Copper-induced aggregation of IgG: a potential driving force for the formation of circulating protein aggregates

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Abstract

Copper is a highly reactive element involved in a myriad of biological reactions. Thus, while essential for mammalian cells, its concentrations must be kept in check in order to avoid toxicity. This metal participates in redox reactions and may exacerbate oxidative stress in aerobic organisms. Nonetheless, the actual driving force of copper-induced cell death is yet unknown. Likely, free copper ions may target different biomolecules which are crucial for the proper functioning of an organism. In this work, we show that free copper induces protein aggregation in serum. The wide set of proteins present in these biological samples are not equally prone to copper-induced aggregation and some, such as albumin, are highly resistant whereas γ-globulins are highly sensitive. The identity of the proteins in the aggregates becomes fairly homogeneous as metal concentrations go as low as 20 μM. The identification of the proteins by mass spectrometry indicates a preponderance of IgG and a minor presence of other different proteins. Therefore, free copper in blood may serve to the formation of circulating protein aggregates with a core of IgG. This may impact on health not only due to the activity of aggregated IgG but also due to the many proteins co-aggregated. Understanding whether the γ-globulin core and the heterogeneous subgroup of proteins elicit differential responses in the organisms requires further research.

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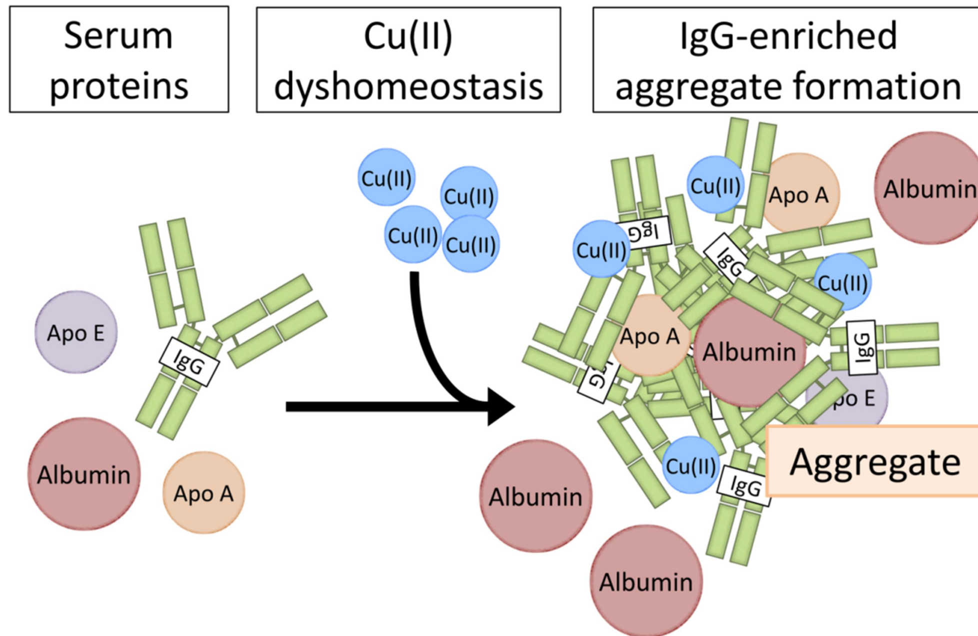
Graphical Abstract



Keywords: copper, protein, aggregates, immunoglobulin, IgG, gamma-globulin

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Introduction

Copper (Cu) is an essential element for mammalian cells. It is employed in the active site of several enzymes which catalyze redox reactions. Within cells, the reducing potential achieved by the high concentration of glutathione would keep any free Cu ion in its reduced form, Cu(I) [1]. The indiscernible occurrence of Cu(I) in the intracellular environment has been corroborated in bacteria [2]. However, due to its reactivity, the intracellular concentration of free Cu(I) must also be kept at negligible levels. Conversely, Cu(II) is the most common oxidation state of this metal in the extracellular environment because of the oxidizing features of this compartment [3]. In plasma, the concentration of total Cu(II) ions, both free and protein-bound, is circa 995 µg/L and nearly 65-90% circulates bound to ceruloplasmin [4]. The remaining metal is loosely bound to albumin and α2-macroglobulin and nearly 1.6 µM Cu(II) circulates in a relatively free state which may further rise, as is the case in Wilson Disease (WD) [5]. This pathology occurs when Cu ions cannot be extruded into the bile by the Cu-pump ATP7B. Therefore, the metal builds up within the hepatocyte where it causes cytotoxicity and upon cell death is ultimately spilled into the bloodstream. A concentration of free Cu(II) in serum higher than 4 µM is considered as positive diagnostic for WD [6]. However, the concentration of free Cu(II) rises up to concentrations as high as 45 µM in untreated patients [7]. However, it may drastically increase with concomitant conditions such as acute liver failure and cirrhosis [8].

While accumulation of Cu within the organism causes toxicity, the molecular events driving tissue and cell damage are not fully understood. Free Cu ions participate in redox reactions and generate oxidative stress. Cu ions react with hydrogen peroxide (H_2O_2) in Fenton like reactions, yielding the highly oxidant hydroxyl radical ($HO^{\cdot}$) which can promote lipid peroxidation and unselectively oxidize and damage essential biomolecules [9]. Some reports have challenged the

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formation of HO· mediated by Cu ions while proposing the existence of a higher oxidation state for Cu [10]. Regardless, no discussion has emerged about the oxidizing potential of this metal in the presence of H_2O_2. Hence, its deleterious effects in Cu dyshomeostasis have usually been linked to oxidation reactions. However, antioxidant therapies have not been proven effective in the treatment of Cu imbalance-related disorders such as WD. Moreover, Cu(II) and Cu(I) are even more harmful for bacteria in anaerobic conditions than in aerobic conditions which indicates that the production of highly reactive species is not their only cytotoxic mechanism [11]. Therefore, alternative toxic pathways should be explored in order to understand Cu toxicity.

Copper ions have been reported to induce protein aggregation and fibril formation *in vitro* [1, 12,13]. In bacteria, Cu(II) exposure induces alterations in the stability of proteins involved in several biological processes [2]. We have previously shown that cells exposed to high concentrations of Cu(II) up-regulate several pathways involved in coping with improperly folded proteins rather than mounting a consistent antioxidant response [1]. Thereby, it is likely that when metal levels reach a certain threshold in a specific scenario, either free Cu(I) or Cu(II) interact directly with proteins, altering their structure and eliciting an organic response focused on restoring proteostasis. Interestingly, protein aggregation is a hallmark event of several neurodegenerative pathologies such as Alzheimer's disease and Parkinson's disease [14]. These pathologies have also been related to Cu imbalances [15-19]. However, understanding the role of Cu ions in complex and multifactorial diseases requires a better understanding of Cu-mediated damage.

Circulating Cu(II) in the bloodstream is bound by several proteins in order to ensure its proper distribution. However, increases in metal concentration may allow its interaction with unwanted protein targets. Due to the heterogeneity of blood proteins, Cu(II) may selectively bind the

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sensitive ones and progressively, as the metal concentration rises, the most resistant proteins. This could contribute to the formation of circulating protein aggregates (CPAs). While CPAs have been found in pathology as well as in physiological conditions, their concentration as well as the identity of the encompassed proteins changes in different conditions. Likely, CPAs formation could be driven by a combination of pro-aggregating stimuli in the medium, such as metal ions, and the increased concentration of prone to aggregation proteins.

Here we show that Cu(II) induces aggregation of rat serum proteins and, as the concentration of Cu(II) decreases, the aggregated pellet becomes enriched in gamma-globulins. Additional proteins are found in the pellet but in low proportion. The γ-globulins have multiple functions involved in neutralization of antigens, signaling pathways, inflammation, etc, which turn active when the γ-globulin binds a target surface on an antigen [20].

Materials and methods

Blood collection and separation of rat serum

The blood of healthy Sprague-Dawley male rats of approximately 200 g was collected for the posterior separation of the serum. Prior to the blood collection, the animals were anesthetized with a single dose of 1 g/kg urethane. Then, the blood was drawn by cardiac puncture with syringe using a 25 Gauge needle and gently put in a 1.5 mL eppendorf. The whole blood was allowed to clot for 30 min and then centrifuged at 5000 g for 10 min. After the separation of the upper phase which corresponds to the rat serum, the sera of five rats were pooled together and stored at -20 ºC.

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Aggregate formation

The samples of either bovine serum albumin (BSA), serum proteins or purified human immunoglobulin G (IgG) at a final concentration of 1.5 mg/mL, were incubated with different concentrations of Cu(II). The aggregation process was carried out in a buffered solution of Tris-HCl 10 mM, pH 7.4 and the incubation took place for the corresponding time at 37 ºC. After incubation, the samples were centrifuged at 21 000 g for 30 min. Then, the supernatant was separated and the pellets were resuspended in distilled water. The corresponding fraction was used in each of the following experiments.

Turbidity assay

Tris–HCl 10 mM was used as the reaction medium and stock solutions of 10 mM $CuSO_4$ were used so as to attain the desired final concentrations in the wells. 1.5 mg/mL BSA or rat serum was added to the well just before starting the measurement. All the solutions were adjusted to pH 7.4. Samples were incubated and analyzed in a Varioskan Flash 96-well plate reader (Thermo Fisher Scientific) at 37 ^{o}C. The absorbance at 405 nm was monitored every 2 min with shaking for 5 sec before initial measurement during 60 min. No turbidity was detected in the control experiments with buffer only, protein only or metal ions in the absence of protein. Turbidity assays were run at least four times for each condition.

Protein content determination

Protein content was determined spectrophotometrically using Folin Reagent [21].

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Separation of proteins

Separation of proteins was carried out using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Samples were diluted with the electrophoresis sample buffer 1:1 v/v. The latter consisted of 20% SDS, 1M Tris-HCl pH 6.8 and 0.5 g/mL sucrose. 1M dithiothreitol (DTT) was added in the cases where reducing conditions were required. Electrophoresis was performed on 10% polyacrylamide under non-reducing conditions, 200 V during 45 min in buffer Tris-/Gly-SDS in Mini Protean Tetra cell System (Bio-Rad. California. USA). The mass of the proteins in the gels were estimated employing Rainbow Molecular weight markers. Code RPN 756 (range: 14.3-220 KDa), serum samples from healthy patients and urine with glomerular disease.

Proteinogram

The corresponding samples were run in cellulose acetate (Cellogel Electrophoresis Co. SRL Milano-Italy). Electrophoresis Buffer: Tris-barbital-sodium (Electra® HR-Helena Laboratories Beaumont, Tx) for 200 V 30 min. Coloration: 0.5% w/v Amido Schwartz suspended in 45% w/v methanol and 10% w/v acetic acid.

Two dimensional- polyacrylamide gel electrophoresis (2-DE)

In the first dimension, protein molecules are resolved depending on their charge: cellulose acetate, 200 V, pH 8.6. In the second dimension, protein separation is performed based on molecular weight using SDS PAGE and Tris-Gly-SDS buffers. The mass of the proteins in the second dimension electrophoresis were estimated employing Rainbow Molecular weight

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markers. Code RPN 756 (range: 14.3-220 KDa), serum samples from healthy patients and urine with glomerular disease.

Coomassie blue staining

The gels were stained with 0.2% w/v Coomassie Brilliant Blue R250 suspended in 0.5% w/v acetic acid and 20% w/v methanol.

Silver staining

The procedure consisted on fixing with 40% w/v methanol and 0.5 mL formaldehyde solution, sensitizing with a solution of sodium thiosulfate 0.02% w/v and silver nitrate 0.1% w/v, developing with sodium thiosulfate (3% w/v sodium carbonate, sodium thiosulfate 0.5 mL formaldehyde). The reaction was stopped with 2 mL glacial acetic acid.

Liquid Chromatography/Mass spectrometry (LC-MS)

Sample preparation for LC-MS: The samples were reduced with 20 mM dithiothreitol (DTT), for 45 min at 56 ^{o}C and alquilated with 50 mM iodoacetamide, for 45 min in the dark. Then, the samples were digested with trypsin overnight. The peptide extraction was performed with acetonitrile. The samples were lyophilized and resuspended with 30 uL trifluoroacetic acid, 0.1% w/v. Then the samples were desalted with ZipTip C18 (Merck). After desalting, the samples were analyzed by nano HPLC coupled to Mass Spectrometry with orbitrap technology. The LC was performed with a liquid chromatography; model EASY nLC 1000 (Thermoscientific). The sample ionization was performed by Electro Spray, employing an EASY-SPRAY ionizer (Thermoscientific) with a spray voltage between 2.5-3.5 kV. The MS was performed employing a mass spectrometer model Q-Exactive (Thermoscientific). The data was analyzed with Proteome Discoverer v2.2 (Thermoscientific) against the Rattus norvegicus UniProt database.

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The precursor mass tolerance was set to 10 ppm and the fragment mass tolerance was 0.5 Da. The proteins were considered as identified when at least two peptides were associated with a particular protein. The protein abundance was calculated with the exponentially modified protein abundance index (emPAI) which correlates with the absolute protein content in a given sample.

Data analysis

The statistical analysis of this data was carried out with the software GraphPad InStat following the recommended settings for the experiments (Anova with Tukey's post-test). The differences and the level of significance were represented with a letter indicating the group which is being compared to in the corresponding figure.

Results and Discussion

Aggregation of BSA and serum proteins by Cu(II)

Free Cu(II) is able to promote aggregation of BSA as well as of serum proteins (Fig. 1A and Fig. 1B). However, there is a threshold of Cu(II) concentration below which BSA does not aggregate (Fig. 1A). In Fig.1B we observe that Cu(II) is also capable of inducing the aggregation of serum proteins. We speculated that the heterogeneous group of proteins in blood would encompass proteins with higher sensitivity to metal-induced aggregation. Indeed, Fig. 1B shows a lower threshold for Cu(II)-induced protein aggregation. The increase in the absorbance at t=0 is likely due to the rapid kinetic of the aggregation process, especially when the concentrations of Cu(II) are high (Fig. 1A and Fig. 1B). Cu(II) does neither increase the absorbance at the t=0 nor throughout the incubation when the experiment is carried out in the absence of a protein sample. In Fig. 1C and Fig. 1D, BSA and serum proteins were precipitated and the pellets and

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supernatants were collected. The protein content was quantified by Lowry in both fractions. Data shown in Fig. 1C corroborates the Cu(II) threshold for BSA aggregation by quantifying the protein content in the pellet and supernatant after separating the aggregated fraction by centrifugation, and results shown in Fig. 1D indicate that much lower concentrations of Cu(II) are sufficient to aggregate serum proteins. Only a minor proportion of serum proteins are aggregated at these concentrations (Fig. 1C). This could be due to the presence of particularly sensitive proteins. Noteworthy, Cu(II) concentrations higher than 600 μM fully aggregate BSA whereas in the case of serum, concentrations as high as 1000 μM only aggregate circa 50% of total proteins (Fig. 1C and Fig. 1D, respectively). Thus, indicating that serum also comprises proteins which are highly resistant to Cu(II) induced aggregation. We expected BSA proclivity to aggregation to be much lower than the most sensitive proteins, which would otherwise be a major hazard due to its high concentration in this fluid.

Analysis of the aggregates by SDS-PAGE and cellulose acetate

The aggregated pellets and the corresponding supernatants of serum proteins incubated with Cu(II) were analyzed by SDS-PAGE stained with coomassie blue (Fig. 2A). The control serum was incubated in the same conditions as the Cu(II) treated samples. After the separation of the supernatant and pellet fraction, no pellet was recovered from the control serum. The bands observed corresponding to serum proteins incubated with high concentrations Cu(II) (800 μM) are fairly similar to the control lanes due to less selective aggregation. Albumin is the protein that is found in highest proportion in serum of healthy rats (circa 55%) [22]. Therefore, the band with the highest intensity in the SDS-PAGE of control serum should correspond to this protein (Fig. 2A, lane 1 and Fig. 2B lane 1). As metal concentrations decrease (200 μM), the pellet becomes enriched majorly in high molecular weight proteins (HMWP) while depleted in

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albumin. Interestingly, while lower concentrations of Cu(II) show induction of the aggregation of mainly HMWP in terms of mass, the silver staining also revealed the presence of few low molecular weight proteins (LMWP) (Fig. 2B). Nonetheless, the content of such LMWP is much lower than the HMWP. To further taper the spectrum of potential proteins in the aggregated fraction, a proteinogram was performed. This is a commonly employed assay to detect changes in specific protein groups known as globulins in serum. According to their migration velocity, the different globulins present in Sprague Dawley rat serum can be divided into 5 fractions as follows: albumin, α1-globulins, α2-globulins, β1/β2-globulins, and γ-globulins [22]. Thereby, the relative protein proportion can be compared to that of the control serum incubated in the absence of Cu(II) in order to distinguish any increase in specific globulin fractions. It must be noted that unlike SDS-PAGE, where SDS is present in the buffer, the proteinogram is carried out in the absence of this detergent. Thus, the migration velocity in the proteinogram depends on the charge/mass ratio of the proteins composing each fraction. Interestingly, the separation of the proteins due to their mass/charge ratio by cellulose acetate showed an enrichment of the aggregated pellets in the γ-globulin fraction (Fig. 2C) which is in agreement with the molecular weight of the preponderant proteins in the SDS-PAGE (Fig. 2A and Fig. 2B). No changes in specific bands (circa 150 kDa, 120 kDa, 100 kDa and 80 kDa) are observed in the supernatant fractions of the Cu(II) exposed proteins. The pellet becomes enriched in specific proteins which correspond to molecular weights of circa 150 kDa (Fig. 2A, lane 4) and some additional bands immediately below it. The additional bands are observable at lower molecular weights between 100-66 kDa. The overall aggregated protein is only a minor fraction of the whole protein content of that particular protein. Therefore, the supernatant remains unaltered, whereas the pellet's proportion of proteins drastically changes (Fig. 2A, Fig. 2B and Fig. 2C). The incubation of

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serum with Cu(II) was also performed in the presence of EDTA where it can be observed that the presence of a chelating agent fully prevents the formation of the aggregate (Fig. 2D). The preventive effect of EDTA on the Cu(II)-induced aggregation of serum proteins takes place when the incubation is performed even in Cu(II) concentrations as high as 800 μM. These results suggest that the metal has to be in a relative free state in order to induce protein aggregation.

Analysis of the aggregates by 2-DE

The high number of bands observed in the re-suspended pellets complicates the outlining of Cu(II)-induced aggregation sensitive proteins. In order to further discriminate the aggregated proteins in the pellet fraction, 2-DE electrophoresis were performed. This experiment shows that the pellet is basically enriched in a single protein at concentrations as low as 20 μM Cu(II) (Fig. 3). Some bands become more intense with lower concentration of Cu(II) because that specific protein is being enriched. However, this occurs not because the protein is being aggregated at lower concentrations but rather because other proteins that should be less sensitive to the metal-induced aggregation decrease in proportion. A control serum was run in parallel so as to show the position and relative band intensities of common proteins found in these biological samples (Fig. 3A). Therefore, the proteins corresponding to bands with higher intensity at 50 μM Cu(II) become more evident. We speculated on the identity of albumin considering that this protein is present in highest concentration in healthy serum and it has a molecular mass of 66 KDa [22]. The highest relative intensity of the band corresponding to this migration distance is evident in a control serum but is found progressively depleted as the concentrations of Cu(II) decrease (Diamond arrow). This is in accordance to the proteinogram of the aggregated fraction (Fig. 2B). Additionally, the migration distance of the majoritarian protein in the sera incubated with low concentrations of Cu(II) (Fig. 3D, Fig. 3E and Fig. 3F) is similar to that of IgG (Full arrow), with

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a molecular mass of 150 KDa. This is in line with the high concentration of γ-globulin observed in the proteinogram of the aggregated fraction (Fig. 2B).

Analysis of the aggregates in reducing conditions

The SDS-PAGE of serum proteins incubated with Cu(II) yields an aggregate which is composed of set of proteins (Fig. 1A, Fig. 1B and Fig. 1C). For this experiment, the serum was incubated with 200 μM Cu(II) and the aggregate was recovered. The sample was prepared for SDS-PAGE and DTT was added in order to cleave the disulfide bonds of the proteins present in the aggregated fraction. This experiment was performed in this particular aggregate because the protein composition is fairly uniform. The proteins present in the aggregate is more defined than that of the supernatant from the control serum (Fig. 2A, Fig. 2B and Fig. 2C) and considering that due to the high molecular weight of the aggregated proteins (Fig. 2A) and the increased γ-globulin fraction (Fig. 2C), we speculated on the enrichment of IgG within the aggregate. Thus, the addition of DTT to the sample loaded in the gel should reduce the disulfide bonds which bind together the whole IgG protein (150 kDa), releasing in this manner the light chains (25 kDa) and the heavy chains (50 kDa) which can be observed in the gel in reducing conditions (Fig. 4, lanes C and D).

Analysis of identity of the aggregated proteins by LC-MS

The most sensitive proteins to 100 μM Cu(II)-induced protein aggregation were identified by LC-MS. For this experiment, the samples were run in SDS-PAGE and stained with coomassie blue so as to identify the migrating proteins (Supplementary Fig. 1A) and the proteins that are not able to enter the gel due to the formation of an aggregate (Supplementary Fig. 1B). The totality of the protein in the aggregate was assayed as we ran the gel and allowed the proteins to briefly migrate within it. The run was stopped after the dye front (bromophenol blue) had

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migrated a distance of 1 cm. Then we separated the migrating fraction (Supplementary Fig. 1A) and the fraction that was not able to migrate due to the formation of aggregates (Supplementary Fig. 1B) by doing a gel excision of 1 cm^2. Both fractions together comprise the great majority of the protein content of the sample as it is observed in the coomassie blue staining (Supplementary Fig. 1A and Supplementary Fig. 1B).

The overall protein composition in the aggregated fractions incubated with concentrations below 100 μM Cu(II) is fairly homogeneous when compared to the groups corresponding to 200 μM Cu(II) and 800 μM Cu(II). Thus, the aggregated proteins with a concentration of 100 μM Cu(II) would give information about the main protein in the aggregate, which seemed to be aggregated also at lower metal concentrations, but also of additional proteins that could have been lost at lower Cu(II) concentrations. In this manner, a broader spectrum of proteins could be available for future studies of the isolated proteins. Here we observe that the most intense band of the gel excise is mainly IgG (Table 1, Fig 5 A). Additionally, we employed LC-MS to identify the proteins that were aggregated in larger mass aggregates which were unable to migrate into running gel (Supplementary Fig. 1B). In this case, the intense band of the SDS-PAGE corresponded also mainly to IgG. However, several additional proteins could be identified. Noteworthy, the size of many of the identified proteins did not correspond to the running distance in the excised gel section, which should be above 220 kDa. In fact, some of these proteins are small such as Apolipoprotein A-I or Apolipoprotein E with a molecular mass circa 30 kDa. We implemented a cut-off for the relative abundance of the proteins of 0.5%.

In summary, results indicate that Cu(II) is capable of inducing the aggregation of IgG from rat serum. The aggregation of IgGs could be due to a combination of their moderate concentration in blood and their inner proclivity to aggregation induced by this metal. The CPAs present

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heterogeneity in their protein content and their relative proportion may change in different physiological conditions as well as in disease. In the case of Cu(II), the metal could drive the formation of the CPA by generating a core of IgG. Notably, some authors have detected the presence of IgG in CPAs [23]. Interestingly, when run in SDS-PAGE, a large part of the aggregate remained at the top the gel. The identification of the proteins in the larger aggregates showed a greater heterogeneity. While IgG continues to be the majoritarian protein, some additional proteins can be detected in lower proportion. The identity of the proteins observed include several LMWP, such as apolipoprotein A-I and apolipoprotein E, which do not match their migration distance in the gel. Despite the larger aggregates, the IgG matching band at 150 kDa is also observable the gel. It must be mentioned that CPAs may not only be an accumulation of inactive proteins. In fact, IgG aggregates can be induced by heat and they are strong activators of the complement cascade [24]. Additionally, the complement activating feature of IgG aggregates is modulated by the size of the aggregate, which adds further complexity to the biology of these circulating structures [25]. The complement cascade plays a crucial role in the defense against pathogens. It is comprised of proteins distributed between the plasma and cell surfaces which become sequentially activated generating proinflammatory mediators and the opsonization of the targeted surfaces. The complement system of proteins can be activated by immune complexes which are constituted by antigen-bound antibodies [26]. These immune complexes trigger the cascade activation which culminates with the opsonization and lysis of the pathogen [27]. It may be possible that Cu-induced aggregated IgGs activate the complement system. Noteworthy, whereas the proinflammatory role of the complement system is well understood, it has recently been related to other functions such as signaling involved in the neurologic development, aging and different diseases [28]. Interestingly, several complement

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proteins were found in the aggregate (Tables 1 and 2, Supplementary Fig. 1) which could hint to a biological activity of CPAs.

It is worth mentioning that one purpose of this work is to set references as to which concentrations of Cu(II) are necessary to aggregate serum proteins. As observed, extremely high concentrations of this metal induce the unselective aggregation of a wide set of proteins but as concentrations of Cu(II) decrease, the aggregated fraction becomes enriched in a more restricted set of proteins (Fig. 2A, Fig. 2B and Fig 3). While the lowest concentrations of Cu(II) employed (20 μM) were enough to yield an aggregate, these concentrations seem high when compared to a physiological situation where the serum concentration of free Cu(II) is near 1.6 μM. It should also be mentioned that Tris, employed as the incubation buffer, is able to coordinate Cu(II) ions which would contribute to a higher threshold for Cu(II)-induced protein aggregation [29]. Furthermore, the incubation of isolated human IgG with Cu(II) leads to the formation of a protein aggregate at low metal concentrations (Fig. 5A and Fig. 5B). Thereby, this work may serve to better understand the participation of Cu(II) in pathology or specific conditions which are not necessarily pathological. For instance, in cases of untreated Wilson Disease, the serum levels of free Cu(II) may rise above 2.3 μM (which is considered a cut-off value for the diagnosis of the disease) [30]. However, Cu(II) levels may increase up to concentrations as high as 45.5 μM whereas upon chelation treatment serum free Cu(II) may be decreased up to normal values [7]. The formation of CPAs should not be attributed merely to free Cu(II) in circulation but rather to the concerted action of several pro-aggregating stimuli. In fact, several other metals have been reported to induce protein aggregation *in vitro* and may play a tip the scale towards a more pro-aggregating environment concomitantly to Cu(II). However, this particular metal could shape the identity of the CPA proteins by its enrichment on Cu(II)-sensitive aggregated proteins.

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Proteins inner tendency to aggregation can be drastically accelerated by several stimuli including metal ions [1, 31, 32]. It is generally accepted that hydrophobic portions of a protein may interact with other exposed hydrophobic patches on other proteins and cause aggregation [33, 34]. However, the precise mechanism driving this process remains obscure. Therefore, identifying the promoting factors and elucidating the mechanisms underlying protein aggregation is a critical matter in complex pathologies involving aggregated proteins [35]. Moreover, metal dyshomeostasis has been related to several disorders such as Alzheimer's disease and Parkinson's disease where protein aggregation is a hallmark of pathology [16-19].

In the bloodstream, the formation of detergent resistant CPAs is observable in young as well as in older patients. However, young patients show significantly lower levels of aggregates [36]. CPAs of healthy plasma present heterogeneity in their composition and some of these proteins should be preponderant. Interestingly, the prone to aggregation proteins, characteristic of several amyloidosis, not only deposit on specific organs but also alter the overall composition of CPAs [23]. Therefore, CPAs may serve as a potential diagnostic tool by reflecting the organ protein proclivity to aggregation, in a sample with ease of collection rather than organ biopsy [37]. The mechanisms behind CPAs generation are not fully understood. Aside from the inner proclivity to aggregation of each protein, several additional stimuli promote aggregation. Cu(II) and other metals could play a role in their formation as they have been reported to induce protein aggregation in several experimental models [1, 13, 38]. Additionally, proteins such as albumin have chaperone functions which, when impaired, may lead to reduced solubility of plasma proteins [39]. Therefore, the formation of CPAs is driven by diverse stimuli that promote protein aggregation which explains why their concentration is increased in different conditions such as aging [36].

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Conclusion

Cu(II) ions are capable of inducing protein aggregation of serum proteins and IgG can be found within the aggregates along with other proteins in minor proportion. Therefore, this metal could be a driving force in the formation of CPAs as well as in shaping the identity of its proteins. It remains to be understood whether these CPAs have biological functions *in vivo* and these functions can be modulated by the identity and proportion of the proteins involved.

Conflict of interest

The authors declare that they have no conflict of interest. None of the authors has anything to disclose regarding the manuscript.

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Data Availability

The data underlying this article are available in the article and in its online supplementary material.

References

1. Saporito-Magriñá C, Musacco-Sebio R, Andrieux Kook, L, Orrego MT, Tuttolomondo M, Desimone M, Boerries M, Borner C, Repetto MG. Copper-induced cell death and the protective role of glutathione: The implication of impaired protein folding rather than oxidative stress. *Metallomics* 2018;**12**:1743-54

2. Wiebelhaus N, Zaengle-Barone JM, Hwang KK, Franz KJ, Fitzgerald MC. Protein Folding Stability Changes Across the Proteome Reveal Targets of Cu Toxicity in E. coli. *ACS Chem Biol* 2020;**16**:214-24

3. Falcone E, Okafor M, Vitale N, Raibaut L, Sour A, Faller P. Extracellular Cu^{2+} pools and their detection: From current knowledge to next-generation probes. *Coord Chem Rev* 2021;**433**: 213727

4. Catalani S, Paganelli M, Gilberti M, Rozzini L, Lanfranchi F, Padovani A, Apostoli, P. Free copper in serum: an analytical challenge and its possible applications. *J Trace Elem Med Biol* 2018;**45**:176-80

5. Walshe, J. Wilson's disease: the importance of measuring serum ceruloplasmin non-immunologicall. *Ann Clin Biochem* 2003;**40**:115-21

6. Roberts E, Schilsky M. Diagnosis and treatment of Wilson disease: An update. *Hepatol* 2008;**47**:2089-211

7. McMillin GA, Travis JJ, Hunt JW. Direct Measurement of Free Copper in Serum or Plasma Ultrafiltrate. *Am J Clin Pathol* 2009;**131**(2):160-5

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8. Barbosa J, Fraga M, Saldarriaga J, Hiroz P, Giostra E, Sempoux C, Ferenci P, Moradpour D. Hepatic manifestations of Wilson's disease: 12-year experience in a Swiss tertiary referral centre. *Swiss Med Wkly* 2018;**148**:w14699

9. Saporito-Magriñá C, Musacco-Sebio R, Acosta JM, Bajicoff S, Paredes-Fleitas P, Boveris A, Repetto MG. Rat liver mitochondrial dysfunction by addition of copper(II) or iron(III) ions. *J Inorg Biochem* 2017;**166**:5-11

10. Pham AN, Xing G, Miller CJ, Waite TD. Fenton-like copper redox chemistry revisited: Hydrogen peroxide and superoxide mediation of copper-catalyzed oxidant production. *J Catal* 2013;**301**:54-64

11. Zuily L, Lahrach N, Fassler R, Genest O, Faller P, Sénèque O, Denis Y, Castanié-Cornet MP, Genevaux P, Jakob U, Reichmann D, Giudici-Orticoni MT, Ilberta M. Copper Induces Protein Aggregation, a Toxic Process Compensated by Molecular Chaperones. *ASM* 2022;**26**: 13(2):e0325121

12. Arnesano F, Scintilla S, Calò V, Bonfrate E, Ingrosso C, Losacco M, Pellegrino T, Rizzarelli, E, Natile G. Copper-Triggered Aggregation of Ubiquitin. *PLoS ONE*; 2009;**4**(9), e7052

13. Capanni C, Taddei N, Gabrielli S, Messori L, Orioli P, Chiti F, Stefani M, Ramponi G. Investigation of the effects of copper ions on protein aggregation using a model system. *Cell Mol Life Sci* 2004;**6**:982-91

14. Giampietro R, Spinelli F, Contino M, Colabufo N. The pivotal role of copper in neurodegeneration: a new strategy for the therapy of neurodegenerative disorders. *Mol Pharm* 2018;**15**:808-20

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15. Rivera-Mancía S, Pérez-Neri I, Ríos C, Tristán-López L, Rivera-Espinosa L, Montes S. The transition metals copper and iron in neurodegenerative diseases. *Chem Biol Interact* 2010;**186**:184-99

16. Bush A. The metallobiology of Alzheimer's disease. *Trends Neurosc* 2003;**26**:207-14

17. Bagheri S, Squitti R, Haertlé T, Siotto M, Saboury A. Role of copper in the onset of Alzheimer's disease compared to other metals. *Front Aging Neurosci* 2018;**9**:446

18. Montes S, Rivera-Mancia S, Diaz-Ruiz A, Tristan-Lopez L, Rios C. Copper and copper proteins in Parkinson`s disease. *Oxid Med Cell Longev*, 2014;147251

19. Davies J, Mercer F, Chen N, Double K. Copper dyshomoeostasis in Parkinson's disease: implications for pathogenesis and indications for novel therapeutics. *Clin Sci (Lond)* 2016;**130**: 565-74

20. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front immunol* 2014;**5**:520

21. Folin O, Ciocalteu V. On tyrosine and tryptophane determinations in proteins. *Biol Chem* 1927;**73**:627-50

22. Zaias J, Mineau M, Cray C, Yoon D, Altman NH. Reference values for serum proteins of common laboratory rodent strains. *J Am Assoc Lab Anim Sci* 2009;**48**(4):387-90

23. Adiutori R, Aarum J, Zubiri I, Bremang M, Jung S, Sheer D, Pike I, Malaspina A. The proteome of neurofilament-containing protein aggregates in blood. *Biochem Biophysics Rep* 2018;**14**:168-77

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24. Füst G, Medgyesi GA, Rajnavölgyi E, Csécsi-Nagy M, Czikora K, Gergely J. Possible mechanisms of the first step of the classical complement activation pathway: binding and activation of C1. *Immunol* 1978;**35**:873-84

25. Doekes G, Vanes L, Daha M. Influence of aggregate size on the binding and activation of the first component of human complement by soluble IgG aggregates. *Immunol* 1982;**45**:705-13

26. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res* 2009;**20**(1):34-50

27. Walport MJ. Complement. *N Engl J Med* 2001;**344**(14):1058-66

28. Warwick CA, Keyes AL, Woodruff TM, Usachev YM. The complement cascade in the regulation of neuroinflammation, nociceptive sensitization, and pain. *J Biol Chem* 2021;**297**(3): 101085

29. Colombo MF, Austrilino L, Nascimento OR, Castellano EE, Tabak M. On the interaction of copper with tris(hydroxymethyl)aminomethane. *Can J Chem* 1987;**65**(4),821-6

30. Brewer GJ, Askari F, Dick RB, Sitterly J, Fink JK, Carlson M, Kluin KJ, Lorincz MT. Treatment of Wilson's disease with tetrathiomolybdate: V. control of free copper by tetrathiomolybdate and a comparison with trientine. *Transl Res* 2009;**154**(2):70-7

31. Tamás MJ, Sharma SK, Ibstedt S, Jacobson T, Christen P. Heavy metals and metalloids as a cause for protein misfolding and aggregation. *Biomolecules* 2014;**4**:252-67.

32. Quintanar L, Domínguez-Calva J, Serebryany E, Rivillas-Acevedo L, Haase-Pettingell C, Amero C, King J. Copper and Zinc ions specifically promote non amyloid aggregation of the highly stable human γ-D crystallin. *ACS Chem Biol* 2016;**11**:263-72

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33. Fink AL. Protein aggregation: folding aggregates, inclusion bodies and amyloid. *Folding and Design* 1998;**3**:R9-R23

34. Chi EY, Krishnan S, Randolph TW, Carpenter JF. Physical stability of proteins in aqueous solution: mechanism and driving forces in nonnative protein aggregation. *Pharm Res* 2003;**20**:1325-36

35. Chong SH, Ham S. Site-directed analysis on protein hydrophobicity. *J Comput Chem* 2014;**35**:1364-70

36. Xia K, Trasatti H, Wymer JP, Colón W. Increased levels of hyper-stable protein aggregates in plasma of older adults. *Age* 2016;**38**(3):56

37. Adiutori R, Puentes F, Bremang M, Lombardi V, Zubiri I, Leoni E, Aarum J, Sheer D, McArthur S, Pike I, Malaspina A. Analysis of circulating protein aggregates as a route of investigation into neurodegenerative disorders. *Brain Commun* 2021;**3**:fcab148

38. Reshmi J, Jissy M, Anu M, Charuvila T, Usha K. Probing the role of Cu(II) ions on protein aggregation using two model proteins. *ACS Omega* 2021;**6**:35559-71

39. Finn TE, Nunez AC, Sunde M, Easterbrook-Smith, S. Serum albumin prevents protein aggregation and amyloid formation and retains chaperone-like activity in the presence of physiological ligands. *J Biol Chem* 2012;**287**:21530-40

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Table 1. Identity and relative abundance of the proteins present in the aggregates formed by serum proteins incubated with Cu(II). The proteins identified correspond to a gel excise comprising proteins in the range 25-225 kDa. The gel excise corresponds to the black box shown in Supplementary Fig. 1A.

Accession	Description	MW [kDa]	Relative abundance (%)
P01835	Ig kappa chain C region, B allele OS=Rattusnorvegicus PE=1 SV=1	11.6	53.7
P01836	Ig kappa chain C region, A allele OS=Rattusnorvegicus PE=1 SV=1	11.7	36.6
P20762	Ig gamma-2C chain C region OS=Rattusnorvegicus PE=2 SV=1	36.5	4.2
P20761	Ig gamma-2B chain C region OS=Rattusnorvegicus GN=Igh-1a PE=1 SV=1	36.5	0.8
P01026	Complement C3 OS=Rattusnorvegicus GN=C3 PE=1 SV=3	186.3	0.5

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Table 2. Identity and relative abundance of the proteins present in the aggregates. The LC-MS was performed on the group of proteins which are not able to enter the gel. The gel excise corresponds to the black box shown in Supplementary Fig. 1B.

Accession	Description	MW [kDa]	Relative abundance (%)
P01835	Ig kappa chain C region, B allele OS=Rattusnorvegicus PE=1 SV=1	11.6	56.8
P01836	Ig kappa chain C region, A allele OS=Rattusnorvegicus PE=1 SV=1	11.7	8.2
P20762	Ig gamma-2C chain C region OS=Rattusnorvegicus PE=2 SV=1	36.5	6.0
Q63041	Alpha-1-macroglobulin OS=Rattusnorvegicus GN=A1m PE=1 SV=1	167	2.6
P20767	Ig lambda-2 chain C region OS=Rattusnorvegicus PE=4 SV=1	11.3	2.3
P04639	Apolipoprotein A-I OS=Rattusnorvegicus GN=Apoa1 PE=1 SV=2	30	1.4
P02770	Serumalbumin OS=Rattusnorvegicus GN=Alb PE=1 SV=2	68.7	1.4
P20761	Ig gamma-2B chain C region OS=Rattusnorvegicus GN=Igh-1a PE=1 SV=1	36.5	1.3
P01946	Hemoglobin sub alpha-1/2 OS=RattusnorvegicusGN=Hba1 PE=1 SV=3	15.3	1.2
P06765	Platelet factor 4 OS=Rattusnorvegicus GN=Pf4 PE=1 SV=1	11.3	1.0
P02650	Apolipoprotein E OS=Rattusnorvegicus GN=Apoe PE=1 SV=2	35.7	1.0
P01026	Complement C3 OS=Rattusnorvegicus GN=C3 PE=1 SV=3	186.3	1.0
P20760	Ig gamma-2A chain Cregion OS=Rattusnorvegicus GN=Igg-2a PE=1 SV=1	35.2	0.9
P14046	Alpha-1-inhibitor 3 OS=Rattusnorvegicus GN=A1i3 PE=1 SV=1	163.7	0.8
P20059	Hemopexin OS=Rattusnorvegicus GN=Hpx PE=1 SV=3	51.3	0.7
Q03626	Murinoglobulin-1 OS=Rattusnorvegicus GN=Mug1 PE=2 SV=1	165.2	0.7
P02091	Hemoglobinsubunit beta-1 OS=Rattusnorvegicus GN=Hbb PE=1 SV=3	16	0.7
Q99PS8	Histidine-richglycoprotein OS=Rattusnorvegicus GN=Hrg PE=1 SV=1	59	0.6
Q01177	Plasminogen OS=Rattusnorvegicus GN=Plg PE=2 SV=2	90.5	0.6
Q6IFW6	Keratin, typeI cytoskeletal 10 OS=Rattusnorvegicus GN=Krt10 PE=3 SV=1	56.5	0.6
P08649	Complement C4 OS=Rattusnorvegicus GN=C4 PE=1 SV=3	192	0.5

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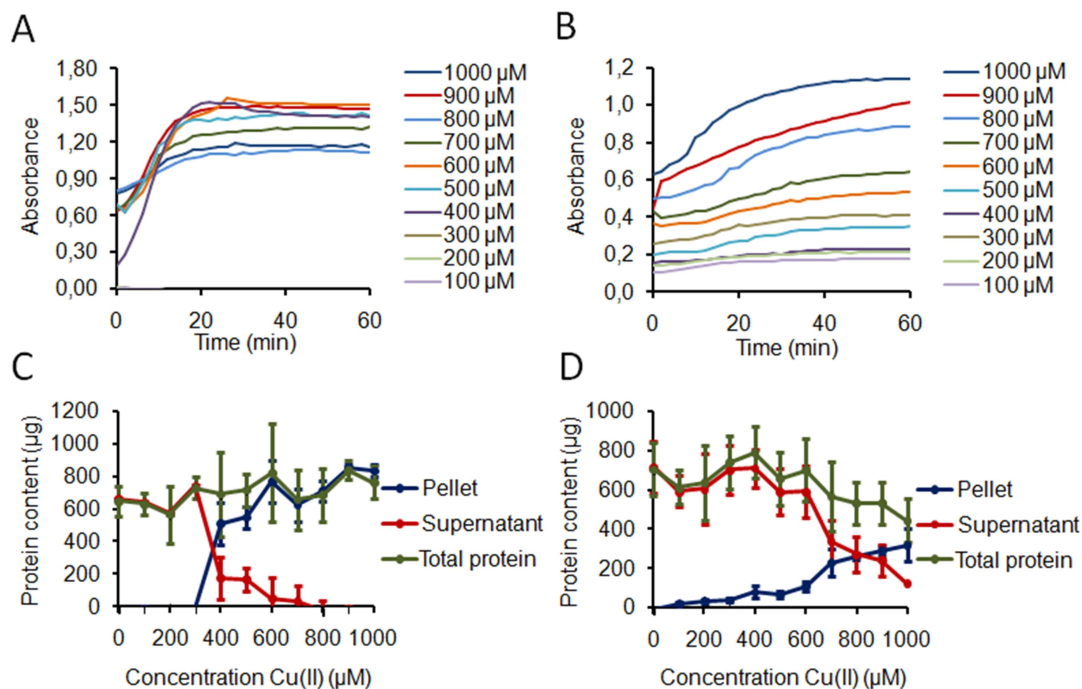

Figure 1. (A) BSA aggregation with different concentrations of Cu(II) assessed by optic density (DO), n=5. (B) Serum protein aggregation with different concentrations of Cu(II) assessed by optic density (DO), n=5. (C) Quantification of protein content in pellet and supernatant fractions after BSA incubation with different concentrations of Cu(II), n=3. (D) Quantification of protein content in pellet and supernatant fractions after serum proteins incubation with different concentrations of Cu(II), n=3. (C) Quantification of protein content in pellet and supernatant fractions after BSA incubation with different concentrations of Cu(II). The protein content of the pellet fraction at Cu(II) concentrations higher than 300 µM is significantly different from that at 0 µM Cu(II) ($p<0,001$) and the protein content of the supernatant fraction at Cu(II) concentrations higher than 300 µM is significantly different from that at 0 µM Cu(II) ($p<0,001$), n=3. (D) Quantification of protein content in pellet and supernatant fractions after serum proteins incubation with different concentrations of Cu(II). The protein content of the pellet fraction at

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Cu(II) concentrations higher than 100 μM is significantly different from that at 0 μM Cu(II) ($p<0,001$) and the protein content of the supernatant fraction at Cu(II) concentrations higher than 700 μM is significantly different from that at 0 μM Cu(II) ($p<0,001$), $n=3$.

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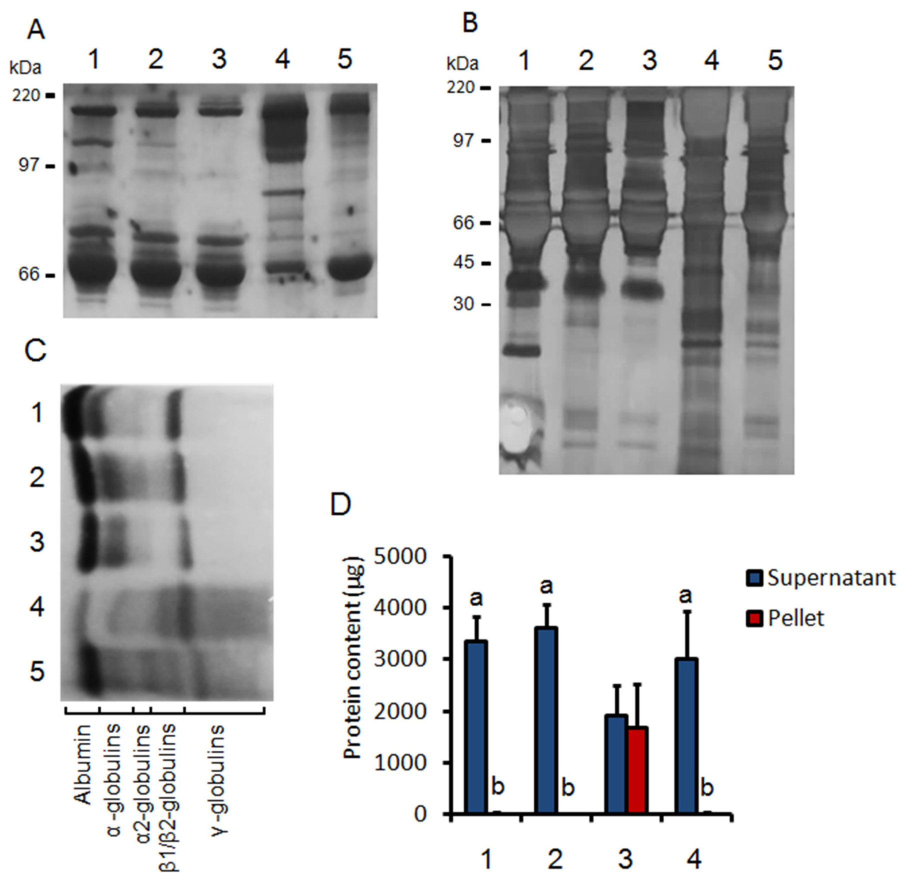

Figure 2. (A) SDS-PAGE stained with coomassie blue of serum proteins incubated with Cu(II). Lane: 1, control serum; 2, supernatant Cu(II) 200 μM; 3, supernatant Cu(II) 800 μM; 4, pellet Cu(II) 200 μM; 5, pellet Cu(II) 800 μM. n =3. (B) SDS-PAGE stained with silver staining blue of serum proteins incubated with Cu(II). Lane: 1, control serum; 2, supernatant Cu(II) 200 μM; 3, supernatant Cu(II) 800 μM; 4, pellet Cu(II) 200 μM; 5, pellet Cu(II) 800 μM. n =3. (C) Proteinogram of serum proteins incubated with Cu(II). Lane: 1, control serum; 2, supernatant Cu(II) 200 μM; 3, supernatant Cu(II) 800 μM; 4, pellet Cu(II) 200 μM; 5, pellet Cu(II) 800 μM. n =3. (D) Protein content of the supernatant and aggregated fraction of serum proteins incubated with Cu(II). Group: 1, control; 2, EDTA; 3, Cu(II) 800 μM; 4, Cu(II) 800 μM with EDTA. n = 5.

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The letter a, indicates significant difference ($p<0.01$) when comparing to 3, supernatant. The letter b indicates significant difference ($p<0.01$) when comparing to 3, pellet.

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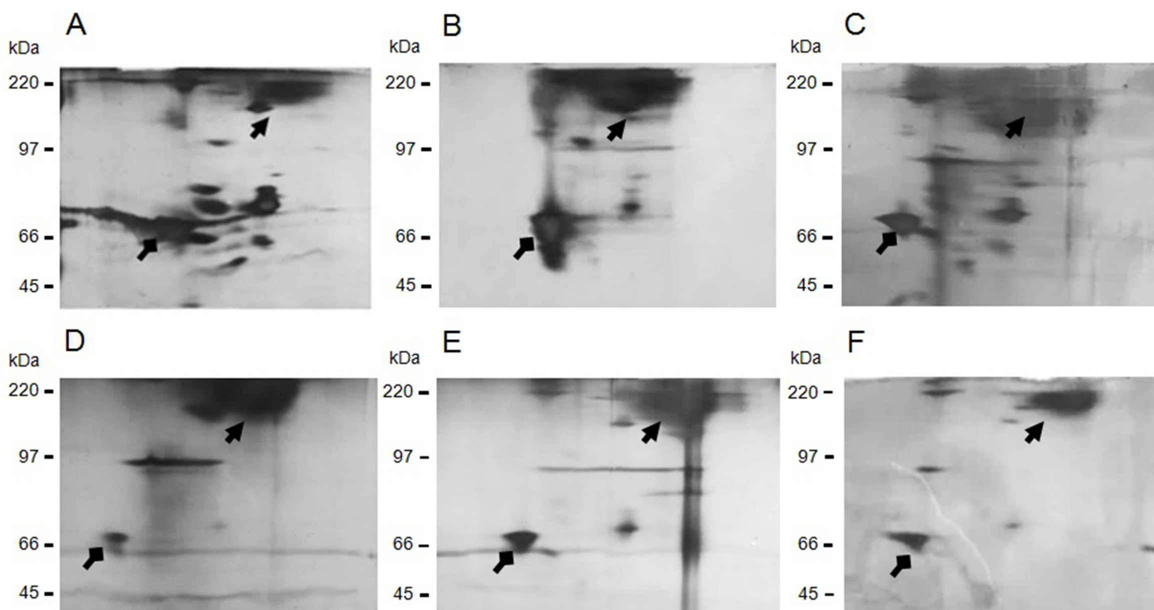

Figure 3. (A) Bidimensional electrophoresis of pellet fractions from serum proteins incubated with different concentrations of Cu(II). A, control serum without added Cu(II); B, pellet Cu(II) 800 μM; C, pellet Cu(II) 200 μM; D, pellet Cu(II) 100 uM; E, pellet Cu(II) 50 μM; F, pellet Cu(II) 20 μM. n=3. Full arrow shows the normal location of IgG. Diamond arrow shows the normal location of Albumin.

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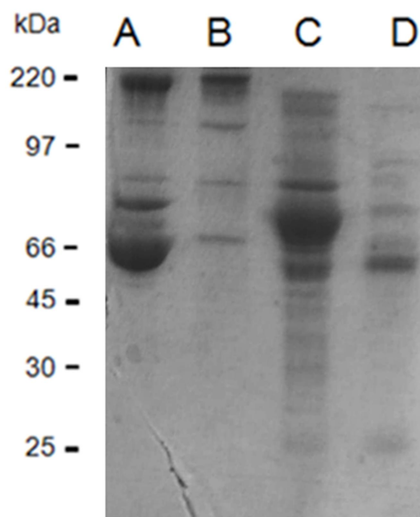

Figure 4. SDS-PAGE in non-reducing and reducing conditions and stained with coomassie blue of serum proteins incubated with Cu(II). Lane: A, control serum; B, pellet Cu(II) 200 μM; C, control serum + DTT; D, pellet Cu(II) 200 μM + DTT. The protein loaded in each lane was: (A), 15 μg, (B), 5 μg; (C), μg; (D), 15 μg. n =3.

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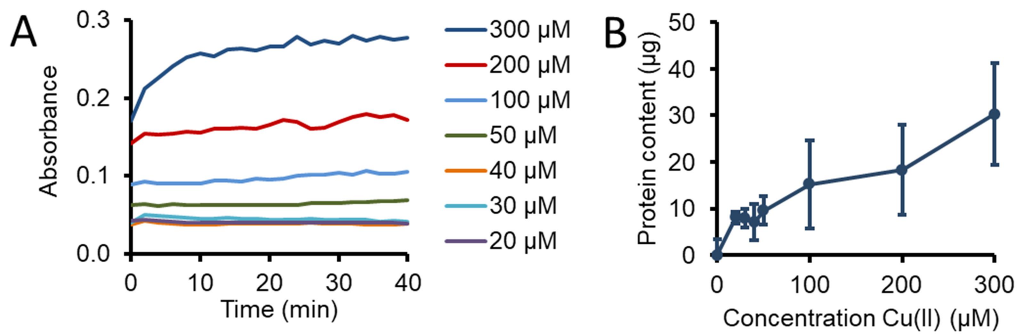

Figure 5. (A) IgG aggregation with different concentrations of Cu(II) assessed by optic density (DO), n=3. (B) Quantification of protein content in the pellet fraction of IgG incubated with different concentrations of Cu(II). The protein content of the pellet fraction at Cu(II) concentrations higher than 20 µM is significantly different from that at 0 µM Cu(II) ($p<0,001$), n=3.

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