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Visual Determination of Serum Copper (II) by Zinc-containing Mesoporous Silica

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The formation of complexes with the chromophore zincon within the nanopores of aminated mesoporous silica was employed to develop a simple colorimetric method for the detection of copper (II) in human serum. In this method, the sedimentation of the aminated mesoporous silica encapsulating zincon allowed for the detection of a distinct color change, resulting in the detection of copper in the 0–6 μM range. Although zincon forms complexes with both copper (II) and zinc (II) in bulk solution, this complexation within the nanopores enabled the selective detection of copper (II). When this method was applied to the measurement of copper (II) in human serum, the measured values were comparable to those obtained by the conventional method using basocuproindisulfonic acid.

1. Introduction

Copper is an essential element for various physiological functions in the body and is necessary for the human body to maintain a delicate balance between deficiency and excess.[1,2] For example, decreased serum copper concentration appears to be a symptom in diseases such as Wilson's disease and chronic ischemic heart disease,[3,4] whereas elevated serum copper concentration is observed in Hodgkin's disease.[5,6] In addition, changes in human serum copper concentration are observed in the course of chemotherapy.[7,8] Therefore, the detection of copper in serum is important.

Conventional techniques for detecting copper ions include the use of fluorescence probes,[9–11] atomic absorption spectrometry,[12–14] inductively coupled plasma mass spectrometry,[15,16] and electrochemical analysis.[17–19] Although these analytical methods have excellent sensitivity, accuracy, and selectivity, they require specialized equipment. Compared with these methods, colorimetric methods are effective because they enable us to obtain test results at the patient's bedside. Colorimetric assays using metal oxides with peroxidase-like activity or peroxidase have been proposed.[20–22] In these assay methods, the reaction from

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3,3′,5,5′-tetramethylbenzidine (TMB) to blue-colored oxide is controlled by copper ions. The intensity of the blue color changes with reaction time, which may lead to erroneous determinations due to the calculation of concentration from the blue intensity. On the other hand, chelating reagents have the potential to develop a colorimetric method for detecting serum copper with simpler experimental manipulations. Although metal colorimetric reagents selective for Cu(I), such as bicinchoninate[23] and bicinchoninic acid (BCA),[24,25] are known, there are few suitable reagents for the selective detection of Cu(II).[26] When Cu(II) is detected using BCA, reduction to Cu(I) by ascorbic acid or potassium sodium tartrate is followed by the formation of orange-yellow (λ_{max} = 485 nm) BCA-Cu(I).[27] Although zincon (2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene) is known as a reagent that can detect Cu(II), Zn(II) also forms complexes with Cu(II), and their complexation stability constants are comparable.[28,29]

In this study, we developed a particulate sensor for the measurement of Cu(II). The particulate sensor was fabricated by encapsulating zincon in the nanopores of mesoporous silica with an aminated surface. Since the nanopores are filled with zincon, the internal volume of the nanopores is considered to be very small. Therefore, small amounts of proteins and low-molecular-weight organic compounds remaining after the removal of these substances are unlikely to penetrate into the nanopores, whereas substances with smaller sizes, such as inorganic compounds, preferentially penetrate into the nanopores. Moreover, by concentrating a large amount of complexes in the nanopores of the microparticles, the color change can be easily observed visually, *i.e.*, the sensitivity of the measurement is expected to be improved. In our approach, we found that Cu(II) could be selectively detected even though zincon, which originally also recognizes zinc, was used.

2. Materials and Methods

2.1 Reagents

Zincon [Fig. 1(a)] was purchased from Dojin Chemical Laboratory. MCM-41, MSU-H, human serum albumin (HSA, lyophilized powder, 97–99%), ɤ-globulin (GG > 99%), and normal human serum (type AB, male, H4522-20ML) were purchased from Sigma-Aldrich. 3-Aminopropyltrimethoxysilane (APS) was purchased from Shin-Etsu Chemical. 3-[(2-Aminoethyl)amino]propyldimethoxymethylsilane (AAPS) [Fig. 1(c)], copper sulfate pentahydrate, and zinc chloride were from Wako Pure Chemical Industries. All other reagents were special grade. Microplates (96-well, round bottom) were purchased from Nunc A/S. Milli-Q water (Millipore, Bedford, MA) was used.

2.2 Apparatus

A Shimadzu UV-Visible Spectrophotometer UV-2500PC was used to measure absorbance. A glass electrode pH meter (Model IOL30 manufactured by Electrochemical Instruments) and a simple pH meter (LAQUAtwin B-71X manufactured by Horiba, Ltd.) were used to measure the pH of aqueous solutions.

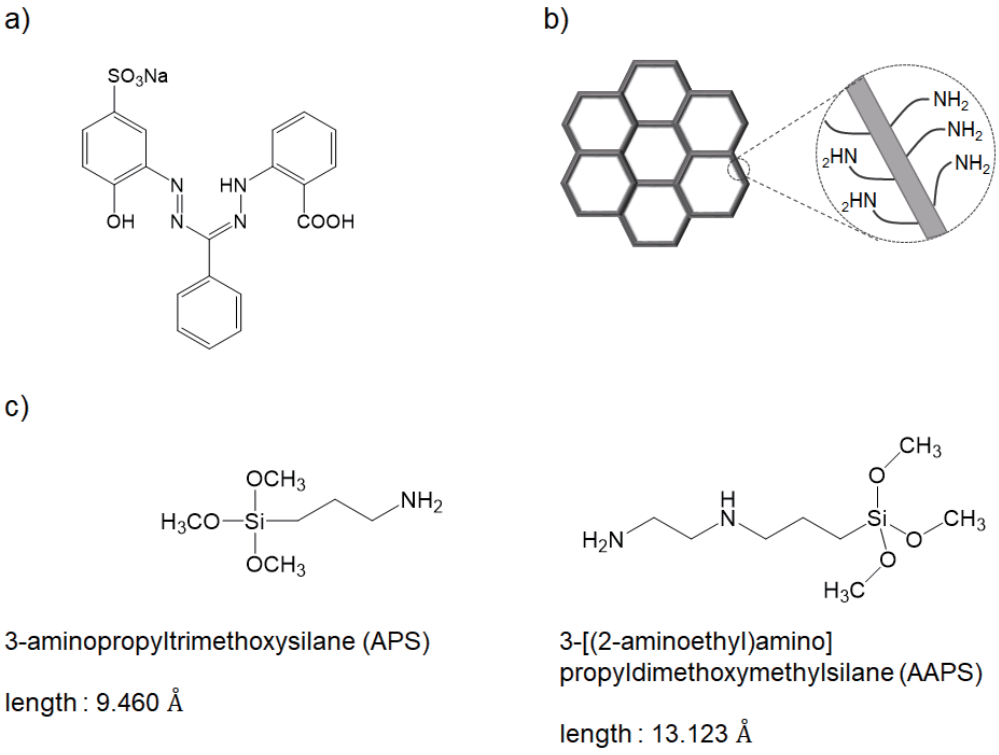

Fig. 1. Chemical structure and schematic of aminated mesoporous silica. (a) Zincon, (b) aminated mesoporous silica, and (c) amino silane coupling agent. Molecular lengths were obtained by 3Dchem.

2.3 Amination of mesoporous silica by silanation treatment

Modified APS MCM-41 (abbreviated as APS-MCM-41), modified APS MSU-H (abbreviated as APS-MSU-H), and modified AAPS MSU-H (abbreviated as AAPS-MSU-H) [Fig. 1(b)] were prepared according to previously published methods.[29] Fifteen milligrams of mesoporous silica, *i.e.*, MCM-41 and MSU-H, were added to 2.4 mL of anhydrous toluene, and the particles were dispersed by the sonication of the suspension. Then, 0.62 g of APS or AAPS was added to this suspension and stirred at 20 °C for 1 h. To purify the aminated mesoporous silica, the supernatant was removed by centrifugation at 2880 g for 5 min. The precipitates were washed by centrifugation with 1.5 mL of anhydrous toluene. The washing procedure was repeated thrice, and the precipitate was dried at 90 °C overnight.

2.4 Zincon encapsulation in nanopores of mesoporous silica

A 20 mg/mL suspension was prepared by adding 20 mg of AAPS-MSU-H or APS-MSU-H to 1000 µL of boric acid buffer. Zincon was dissolved in boric acid buffer to a concentration of 1 mM and used as a stock solution. A solution of 0.05 mM zincon was prepared by diluting a stock solution with boric acid buffer. A portion of 700 µL of 0.05 mM zincon solution was added to a portion of 700 µL of 20 mg/mL AAPS-MSU-H or APS-MSU-H suspension, followed by stirring and then incubation in the dark at room temperature for 60 min. After incubation, the supernatant

was collected by centrifugation for 5 min (17968 *g*, 20 °C). The precipitates were washed by adding 1400 μL of boric acid buffer, and the supernatant was recovered by centrifugation. The absorption spectrum of the zincon solution was measured using the Shimadzu UV-Visible Spectrophotometer UV-2500PC. The ratio of absorbance values at 467 nm with and without mesoporous silica was used to estimate the incorporation rate into the mesoporous silica.

2.5 Deproteinization of human serum

Four hundred microliters of human serum was added to 100 μL of 1 M hydrochloric acid, followed by heating at 95 °C for 5 min. The solution was cooled to room temperature and mixed with 100 μL of 100 (w/v)% trichloroacetic acid, and the supernatant was collected by centrifugation at 18000 g for 5 min. pH was adjusted to 8.5–10.0 by adding 140 μL of the pH adjuster, which is a mixture of 176 μL of 2.83 M sodium hydroxide solution and 200 μL of 500 mM boric acid buffer solution, to 150 μL of the collected supernatant.

2.6 Experimental procedure for semiquantitative determination of copper (II) ions by visual inspection

$CuSO_4 \cdot 5HO$ was dissolved in Milli-Q to a concentration of 1 mM (stock solution), and then 200 μl of 0.05 M HCl was added to the 1 mM stock solution. This solution was diluted with ammonia buffer as needed to observe concentration-dependent blue coloration. Two hundred and fifty-five microliters of copper sulfate solution diluted in ammonia buffer or human serum after deproteinization was transferred to the wells of 96-well microplates. Forty-five microliters of zincon-encapsulated aminated mesoporous silica, *i.e.*, APS-MSU-H and AAPS-MSU-H, prepared by suspending in ammonia buffer to 10 mg/ml, was added to the wells to give a total volume of 250 μl in the wells. The solution in the wells was thoroughly mixed with a micropipette and incubated for 2 h. The wells were photographed with a smart phone camera.

2.7 Preparation of serum sample

Six hundred microliters of human serum that had been pretreated for deproteinization was added to 280 μL of 8.45 mM ascorbic acid solution, 100 μL of boric acid buffer, 140 μL of 1 mM BCA, and 100 μL of 2.83 M NaOH. The mixture was stirred and then the absorption spectrum was measured. The concentration of copper in serum was calculated using a calibration curve from the absorbance at 485 nm based on complex formation with BCA.

3. Results and Discussion

3.1 Enrichment of complex of zincon with Cu (II) ions within nanopores of aminated mesoporous silica

The enrichment of zincon complexes formed with copper (II) in the nanopores of mesoporous silica is expected to enhance the sensitivity of visually determining the color change. Figure 2

shows the color change after copper (II) was added to 50 µM zincon solution. In ammonia buffer solution (pH 8.8), the color of the copper (II) solution changed from red to blue with increasing copper (II) concentration owing to the complexation with zincon [Fig. 2(a)]. On the other hand, APS-MCM-41, *i.e.*, aminated mesoporous silica, was added to a solution containing zincon and copper (II), causing the blue tone of the suspension at 0 min to become weaker than that of the complex alone [Fig. 2(b)]. However, about 2 h after the addition of copper (II), APS-MCM-41 spontaneously sedimented, imparting a more distinct blue color to the sediment [Fig. 2(b)]. The complexes formed with zincon and copper (II) were enriched in the nanopores since the absorption spectrum of the supernatant solution did not show any peaks around 600–620 nm, *i.e.*, absorption bands of the complexes (Fig. 3). Aminating the surface of mesoporous silica is

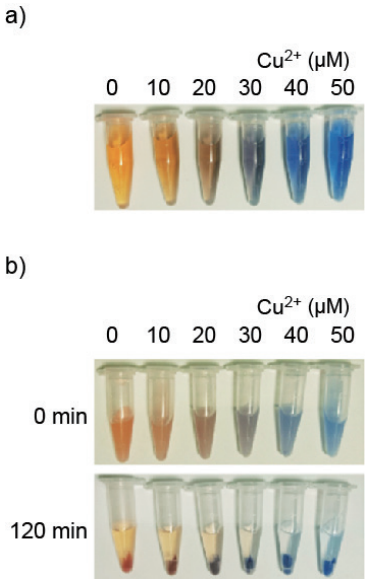

Fig. 2. (Color online) Photos of a series of color intensities for Cu(II)-zincon complexes. (a) Images of 50 µM zincon and 25 µM copper (II) in 10 mM NH_3-NH_4Cl buffer solution (pH 8.8). Images immediately (b) and at 120 min after MCM-41 particles were added to the solution to give a concentration of 5 mg/mL.

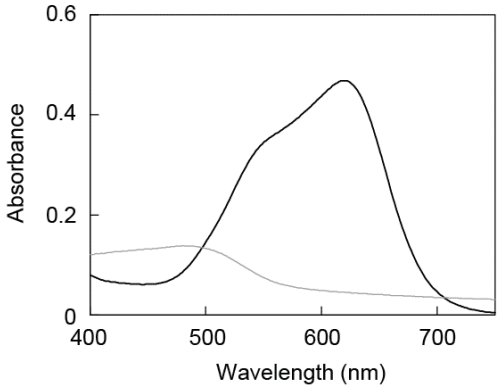

Fig. 3. Absorption spectrum of copper (II)-zincon complex. 25 µM zincone and 25 µM copper (II) were dissolved in 10 mM NH_3-NH_4Cl buffer solution (pH 8.8) to prepare complex solutions. Absorption spectrum of the complex solution without APS-MCM-41 (gray line). Absorption spectrum of the supernatant after adding APS-MCM-41 to give a concentration of 5 mg/mL (black line).

likely to enrich zincon with sulfo and carboxy groups in its nanopores through electrostatic interaction. These results indicate that both the enrichment effect of the blue complex in the nanopores of APS-MCM-41 and the aggregation effect of the APS-MCM-41 particles are essential for the distinct color tone of the sediments.

3.2 Zincon encapsulation in nanopores of aminated mesoporous silica

The high sensitivity of the measurement method poses a challenge in colorimetric analysis, where the concentration of analytes is estimated visually, as opposed to analytical techniques that involve expensive and large equipment. In our approach, a large number of complexes between copper (II) and zincon in the nanopores may increase the sensitivity of the visual detection. Therefore, we decided to investigate the development of a colorimetric method for the detection of copper (II) using MSU-H with a pore diameter of 11 nm instead of MCM-41 with a pore diameter of approximately 2 nm. In addition, the possibility of encapsulating only the ligand zincon, rather than the complex of zincon and copper (II), in the nanopores of aminated-MSU-H is also important to investigate. If copper (II) and zincon can form complexes in the nanopores, then the aminated-MSU-H containing zincon itself would be treated as an alternative material to the reagent for copper (II) detection.

Therefore, the incorporation ratio of zincon into aminated MSU-H was estimated from the absorption spectrum (Fig. 4). The absorbance at the maximum absorption wavelength of zincon was barely observed in the supernatant of the suspension containing AAPS-MSU-H and zincon. This suggests that almost all of the zincon molecules were incorporated into the nanopores of mesoporous silica, with an estimated uptake rate of about 95%. Moreover, the absorption spectrum of the supernatant after resuspending the zincon-encapsulated mesoporous silica showed that the zincon encapsulated in AAPS-MSU-H almost did not leak out. On the other hand, the incorporation ratio of zincon into APS-MSU-H was about 25%, and zincon molecules leaked out with each resuspension. The APS and AAPS used to aminate the pore surface of mesoporous silica differ in chain length. The longer the chain length, the larger the volume of the silanating agent occupying the interior of the pore, suggesting that the encapsulated zincone is less likely to leak out. With the non-aminated MSU-H, zincon could not able to be encapsulated.

3.3 Complexation of zincon with Cu(II) in nanopores of aminated mesoporous silica

The incorporation of zincon into the nanopores is predicted to involve electrostatic interactions between the carboxy groups of zincon and the amino groups on the surfaces of the nanopores. In particular, since carboxy groups are functional groups involved in coordination bonds, the ability of zincon to form complexes with copper (II) in the nanopores needs to be demonstrated.

Zincon was added to the AAPS-MSU-H suspension at concentrations of 0.05 and 0.1 mM to enrich the zincon into its nanopores. In the case of 0.05 mM, the color change based on the

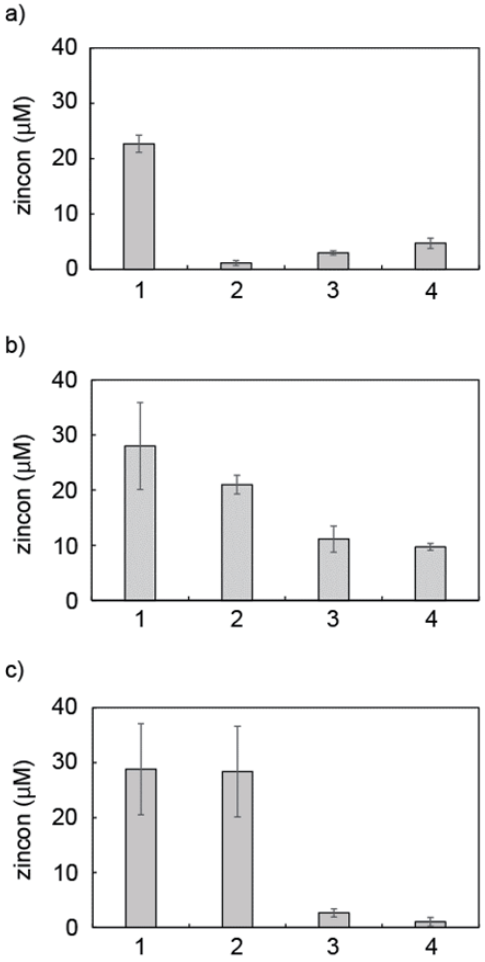

Fig. 4. Leakage of encapsulated zincon from mesoporous silica. The 25 μM zincon solution was prepared in 10 mM NH_3-NH_4Cl buffer solution (pH 8.8). Each mesoporous silica, *i.e.*, (a) AAPS-MSU-H, (b) APS-MSU-H, or (c) MSU-H, was added into the zincon solution to give a concentration of 5 mg/mL. 1. Before adding mesoporous silica., *i.e.*, zincon solution. 2. Supernatant after the suspension of mesoporous silica. 3. Supernatant after the resuspension of mesoporous silica. 4. Supernatant after the suspension of mesoporous silica twice. Zincon concentrations were estimated from a calibration curve. The calibration curve was prepared by measurements of absorbance at 476 nm ($y = 12.93x - 0.0023$, $R^2 = 0.9998$, $n = 3$). Averages of zincon concentration are plotted. Error bars indicate mean ± standard deviation ($n = 3$).

complex formation was observed by adding copper (II) ions to the zincon-incorporated AAPS-MSU-H [Fig. 5(a)]. A slight color change was observed even with 1 μM copper (II) ions, and the higher the concentration of copper ions, the more clearly the blue color tone could be judged. These results indicate that complexes can be formed even in nanopores. The resuspension of the AAPS-MSU-H particles after centrifugal collection indicated that the complexes did not leak from the nanopores, since they did not exhibit absorption wavelength peaks attributable to the complexes in the supernatant solution (Fig. 6). This is important in semiquantifying the concentration of few copper (II) ions by visual color change. On the other hand, when zincon was encapsulated at a concentration of 0.1 mM, the addition of copper (II) did not change the color tone. The relatively large number of molecules of non-complexed zincone in the nanopores

probably hindered the visual detection of color changes. In contrast, for zinc (II), no color change was observed in the suspension of zincon-incorporated AAPS-MSU-H, with the precipitate remaining orange, reflecting the color tone of free zincon (Fig. 5). The pH in mesoporous silica pores with positively charged inner walls is reported to be lower than that in the bulk pore.[30] In this study, copper (II), which complexes at low pH, is probably more selectively complexed with zincon, rather than zinc (II), because of the lower pH in the pores. These results indicate that AAPS-MSU-H incorporating zincon can selectively recognize copper (II), but not zinc (II). In general, studies of chelating agents or ligands have shown that metal–ligand complexes depend on the ring size formed during chelation, the number of rings formed, the basicity of the chelating agent, the nature of the donor metal, and the central metal ion.[31] However, the enhancement of selectivity between metal ions with similar valences, number of ligands, and ion sizes, such as copper and zinc ions, is difficult to achieve. Our approach of using nanopores to improve selectivity may be a possible means of overcoming these challenges.

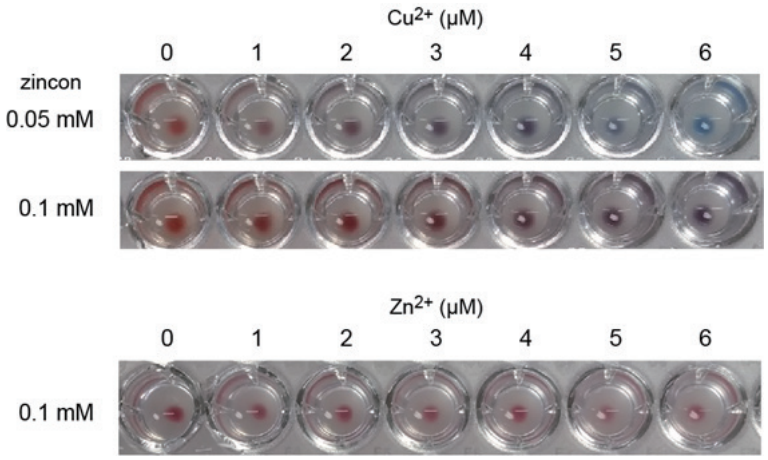

Fig. 5. (Color online) Comparison of a color tone caused by formation of a zincon complex with copper (II) or zinc (II) in nanopores of AAPS-MSU-H. Zincon at concentrations of 25 and 50 μM was encapsulated within the nanopores of AAPS-MSU-H.

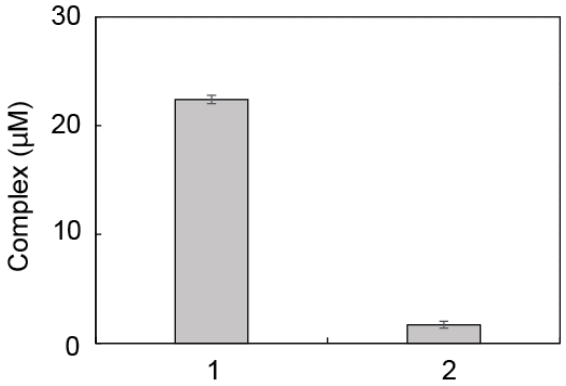

Fig. 6. Leakage of encapsulated copper (II)–zincon complex from 5 mg/mL AAPS-MSU-H suspended in 10 mM NH3-NH4Cl buffer solution (pH 8.8). Zincon concentrations were estimated from a calibration curve. The calibration curve was prepared by measurements of absorbance at 600 nm ($y = 20.314x + 0.0141$, $R^2 = 0.9992$, $n = 3$). Averages of zincon concentration are plotted. Error bars indicate mean ± standard deviation ($n = 3$). (1) Without AAPS-MSU-H. (2) Supernatant of AAPS-MSU-H suspension.

3.4 Visual semiquantitative determination of copper (II) in normal human serum

Various biomolecules coexist in human serum, and the possibility that these components interfere with the complex formation cannot be denied. For example, under pH 9, HSA with isoelectric point (pI) 4.7 and GG with pI 6.0–8.5 are negatively charged and may interact electrostatically with the positively charged surface of AAPS-MSU-H. This may inhibit the penetration of Cu(II) ions into the pores. Therefore, after deproteinization with organic acid was performed, this pretreated solution was added to a suspension of AAPS-MSU-H encapsulated with zincon. The concentration of copper in human serum was determined by comparison with a standard sequence of copper (II) at about 1–2 μM (Fig. 7). Since the serum was diluted 2.8-fold

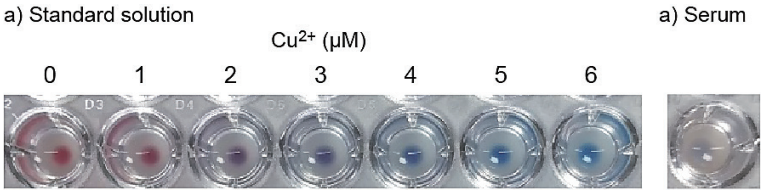

Fig. 7. (Color online) (a) Photographs of the visual assay of human serum and 0–2 μM copper (II) in 10 mM NH_3-NH_4Cl buffer solution (pH 8.8). (b) The assay was performed after the deproteinization of human serum at fourfold dilution.

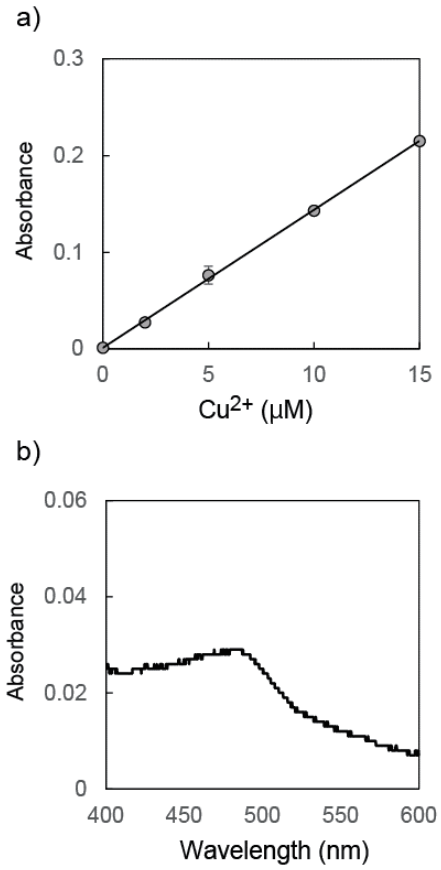

Fig. 8. Measurement of serum copper (II) by BCA method. (a) Calibration curve prepared with standard solution of copper (II). (b) Absorption spectrum of serum copper (II) measured by BUA method.

by the deproteinization operation, the undiluted human serum contained copper at approximately 3–4 μM when the dilution factor was taken into account.

The accuracy of this method was examined by absorbance spectrophotometry using BCA.[32,33] Since BCA is light yellow in color at pH 4.0–10.0, serum was adjusted to pH 4.0–10.0 with sodium hydroxide after deproteinization. The absorbance measurement of serum copper revealed a peak of serum copper around 485 nm (Fig. 8). The concentration of serum copper was calculated to be 3.0 ± 0.6 μM, which is comparable between the BCA method and this method.

4. Conclusion

The amination of mesoporous silica MSU-H allowed zincon to be encapsulated in its nanopores. This resulted in the formation of blue complexes with copper (II) in the nanopores. In addition, Zn(II) complexing with zincon was not detected by this method using nanopores. Therefore, AAPS-MSU-H containing zincon can be a new material for the selective detection of copper (II). Using this material, we have developed a highly sensitive method for the colorimetric analysis of copper (II) by combining a large amount of blue complexes in the nanopores with the sedimentation and aggregation of MSU-H. Since zincon is encapsulated within the nanopores, copper (II) could be detected with a minimal effect from low-molecular-weight organic compounds remaining after protein removal, although the removal of some proteins that might block the entrance of the nanopores with a few molecules is necessary. This may be a useful and simple diagnostic method at the patient's bedside for Wilson's disease and chronic ischemic heart disease, in which decreased serum copper (II) concentration is observed.

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