Electronic Supplementary Material (ESI) for Analytical Methods.

Quantification Signalling via Transition of Solution Inhomogeneity: Determination of Iron Contents in Human Serum by the Naked Eye

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1. Experimental Section.

Chemicals. All reagents were ACS grade or better and used without further purification. Iron chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), potassium chromate, sodium hydroxide, and imidazole were purchased from Sigma-Aldrich. Copper chloride dihydroxide ($CuCl_2 \cdot 2H_2O$) was purchased from Nihon Shiyaku Industries. Nickel chloride hexahydrate ($NiCl_2 \cdot 6H_2O$) and hydrochloric acid were from Merck. Other metal ions (chloride salts, > 98%) were from SHOWA, including Na^+, K^+, Mg^{2+}, Ca^{2+}, and Zn^{2+}. D-Penicillamine was purchased from Acros, and ferrozine was from Alfa Aesar. Water used in all experiments was doubly distilled and purified by Millipore-Q system. The real serum sample, Trace Elements Serum L-1, was purchased from Seronorm^{TM}. The serum sample contains 100 mM Na^+, 5 mM K^+, 2.5 mM Ca^{2+}, 1 mM Mg^{2+}, 20 μM Cu^{2+}, 13 μM Zn^{2+}, 0.7 μM Ni^{2+}, and 0.4 μM Cr^{2+}, according to the information provided by the supplier. As what described in the caption of Figure 3 for the human serum sample, 25.6 μM Fe^{3+} was determined locally by ICE-AES.

Apparatus. The apparent sizes and surface charge of the flocculates were measured by using a ZetaSizer (Nano-90S, Malvern). A handy laser pointer (< 1 mW) purchased from NovaStar (Taipei, Taiwan) was used to manifest the Tyndall effect. The UV-vis spectrophotometer (UV 300, UNICAM) was employed to figure out the mechanism. X-ray Absorption Near-Edge Structure (XANES) and Extended X-ray Absorption Fine Structure (EXAFS) data were collected at beamline 17C1 of NSRRC (the National Synchrotron Radiation Research Centre, Hsinchu, Taiwan). The Fe K-edge and Cu K-edge spectra were obtained in transmission and fluorescence mode, respectively.

Preparation of the metal ion solutions. Stock solutions of the metal ions were prepared by dissolving the corresponding chloride salts, including Na^+, K^+, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{3+}, Zn^{2+}, Ni^{2+}, and Cr^{2+}, with Millipore-Q purified water (18.2 MΩ·cm). The concentrations of the stock solutions were all 10 mM except Na^+, and were diluted to the concentration as those in human serum. The concentration of Na^+ was 100 mM. The solution of Fe^{3+} was prepared freshly to avoid the $Fe(OH)_3$

precipitate. The concentration of Fe^{3+} prepared was confirmed by titration with EDTA (ethylenediaminetetraacetic acid disodium salt dihydrate, $C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$).

Preparation of the imidazole buffer solution. To prepare 100-mL imidazole buffer (50 mM), 0.34 g of imidazole was weighed and dissolved in Milli-Q water. Hydrochloric acid and sodium hydroxide were used to adjust the solution pH with the aid of a pH-meter (Orion, model 410A).

Preparation of the real serum sample. The serum samples (Trace Elements Serum L-1, Seronorm^{TM}, Billingstad, Norway) were subjected to digestion by hot HNO_3 for 2 h.[1] The digested sample was neutralised by a 1-M NaOH solution and through a filter (pore size 0.1 μm, Millipore) subsequently. Finally, a suitable amount of water was added to the filtrated solution to reach the original volume as well as $[Fe^{3+}]$ of the serum samples. The serum sample for ICP-AES (S-35, Kontron) was diluted by 1% HNO_3[2] and the initial $[Fe^{3+}]_0$ was found to be 25.6 μM. To analyse the real sample, an aliquot of 0.50-mL serum was introduced into 2.2 mL of imidazole buffer (18.5 mM, pH 6.20) such that the final solution became 2.7 mL and contained 5.9-μM penicillamine.

2. General aspects of the adjustment of the iron(III)-responsive level.

The iron concentration level to which the sensing scheme responds can be tuned by the amount of penicillamine, as what has been discussed for Entry 10 (Table 1) and for the comparison made between Figures 1 and 3 in the main text. Note that the protocol involves the pre-determined time for the flocculates becoming observable. Therefore, in principle, the level to be quantified visually can be adjusted by experimental factors that accelerate or retard the polymerisation or flocculation, such as variables associated with (1) iron-imidazole complexation and (2) aggregation between cross-linked complexes.

Figure S1 demonstrates that solution pH, ionic strengths, and reaction temperature affect the growth of flocculates. The apparent sizes were monitored by dynamic light scattering (ZetaSizer, Nano-90S, Malvern). The complexation reaction is affected by the deprotonation degree of imidazole and thus the solution basicity. Figure S1a shows that a faster growth rate is found in a more basic solution. Aggregation between polymerised complexes can be retarded by electrostatic repulsion which can be reduced in solutions with a larger ionic strength (Figure S1b). Temperature is another apparent factor on the aggregation (Figure S1c). For the purpose of adjusting the pre-determined time, the experimental conditions in Figure S1 are not exhaustedly examined. Figure S1 simply demonstrated that the interplays between these parameters can be fine-tuned and result in an optimised protocol.

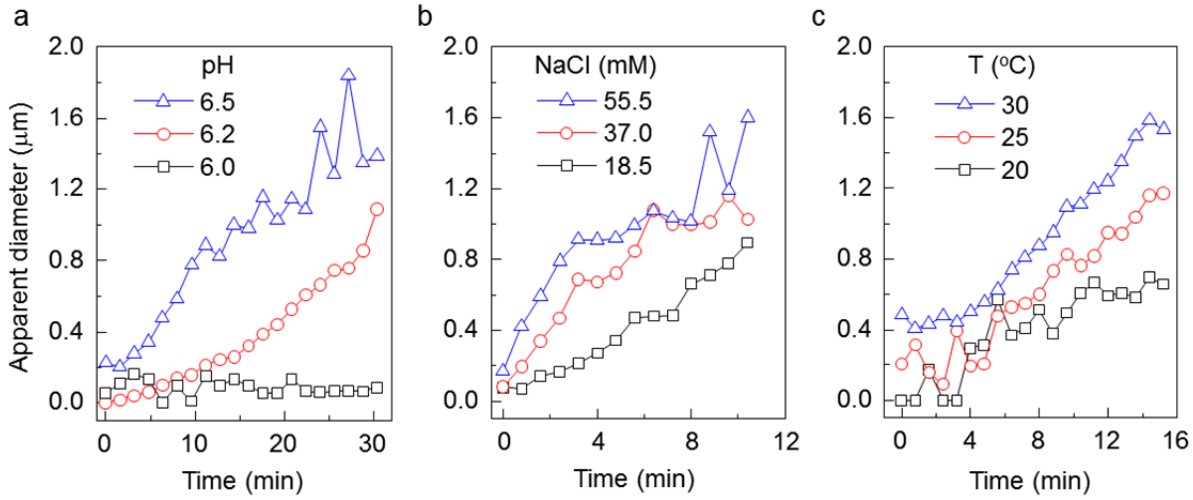

Figure S1. The effect of (a) pH, (b) ionic strength, and (c) temperature on the flocculation. The solution contained imidazole, penicillamine, and Fe^{3+}. Specifically, unlike the protocol presented in the main text, the solutions do not have Cu^{2+}. For the purpose of monitoring the growth for a longer duration, the concentration of Fe^{3+} (0.18 mM) is lower than that shown in Figure 1 (0.46 mM Fe^{3+}). Evolution of the apparent diameters of flocculates was recorded by DLS (dynamic light scattering, Nano-90S, Malvern). The results show that the formation of flocculates is faster in an environment of higher pH, ionic strength, and temperature. General experimental conditions: buffer, 18.5 mM imidazole (pH 6.20); penicillamine, 0.74 mM; temperature, 25 °C.

3. The insignificant influence of $[Cu^{2+}]_0$ variations on the sensing performance.

In the main text, it is demonstrated that the presence of Cu^{2+} accelerates the flocculate formation. The samples of human serum contain Cu^{2+} and Fe^{3+} with concentrations on the same order of magnitude. Therefore, the addition of Cu^{2+} is unnecessary. However, how the sample-to-sample variations of Cu^{2+} concentrations affect the determination of Fe^{3+} should be addressed. To clarify the concerns, Figure S2 is re-plotted from Figure 2 and shows that the behaviours do not depend on the mole ratios. Specifically, the pre-determined time to indicate 1-mM Fe^{3+} lies at 3 min (Figure 2 in the main text) where the relative concentrations of Fe^{3+} to Cu^{2+} spread in a wide range. Therefore, the influence of $[Cu^{2+}]_0$ variations in the sample is insignificant for the sensing performance.

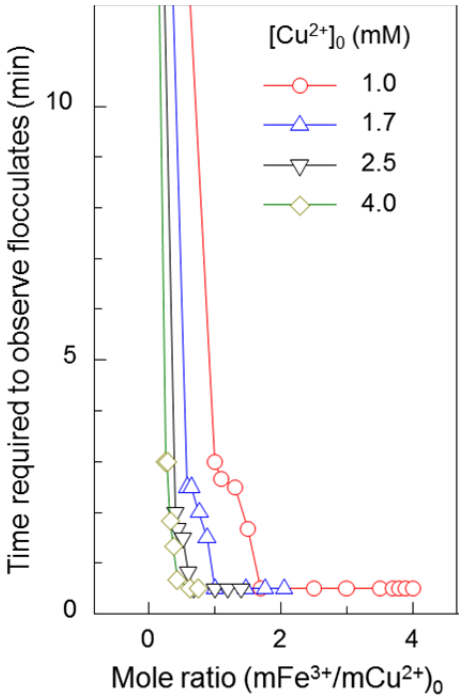

Figure S2. Time required for flocculates becoming apparent as a function of mole ratio of Fe^{3+} to Cu^{2+}. This Figure is re-drawn from Figure 2 of the main text. $[Fe^{3+}]_0$ and $[Cu^{2+}]_0$ ranged from 0.5–4.0 mM and 1.0–4.0 mM, respectively, in the 0.5-mL sample solution. The total volume of solution was 2.7 mL so the final concentration of $[Fe^{3+}]_0$ and $[Cu^{2+}]_0$ were diluted by 27/5 fold. For $[Fe^{3+}]_0$ below a certain level (*e.g.*, ~1.0 mM for this assay composition), the solution remained clear for a very long time (> 30 min) which was out of the vertical scale of Figure S2. Other experimental conditions are the same as described in Figure 1.

4. Formation mechanism and the composition of flocculates: supportive findings for (1) the oxidation states of Fe^{3+} and Cu^{2+} and for (2) the presence of disulphide bridges.

It is well known that thiols react with Fe^{3+} and Cu^{2+} and become disulphides, Fe^{2+}, and Cu^{+}. Penicillamine is a thiol and the associated redox reactions involved in the sensing scheme are elaborated in the main text. Equations 3 and 4 describe, respectively, the cases without and with the participation of Cu^{2+}. Entries 6 and 10 of Table 1 represent the former and Entry 11 exemplifies the latter. The resulted disulphides bridge metal complexes and facilitate the formation of flocculates.

$$Fe^{3+}\text{–RSH (purple)} \rightarrow Fe^{2+} + \tfrac{1}{2}\text{RSSR} + H^{+}\text{ (clear)} \quad (3)$$

$$Cu^{2+} + \text{RSH} \rightarrow Cu^{+} + \tfrac{1}{2}\text{RSSR} + H^{+} \quad (4)$$

The Equations are proposed based on the following assessment. The reduction reactions are examined by Fe^{2+}–specific ferrozine (Figure S3) and Cu^{+}–specific bcs (bathocuproinedisulphonic acid disodium salt, Figure S4). The presence of disulphide linkers are supported by the dissolution of flocculates *via* the addition of ascorbic acid which presumably turns disulphides into thiols (Figure S5).

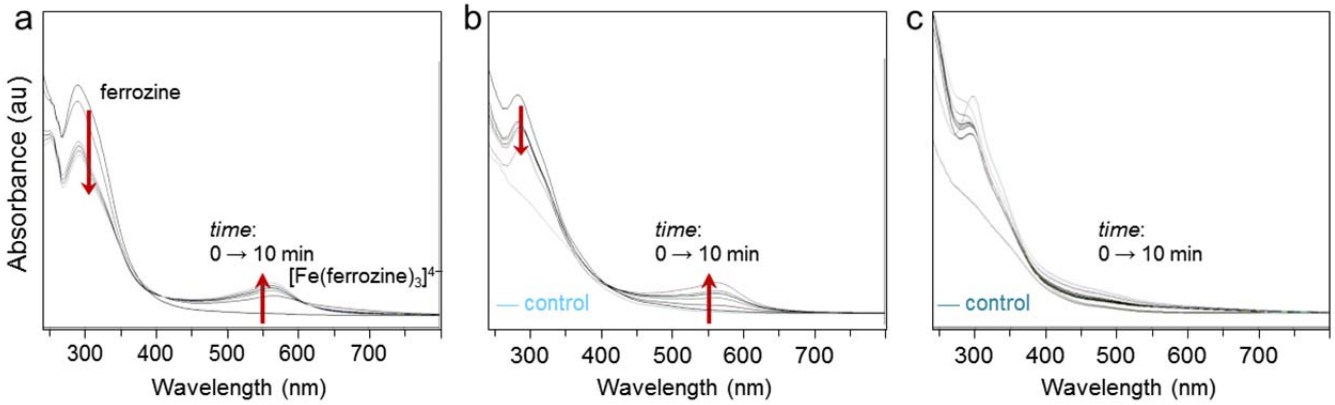

Figure S3. Mechanistic studies on the metal-penicillamine reactions under the conditions of Entries (a) 6, (b) 10, and (c) 11. Specifically, prior to the introduction of penicillamine, the cuvets contained ferrozine and a) Fe^{3+}, b) Fe^{3+} and imidazole, and c) Fe^{3+}, imidazole, and Cu^{2+}. For Panel c (*i.e.*, Entry 11), the introduced solution contained Fe^{3+} and Cu^{2+}. After penicillamine was added, the spectra were acquired and overlaid to show the evolution of ferrozine (at 290 nm)[3] and $[Fe^{II}(ferrozine)_3]^{4-}$ (at 562 nm). In Panels a and b, the decrease in intensity at 290 nm and the concomitant increase at 562 nm indicate the formation of Fe^{2+}. Note that in the case with imidazole (Panel b, Entry 10), the growth at 562 nm is slower than that without imidazole (Panel a, Entry 6), consistent with the scenario that Fe^{3+}–imidazole complexation retards the reduction of Fe^{3+} by penicillamine. Panel c (Entry 11) shows no change at 562 nm, yet a slight increase at ~470 nm for $[Cu^{I}(ferrozine)_2]^{3-}$.[3] The results justify the proposed redox reactions and demonstrate that penicillamine preferentially reduces Cu^{2+} over Fe^{3+}. Therefore, in the protocol (Entry 11), the formation of Fe^{2+} is unlikely, consistent with the results of XANES and EXAFS. In Panels b and c, the featureless spectra denoted *control* were obtained from solutions of Fe^{3+} and imidazole buffer to manifest that the peaks at 290 nm and 562 nm were associated with ferrozine and $[Fe^{II}(ferrozine)_3]^{4-}$. General experimental conditions: ferrozine, 0.07 mM; penicillamine, 0.74 mM; buffer, 18.5 mM imidazole (pH 6.20); Fe^{3+}, 0.46 mM; Cu^{2+}, 0.31 mM; temperature, 25 °C.

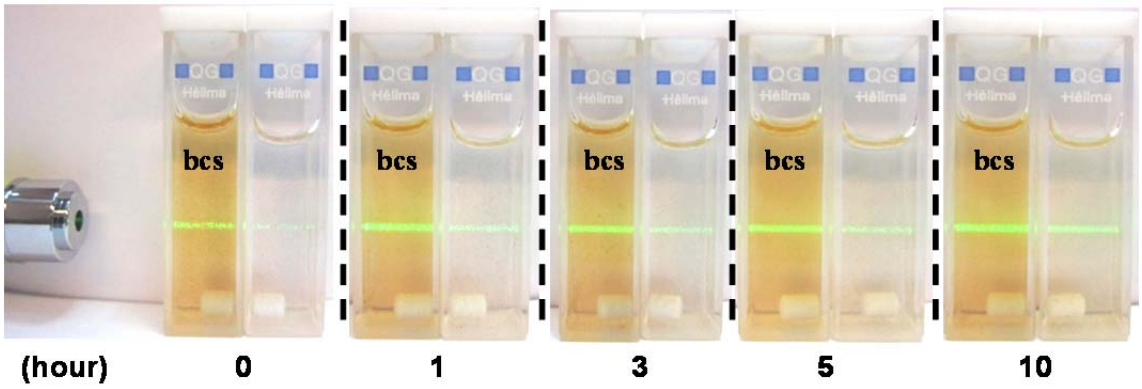

Figure S4. Verification of Cu^{+} presence in the sensing scheme *via* the formation of $[Cu^{I}(bcs)_2]^{3-}$ complex. Bathocuproinedisulphonic acid, bcs,[4] is a widely used Cu^{+}–specific chelator. The presence of Cu^{+} can be read by the formation of yellow $[Cu^{I}(bcs)_2]^{3-}$ complex.[5] The solutions in the vials were the sensing assay, *i.e.*, Entry 11. Only the left ones contained bcs (concentration: twice that of Cu^{2+}) while the right ones did not and thus served as the reference. The yellowish colour appeared upon the addition of a 0.5-mL sample aliquot which had Fe^{3+} and Cu^{2+}, manifesting the formation of Cu^{+}. If Cu^{+} complexed with the ligands and cross-linked the flocculates together, bcs would presumably extract Cu^{+} from the complexes and break the networks, leading to dissolution of the flocculates. However, the flocculates did not dissolve and the vial with bcs exhibited the same Tyndall effect as that of the reference after 10 hrs. The results suggest that the copper in the flocculates is not Cu^{+}, consistent with the findings of X-ray absorption spectroscopy.

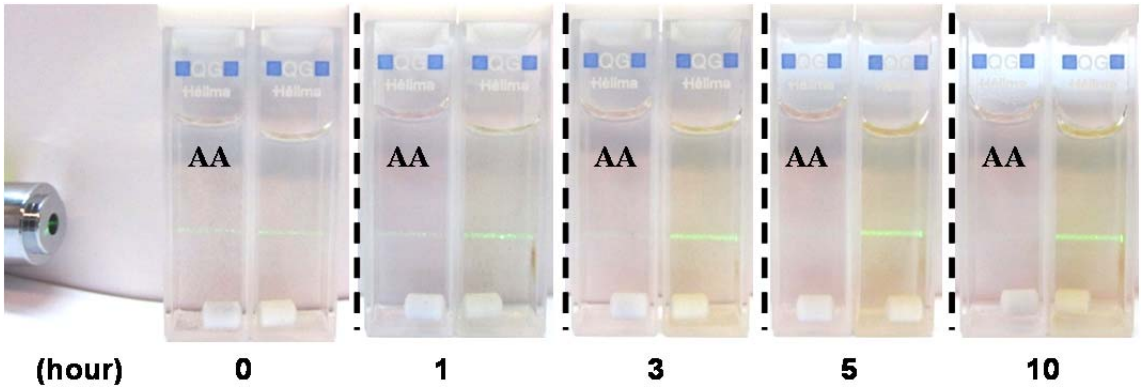

Figure S5. Dissolution of the flocculates *via* the addition of ascorbic acid. The left vials contained ascorbic acid (AA), an oxidant known able to oxidise disulphides.[6-9] Similar to Figure S4, the right vials did not have AA and served as the reference. AA was introduced 1 min after the flocculates became observable. The intensity of the scattered laser beam was weakening and eventually unobservable, suggesting the dissolution of flocculates by transforming the disulphide linkers into thiols. Solutions: Fe^{3+}, 0.46 mM; buffer, 18.5 mM imidazole (pH 6.20); penicillamine, 0.74 mM; Cu^{2+}, 0.31 mM; AA, 0.74 mM; temperature, 25 ^{o}C.

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