



HAL
open science

Lipid molecules induce the cytotoxic aggregation of Cu/Zn superoxide dismutase with structurally disordered regions

Inhee Choi, Young In Yang, Hyeon Don Song, Jeong Seon Lee, Taewook Kang, Jung-Joon Sung, Jongheop Yi

► **To cite this version:**

Inhee Choi, Young In Yang, Hyeon Don Song, Jeong Seon Lee, Taewook Kang, et al.. Lipid molecules induce the cytotoxic aggregation of Cu/Zn superoxide dismutase with structurally disordered regions. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2010, 1812 (1), pp.41. 10.1016/j.bbadis.2010.09.003 . hal-00642445

HAL Id: hal-00642445

<https://hal.science/hal-00642445>

Submitted on 18 Nov 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Lipid molecules induce the cytotoxic aggregation of Cu/Zn superoxide dismutase with structurally disordered regions

Inhee Choi, Young In Yang, Hyeon Don Song, Jeong Seon Lee, Taewook Kang, Jung-Joon Sung, Jongheop Yi

PII: S0925-4439(10)00203-6
DOI: doi: [10.1016/j.bbadis.2010.09.003](https://doi.org/10.1016/j.bbadis.2010.09.003)
Reference: BBADIS 63161

To appear in: *BBA - Molecular Basis of Disease*

Received date: 1 May 2010
Revised date: 18 August 2010
Accepted date: 7 September 2010



Please cite this article as: Inhee Choi, Young In Yang, Hyeon Don Song, Jeong Seon Lee, Taewook Kang, Jung-Joon Sung, Jongheop Yi, Lipid molecules induce the cytotoxic aggregation of Cu/Zn superoxide dismutase with structurally disordered regions, *BBA - Molecular Basis of Disease* (2010), doi: [10.1016/j.bbadis.2010.09.003](https://doi.org/10.1016/j.bbadis.2010.09.003)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Lipid molecules induce the cytotoxic aggregation of Cu/Zn superoxide
dismutase with structurally disordered regions**

**Inhee Choi^a, Young In Yang^a, Hyeon Don Song^a, Jeong Seon Lee^b, Taewook Kang^c, Jung-Joon
Sung^b, and Jongheop Yi^{a,*}**

^aSchool of Chemical and Biological Engineering, Institute of Chemical Processes, Seoul National
University, Seoul 151-742, Korea,

^bDepartment of Neurology, and Seoul National University College of Medicine, Seoul 110-744,
Korea

^cDepartment of Chemical and Biomolecular Engineering, Sogang University, Seoul, 121-742, Korea

*Address correspondence to: Jongheop Yi, Phone: +82-2-880-7438; Fax: +82-2-885-6670; E-mail:
jyi@snu.ac.kr

Abstract

Cu/Zn-superoxide dismutase (SOD1) is present in the cytosol, nucleus, peroxisomes and mitochondrial intermembrane space of human cells. More than 114 variants of human SOD1 have been linked to familial amyotrophic lateral sclerosis (ALS), which is also known as Lou Gehrig's disease. Although the ultimate mechanisms underlying SOD1-mediated cytotoxicity are largely unknown, SOD1 aggregates have been strongly implicated as a common feature in ALS. This study examined the mechanism for the formation of SOD1 aggregates *in vitro* as well as the nature of its cytotoxicity. The aggregation propensity of SOD1 species was investigated using techniques ranging from circular dichroism spectroscopy to fluorescence dye binding methods, as well as electron microscopic imaging. The aggregation of SOD1 appears to be related to its structural instability. The demetallated (apo)-SOD1 and aggregated SOD1 species, with structurally disordered regions, readily undergo aggregation in the presence of lipid molecules, whereas metallated (holo)-SOD1 does not. The majority of aggregated SOD1s that are induced by lipid molecules have an amorphous morphology and exhibit significant cytotoxicity. The lipid binding propensity of SOD1 was found to be closely related to the changes in surface hydrophobicity of the proteins, even at very low levels, which induced further binding and assembly with lipid molecules. These findings suggest that lipid molecules induce SOD1 aggregation under physiological conditions and exert cytotoxicity, and might provide a possible mechanism for the pathogenesis of ALS.

Keywords: Amyotrophic lateral sclerosis; aggregation; Cu/Zn-superoxide dismutase; cytotoxicity; lipid molecules; Lou Gehrig's disease

1. Introduction

Cu/Zn superoxide dismutase (SOD1) [1], a 32 kDa homodimeric protein comprised of two identical subunits arranged in eight β -strands connected by seven loops, is found in the cytosol, nucleus, peroxisomes, lysosomes, and mitochondrial intermembrane space of eukaryotic cells. It functions primarily as an antioxidant enzyme to reduce the steady-state concentration of superoxide anions. However, structural disorders in SOD1, such as mutations and aggregation, have been linked to the development of familial amyotrophic lateral sclerosis (ALS). ALS is a progressive, fatal neurodegenerative disease that mainly affects the motor neurons in the brain stem and spinal cord. Recent studies have shown that SOD1 aggregates are present in the tissues from ALS patients [2, 3], ALS-SOD1 transgenic mice [4,5], as well as in cell culture models, which indicates that SOD1-associated familial ALS is a protein conformational disorder that is similar to other neurodegenerative diseases in which protein aggregates play a role [6]. Although the ultimate mechanisms underlying SOD1-mediated cytotoxicity are unknown, SOD1 aggregates have been strongly implicated as a causative factor in the disease. Many experimental studies have attempted to define the critical factors leading to SOD1 aggregation because the aggregation of SOD1 is a complex multistep process consisting of dimer dissociation, loss of metals, conformational changes, nucleation and accretion, and involves a variety of cellular components [7,8]. In this regard, it is generally accepted that altering the physiological conditions, such as increasing the temperature, lowering the pH, adding a metal chelator, reduction of disulfide linkages, and oxidative modification, can affect the formation of SOD1 aggregates [8-11]. Recently, the formation of SOD1 aggregates as a result of interactions with other cellular components was also suggested [12,13]. Considering the ubiquitous distribution of SOD1 in a cell (i.e. cytosol, nucleus, peroxisomes, lysosomes, and mitochondrial intermembrane space), it would not be surprising that aberrant SOD1 species can interact with other cellular components, which may eventually be involved in the formation of heterogeneous aggregates without perturbing physiological conditions.

In this study, we examined the *in vitro* SOD1 aggregation in the presence of lipid molecules as a possible aggregation model system for ALS. Lipid molecules serve as mediators for signaling and inflammation during the progression of neurodegeneration. Although SOD1 is frequently in contact with lipids, the interactions with SOD1 have not been investigated extensively compared to other disease linked proteins [14-16]. The lipid molecule-associated behavior of proteins can be generally derived from interactions between the lipid molecules and proteins, and the extent of these interactions would be expected to promote the lipid molecule mediated abnormal assembly of SOD1. These findings suggest that lipid molecules promote the abnormal assembly and cytotoxic aggregation of SOD1. This aggregation was found to be strongly related to the structural instability and affinity of SOD1 for binding to lipid molecules. These results show that lipid molecules, acting as a promoter, lead to a significant acceleration of SOD1 aggregation, as evidenced by fluorescence dye binding and the negatively-stained electron micrographs. The cytotoxicity of the resulting aggregates was also evaluated through cellular viability measurements.

2. Materials and methods

2.1. Chemicals

Bovine serum albumin (BSA), DPPC (1,2-dipalmitoyl-sn-glycero-3-Phosphocholine), POPC (1-palmitoyl-2-oleoyl-phosphatidylcholine), cholesterol, Thioflavin-T (ThT) and 8-Anilino-1-naphthalene-sulfonic acid (ANS) were purchased from Sigma Chemical Co. 2,2,2-trifluoroethanol (TFE) was supplied by Aldrich Chemical Co. All chemicals were used as received without further purification. All water was purified using a Milli-Q water system (18 M Ω , Millipore).

2.2. Purification of SOD1

The proteins were prepared using the purification protocol reported elsewhere [17-20]. Human SOD1 genes encoding the wild type enzyme were cloned into the pET23b(+) (Novagen) vector and the proteins were expressed in *E. coli*. The cultures were induced by the addition of 0.5 mM isopropyl b-D-thiogalactopyranoside for 3 to 6 hr at 30 °C. The cells were then lysed by sonication in a buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 1 mM phenylmethylsulfonyl fluoride (PMSF). The proteins were eluted with a linear gradient of ammonium sulfate (0.75-0 M) in 50 mM sodium phosphate (pH 7.0), 150 mM NaCl, 0.1 mM EDTA and 0.25 mM DTT. Wild type (WT) SOD1 was released with high specificity from the column between 1.3 and 0.8 M ammonium sulfate. Apo type SOD1 was produced by the demetallation of Zn and Cu.

For the pre-formation of SOD1 aggregates, purified WT apo SOD1 molecules were dissolved in acidic phosphate buffered saline (PBS) solution (pH 5.4) at a concentration of 0.1 mg/ml, followed by the previously reported protocol with a solution containing 20 % (v/v) TFE [21].

2.3. Preparation of lipid vesicles

To prepare all lipid vesicles, a 40 mg of lipids was dissolved in 4 mL chloroform in a small vial, agitated and exposed to three freeze-thaw cycles using liquid nitrogen. The organic solvent was

evaporated with a N₂ stream, leaving a thin lipid layer on the inner wall of the vial. The dried lipid films were resuspended in a 25 mM PBS solution (pH 7.0) at a concentration of 0.1 mg/mL. The suspension was sonicated at low amplitude in an ice bath for 30 min. Sonication was carried out at 5 minute intervals to avoid an excessive increase in suspension temperature. The resulting vesicle solution was stored at 4 °C for 1 hr prior to use.

2.4. Incubation of proteins with lipid vesicles

To determine the effect of the interaction of lipid molecules with SOD1 on aggregation, SOD1 was incubated with the DPPC (a neutral, saturated lipid) vesicles in PBS at 37.5 °C. The proteins, apo SOD1, holo SOD1, SOD1 aggregates and BSA, were dissolved in 25 mM PBS at a concentration of 0.1 mg/ml and added to the vesicle solution to yield incubation mixtures with a SOD1 to lipid molar ratio of 1:45. As controls, proteins without DPPC vesicles were also incubated under the same conditions at 37.5 °C. All samples were incubated without agitation. At each time point, an aliquot of each sample was taken and the presence of aggregates was determined using the analytical methods described below. To further understand the relevance of lipid molecule-promoted SOD1 aggregation, the above mentioned procedures were also performed with POPC (a neutral, unsaturated lipid molecules) vesicles and lipid vesicles containing cholesterol.

2.5. Circular Dichroism spectroscopy

The secondary structures of the proteins were examined by circular dichroism (CD) spectroscopy (Jasco J-715 spectropolarimeter, USA) equipped with a Naslab temperature controller. A 0.1-cm quartz cell was used for the measurements and the CD spectra were recorded from 190 to 250 nm. All CD measurements were carried out using the following parameters: 1 nm bandwidth, 50 nm/min run speed, 1 nm step size, 8.3 s response time and an average of three runs.

2.5. Fluorescence spectroscopy

To monitor the formation of SOD1 aggregates, the ThT fluorescence of the incubated samples was measured by fluorescence spectroscopy (LS 55, PerkinElmer, UK). ThT is associated rapidly with aggregated structures, giving rise to a new excitation maximum at approximately 450 nm and enhanced emission at around 482 nm. Therefore, previous studies employed the ThT binding characteristics to identify the aggregation propensity of SOD1 [9,21,22]. At each time point, 150 μ L of each sample was mixed with 15 mL of 10 μ M ThT in PBS, and the fluorescence emission intensity was determined at least 3 times for each sample.

ANS fluorescence [12, 23] was used to determine the surface hydrophobicity of the protein. The ANS fluorophore (final concentration 20 μ M) was incubated at 37 °C with the proteins in 25 mM PBS. After excitation at 360 nm, the emission spectra were recorded between 400 and 700 nm.

2.6 Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to characterize the morphology of the resulting SOD1 aggregates. The samples (150 μ L aliquots) were incubated overnight on 400-mesh carbon-coated formvar copper grids. The grids were air-dried and stained with a 1 % (wt/vol) solution of uranyl acetate. Specimens were viewed using a LIBRA energy-filtering transmission electron microscope at an accelerating voltage of 120 kV. The images were digitized using a Gatan slow-scan CCD camera and analyzed using a Gatan Pigtal micrograph.

2.7. Cell culture and Toxicity Assay

The cytotoxicity of the prepared protein samples was measured using a simple cell viability assay. The PC-12 cell line, which was derived from *Rattus norvegicus* pheochromocytoma (KCLB No, 21721), was obtained from the Korean Cell Line Bank (<http://cellbank.snu.ac.kr>). The PC-12 cells were grown in RPMI-1640 media supplemented with 10 % fetal bovine serum and 1 % antibiotics in a humidified atmosphere containing 5 % CO₂ at 37 °C. The cells were cultured on poly-L-Lysine coated plates and slides.

The cell viability was determined by a MTT assay for the mitochondrial function, which is based on the conversion of a tetrazolium salt into an insoluble formazan product by various dehydrogenases in the mitochondria. The cells were plated at approximately 10^6 cells/ml, grown to 80 % confluence, and exposed to the prepared protein samples for 24 hr. At the end of incubation, the cells were treated with the MTT solution for 30 min at 37 °C in a cell culture incubator. The resulting blue-colored formazan product was dissolved in 1 ml of acidified isopropanol, and the absorbance was measured at 570 nm using a VersaMax tunable Microplate reader (Molecular Devices Corporation, USA).

3. Results

3.1. Structural instability of metal-free SOD1

The propensity of a native protein to undergo aggregation is a complex property that involves a range of biophysical and physicochemical parameters, including the net charge, surface hydrophobicity, and intrinsic secondary structure [24]. Therefore, aggregated SOD1 species would be expected to have different structural and physicochemical properties from proteins in the native state. Before measuring the interactions between the SOD1 and lipid molecules, the structural and superficial properties of SOD1s were examined under different conditions by CD spectroscopy and ANS fluorescence spectroscopy.

Fig. 1A shows the CD pattern for fully metallated (holo) SOD1 (①), which shows the characteristic peak for an abundant β -sheet structure. Note that the secondary structure of holo SOD1 is comprised mainly of 60 % β -sheets and 30 % random coils with the remainder being α -helices [25]. On the other hand, the spectrum of apo SOD1 (②) shows a broad characteristic peak due to the structural changes caused by the absence of Cu^{2+} and Zn^{2+} , which play a role in the catalytic and structural cores of SOD1, respectively. The structure of SOD1 becomes ordered as a result of Cu^{2+} and Zn^{2+} coordination [1]. Importantly, these disordered regions as a result of the metal-free state of SOD1 have been shown to be involved in initiating protein aggregation, and in some cases, are believed to become integrated into the aggregate core [8,24,26]. This is also supported by observations of apo SOD1 species that had been incubated for 2 months at room temperature. In this case, the characteristic CD patterns (③) were broadened compared to the patterns of the freshly prepared apo SOD1. The characteristic pattern of SOD1 totally disappeared (④) under more severe conditions, such as treatment with TFE (an aggregation-enhancing agent) [18,21]. Apo SOD1 species readily assemble into largely aggregated structures under destabilizing conditions [8,9,13,21]. The CD spectra clearly show the structural changes in SOD1 during the aggregation progress. In a previous study [18], the unique secondary structures of native state SOD1 were lost within 2 days under the TFE treatment conditions. These conformational changes in the case of the metal-deficient state and/or

other destabilizing conditions are generally accompanied by changes in the superficial properties of the proteins, e.g. hydrophobicity. The ANS fluorescence results (Fig. 1B) are consistent with the hydrophobic residues being exposed as the result of conformational changes because the ANS dye is a probe for identifying the extent of exposed hydrophobic surfaces. As shown in Fig. 1B, holo SOD1 did not show any significant ANS fluorescence enhancement from the base line for a control sample (ANS dye only), whereas substantial ANS fluorescence enhancement was observed for apo SOD1. This can be attributed to a conformational change due to the removal of metal ions from its structure, and is in good agreement with data that appeared in a previous report [27]. Moreover, the ANS fluorescence enhancements were more prominent in the case of lengthy incubation (2 months) and a TFE treatment of apo SOD1. Obviously, this enhancement is caused by conformational changes in SOD1, resulting in the exposure of more hydrophobic residues at the surfaces during the aggregation process.

3.2. Time-resolved Analysis of Lipid Molecule-Induced SOD1 Aggregation

Three types of SOD1 variants, apo SOD1, holo SOD1 and as-prepared SOD1 aggregate, were incubated with DPPC lipid vesicles to examine the lipid molecule-induced aggregation propensity of SOD1. As a control protein, BSA, which contains abundant α -helices unlike SOD1, was also examined. DPPCs are neutral, saturated lipid molecules that are relatively unreactive compared to other types of lipid molecules (i.e. cationic, anionic and/or unsaturated lipid molecules). For example, the electrostatic interactions between proteins and lipid molecules are also involved in protein aggregation because the net charge of the protein can be altered by conformational changes [24, 28]. However, the charge of lipid molecules is not a shared property for all types of lipid molecules. Although many other parameters can affect interactions between proteins and lipid molecules [28, 29], in this study, we focused on the examining whether lipid molecule-induced protein aggregation can be induced by the general nature of lipid molecules, i.e., amphiphilicity. After incubation with DPPC lipid vesicles, the aggregated SOD1 was sampled and then subjected to ThT fluorescence

measurements. ThT [9,21,22] is a fluorescence probe used to detect the presence of aggregates and the amount of aggregated proteins, compared to the initial state of each sample (Supplement, Fig. S1). Fig. 2A shows the results for time-resolved analysis of the process of SOD1 aggregation. Apo SOD1 and an as-prepared SOD1 aggregate, when incubated with lipid molecules, showed a higher propensity for aggregation than holo SOD1 or BSA depending on the incubation time. Native holo SOD1 and BSA, which are the stable forms, did not show any significant changes in fluorescence intensity. This suggests that they interact minimally or not at all with lipid molecules. In the case of apo SOD1, which is the unstable form due to the absence of Zn^{2+} as a structural core, an increase in fluorescence intensity was observed during incubation. Similarly, a significant increase in fluorescence intensity was also observed for an as prepared SOD1 aggregate. Based on control measurements, lipid molecules also have a propensity to bind to ThT; however, no significant change in the time-resolved fluorescent intensities was observed (Supplement, Fig. S2). Therefore, the addition of lipid molecules to all protein samples generally induced an increase in ThT fluorescent intensities in the initial state (0 day). However, the magnitude of the increase was not the same for all samples. Thus, the initial large increase for an aggregated SOD1 can be attributed to a rapid association between aggregated SOD1 and lipid molecules. These results suggest that the conformational changes in SOD1s, which are observed in apo SOD1 and its aggregate, can promote further aggregation by assembling with lipid molecules. The propensity of lipid molecule-induced aggregation was readily confirmed by the enhanced ThT fluorescence intensity after 5 days incubation (Fig. 2B).

3.3. Changes in Hydrophobicity during Lipid Molecule-Induced SOD1 Aggregation

The hydrophobicity of SOD1 aggregates assembled with lipid molecules might be altered to some degree owing to their amphiphilicity, which is a unique property of lipid molecules. The hydrophobicity of the prepared samples was measured to obtain clear evidence of an assembly with lipid molecules. After incubation for 5 days, changes in ANS fluorescence intensities were observed

before and after incubation with lipid molecules, as shown in Fig. 3. A plot for a control sample shows the intrinsic fluorescence of ANS dye (without lipid) and ANS fluorescence of lipid molecules (with lipid), respectively. This result shows that lipid molecules induce an enhancement in ANS fluorescence. This can be attributed to the adsorption of ANS dyes to the hydrocarbon chains in lipid molecules [30-32]. Interestingly, apo SOD1s with lower ANS intensities, compared to lipid molecules, showed enhanced ANS fluorescence, and that of the SOD1 aggregate with higher ANS intensities showed a decrease in intensity after incubation with the lipid molecules. These slight discrepancies are not due simply to the co-existence of two components remaining unassembled in the mixtures considering the protein to lipid molar ratio of the incubation mixtures, 1:45. Specifically, it is due to changes in the surface hydrophobicity of the newly formed structures formed from the assembly between lipids and proteins. Native holo SOD1 and BSA, which are the stable forms, did not show any significant changes in ANS fluorescence intensity. Therefore, these changes in ANS fluorescence intensities can be interpreted as changes in the hydrophobicity of the proteins caused by further binding and the formation of assemblies with lipid molecules.

3.4. Morphological Analysis of Lipid Molecule-Induced SOD1 Aggregates

Negatively stained samples were observed by TEM to visualize the protein structures and examine the morphology of the resulting structures. In the case of apo SOD1, which had been incubated in PBS buffer for 2 weeks, no visible macrostructures, except for a few small structures, were observed (Fig. 4A). In the case of SOD1 aggregate (Fig. 4C) prepared by the TFE treatment, some individual aggregates ranging in size from several hundreds of nanometers were observed frequently. In particular, apo SOD1, which had been incubated with lipid molecules, formed large, amorphous network structures (Fig. 4B). Dramatic growth of these structures (Fig. 4D) was also observed in the case of the SOD1 aggregate that had been incubated with lipid molecules. On the other hand, no visible protein aggregates were observed in the BSA, BSA-lipid, lipid only samples (Supplement, Fig. S3). The ThT binding propensities for the resulting aggregated structures were visualized by

fluorescence imaging (insets of each TEM image). The fluorescence images are completely consistent with the morphological properties revealed from the TEM images. In particular, large fluorescence spots, observed in the SOD1 aggregate samples that had been incubated with lipid vesicles, support the existence of enormous aggregates formed from the further assembly with lipid molecules.

From the results, it was concluded that favorable interactions between lipid molecules and denaturated proteins (i.e. apo SOD1 and as prepared SOD1 aggregate) promotes the lipid molecule-mediated abnormal assembly of denaturated SOD1s. As a control, all data (Fig. 2 and Supplement, Fig. S4) obtained from structurally ordered holo SOD1 strongly support this finding. After incubation of holo-SOD1 with or without lipid vesicles, their structural and superficial properties did not change, unlike those of apo SOD1 (metal-deficient).

3.5. Cellular toxicity of Lipid Molecule-Induced SOD1 Aggregates

Having established that SOD1 variants with structurally disordered regions (i.e. apo SOD1 and as prepared SOD1 aggregate) have stronger affinity to lipid molecules than the native-state-stable protein forms (i.e., holo SOD1 and BSA) and consequently form larger protein aggregates assembled with lipid molecules, their cellular toxicity was examined using a cell viability assay. Aliquots of the samples that had been incubated in the presence or absence of lipid molecules were diluted with the culture medium, and added directly to the cultures of PC-12 cells. PC-12 cells are a cell line originating from the pheochromocytoma in rats, and are commonly used as a research model for examining neurodegenerative diseases and spinal cord injuries [33-35]. After incubation, the toxicity was evaluated using a MTT assay. As shown in Fig. 5, the SOD1 aggregates incubated with lipid molecules as well as the SOD1 aggregate themselves are quite toxic and clearly reduced the cell viability (*). In contrast, no significant decrease in viability was detected when the cells were exposed to most of the incubated samples containing only proteins and/or only lipids. They showed a cell viability of more than 80 %, as shown in control samples. However, the aggregates formed from the

incubation of apo SOD1 with lipid significantly reduced the cell viability by up to 55 % (*). This suggests that lipid-induced SOD1 aggregates are quite toxic to cells.

3.6. Relevance of Lipid Molecule-Induced SOD1 Aggregates

To identify the relevance of this lipid molecule-promoted SOD1 aggregation, apo SOD1 was also incubated with POPC, a neutral, unsaturated lipid molecule. After incubating apo SOD1 with different lipid vesicles, the amorphousness of the resulting structures was determined from CD spectra (Fig. 6A). Similarly, the negatively stained electron micrographs also revealed the presence of large amorphous networked structures of SOD1 (Fig. 6B). Moreover, we performed additional analyses for apo SOD1s that have been incubated with different lipid vesicle compositions, which are prepared by inserting cholesterol molecules to lipid vesicles, in an attempt to mimic the raft domains of real cell membranes. Analogous results, structural variations (Fig. 7A) and resulting amorphous morphologies (Fig. 7B), were also obtained for these types of lipid vesicles. This strongly suggests that lipid molecules can promote aggregation of SOD1s with abundant hydrophobic parts exposed by structural disordering, due to the amphiphilic property as a general feature of lipid molecules. Fig. 8 shows a schematic description of the molecular mechanism for the lipid molecule-associated SOD1 aggregation. Based on our findings, it appears that the structurally disordered SOD1 variants (apo SOD1 and aggregated SOD1) with abundant hydrophobic regions can be readily inserted to the inner space of lipid vesicles, disrupt them, and consequently become assembled with the hydrophobic tails of lipid molecules.

4. Discussion

This study examined a molecular mechanism involving lipid molecules in the formation of SOD1 aggregates, as well as the cytotoxicity of these aggregates. *In vitro* biophysical analyses showed that apo SOD1 and aggregated SOD1 species, which undergo conformational changes from their native structure, aggregated readily in the presence of lipid molecules, even under physiological conditions. Conformational changes in SOD1s result in an increase in surface hydrophobicity, which promotes binding to lipid molecules with amphiphilic properties. This is consistent with results observed for structurally ordered proteins (i.e., holo SOD1 and BSA), showing no significant formation of lipid-associated aggregates. That is, the formation of lipid-associated aggregates can be attributed to differential interactions between lipid molecules and denatured (or native) proteins. Therefore, the extent of these interactions promotes the lipid molecule-mediated abnormal assembly of denatured SOD1s.

It is important to note that interactions between proteins and lipid molecules can be controlled by many factors. G. P. Gorbenko, et al. reported that these interactions are controlled by not only the general physicochemical characteristics of a membrane (i.e., its phase state, bilayer curvature and elasticity, surface charge, and degree of hydration), but also the exact chemical nature of membrane lipids (i.e., the extent of acyl chain unsaturation, conformation and dynamics of lipid head groups and acyl chains, and protein–lipid selectivity arising from factors such as the hydrophobic matching) [28]. In addition, the role of lipid rafts in the protein aggregation has been also emphasized in some proteins [29].

In this regard, we focused on the aggregation induced by the general nature of lipid molecules, which is shared between all of lipids, that is “amphiphilicity”. We concluded that other parameters, as mentioned above, can also accelerate or decelerate aggregation to some extent, compared with our present observation results. To address this, first of all, DPPC (a neutral, saturated lipid molecule), that is relatively inactive compared to other types of lipid molecules, was selected as a component of model membrane system. Furthermore, we performed additional analyses with other different types of

lipid vesicles including unsaturated lipids (POPC) and lipid rafts (lipid vesicles containing cholesterol), in an attempt to identify the relevance of this lipid molecule-promoted SOD1 aggregation. Similar results (Fig. 6 and Fig. 7), structural variations and resulting amorphous morphologies, were also observed for these types of lipid vesicles. These findings suggest that lipid molecules can promote the aggregation of SOD1s with abundant hydrophobic parts exposed by structural disordering, due to their amphiphilic properties as a general nature of lipid molecules.

Since lipid molecules are the main components of the cellular membrane, the proposed molecular mechanism of SOD1 aggregation can be directly associated with the membrane-related behavior of SOD1s, such as membrane binding and membrane disruption. In particular, membrane linked SOD1 aggregates can be formed by the direct binding of SOD1 species to membranes, and/or the assembly of SOD1 with detached lipid molecules. Consequently, these assembled macrostructures would be expected to have an effect on cell viability, for example, homeostasis and permeability [14,36,37]. It has been extensively reported that interactions between proteins and lipid molecules may play a crucial role in determining the membrane-associated cytotoxicity of many other proteins that have been implicated in conformational diseases [14-16,36,37].

Recently, the hypothesis that SOD1 variants might aggregate on mitochondrial membrane surfaces has received support by the demonstration that SOD1 aggregates are attached to the cytoplasmic face of the mitochondrial membrane in transgenic ALS mice [4,5,24,38]. Interestingly, it has been reported that SOD1 may form pore structures in membranes [39,40]. Furthermore, we observed abnormal ion permeability in real cell membranes induced by aggregated SOD1 (Choi. I et al., unpublished data). The findings reported herein suggest that an aggregate with abundant hydrophobic parts, induced by conformational changes, can be readily inserted into the inner space of a membrane and assembled with the hydrophobic tails of lipid molecules, in a similar manner to that described in Fig. 8. That is, defects within a membrane can be formed by the detachment of lipid molecules that are co-assembled with structurally disordered SOD1 species. Such behaviors of lipid membranes provide a plausible explanation for the abnormal aggregation and resulting cytotoxicity of disordered SOD1 variants. We

expect that, given the findings presented herein, the lipid molecule-induced aggregation of SOD1 may be one of the possible mechanisms for the pathogenesis of ALS.

Acknowledgements

This study was supported by a grant (no. R01-2006-000-10239-0) from the Basic Research Program of the Korea Science & Engineering and a grant (101-082-032) from the Ministry of Environment as "The Eco-technopia 21 project". This research was also supported by the WCU (World Class University) program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (R31-10013).

References

- [1] J.S. Valentine, P.A. Doucette, S.Z. Potter, Copper-Zinc superoxide dismutase and amyotrophic lateral sclerosis, *Annu. Rev. Biochem.* 74 (2005) 563-593.
- [2] L.I. Bruijn, M.K. Houseweart, S. Kato, K.L. Anderson, S.D. Anderson, E. Ohama, A.G. Reaume, R.W. Scott, D.W. Cleveland, Aggregation and motor neuron toxicity of an ALS-linked SOD1 mutant independent from wild-type SOD1, *Science* 281 (1998) 1851-1854.
- [3] T. Koide, S. Igarashi, K. Kikugawa, R. Nakano, T. Inuzuka, M. Yamada, H. Takahashi, S. Tsuji, Formation of granular cytoplasmic aggregates in COS7 cells expressing mutant Cu/Zn superoxide dismutase associated with familial amyotrophic lateral sclerosis, *Neurosci. Lett.* 257 (1998) 29-32.
- [4] C.M. Higgins, C. Jung, Z. Xu, ALS-associated mutant SOD1^{G93A} causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes, *BMC Neurosci.* 4 (2003) 16:1-14.
- [5] J. Liu, C. Lillo, P.A. Jonsson, V.C. Vande, C.M. Ward, T.M. Miller, J.R. Subramaniam, J.D. Rothstein, S. Marklund, P.M. Andersen, T. Brännström, O. Gredal, P.C. Wong, D.S. Williams, D.W. Cleveland, Toxicity of familial ALS-linked SOD1 mutants from selective recruitment to spinal mitochondria, *Neuron* 43 (2004) 5-17.
- [6] C.M. Dobson, Protein folding and misfolding, *Nature* 426 (2003) 884-890.
- [7] F. Zhang, H. Zhu, Intracellular conformational alterations of mutant SOD1 and the implications for fALS-associated SOD1 mutant induced motor neuron cell death, *Biochim. Biophys. Acta.* 1760 (2006) 404-414.
- [8] S.D. Khare, M. Caplow, N.V. Dokholyan, The rate and equilibrium constants for a multistep reaction sequence for the aggregation of superoxide dismutase in amyotrophic lateral sclerosis, *Proc. Natl. Acad. Sci. USA.* 97 (2004) 12571-12576.
- [9] L. Banci, I. Bertini, A. Durazo, S. Girotto, E.B. Gralla, M. Martinelli, J.S. Valentine, M. Vieru, J.P. Whitelegge, Metal-free superoxide dismutase forms soluble oligomers under physiological

- conditions: A possible general mechanism for familial ALS, *Proc. Natl. Acad. Sci. USA.* 104 (2007) 11263-11267.
- [10]M. DiDonato, L. Craig, M.E. Huff, M.M. Thayer, R.M.F. Cardoso, C.J. Kassmann, T.P. Lo, C.K. Bruns, E.T. Powers, J.W. Kelly, E.D. Getzoff, J.A. Tainer, ALS mutants of human superoxide dismutase form fibrous aggregates via framework destabilization, *J. Mol. Biol.* 332 (2003) 601-615.
- [11]E. Gaggelli, H. Kozlowski, D. Valensin, G. Valensin, Copper homeostasis and neurodegenerative disorders (Alzheimer's, Prion, and Parkinson's diseases and amyotrophic lateral sclerosis), *Chem. Rev.* 106 (2006) 1995-2044.
- [12]W. Jiang, Y. Han, R. Zhou, L. Zhang, C. Liu, DNA is a template for accelerating the aggregation of copper, zinc superoxide dismutase, *Biochemistry* 46 (2007) 5911-5923.
- [13]Y.-J. Kim, R. Nakatomi, T. Akagi, T. Hashikawa, R. Takahashi, Unsaturated fatty acids induce cytotoxic aggregate formation of amyotrophic lateral sclerosis-linked superoxide dismutase 1 mutants, *J. Biol. Chem.* 280 (2005) 21515-21521.
- [14]E.Y. Chi, S.L. Frey, K.Y.C. Lee, Ganglioside GM1-mediated amyloid-beta fibrillogenesis and membrane disruption, *Biochemistry* 46 (2007) 1913-1924.
- [15]J. Madine, A.J. Doig, D.A. Middleton, The aggregation and membrane-binding properties of an α -synuclein peptide fragment, *Biochem. Soc. Trans.* 32 (2004) 1127-1129.
- [16]J. Kazlauskaitė, N. Sanghera, I. Sylvester, C. Ve'nien-Bryan, T.J.T. Pinheiro, Structural changes of the Prion protein in lipid membranes leading to aggregation and fibrillization, *Biochemistry* 42 (2003) 3295-3304.
- [17]T. Kang, S. Hong, I. Choi, J.-J. Sung, Y. Kim, J.-S. Hahn, J. Yi, Reversible pH-driven conformational switching of tethered superoxide dismutase with gold nanoparticle enhanced surface plasmon resonance spectroscopy, *J. Am. Chem. Soc.* 128 (2006) 12870-12878.

- [18]S. Hong, I. Choi, S. Lee, Y.I. Yang, T. Kang, J. Yi, Sensitive and colorimetric detection of the structural evolution of superoxide dismutase (SOD1) with gold nanoparticles, *Anal. Chem.* 81 (2009) 1378-1382.
- [19]S. Lee, I. Choi, S. Hong, Y.I. Yang, J. Lee, T. Kang, J. Yi, Highly selective detection of Cu^{2+} , utilizing specific binding between Cu-demetallated superoxide dismutase 1 and the Cu^{2+} ion via surface plasmon resonance spectroscopy, *Chem. Commun.* (2009) 6171-6173.
- [20]J.M. McCord, I. Fridovich, Superoxide dismutase: An enzymic function for erythrocyte (hemocuprein), *J. Biol. Chem.* 244 (1969) 6049-6055.
- [21]P.B. Stathopoulos, J.A.O. Rumfeldt, G.A. Scholz, R.A. Irani, H.E. Frey, R.A. Hallewell, J.R. Lepock, E.M. Meiering, Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis show enhanced formation of aggregates in vitro, *Proc. Natl. Acad. Sci. USA.* 100 (2003) 7021-7026.
- [22]L. Banci, I. Bertini, M. Boca, S. Girotto, M. Martinelli, J.S. Valentine, M. Vieru, SOD1 and amyotrophic lateral sclerosis: Mutations and oligomerization, *PLoS ONE* 3 (2008) e1677.
- [23]A. Tiwari, Z. Xu, L.J. Hayward, Aberrantly increased hydrophobicity shared by mutants of Cu,Zn-superoxide dismutase in familial amyotrophic lateral sclerosis, *J. Biol. Chem.* 280 (2005) 29771-29779.
- [24]B.F. Shaw, J.S. Valentine, How do ALS-associated mutations in superoxide dismutase 1 promote aggregation of the protein?, *Trends Biochem. Sci.* 32 (2007) 78-85.
- [25]H. Nagami, N. Yoshimoto, H. Umakoshi, T. Shimanouchi, R. Kuboi, Liposome-assisted activity of superoxide dismutase under oxidative stress, *J. Biosci. Bioeng.* 99 (2005) 423-428.
- [26]S.M. Lynch, S.A. Boswell, W. Colón, Kinetic stability of Cu/Zn superoxide dismutase is dependent on its metal ligands: Implications for ALS, *Biochemistry* 43 (2004) 16525-16531.
- [27]A. Tiwari, A. Liba, S.H. Sohn, S.V. Seetharaman, O. Bilsel, C.R. Matthews, P.J. Hart, J.S. Valentine, L.J. Hayward, Metal deficiency increases aberrant hydrophobicity of mutant

- superoxide dismutases that cause amyotrophic lateral sclerosis, *J. Biol. Chem.* 284 (2009) 27746-27758.
- [28]G.P. Gorbenko, P.K.J. Kinnunen, The role of lipid–protein interactions in amyloid-type protein fibril formation, *Chem. Phys. Lipids* 141 (2006) 72-82.
- [29]T.J.T. Pinheiro, The role of rafts in the fibrillization and aggregation of prions, *Chem. Phys. Lipids* 141 (2006) 66-71.
- [30]F. Conti, F. Malerba, Fluorescence signals in ANS-stained lipid bilayers under applied potentials, *Biophysik* 8 (1972) 326-332.
- [31]G.L. Jendrasiak, T.N. Estep, The interaction of 1-anilino-8-naphthalenesulfonate with thyroid lipids and membranes: A nuclear magnetic resonance study, *Chem. Phys. Lipids* 19 (1977) 323-337.
- [32]H. Merkle, W.K. Subczynski, A. Kusumi, Dynamic fluorescence quenching studies on lipid mobilities in phosphatidylcholine-cholesterol membranes, *Biochim. Biophys. Acta.-Biomembranes* 897 (1987) 238-248.
- [33]K. Seth, A.K. Agrawal, M.H. Aziz, A. Ahmad, Y. Shukla, N. Mathur, P.K. Seth, Induced expression of early response genes/oxidative injury in rat pheochromocytoma (PC12) cell line by 6-hydroxydopamine: implication for Parkinson's disease, *Neurosci. Lett.* 330 (2002) 89-93.
- [34]D. A. Harris, Cellular biology of prion diseases, *Clinic. Microbiol. Rev.* 12 (1999) 429-444.
- [35]S.H. Kim, Y. Shi, K.A. Hanson, L.M. Williams, R. Sakasai, M.J. Bowler, R.S. Tibbetts, Potentiation of amyotrophic lateral sclerosis (ALS)-associated TDP-43 aggregation by the proteasome-targeting factor, ubiquilin 1, *J. Biol. Chem.* 284 (2009) 8083-8092.
- [36]M.F.M. Engel, L. Khemtémourian, C.C. Kleijer, H.J.D. Meeldijk, J. Jacobs, A.J. Verkleij, B. Kruijff, J.A. Killian, J.W.M. Höppener, Membrane damage by human islet amyloid polypeptide through fibril growth at the membrane, *Proc. Natl Acad. Sci. USA.* 105 (2008) 6033-6038.
- [37]R. Friedman, R. Pellarin, A. Caflisch, Amyloid aggregation on lipid bilayers and its impact on membrane permeability, *J. Mol. Biol.* 387(2009) 407-415.

- [38]J. Zhai, A-L. Ström, R. Kilty, P. Venkatakrisnan, J. White, W.V. Everson, E. J. Smart, H. Zhu, Proteomic characterization of lipid raft proteins in amyotrophic lateral sclerosis mouse spinal cord FEBS J. 276 (2009) 3308-3323.
- [39]J. Chung, H. Yang, M.D. de Beus, C.Y. Ryu, K. Cho, W. Colon, Cu/Zn superoxide dismutase can form pore-like structures, Biochem. Biophys. Res. Commun. 312 (2003) 873–876.
- [40]S.S. Ray, R.J. Nowak, K. Strokovich, R.H. Brown Jr., T. Walz, P.T. Lansbury Jr., An intersubunit disulfide bond prevents in vitro aggregation of a superoxide dismutase-1 mutant linked to familial amyotrophic lateral sclerosis, Biochemistry 43 (2004) 4899-4905.

Figure 1. Structural and superficial properties of the proteins. (A) Secondary structures of the proteins characterized by CD spectra. (B) Surface hydrophobicity of the proteins measured by ANS fluorescence.: Freshly prepared holo SOD1 (○), Freshly prepared apo SOD1 (◐), Apo SOD1 long-timely incubated under a physiological condition (◑), and SOD1 aggregates prepared by treatment with TFE under acidic conditions (●).

Fig. 1

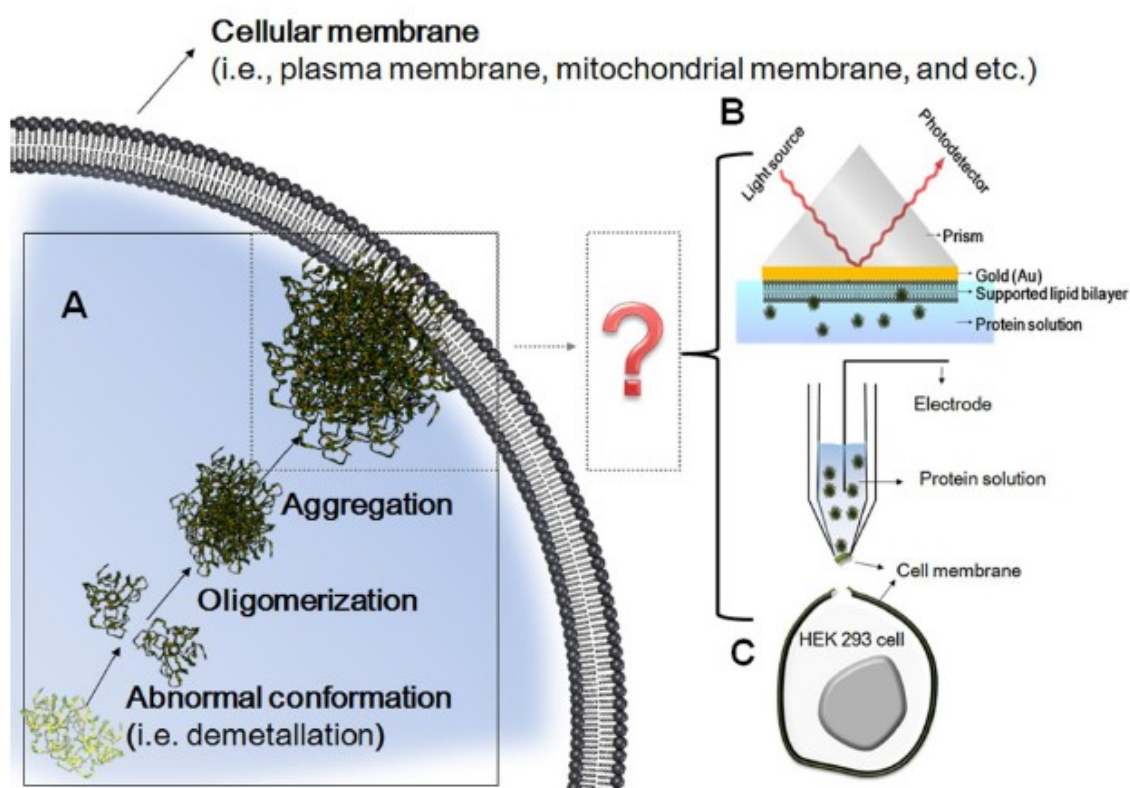


Figure 2. Time-resolved ThT fluorescence measurement of the samples. (A) The data set displayed as a function of the incubation time. (◆) SOD1 aggregates incubated with lipid vesicles, (●) Apo SOD1 incubated with lipid vesicles, (●) Holo SOD1 incubated with lipid vesicles, (■) BSA incubated with lipid vesicles, (◇) SOD1 aggregate, (○) Apo SOD, (○) Holo SOD1, and (□) BSA. (B) ThT fluorescence enhancement of each sample after 5 days incubation.

Fig. 2

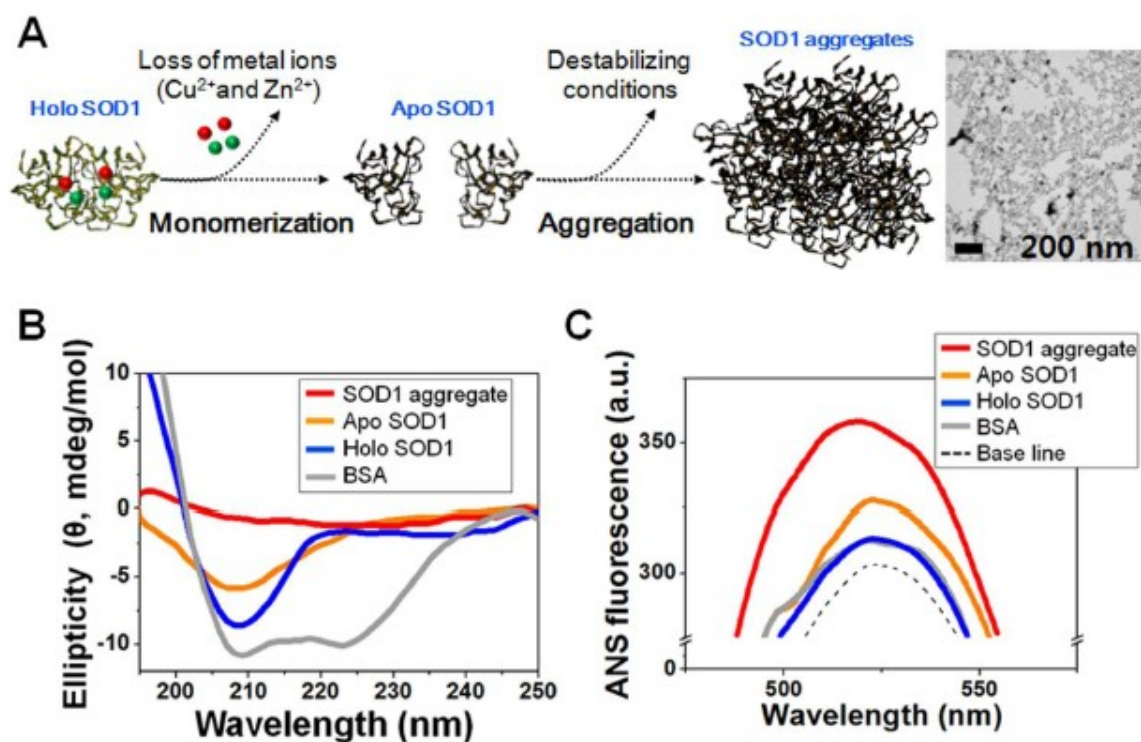


Figure 3. ANS fluorescence measurement for changes in hydrophobicity during lipid molecule-induced SOD1 Aggregation: Incubation without lipid (white box) and incubation with lipid molecules (gray box). A plot for a control sample shows the intrinsic fluorescence of ANS dye (without lipid) and ANS fluorescence of lipid molecules (with lipid), respectively.

Fig. 3

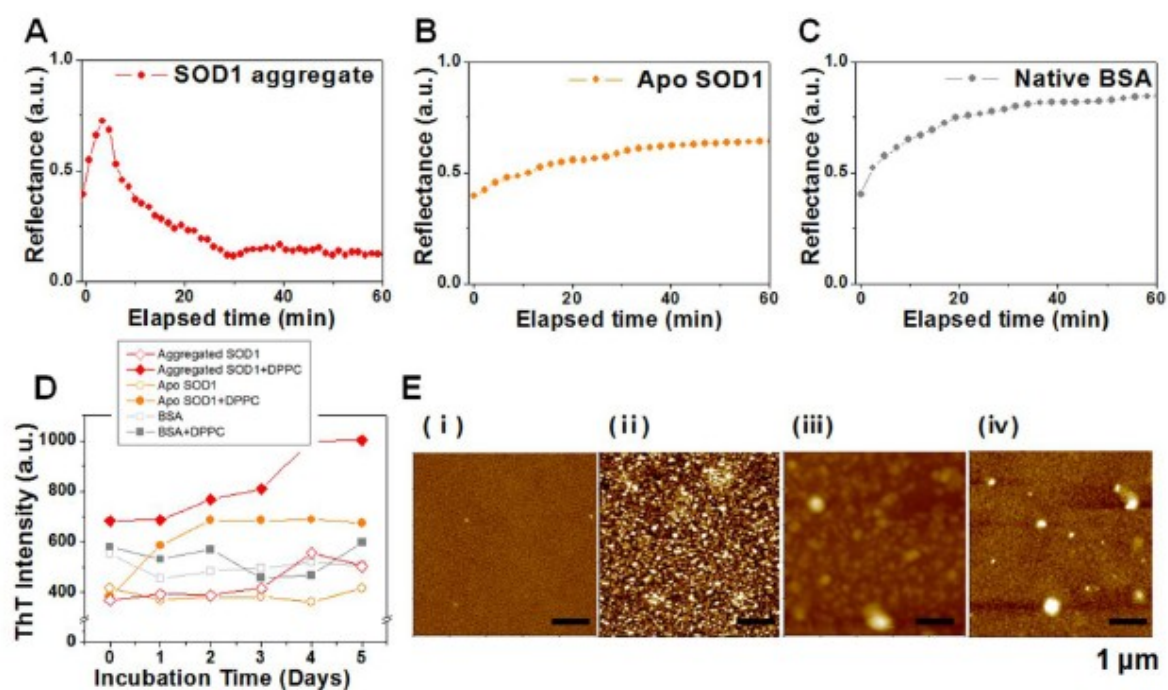


Figure 4. TEM images and ThT fluorescence images for the incubated samples. (A) Apo SOD1 monomer incubated without lipid vesicles. (B) Apo SOD1 incubated with lipid vesicles. (C) SOD1 aggregate incubated without lipid vesicles. (D) SOD1 aggregates incubated with lipid vesicles. The scale bar is 100 nm.

Fig. 4

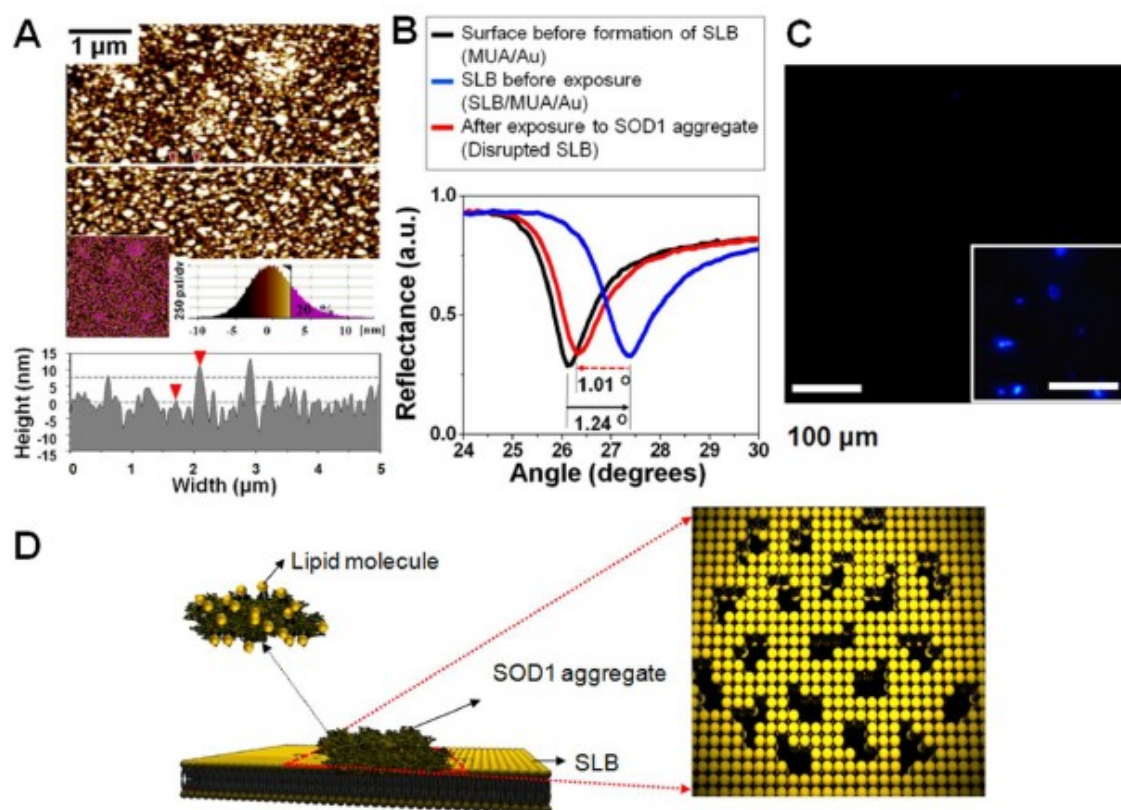


Figure 5. Cellular toxicity of lipid molecule-induced SOD1 aggregates: Incubation without lipid vesicles (white box) and incubation with lipid vesicles (gray box). The cytotoxicity was assessed using an MTT reduction assay. The data is expressed as the average percentage of MTT reduction \pm S.D. relative to the cells treated with the medium alone from 5 wells. *Showing a significant decrease in cell viability compared to the other samples.

Fig. 5

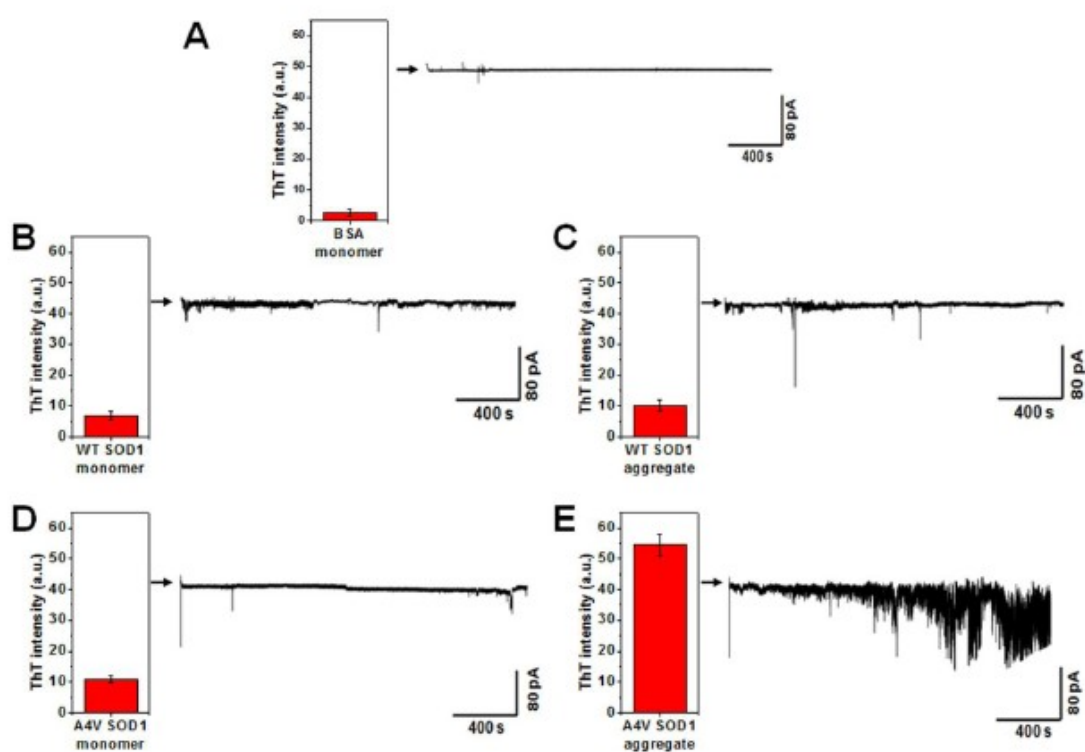


Figure 6. Apo SOD1s incubated with POPC lipid vesicles. (A) CD spectra: apo SOD1 (open circle) and apo SOD1 incubated with POPC (closed circle). (B) TEM image. The scale bar is 200 nm.

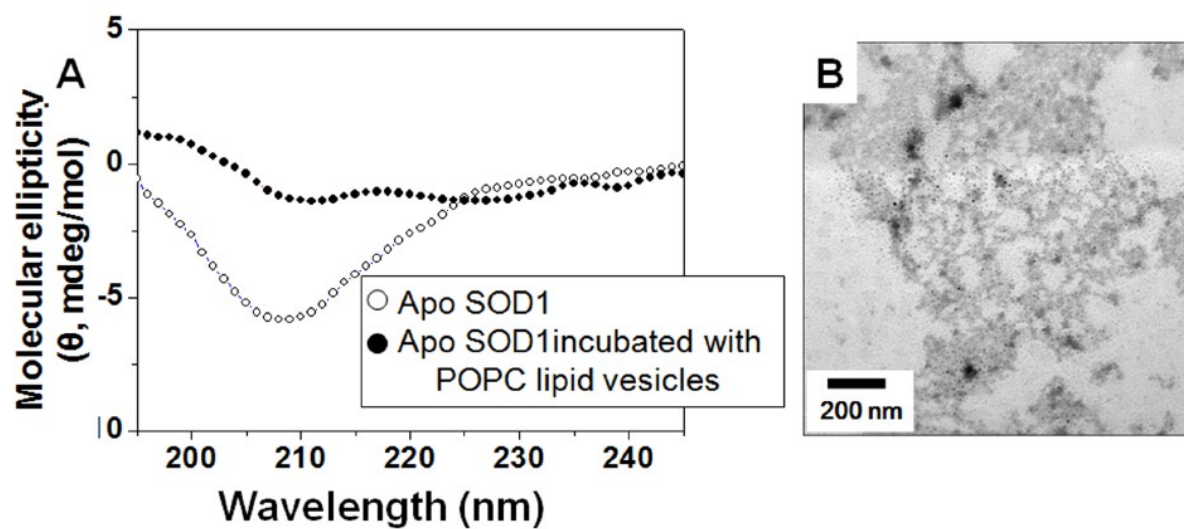


Figure 7. Apo SOD1s incubated under different lipid vesicle compositions. (A) CD spectra: Holo SOD1 (open circle) and apo SOD1 (gray closed circle) are control samples. All closed black symbols indicated the apo SOD1 incubated under different lipid vesicle compositions, which are prepared by insulating cholesterol molecules to lipid vesicles.; lipid only (○), lipid : cholesterol = 1 : 1 (●), and lipid : cholesterol = 2 : 1 (▲). (B) Representative TEM images of resulting structures incubated under different lipid vesicle compositions. The scale bar is 500 nm.

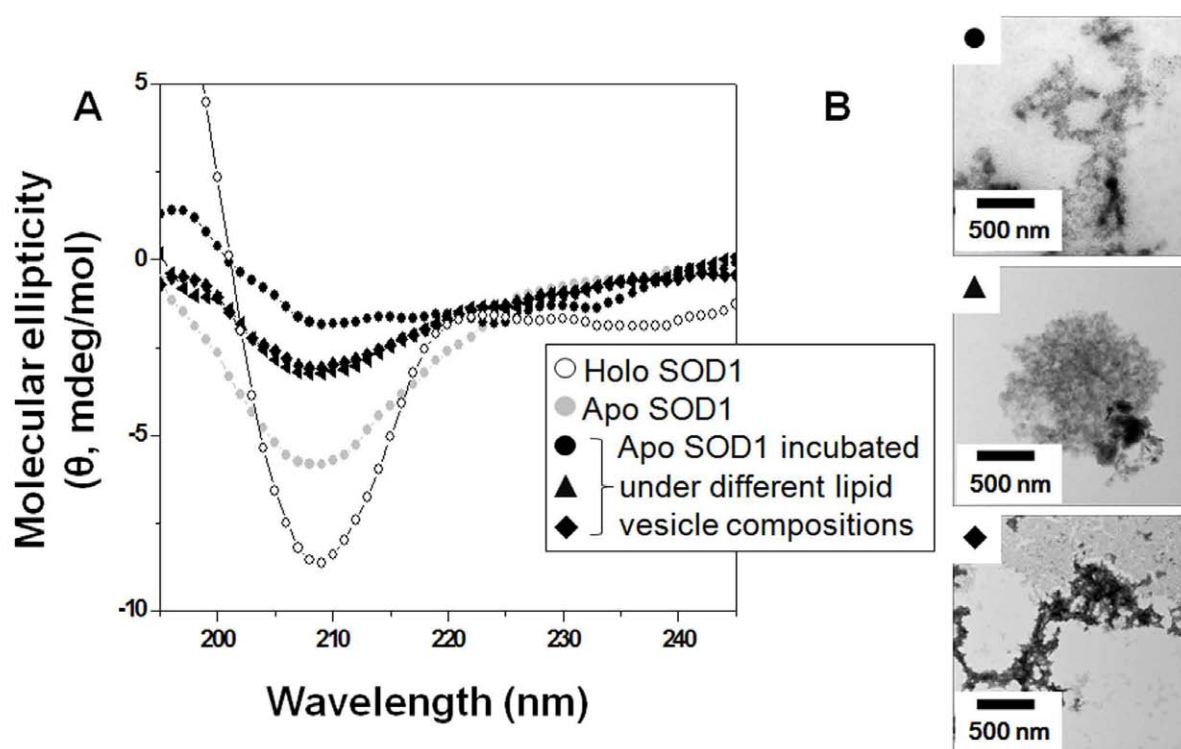
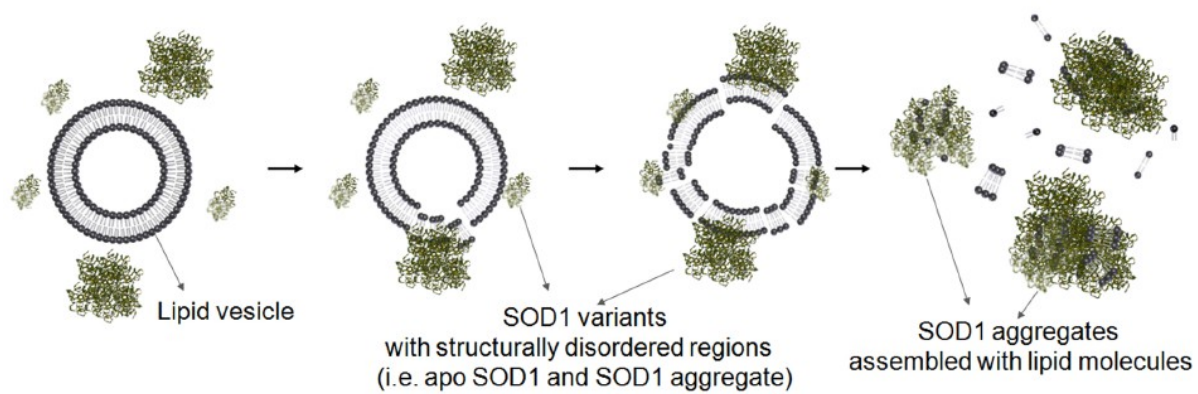


Figure 8. A schematic description of the lipid molecule-associated SOD1 aggregation.



ACCEPTED MANUSCRIPT

Supporting data

Figure S1. Comparison of the ThT fluorescence intensities of the SOD1 aggregate with apo SOD1

Figure S2. Time-resolved fluorescent intensities for a lipid only sample

Figure S3. TEM images of the incubated control samples. (A) BSA monomer only. (B) BSA monomer + DPPC lipid molecules. (C) DPPC lipid only. The scale bar is 100 nm.

Figure S4. Structurally ordered holo SOD1s maintain their secondary structures and surface properties, after incubation with/without lipid vesicles. (A) Secondary structures of the proteins characterized by CD spectra.; Freshly prepared holo SOD1 (●), holo SOD1 long-timely incubated without under a physiological condition (●), holo SOD1 incubated with lipid molecules under a physiological condition (●), and as prepared SOD1 aggregates (--). (B) Surface hydrophobicity of the proteins measured by ANS fluorescence.