Electronic Supplementary Information

Separation of Metalloproteins using Novel Metal Ion Contaminant Sweeping Technique and Detection of Protein-Bound Copper by A Metal Ion Probe in Polyacrylamide Gel Electrophoresis: Distribution of Copper in Human Serum

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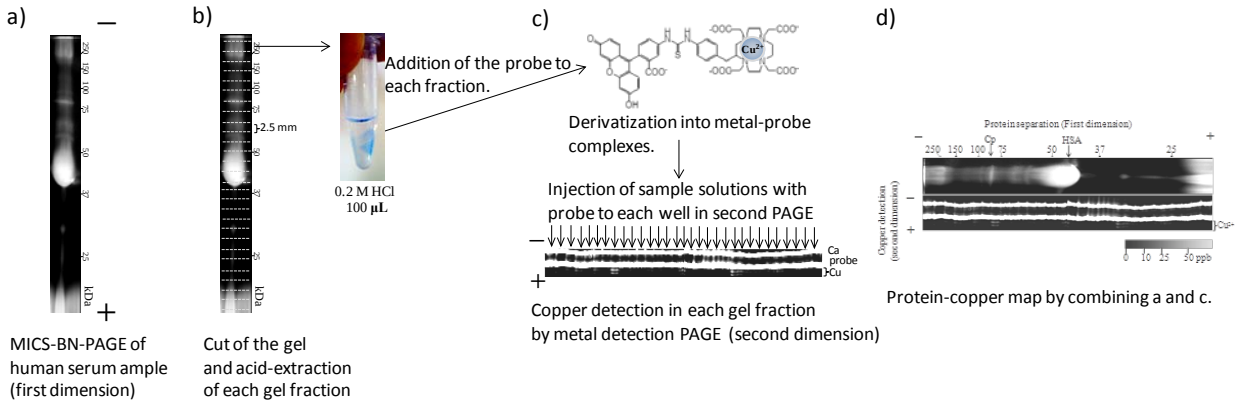

Figure S-1. Outline of procedure for protein-copper mapping by MICS-BN-PAGE/metal detection PAGE.

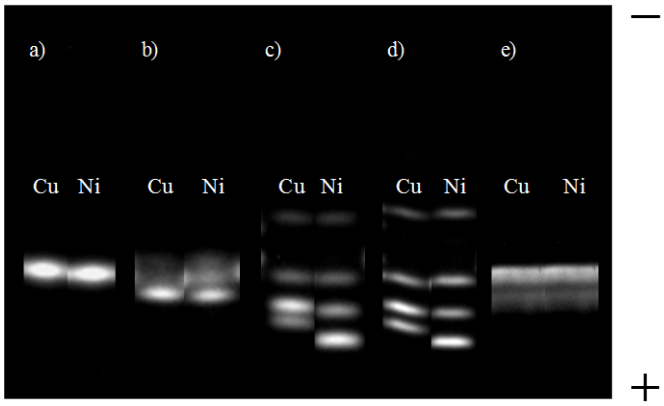

Figure S-2. The effect of gel concentration on the separation of metal-probe complexes. a) 5 %T/2.7 %C, b) 10 %T/2.7 %C, c) 20 %T/2.7 %C, d) 30 %T/2.7 %C, e) 5 %T/10 %C. Sample, $[Cu^{2+}] = [Ni^{2+}] = 1.0 \times 10^{-6}$ M, [probe] = 1.0×10^{-5} M, [Tris-HCl] = 75 mM (pH 8.8), [Glycerol] = 2.5 %v/v. The pore radii were estimated at 101, 71, 50, 40 and 29 nm for 5%T, 10%T, 20%T, 30%T (2.7 %C) and 5%T (10%C), respectively, using the equation by Holms and Stellwagen;[59,60]

$$r_p \text{ (nm)} = 231\ T^{0.51} \text{ (for 3\%C)} \qquad \text{(S1)}$$

$$= 73.7\ T^{0.58} \text{ (for 10\%C)} \qquad \text{(S2)}$$

here, r_p and T are pore radius and total gel concentration (%T), respectively.

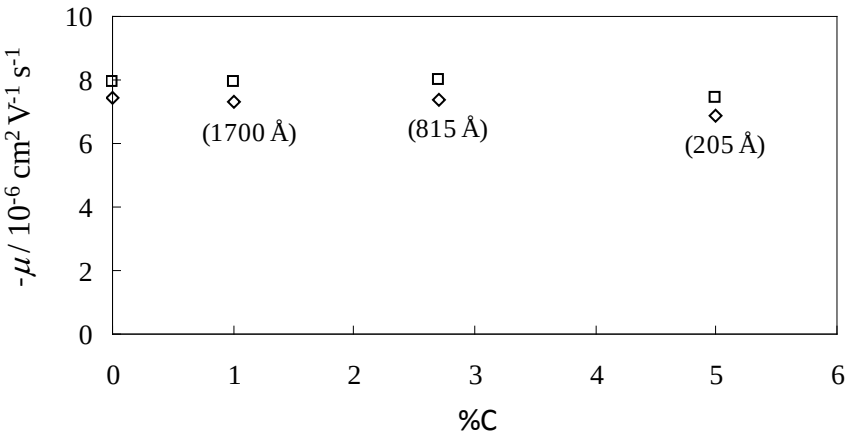

Figure S-3. The effect of the degree of cross-linkage (%C) on the mobility of metal-probe complexes. ◊, probe; □, Co^{2+}-probe complex. 30%T, 0–5%C. The values in parentheses are the estimated pore radii based on Refs. 59 and 60.

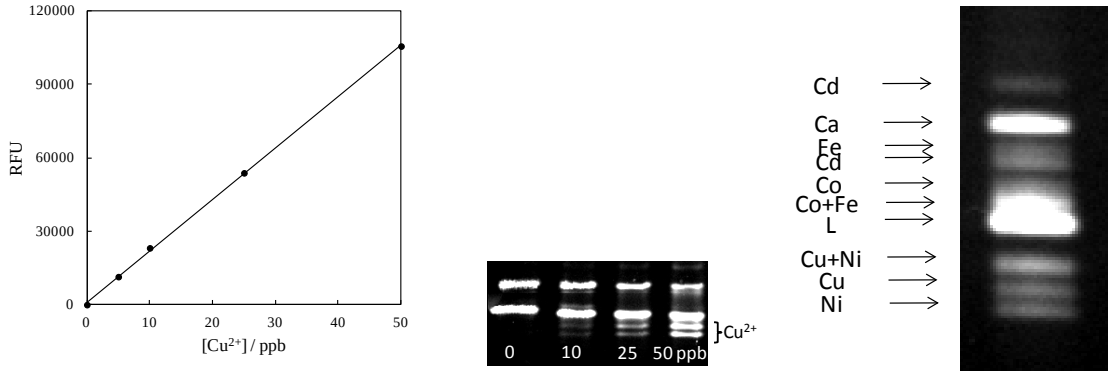

Figure S-4. A calibration curve for Cu^{2+} (left), typical electropherograms of Cu^{2+} with various concentrations (middle) and of a mixed solution including metal ions (Cu^{2+}, Fe^{2+}, Ni^{2+}, Co^{2+}, Cd^{2+}) each of 10 nM (right). The signal strength in the calibration curve using a CCD camera with a transilluminator is RFU = 2.1×10^3 x(ppb) + 1.0×10^3. Although a slight intercept corresponding to 0.46 ppb was observed,

no band originated from metal ions was actually observed in the blank. Since the blank value was apparent caused mainly by dark-light current, the blank value is very stable.

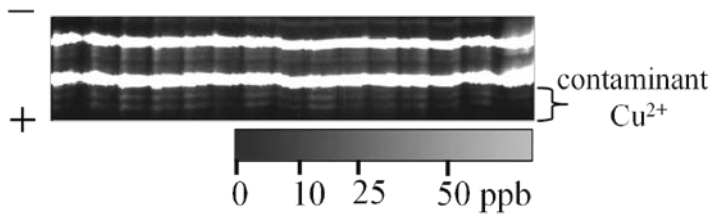

Figure S-5. Copper detection in gel fractions in BN-PAGE with water sample injection.

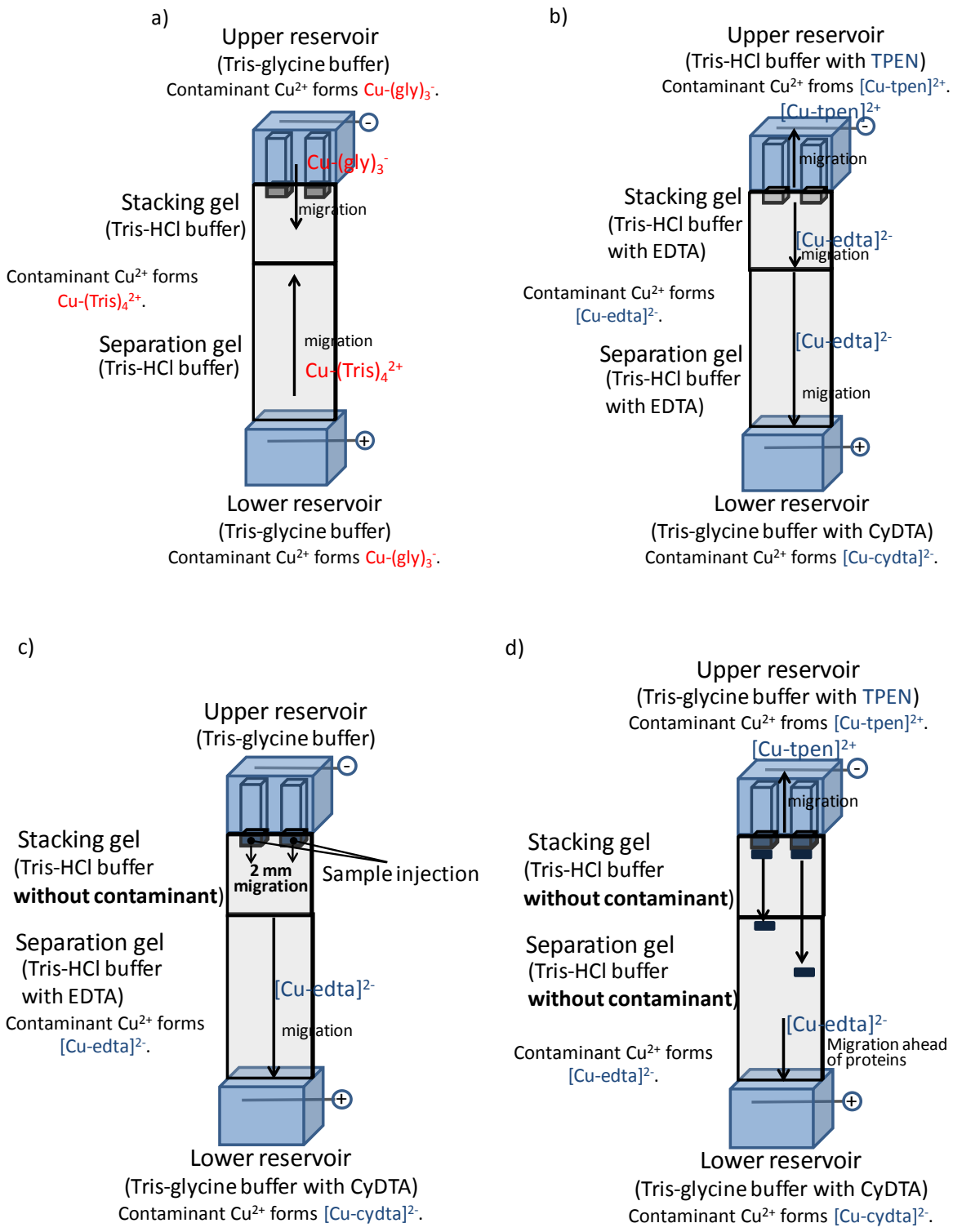

Figure S-6. Outline of procedure for metal ion contaminants sweeping (MICS)-PAGE. a) Normal PAGE. The contaminant copper complexes in the buffer and gel migrate into the gel, resulting in a mix with

sample proteins during separation. b–d) MICS method. b) Conditioning to prepare a contaminant-free stacking gel. The contaminant copper ion complexes with TPEN and EDTA migrate towards the upper and lower directions, respectively. c) Sample injection. Sample solutions are injected in TPEN-free upper buffer, so that no ligand exchange reaction occurs (Cu-protein + TPEN → Cu-TPEN + protein). Then, samples are migrated into the stacking gel for 2 mm to avoid contact with TPEN during protein separation (d). d) Sample migration in contaminant Cu-free stacking and separation gel. No contaminant Cu in the upper reservoir migrates into the gel, and the contaminant Cu in the separation gel migrates ahead of the protein bands during separation.

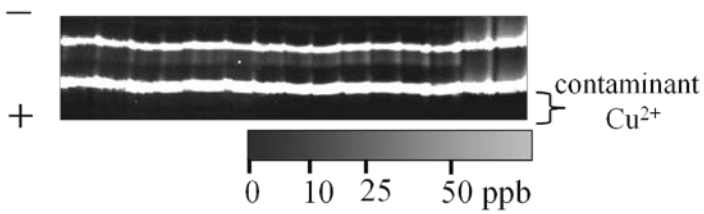

Figure S-7. Blank measurements for MICS-BN-PAGE.

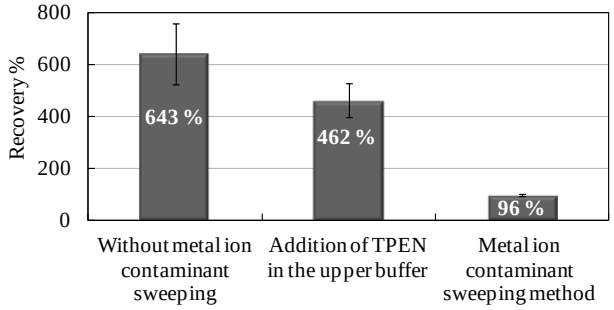

Figure S-8. Effect of addition of TPEN in the upper buffer solution and EDTA in the gel and the lower buffer solution on Cu recovery of a human serum sample.

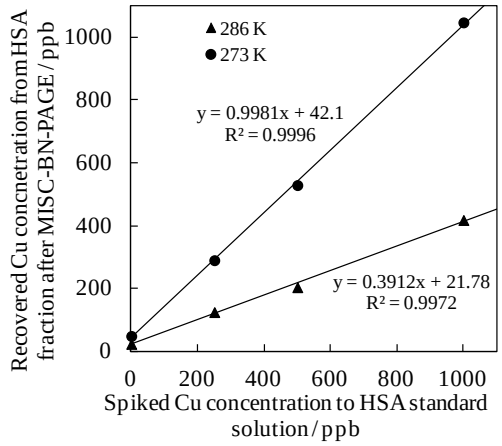

Figure S-9. Spiking test of HSA-Cu in MICS-BN-PAGE/metal detection PAGE mapping.

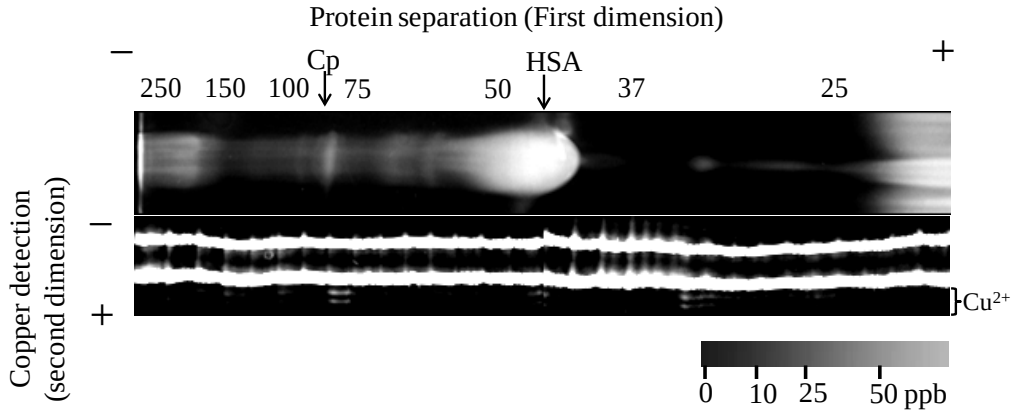

Figure S-10. Protein-copper map of a human serum sample (a healthy male, age 26) after four freeze-thaw cycles by MISC-BN-PAGE/metal detection PAGE.

Table S-1 Concentration of Cu and Cd in standard protein solutions and a human serum sample, determined by metal detection PAGE using FTC-ABDOTA and ICP-AES.

Standard protein solution (metal ion detected)	Cp (Cu)	SOD (Cu)	HSA (Cu)	MT[a] (Cd)	human serum[b] (Cu)
Concentration of proteins / M (ppb)	1.3×10^{-7} (17)	3.2×10^{-7} (10)	3.1×10^{-4} (21,000)	1.0×10^{-6} (7.0)	—
Metal concentration determined by this method / M (ppb)	5.2×10^{-7} (33)	5.3×10^{-7} (34)	7.7×10^{-7} (49)	5.1×10^{-7} (57)	1.1×10^{-6} (67)
Metal concentration determined by ICP-AES/M (ppb)	5.3×10^{-7} (34)	5.3×10^{-7} (34)	8.0×10^{-7} (50)	7.0×10^{-7} (78)	1.1×10^{-6} (69)

[a] MT-I from rabbit liver, metal content: Cd, Zn ~7 %. [b] a healthy male, age 24. The serum was diluted by a factor of 10.

Table S-2 Contamination of Cu (upper) and Fe (lower) in reagents employed.[a]

	Contaminant Cu concentration / ppb	Contaminant Cu concentration (with the addition of ion-exchange resin)[b] / ppb
Stock solution		
pH buffer stock solution of 250 mM Tris-1.92 M glycine	4.1 ± 0.4 < 1	3.3 ± 0.3 < 1
Monomer stock solution of 60 %T AA/ 2.7 %C Bis	30 ± 3 109 ± 14	23 ± 8 52 ± 5
Stacking gel buffer stock solution of 0.5 M Tris-HCl (pH 7.4)	5 ± 2 1 ± 2	3.4 ± 0.8 < 1
Separation gel buffer stock solution of 1.5 M Tris-HCl (pH 8.8)	5.9 ± 0.4 9.9 ± 0.3	4.5 ± 0.2 1.8 ± 0.7
Solution and gel employed[c]		
Stacking gel employed in this study	2.6 ± 0.3 7.3 ± 0.9	1.9 ± 0.5 3.5 ± 0.3
Separation gel employed in this study	5.7 ± 0.5 19 ± 2	5 ± 1 9.0 ± 0.8
Migration buffer of 6.3 mM Tris-48 mM glycine	0.10 ± 0.4 < 0.025	0.083 ± 0.008 < 0.025

[a] measured by ICP-AES. [b] sufficient amount of ion-exchange resin, OT-71, was added to the stock solutions to remove serious metal contamination. [c] calculated from the results of contaminant copper ion concentrations in stock solutions.

Table S-3 Detail data of Cu distribution in human serum samples obtained by MICS-BN-PAGE/metal detection PAGE (n = 10)[a]

Sample	Total Cu / ppb	Total Cu found / ppb (recovery %)	Cp fraction / ppb (distribution %)	HSA fraction / ppb (distribution %)
Female, age 25	760	728 (96)	728 (100)	n.d.
Male, age 48	749	735 (98)	735 (100)	n.d.
Female, age 32	792	820 (104)	813 (99.1)	7.2 (0.88)
Male, age 56	738	742 (101)	742 (100)	n.d.
Male, age 27	833	799 (96)	799 (100)	n.d.
Male, age 26	715	701 (98)	701 (100)	n.d.
Female, 31	728	720 (99)	720 (100)	n.d.
Female, 40	748	731 (98)	731 (100)	n.d.
Male, 42	812	794 (98)	790 (99.6)	3.5 (0.44)
Male, 56	765	786 (103)	786 (100)	n.d

[a] determined by ICP-AES.

1. 1 Estimation of uptake of contaminant copper ions into free HSA during separation.

There are metal contaminants at sub–ppb levels in separation buffers employed in LC and CE (an example of the contaminant concentration level in normal pH buffer reagents of analytical grade is shown in Table S2; also, see Ref. 3). Here, when copper contaminants of 0.5 ppb (7.8 nM) from the separation buffers and instruments exist in LC, uptake of the contaminant Cu into free HSA occurs. The affinity of albumin to Cu ion has been reported by several researchers as $K_{\text{alb-Cu}} = 10^{12}–10^{16}\ M^{-1}$. Since 0.5 ppb of copper is steadily supplied to the HSA band from the mobile phase during separation, a constant concentration of 0.5 ppb free Cu^{2+} can be assumed in the band. The ratio of free and Cu-bound HSA under these conditions using $K_{\text{alb-Cu}}$,

$$\frac{[\text{HSA-Cu}]}{[\text{HSA}]} = 10^{3}\sim10^{7}$$

was obtained. This suggests that almost all contaminant copper supplied is taken up into HSA to become $10^{-12}–10^{-16}$ M free Cu if the HSA-Cu complex formation is sufficiently fast (Cu^{2+} complex formation is generally very fast at $k_f = \sim10^{10}\ s^{-1}M^{2}$).[74] The elution volume of HSA was generally 100 times greater than the sample volume in SEC and ion-exchange chromatography (see Ref. 26, 28 and M. C. Linder et al., *Am. J. Clin. Nutr.* **1998**, 67, 965S–9714S, etc.). The contaminant Cu was concentrated in the HSA band by up to 100-fold, i.e. 50 ppb (0.78 μM). Since the average of total copper concentration in serum is 750 ppb (see Ref. 2), the contribution of contaminant Cu is around 7%. In general, the concentration of the elution electrolyte for SEC and IC is very high (~1 M), this estimation of the contamination level might be optimistic. It can

be concluded that the contamination of Cu from separation buffers and instruments has considerable influence on determining the Cu distribution in serum.

1.2 Estimation of contaminant metal concentration in MICS-BN-PAGE.

In PAGE, the supply of contaminant Cu during separation occurs by migration since only the water molecules in the gel and the separation buffer do not move (the contaminants are supplied by flow in the case of LC). Contaminant Cu ions are masked by EDTA and TPEN and do not migrate into the gel in MICS-BN-PAGE. Free Cu concentration can be estimated by equilibrium calculations using stability constants of Cu-EDTA[64] and Cu-TPEN.[73] Based on conditional stability constants at pH 7.5 ($K_{Cu\text{-}edta}$' = $10^{16.1}$ M^{-1}, $K_{Cu\text{-}tpen}$' = $10^{20.3}$ M^{-1}), a contaminant of 7.8 nM Cu (0.5 ppb) is ideally lowered to below 10^{-13} M (6.5 ppq) in the presence of 10 μM EDTA and TPEN.