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Development of an Albumin Copper Binding (ACuB) Assay to Detect Ischemia Modified Albumin

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Myocardial ischemia (MI) induces many changes in the body, including pH decrease and electrolyte imbalance. No obvious symptoms of MI appear until irreversible cellular injuries occur. Since early treatment is critical for recovery from ischemia, the development of reliable diagnostic tool is demanded to detect the early ischemic status. Ischemia modified albumin (IMA), formed by cleavage of the last two amino acids of the human serum albumin (HSA) N-terminus, has been considered so far as the most trustworthy and accurate marker for the investigation of ischemia. IMA levels are elevated in plasma within a few minutes of ischemic onset, and may last for up to 6 h. In the present study, we developed a novel assay for the examination of IMA levels to ameliorate the known albumin cobalt binding (ACB) test established previously. We observed a stronger copper ion bound to the HSA N-terminal peptide than cobalt ion by HPLC and ESI-TOF mass spectrometric analyses. The copper ion was employed with lucifer yellow (LY), a copper-specific reagent to develop a new albumin copper binding (ACuB) assay. The parameters capable of affecting the assay results were optimized, and the finally-optimized ACuB assay was validated. The result of the IMA level measurement in normal *versus* stroke rat serum suggests that the ACuB assay is likely to be a reliable and sensitive method for the detection of ischemic states.

Keywords Albumin copper binding (ACuB) assay, ischemia modified albumin, serum IMA levels in stroke rat models, 96-well volume reaction

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Introduction

Myocardial ischemia (MI) results from an impairment of the coronary blood supply, in most cases, due to a blockage of the blood flow. As this ischemic state persists, (*i.e.*, oxygen supply is stopped) metabolic changes occur, such as a decrease of ATP and/or pH and accumulation of lactate, which may lead to an imbalance of intracellular electrolytes. Such stressful conditions without treatment may cause irreversible cellular injury, and eventually infarction.[1,2] Despite the serious problems caused by MI, no specific symptoms appear, and therefore most MI patients are unaware of their illnesses until it becomes irreversible.[3] Fortunately, some patients feel symptoms such as throbbing chest pains and rush themselves into the emergency department. However, not all patients with severe pain are seriously ill and require expensive and intensive care. Electrocardiograms (ECGs) are initially taken in order to diagnose the patients' status. About 80% of the patients with abnormal ECG results such as elevation in the ST-segment have myocardial infarction and only half of the patients with chest pain show abnormalities in their cardiograms. Thus, many are mistakenly discharged and in consequence the delayed treatment may lead to irrevocable results.[4]

In addition to abnormal ECGs, biochemical markers, such as creatine kinase-MB isoenzyme (CK-MB) and cardiac troponins, are also used to assess coronary diseases. Cardiac troponins are mainly observed due to their organ-specificity and sensitivity toward myocardial infarction. However, current trends in examining coronary syndromes suggest that these traditional markers are not so ideal for an early diagnosis or the selection of appropriate therapy.[5] Instead, the testing of multi-markers is regarded as being more reliable than a single-marker analysis, providing a better risk stratification for mortality.[6] Metabolomics in various types of ischemia are also on the rise,[7,8] but these techniques usually require expensive equipment, and may need professional handling.[9] Amongst the many identified ischemic biomarkers, the most interest has grown regarding the ischemia modified albumin (IMA) since its discovery. IMA is easily detected, and is thus useful in the early identification of patients with acute coronary syndrome who complain of chest pains with or without abnormal ECGs.[10]

Simply put, IMA is a structurally modified human serum albumin (HSA) by ischemic stress. HSA is the most abundant protein in the human body (~5% (w/v), ~5 g HSA in 100 mL of human blood) and weighs about 66.5 kDa with 585 residues.[11]

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One of the main functions of HSA is to carry around various ligands in the body. It is widely known to bind to several metal ions, such as Ca^{2+}, Co^{2+}, Co^{3+}, Ni^{2+}, Cu^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pt^{2+}, and Hg^{2+}. The three major metal binding sites are the N-terminal binding site (NTS), Cys34 binding site, and the primary multi-metal binding site or Cadmium site A (MBS-A).[12] The NTS is the first metal binding site for divalent transition metals, such as Co^{2+}, Cu^{2+}, and Ni^{2+}. These metals bind to the N-terminus region, Asp-Ala-His-Lys (human), with the first three amino acids being essential for its strong affinity.[13] When MI occurs, IMA is formed by the cleavage of two amino acids at the N-terminus (Asp-Ala), which results in the loss of metal-binding ability. IMA has already been proven to have low binding affinities toward metal ions that may strongly bind to the normal HSA.[13]

IMA levels rise within a few minutes of cardiac ischemia and may persist for up to 6 h.[14] Because IMA is formed before necrosis, it is recognized as an excellent biomarker for early detection of MI at a reversible state. For a comparison, the cardiac troponin level increases when irreversible cellular damage is already in place, making it a relatively 'late' marker for ischemia.[15] Although the primary purpose is to detect coronary diseases, many reports on elevated IMA levels in other diseases, such as stroke, have also been published.[16,17] This implies that IMA-related research may expand its investigation into interpreting numerous types of ischemic states. A few studies have questioned the *in vitro* stability of IMA and pointed out the need for a rapid analysis after the collection of blood samples.[18,19] However, in spite of the above-mentioned unresolved problems, the measurement of IMA is still the only clinical test approved by the American Food and Drug Administration (FDA) since 2003 for detecting MI.[10]

Based on the fact that IMA has a lower Co^{2+}-binding capacity than the normal HSA,[13] Bar-Or *et al.* established a test called the albumin cobalt binding (ACB) test in 2000. Briefly, cobalt is mixed with serum samples, and then dithiothreitol (DTT) is used to detect the remaining free cobalt ions after binding to the normal HSA. More free cobalt forms the complex with DTT showing an increase in absorption at 470 nm if the sample contains low levels of the normal HSA (*i.e.* IMA levels are high).[20] Also Bar-Or *et al.* proposed different parameters, such as pH and reagents that may affect the mode of action of the assay, but further studies seem to be necessary.[21] The ACB test is currently used in many emergency departments to rapidly decide whether to rule out ischemia in patients with severe chest pains.[15,22]

Interestingly, in 2007, Mothes *et al.* first observed that the major cobalt binding site is not at the N-terminus. According to this study, cobalt binds to three distinct sites of HSA; principally at site A (His 67 as the ligand) and site B (not yet localized) and weakly at the NTS.[23] It has been previously pointed out that Cu^{2+} and Ni^{2+} preferentially bind to the NTS, with the dissociation equilibrium constants of the metal-HSA complex being 6.7×10^{-17} and 2.5×10^{-10} M, respectively. In contrast, the dissociation constant for the Co^{2+}-HSA complex was 1×10^{-4} M, indicating that Cu^{2+} has the highest binding affinity toward the N-terminus of HSA.[12] Since it was found that Cu^{2+} and Ni^{2+} were the major metal ions that bind to the NTS, it was evident that the ACB test needed to be modified with different metal ions for better sensitivity.

In this study, the copper-binding characteristic of HSA was utilized to develop a novel assay for detecting IMA. Based on a literature search, some copper ion detecting reagents were examined. Among them, lucifer yellow carbohydrazide (LY-CH, to be referred as 'LY') was selected as a selective and sensitive reagent to detect free copper ions.[24] We confirmed that LY was indeed copper-specific and validated the assay in development. The newly set-up albumin copper binding (ACuB) assay was able to detect differences in the IMA level of between normal and stroke-induced rat models which may provide a more accurate information on IMA status than the existing ACB test.

Experimental

Materials

Tetrapeptide of HSA N-terminus (DAHK) was synthesized and purchased from Peptron, Inc. (Korea). Cobalt chloride ($CoCl_2$), copper chloride ($CuCl_2$), nickel chloride ($NiCl_2$), ferrous chloride ($FeCl_2$), lyophilized HSA, ammonium formate and lucifer yellow–carbohydrazide (LY) dilithium salt were purchased from Sigma (USA). Sodium dihydrogen phosphate (NaH_2PO_4) was purchased from Junsei Chemical Co., Ltd. (Japan), sodium hydroxide (NaOH) was from Samchun chemicals (Korea), and phosphoric acid (H_3PO_4) was purchased from Santoku Chemical Industries Co., Ltd. (Japan).

Interactions between Co^{2+}, Cu^{2+}, Ni^{2+}, and Fe^{2+} and the NTS peptide of HSA

The tetrapeptide of the NTS (DAHK, MW 469) was incubated with $CoCl_2$, $CuCl_2$, $NiCl_2$, and $FeCl_2$, and analyzed through high-performance liquid chromatography (HPLC). First, 0.1 mM tetrapeptide and 0.1 mM metal ion were mixed and incubated for 10 min. To compare the different affinities of each metal toward the peptide, the competition between two ions was observed after incubation. The peptide and one metal were incubated for 10 min, and then another metal was added and incubated for another 10 min. All reactions were performed in the mobile phase, which was selected as 50 mM NaH_2PO_4 (pH 7.4) in order to mimic *in vivo* conditions. The HPLC running conditions were as follows: the mobile phase was run in an isocratic mode with a flow rate of 1 mL/min through the column Synergi 4U Fusion-RP 80 Å (C18, 250×4.6 mm, 4 μm) (Phenomenex, USA), the sample injection volume was 20 μL, and the sample detection wavelength was set at 220 nm. Agilent 1100 Series (Agilent Technologies, USA) was used to detect the samples and Agilent ChemStation for LC 3D (Agilent Technologies, USA) was used to interpret the results.

To confirm that the incubation of peptide and metal ions produced peptide-metal complexes, they were analyzed with electrospray ionization–time of flight–mass spectrometry (ESI-TOF-MS). A final amount of 5 pM tetrapeptide and 5 pM metal ions was incubated for 10 min and analyzed. The conditions for MS analyses were as follows: 5 mM ammonium formate (pH measured as 6.93) as the mobile phase in an isocratic flow of 0.4 mL/min, positive mode, gas temperature 350°C, ESI Vcap voltage 4000 V and sample injection volume 2 μL. 6220 Accurate–Mass TOF LC/MS (Agilent Technologies, USA) was used to detect the peptide-metal complexes, and the results were analyzed with MassHunter Workstation Software, Qualitative Analysis (Agilent Technologies, USA).

HSA preparations

Lyophilized HSA (Sigma, USA) was dissolved in double-distilled water (DDW) to make a concentration of HSA, 4% (w/v), and then defatted by treatment with 8 M urea, followed by dialysis using snakeskin dialysis tubing (10K MWCO, Thermo Scientific, USA) in 50 mM NaH_2PO_4 (pH 7.4) with stirring overnight at 4°C and concentration using a pierce concentrator (7 mL/9K MWCO, Thermo Scientific, USA).

During this dialysis process, HSA was refolded and defatted to be ready for assay without interference by bound fatty acids to HSA. After concentration of the defatted HSA, we collected circular dichroism (CD) spectra of the original and defatted HSA solution to conform whether the refolded HSA adopted the α-helix conformation. The defatted HSA showed a typical CD type of α-helix (Fig. S-1, Supporting Information). The 4% (w/v) stock solution of the defatted HSA was prepared by the absorbance measured at 280 nm and the extinction coefficient provided by the ProtParam tool of ExPASy program.

Set up of albumin copper binding (ACuB) assay

The optimal excitation and emission wavelengths of LY were scanned. The absorbance was read with a multi-functional microplate reader Infinite® 200 PRO (Tecan, Switzerland) and analyzed using i-control™ software (Tecan, Switzerland). The fluorescence was measured with a fluorescence microplate reader Spectra MAX Gemini EM (Molecular Devices, USA) and analyzed using SoftMax Pro Software (Molecular Devices, USA). All absorbance and fluorescence values were read as absorbance units (ABSU) and relative fluorescence units (RFU). Then, the RFUs of LY were observed with or without copper in buffers of different pH conditions. Reaction buffers of 50 mM NaH_2PO_4 with pHs from 1.5 – 12.4 were prepared using a concentrated solution of NaOH or H_3PO_4.

As the final setting, 0.004 µmol LY and 0.025 µmol $CuCl_2$ were mixed in a final volume at a concentration of 200 µL 50 mM NaH_2PO_4 (pH 7.4). Then the effects of incubation times were observed between each reagent. LY and copper, HSA and copper, and lastly HSA-Cu and LY were mixed for various duration times from 0 to 60 min.

Each reagent was mixed in different sequences to confirm that the mixing order of the reagents did not affect the outcomes. The first set was added in the order of copper, LY and HSA and the second set was added in the order of copper, HSA and LY.

Validation of the ACuB assay

The specificity of LY toward copper was confirmed by mixing different metal ions together with copper and LY. 0 – 0.002 µmol Cu^{2+} in the presence of 0 – 0.02 µmol Co^{2+}, Fe^{2+} and Ni^{2+} were mixed with 0.004 µmol LY. Then, the linearity of the final set-up ACuB assay was validated; 0.025 µmol $CuCl_2$ and 1.5 – 5.5% (w/v) HSA were incubated for 10 min and then 0.004 µmol LY was added.

Detection of IMA levels in stroke and normal rat models by the ACuB assay

Normal and stroke-induced rat models were sacrificed to confirm that the ACuB assay was capable of analyzing *in vivo* samples. Although IMA is currently used to detect early myocardial ischemia, stroke is also reported to increase the IMA levels.[16] Briefly, anesthesia was induced in Sprague Dawley rats (255 – 270 g) with 3% (w/v) isoflurane (Hana Pharm., South Korea), and a middle cerebral artery occlusion (MCAo) was performed. The common, external and internal carotid arteries (CCA, ECA, and ICA, respectively) were tied in the written order and a 4-0 silicon suture was inserted from the ICA up to the MCA. The suture was removed to induce reperfusion after 90 min and the incision was closed. After 24 h, the animals were scored by the neurological severity score (NSS)[25] for confirmation of stroke induction and then their blood samples were collected from the heart under zoletil (0.1 mL/100 g) (Virbac, France) anesthesia. The collected blood from normal or stroke-induced rat models was centrifuged at 3000 rpm for 15 min to prepare serum samples. A volume of 40 µL of each serum sample was applied to the final optimized ACuB assay method to detect IMA levels, as mentioned in the result section of 3.3. Validation of the ACuB assay. The results of the ACuB assay were compared with those of the ACB test, which was performed exactly as described previously.[20]

Statistical analysis

All experiments were performed at least three times and all data are expressed as the mean ± standard deviation. Statistics were calculated by one-way analysis of the variance (ANOVA) with GraphPad Instat Version 3.10 (GraphPad Software, USA) and the differences between two values were considered to be statistically different when $p < 0.05$.

Results and Discussion

Binding of metal ions to the N-terminal peptide of HSA

Since it has already been reported that many divalent transition metal ions, such as Co^{2+}, Cu^{2+} and Ni^{2+}, are capable of binding to the NTS of human albumin,[12,13] choosing the appropriate ion seemed to be necessary for the precise detection of IMA. In previous studies, octapeptide or tetrapeptide that originated from the NTS of human albumin was utilized to characterize the Co^{2+} and Ni^{2+} binding amino-acid residues of the N-terminus of human albumin by HPLC, LC-MS and ^{1}H NMR analyses.[13] We synthesized tetrapeptide with a sequence of the albumin N-terminus (Asp-Ala-His-Lys, DAHK) and analyzed mixtures of the peptide and each of the metal ions using HPLC. Based on the retention time of the free peptide peak in Fig. 1a, we were able to designate the peaks in Figs. 1b – 1e either as peptide-metal complexes or left-over free peptides. The formation of peptide-metal complexes was confirmed through MS analyses as potassium and sodium salt (Fig. S-2, Supporting Information).

Since we observed that cobalt, copper, iron and nickel ions could interact with the tetrapeptide, we next sought for the metal ion with the strongest binding capacity. When cobalt and peptide were mixed first and copper was next added, both peptide-Co^{2+} and peptide-Cu^{2+} peaks were observed (Fig. 2a). When the metal ions were mixed with the peptide in the reverse order, only a peptide-Cu^{2+} peak was detectable (Fig. 2b). Because cobalt could not bind with the peptide when copper was already present, we concluded that copper had a greater affinity toward the tetrapeptide of the human albumin N-terminus. Other metal ions were analyzed in the same way as indicated in Fig. S-3 (Supporting Information). The intensity order of binding affinities toward the tetrapeptide was $Cu^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+}$. This result is consistent with the dissociation equilibrium constants of the metal-HSA complex; 6.7×10^{-17} M for Cu-HSA, 2.5×10^{-10} M for Ni-HSA and 1×10^{-4} M for Co-HSA.[23] The normal serum copper level was reported to be 115 µg/dL which is the third most abundant trace element in the body following zinc and iron. Based on the much stronger binding affinity of Cu^{2+} to the NTS of human HSA than Co^{2+} and the high serum copper concentration, the copper-bound HSA may exist in a normal condition. The added cobalt in the ACB test cannot bind to the already copper-bound HSA, which leads to more free remaining cobalt and a false increase in ABSU, which implicates IMA. Therefore, Cu^{2+} is suitable for the measurement of IMA instead of Co^{2+}, and consequently copper instead of cobalt was chosen for a further investigation of the indirect IMA level measurement.

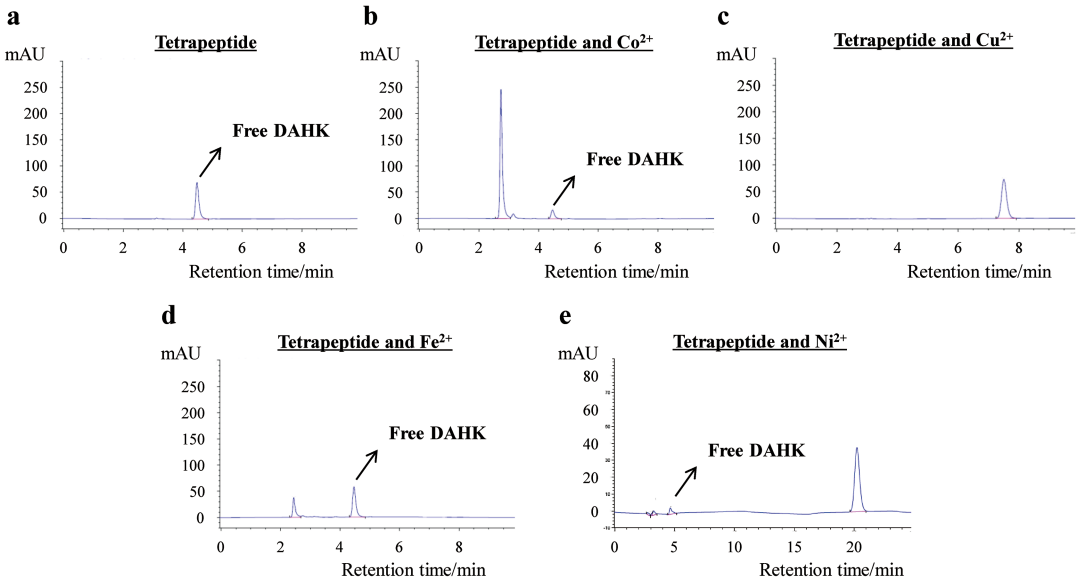

Fig. 1 Chromatograms of HPLC analyses for peptide-metal interactions. (a) 0.1 mM tetrapeptide was analyzed to determine the retention time of free peptide. (b – e) 0.1 mM tetrapeptide and equal amounts of $CoCl_2$, $CuCl_2$, $NiCl_2$, and $FeCl_2$ were mixed with incubation for 10 min and analyzed. All experiments were performed at least three times.

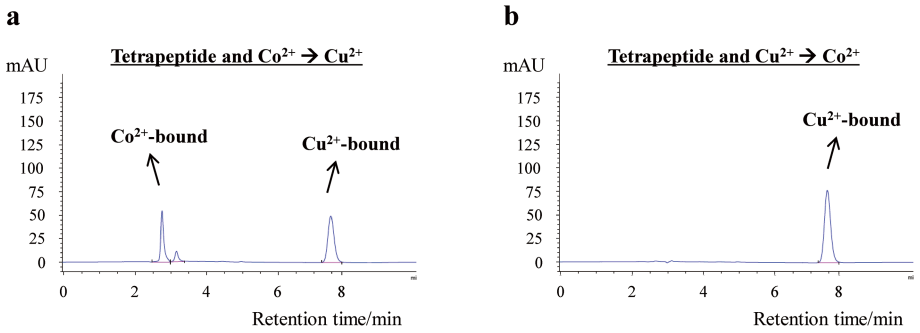

Fig. 2 Chromatograms of HPLC analyses for metal affinity toward albumin N-terminal tetrapeptide. The mixture samples were prepared as follows: 0.1 mM tetrapeptide and 0.1 mM $CoCl_2$ and $CuCl_2$ were mixed in the written order with incubation for 10 min, and then the samples were analyzed.

Optimization of ACuB assay

LY was chosen as a copper-selective detecting agent among numerous previously reported reagents capable of detecting copper.[24] In order to select the optimal excitation and emission wavelengths of LY, its spectrum was scanned (Fig. S-4, Supporting Information) and wavelengths were set with 430 nm for excitation and 540 nm for emission. Then, the effects of the pH on the LY fluorescence and its interaction with copper were observed. As shown in Fig. S-5a (Supporting Information), LY itself was relatively stable, except under strongly basic conditions. Further experiments were performed in a buffer condition of pH 7.4 because Cu-induced quenching of LY fluorescence occurred the most at the neutral state (Fig. S-5b, Supporting Information) and it was also the closest to the physiological pH.

A Stern–Volmer relationship was previously described to explain many enhancing/quenching mechanisms of fluorescence.[27] Figure 3 shows that Cu-induced LY quenching did not fit the Stern–Volmer equation, but F_0/F *versus* copper represented a sigmoidal curve. Copper in the amount of

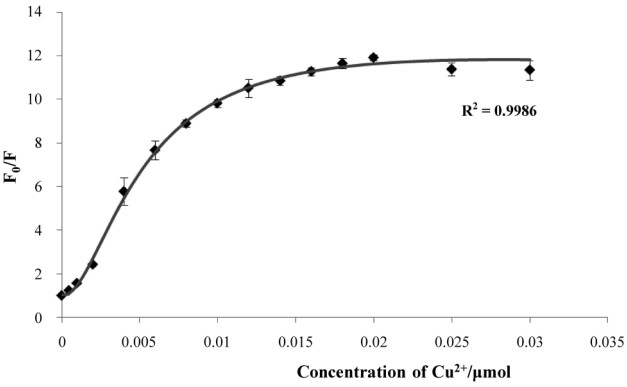

Fig. 3 Cu^{2+}-induced quenching of LY fluorescence. 0.004 μmol LY and 0 – 0.03 μmol $CuCl_2$ were mixed and RFUs were detected. F_0 stands for the initial LY fluorescence without copper and F stands for the quenched fluorescence. All experiments were performed at least three times. Dots represent the mean ± standard deviation, and R^2 indicates the reliability of the trend line.

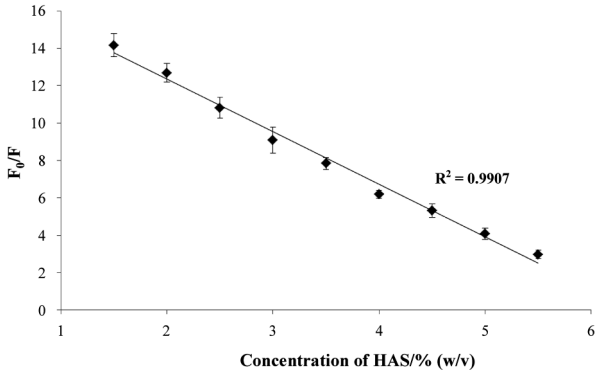

Fig. 4 Linearity of the ACuB assay. 0.025 µmol $CuCl_2$ were incubated with 1.5 – 5.5% (w/v) HSA for 10 min, and RFUs were measured after adding 0.004 µmol LY. All experiments were performed at least three times. Dots represent mean ± standard deviation, and a solid line represents the trend line of all dots. R^2 indicates the reliability of the trend line.

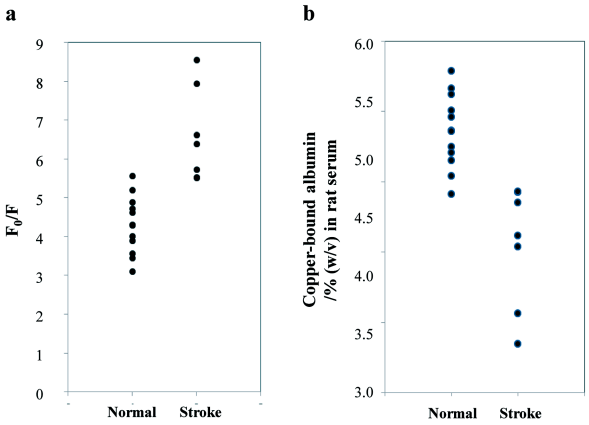

Fig. 5 Analysis of *in vivo* samples through the ACuB assay. (Normal $n = 12$, Stroke $n = 7$) (a) Samples were examined with the ACuB assay. (b) The results of (a) were converted into percentage of normal albumin in the rat serum. All experiments were performed at least three times.

0.025 µmol seemed to be sufficient to fully quench the LY fluorescence.

Lastly, the incubation times were optimized between each reagent. None of the interactions between LY *versus* copper, HSA *versus* copper and LY *versus* HSA-Cu mixture were affected by the incubation times (Fig. S-6, Supporting Information). Therefore, the HSA sample and copper were mixed and incubated for 10 min to induce sufficient interaction, and the fluorescence was measured immediately after adding LY. The mixing order of each reagent was also observed, and it was confirmed that LY was not affected by the albumin-bound copper (Fig. S-7, Supporting Information).

Validation of the ACuB assay

The finally optimized ACuB assay was set as follows: incubation of 40 µL sample and 10 mM $CuCl_2$ 2.5 µL for 10 min, followed by the addition of 1 mM LY 4 µL and the detection of fluorescence at an excitation wavelength of 430 nm and an emission wavelength of 540 nm. The LY specificity toward copper was first observed in order to validate the established assay. Excess metal ions had some influence on LY fluorescence (Fig. S-8, Supporting Information), but the extent could be ignored since an excess amount of copper was to be added in the assay. Then, the linearity of 1.5 – 5.5% (w/v) HSA as samples was confirmed with a good reliability, as shown in Fig. 4. Since the HSA level in blood is about 5% (w/v) (~5 g HSA in 100 mL of human blood) at normal state,[11] the detection range was considered sufficient. The results indicated that the newly developed ACuB assay is reliable to detect ischemia-induced IMA in blood. There are copper-carrying proteins in human serum, such as ceruloplasmin, albumin and transcuprein listed in order of association with copper.[28] Among them, ceruloplasmin caries about up to 95% of the total copper in normal human plasma by containing 6 copper atoms per each molecule of ceruloplasmin.[29,30] The average concentration of ceruloplasmin in normal serum is 0.0316 ± 0.0120 g/dL, which is more than 158-times lower than normal HSA concentration (~5 g/dL).[11] Therefore, the taken human serum sample for each reaction of the ACuB assay would contain an extremely small amount of ceruloplasmin compared to albumin. Moreover, human ceruloplasmin exists as a copper bound form because apoenzyme without copper binding is unstable and largely degraded in hepatocyte.[32] It is very likely that ceruloplasmin contained in serum sample of the ACuB assay is already occupied by copper. No interfering by ceruloplasmin is expected during the ACuB assay. Therefore, the optimized ACuB assay is applicable for the serum sample without further depletion of copper-carrying proteins.

Detection of serum IMA levels in rat stroke samples through ACuB assay

We tested the normal *versus* the stroke rat serum to confirm whether the newly developed ACuB assay was able to detect IMA levels *in vivo*, since it has been reported that although the amino acid level of rat serum albumin is different from that of HSA,[33] rat serum albumin possesses a preferential Cu^{2+} binding site in the N-terminal last four residues (N-Glu-Ala-His-Lys (GenBank accession number: NM_134326.2, NM_001082344.1) like HSA.[34,35] Figure 5a shows the results of the *in vivo* samples; those of normal models presented high F values whereas those of stroke models presented low values. This suggests that LY fluorescence was more readily quenched by the albumin-unbound free copper ions in the latter group. However, ACB tests of the same samples were unable to give positive results in this study (Fig. S-9, Supporting Information). Although previous reports have assumed that the ACB test was applicable to rat models,[36,37] the results obtained from this study showed higher ABSU values (> 0.8) than those reported for normal human plasma (normally < 0.4)[20] and even higher values than those in stroke models (~0.7). This may be attributed to the fact that albumin N-terminus is not a major cobalt binding site.[23] Moreover, mammalian albumins form a tight complex with copper[38] and copper binds to bovine, rat, and human albumin N-terminus on a mole per mole basis.[34,39,40] Based on those observations, it is likely that copper will present more precise results in detecting albumin N-terminal modifications than cobalt. The F_0/F values were converted into the actual amount of copper-bound albumin *via* the equation from the trend line of Fig. 4. As shown in Fig. 5b, the percentages of normal serum albumin in stoke-induced rats were 4.04 ± 0.430% (w/v) (n = 7) and those in normal rats were 4.86 ± 0.262% (w/v) (n = 12). The novel assay was able to detect low levels of normal albumin in stroke-induced animal samples. The difference between IMAs induced by stroke and by cardiac diseases has not yet been described, but they gave similar trends in measuring IMA levels.[16] Therefore, the ACuB assay results of *in vivo* samples

in Fig. 5 may be considered reliable. Further studies with more samples are warranted to determine whether the ACuB assay can be considered to be a reliable and sensitive method for detecting stroke-induced ischemic states.

Conclusions

As an early and accurate diagnosis of MI is considered to be critical for its prognosis, the importance of precise examination has been emphasized. Currently, there are no standard diagnostic methods, but the detection of IMA levels has been on the rise since the establishment of the ACB test by Bar-Or *et al.* Despite its usefulness in emergency departments, Mothes *et al.* revealed that cobalt was not an ideal reagent for the detection of abnormal albumin. Therefore, in this study, a novel method for measuring IMAs was developed and validated using copper rather than cobalt. Further investigation seems to be necessary, but this innovative analytical assay is anticipated to develop into a facile and valuable technology in related fields.

Acknowledgements

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Supporting Information

Supporting Information includes nine figures and is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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