted to a 1 \( \mu \text{M} \) peptide solution in MES buffer and the difference between fluorescence intensity relating to the starting intensity determined. From a six fold titration study the standard deviation of the change in fluorescence intensity with the addition of blank solution is determined and used as the \( \sigma \) value (1.28).\(^2\)
Figure S17 | Full $I$-$V$ characteristics of peptide 1 in a nanopore. a, $I$-$V$ titration response of immobilized with peptide 1-nanopore on exposure to different concentration of CuSO4 in the 0.1 м KCl solution. b, Reversible complexation/decomplexation of Cu$^{2+}$-ions.
Figure S18 | Fluorescence microscopy of immobilized nanopore with Ni(II) or Zn(II). 

a. Fluorescence images after addition of Ni(II) and decrease in fluorescence intensity (median) with addition of NiSO$_4$ (0 – 1000 µM, MES buffer pH 6.50).  
b. Fluorescence images after addition of 100 mM ZnSO$_4$ solution. Recovery steps are performed with 100 mM EDTA solution.  
c. Graphical illustration of fluorescence intensities according to different concentrations of NiSO$_4$.  
d. Graphical illustration of fluorescence intensities according to application of ZnSO$_4$ at concentration of 100 mM and two recovery steps with 100 mM solution of EDTA (fluorescence excitation at $\lambda_{Ex}$ 488 nm, emission detected at $\lambda_{Em}$ 515-530 nm).
Figure S19 | RP-HPLC calibration of NMR samples. a. Calibration of peptide 1 representing correlation between the peak area vs. concentration (mM). HPLC conditions: gradient of 5 – 40% eluent B in 20 min., detection at 494 nm. b. Calibration of peptide 2 representing correlation between the peak area vs. concentration (mM). HPLC conditions: 0% eluent B for 4 minutes followed by a linear gradient to 30% eluent B within a total of 20 minutes, detection at 214 nm.

Experimental Section

Solid phase peptide synthesis and peptides characterization. (5/6) FAM-DAP-β-Ala-His-PEG₄(1) and Ac-DAP-β-Ala-His-PEG₄(2) were synthesized following the standard Fmoc-SPPS using chlorotrityl chloride resin. After PEG₄ coupling the resin was capped with 8.5:1.0:0.5 dichloromethane (DCM)/methanol/N-ethyl-N-(propan-2-yl)propan-2-amin (DIEA) followed by coupling of Fmoc-His(Trt)-OH, Fmoc-β-Ala-OH, Fmoc-Dap(Boc) and 5/6-carboxyfluorescein (fluorophore only for 1) correspondingly. Deprotection was obtained by 20% piperidine in N,N-Dimethylformamide (DMF) and the coupling efficiency was monitored by UV-Vis – spectrometry at 301 nm. The peptide 2 was acetylated at the N-terminus with acetic anhydride. The final cleavage of both peptides 1 and 2 from the resin was carried out in 2/2/48/48 triisopropylsilane(TIPS)/water/trifluoroacetic acid(TFA)/DCM and agitated for 1 h at room temperature. RP-HPLC purification was performed on a C18 column (MultoKrom 100-5.250 x 20 mm, 100 Å pore diameter, 5.0 µm particle size) using a linear gradient of 5% to 40% of eluent B (eluent A: water (0.1% TFA) and eluent B: acetonitrile (0.1% TFA)) in 60 min (Figure S1). The molecular mass was confirmed by ESI-MS. Collected fractions were combined, freeze-dried and stored at –28°C. Purity of the collected fractions were confirmed by analytical RP-HPLC on a Waters XC e2695 system (Waters, Milford, MA, USA) employing a Waters PDA 2998 diode array detector equipped with ISAspher 100-3 C18 (C18, 3.0 µm particle size, 100 Å pore size, 50×4.6 mm, Isera GmbH, Düren, Germany). Peptide 1 was eluted with a linear gradient of 5% - 40% eluent B with a flow rate of 1 ml/min within 20 minutes. Peptide 2 was eluted with an isocratic method at 0% eluent B for 5 minutes followed by a linear gradient to 30% eluent B within 20 minutes in total at a flow rate of 1 ml/min. Chromatograms were extracted at 214 nm. The molecular weight of the purified peptides as well as complexation of peptide 1 and 2 with Cu(II), Ni(II) was confirmed by ESI mass spectrometry on a TOF-Q impact II spectrometer (Bruker Daltonik GmbH, Bremen, Germany) and calibrated using Bruker’s ESI-Tune-Mix.

Preparation of hybrid PET foils. Conical nanopores in polyethylene terephthalate (PET) membranes were fabricated through asymmetric chemical etching of latent ion tracks at the linear accelerator UNILAC (GSI Helmholtz Centre for Heavy Ion Research, Darmstadt, Germany). The chemical track-etching process was performed in a custom-made conductivity cell. The detailed chemical asymmetric track-etching process can be found elsewhere. [3] The carboxylic acid groups on the pore surface were first activated through carbodiimide coupling and peptide was immobilized to nanopores by standard peptide coupling protocol. [4]
Current-voltage measurements. The IV-measurements were performed according to the protocol of Ali et al. at 25°C. For this purpose, as-prepared nanopore membrane is clamped between two chambers of a home-made measuring cell. Both cells are filled with electrolyte solution (0.1 M KCl, MES buffer, pH 6.5 or Sigmatrix urine diluent) and a pair of Ag/AgCl electrodes connected to a picometer/voltage source (Keithley 6487, Keithley instruments, OH) are mounted in each chamber. Here, the positive pole is located on the tip side of the foil and the negative one is placed on the bulk side. Then, the measurement cell is placed in a faraday cage and additionally connected to the ground. For IV-record, a scanning triangular voltage is applied from -1 V to 1V by the use of Labview 6.1 (National Instruments).

NMR calibration. All following peptide concentrations were determined by RP-HPLC using a calibration curve with a peptide solution quantified through NMR experiments (Figure S18). As an external standard, N-acetyl alanine was used. The 1H integrals of the protons within the acetyl-group of N-acetyl alanine were compared to the protons of the aromatic imidazole protons of histidine (peptide 1) and the acetyl group at the N-terminus of peptide 2. The sample (water + 10% D2O, 25°C) was then used as the calibration standard for the RP-HPLC.

pH UV-Vis spectrophotometric titration. pH titration studies were performed using a 1 mM peptide solution in deionized water at 25°C. 1 equivalent of the according metal ion, Cu(II) or Ni(II), was added and the pH lowered to pH < 3 using 0.1 mM HCl. Adding small aliquots of 0.1 mM NaOH the pH was increased and with each step a spectrum recorded. For the optimal formation of the metal-peptide complex, pH 8.0 was evaluated for copper complexation, pH 10.55 for nickel complexation.

Fluorescence titration experiments peptide 1. Fluorescence titration was performed using 0.1 µM peptide solution in 100 mM phosphate buffer, pH 8.0 at 25°C. Aliquots containing 0.1 equivalents of CuSO4 were added and after each step a spectrum recorded. For NiSO4 CAPS buffer pH 10.5 was used, respectively. For fluorescence titration studies aiming at the selectivity towards copper(II) ions MES buffer pH 6.5 was used. To a 1 µM peptide 1 solution small aliquots of a 100 mM CuSO4 solution (final concentration 1.30 µM – 24.7 µM) and a NiSO4 solution (final concentration 0.13 mM – 1.33 mM) were added. λemission was determined to be 518 nm at fluorescence excitation at λex 494 nm. The Stern-Volmer plot and a diagram of log([F0 – F]/F) vs log(Q) (Q = concentration of quencher, F0 = fluorescence intensity at Q = 0, F = fluorescence intensity at Q > 0) the Stern Volmer constant, quenching constant and binding constant were determined.

UV/Vis spectrophotometric titration of peptide 2. UV-Vis titration was performed using 100 mM TRIS buffer pH 8.0 and 100 mM TRIS buffer pH 10.55 at 25°C. To a 1 mM peptide solution 0.1 equivalents of Cu(II)/Ni(II) solution were added to a total of 1.4 equivalents and each time a spectrum recorded. The pH was monitored and adjusted using 0.1 mM NaOH. Identiically to fluorescence spectroscopy UV-Vis titration was performed using 100 mM MES buffer at pH 6.5. To a 1 mM peptide solution 2 small aliquots of a CuSO4 solution (0.06 mM – 3.1 mM) and a NiSO4 solution (0.63 – 18.75 mM) were added resulting in the increase of the absorption at λmax = 552 (Cu2+) and λmax = 394 (Ni2+) due to the formation of the peptide-heavy metal complex. Using the Benesi-Hildebrand method, binding constants were determined.

Confocal Laser Scanning Microscopy (CLSM). Titration experiments were designed as followed: the PET foil was fixated on an object holder making it possible to investigate one defined, tagged pore. Onto the object holder different concentrations of a CuSO4/NiSO4 solution (10 µM – 100 µM) are pipetted and an image recorded using a 488 nm laser. To remove all heavy metal a 1 mM EDTA solution is added followed by a washing step using 10 mM MES buffer. For “turn on-off” characteristics the following set-up was done iteratively: 100 µM Cu2+ were added an image recorded, copper washed off using 1 mM EDTA solution and MES buffer pH 6.5 and after each washing step an image recorded.
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