

**Research paper****Synthesis and Biological Evaluation of Metal Oxide Nanoparticles on Superoxide Dismutase in Alzheimer's Disease**

*Fateme Mirzajani*<sup>1,2\*</sup> , *Narges Parniaei*<sup>2</sup>, *Paniz Nasiri*<sup>1,2</sup>, *Taha Nami*<sup>1</sup>,  
*Paria Kamali*<sup>2</sup>, *Nafyseh Poormeydany*<sup>2</sup>

<sup>1</sup>Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran

<sup>2</sup>Protein Research Center, Shahid Beheshti University, Tehran, Iran

[f\\_mirzajani@sbu.ac.ir](mailto:f_mirzajani@sbu.ac.ir)

**Article info:****Article history:**

Received: 21/01/2026

Accepted: 22/02/2026

**Keywords:** Magnetic iron oxide nanoparticles, Zinc oxide nanoparticles, Copper oxide nanoparticles, Superoxide dismutase, Oxidative stress and Alzheimer's disease

**Abstract**

In recent years, the growing application of metal oxide nanoparticles in medicine, industry and consumer products has led to an increase in human exposure to these materials (whether intentional or unintentional). On the other hand, oxidative stress is one of the key axes in the pathophysiology of Alzheimer's disease and other neurodegenerative disorders, and changes in the activity/structure of antioxidant enzymes can play a reinforcing role in this cycle. Authoritative reviews have shown that oxidative stress is intertwined with metabolic disorders, neuroinflammation and protein/lipid damage in Alzheimer's. In this study, magnetic iron oxide nanoparticles (MONPs), copper oxide (CuO NPs) and zinc oxide (ZnO NPs) were synthesized and then their interaction with the enzyme superoxide dismutase (SOD) was investigated in a statistical design (CCD). A colorimetric kit based on the production of superoxide by xanthine oxidase and the reduction of tetrazolium salt to formazan (reading around 450 nm) was used to measure SOD activity. In addition to activity, the physicochemical changes of nanoparticles as well as the structural changes of SOD after interaction were evaluated by DLS/zeta, UV-Vis, SEM, XRD, CD and SDS-PAGE (to examine the protein crown and approximate separation of soft/hard crown). The results showed that the changes in SOD activity are not severe and destructive in most conditions, but the type of nanoparticle and the contact time/ratio can create different patterns in protein structure and nanoparticle aggregation behavior; the corona phenomenon is also visible/confirmed as a new biological identity of the nanoparticle.

**1. Introduction**

Nanotechnology provides materials on the scale of 1–100 nm, dramatically increasing the surface-to-volume ratio, which decouples the reactive behavior and biological interactions from the bulk.

Increased surface area can lead to advantages such as improved catalysis, special optical/magnetic properties, and drug delivery applications [1], but at the same time increases the possibility of increased production of reactive oxygen species (ROS) and disruption of the redox balance [2].

## Synthesis and Biological Evaluation of Metal Oxide Nanoparticles

Nanotoxicological reviews have shown that metal nanoparticles and metal oxides can increase ROS directly (e.g., from surface pathways, lattice defects, photochemistry) or indirectly (stimulation of cellular pathways, inflammation), and this effect depends on the dose, metal species, and environmental conditions. In the environment, a nanoparticle is almost never alone. Upon contact with serum/plasma or even simpler protein environments, a layer of biomolecules settles on the surface of the nanoparticle, called the protein crown/corona [3]. This corona can be divided into two functional parts (a hard crown with stronger and more persistent binding, and a soft crown with weaker and more exchangeable binding), and its nature depends on the size, surface charge, surface chemistry, and curvature of the nanoparticle, as well as the composition of the environment. From a biological perspective, the corona constitutes the real identity of the nanoparticle and can alter cell entry, inflammatory pathways, and even toxicity; therefore, studying the effect of a nanoparticle on a key protein without considering the corona gives an incomplete picture [4].

On the other hand, Alzheimer's disease is a progressive neurodegenerative disorder associated with amyloid-beta accumulation, tau pathology, neuroinflammation, and mitochondrial dysfunction. In the authoritative review by Butterfield and Halliwell, oxidative stress is introduced not only as a consequence, but also as one of the engines amplifying the damage cycles in Alzheimer's disease; in particular, through lipid/protein damage, altered energy pathways, and interaction with neuroinflammation [5]. At the animal model level, manipulation of antioxidant systems can also affect the severity of Alzheimer's pathology; for example, reduction of SOD2 in transgenic models can exacerbate Alzheimer-like phenotypes, and conversely, increase of SOD2 in some models has been shown to reduce superoxide and improve cognitive/plaque deficits [6]. The enzyme superoxide dismutase, as the first line of defense against superoxide anion, regulates redox balance by catalyzing the dismutation of superoxide to oxygen and hydrogen peroxide [7, 8]. The Cu/Zn-SOD form (SOD1) is a homodimer in which copper and zinc ions play key roles in each subunit in the active site [9]; In humans, copper binding is usually described with histidines

His46, His48, His63 and His120 and zinc binding with His63, His71, His80 and Asp83, with His63 acting as a bridging ligand for both metals. This metal-centered architecture makes it possible for metal oxide nanoparticles (especially those that can release ions or react with surface groups) to potentially affect SOD structure/activity, even if the effect is small at the apparent activity level [8]. In humans, there are also reports of reduced SOD1 in the brains of individuals with Alzheimer's disease, suggesting that alterations in this axis may also be relevant in human pathology [10].

There is a broad consensus on the role of oxidative stress in Alzheimer's disease, but reports on the direction and magnitude of SOD changes in tissue/serum/red blood cells of patients are not always consistent, with some studies reporting decreases, some increases, and some no change; this heterogeneity highlights the importance of examining the contact conditions and biological matrix [11]. On the other hand, many works report the cytotoxicity of nanoparticles, but the link between the physicochemical properties of the nanoparticle, corona formation and changes after protein contact, and the functional output of a key antioxidant enzyme such as SOD has been less studied in a multivariate and systematic design [12]. The corona concept suggests that the cell does not see what we call pure nanoparticle in the laboratory glass, but rather nanoparticle + corona [13, 14]; this biological identity influences interactions and pathways of toxicity. Therefore, the main gap is how contact time, nanoparticle to protein ratio, protein concentration and metal oxide type simultaneously shape the activity and structural outputs of SOD and whether these relationships can be used to build a closer picture of real exposures (especially occupational exposure) [15]. The aim of this study is to reconstruct and systematically analyze the interaction of three widely used metal oxide nanoparticles (MONPs, CuO NPs, ZnO NPs) with the SOD enzyme as one of the core antioxidant defenses of the body, in such a way that both the functional output (enzyme activity under different conditions) and the structural/physicochemical output (nanoparticle changes, corona formation, changes in protein secondary structure, and band patterns) can be interpreted complementary. The scope of the research includes synthesis and

characterization of nanoparticles, CCD experiment design and analysis of variance, selection of optimal points for instrumental analyses, assessment of cytotoxicity with MTT in PC12, and an exploratory human study of changes in oxidative stress markers in individuals exposed to nanoparticles.

This research aims to demonstrate that even if SOD activity remains apparently stable, subtle structural changes or changes in nanoparticle corona/aggregation can suppress biological pathways associated with oxidative stress, a stress that is of dual importance in the context of diseases such as Alzheimer's.

## **2. Experimental**

### **2.1. Materials**

The Chemicals including copper sulfate, zinc sulfate Iron hydroxide, Hydrochloric Acid, Nitric Acid, Phosphate Buffered Saline, Acrylamide, Tris Base, Tris-HCl, SDS, Ammonium Persulfate, TEMED, Glycine, Loading Dye, CBC-G250, Acetic Acid, Phosphoric Acid, Sodium Thiosulfate, Sodium Carbonate, 37% Formaldehyde, Mueller-Hinton Broth Medium and Agar Powder were all purchased from MERK Co. (Germany). Trisodium Citrate, Bovine Serum Albumin and Dithiothreitol (DTT) were purchased from Sigma-Aldrich Co. (Germany) and 96% ethanol along with methanol were purchased from Dr. Mojallali Chemicals Co. (Iran). The Superoxidase protein which was a protein fraction was gifted by Prof. Wilson's research team from Wollongong University and the Superoxidase quantification ELISA kit was purchased from Proteintech Group, Inc (New Zealand Branch). HPLC grade water was used in all of the stages.

### **2.2. Metal oxide Nanoparticles Synthesis and Characterization Methods**

The synthesis of MONPs was carried out by the coprecipitation method, because this method allows the production of magnetic particles at the nanoscale with common equipment and has good control over variables such as pH, temperature and stirring speed. In this method, divalent and

trivalent iron salts were dissolved in an aqueous medium and a precipitate of magnetic particles was formed by adding an alkaline agent [16]. To reduce the tendency of magnetic particles to agglomerate, surface modification with a stabilizer (such as citric acid) was used to increase colloidal stability by creating a surface charge and steric barrier [13].

After the formation of the precipitate, the samples were washed, separated and dried and prepared for subsequent tests as a stable suspension in a suitable buffer.

The synthesis of CuO NPs was carried out by the chemical reduction route to produce particles with an active surface and the possibility of appropriate repetition [17]. The copper precursor solution was reacted under controlled conditions in the presence of a reducing agent (such as ascorbic acid) and, together with a capping/stabilizing agent (such as starch), led to the formation of nanoparticles. At this stage, control of the pH and the order of addition of materials was important, because rapid changes in pH or excessive growth could increase the size or cause the formation of unwanted phases. After synthesis, the particles were separated from impurities by washing and prepared as dry powder or suspension for further steps.

The synthesis of ZnO NPs was based on the formation of precipitation in an alkaline medium and then stabilization with a polymer such as PVP to prevent strong adhesion and improve the stability of the suspension [18]. ZnO is known as a semiconducting oxide and its behavior is sensitive to environmental conditions, so during the synthesis, efforts were made to maintain uniform conditions of temperature, reaction time and stirring intensity.

Finally, the particles were washed, dried, and prepared for biological assays by redispersion in buffer.

After synthesis, basic characterization of the nanoparticles was performed to confirm phase formation and investigate colloidal behavior. UV-Vis spectra were used as optical fingerprints to observe absorption peaks and investigate changes after interaction with protein, as changes in peak intensity/shift could be indicative of aggregation, changes in surface dielectric environment, or phase changes.

## Synthesis and Biological Evaluation of Metal Oxide Nanoparticles

Hydrodynamic size and size distribution were measured by DLS and surface charge by zeta potential to assess the relative stability of the suspensions; interpretation of these data is based on the assumption that higher absolute zeta potentials generally indicate greater electrostatic repulsion and less tendency to aggregate. Morphology and shape were assessed by SEM to observe differences in sphericity/polygonality/clustering and evidence of aggregation.

XRD was used to investigate the crystal structure and phase, as the diffraction patterns allow comparison with standard phases and show that the material has transitioned from an amorphous state to the expected crystalline state.

### 2.3. Statistical Study Methods

To systematically study the interaction of SOD with nanoparticles, a Central Composite Design statistical design was performed in Design-Expert software to investigate the main effects and

interaction effects of three quantitative factors including interaction time, nanoparticle to enzyme ratio and enzyme concentration, along with the qualitative factor nanoparticle type in a predictable framework.

The logic of CCD is that instead of varying a single factor, multiple levels are defined for the factors so that both linear and nonlinear/interaction effects can be detected and finally the statistical significance of the model and coefficients can be measured by analysis of variance.

In each treatment, specific amounts of enzyme, nanoparticles and buffer were combined in vials, controlled mixing was performed and then the samples were incubated at 37°C for defined times to create conditions closer to the biological state. After incubation, SOD activity was measured according to the kit instructions; In this method, superoxide is produced by xanthine oxidase, the tetrazolium salt is converted to formazan, and the absorbance is read at around 450 nm, while the presence of SOD inhibits color intensity.

**Table 1** Factors and their levels defined for the designed interaction experiments

Factors	Units	- $\alpha$	-1	0	1	+ $\alpha$
Time	min	10	45	97.5	150	185
Superoxidase Concentration	mg/ml	2	7.5	16.25	25	30.5
Superoxidase/MONPs Concentration	%	10	20	35	50	60
Nanoparticle type			MONPs	CuONPs	ZnONPs	

The effect of nanoparticle exposure was also studied through a human-based test. The participants were selected among 20-65 years old male and female individuals and got separated into two groups of constantly exposed and unexposed (control groups) to nanoparticles.

Standardized questionnaires were also completed by each individual and based on the responses each group further got divided into two subgroups based on the presence or absence of familial AD history.

Blood samples from participants were collected based on ethical protocols and plasma Superoxidase concentration of each sample was assessed.

The resulting data along with the data collected from the questionnaires were analyzed using IBM SPSS Statistics (26.0).

### 2.4. Superoxidase Characterization Methods

To select the optimal conditions for further analysis, the software optimization module was used to extract the point(s) that showed the most differentiation or the most likely interaction between the nanoparticle and the enzyme. Then, additional tests were performed under those conditions to look at structural and physicochemical effects in addition to the activity number.

Changes in the secondary structure of the enzyme were examined by CD and the data were submitted to analytical tools to estimate the contribution of alpha/beta/turn helices; the importance of this step is that small changes in secondary structure can affect stability, folding and even access to the active site, especially in metal-dependent enzymes

such as SOD, where the geometry of the active site depends on the protein's binding network. Structural information on the SOD1 metal site (key histidines and Asp83) is traceable in structural/reviewed sources and strengthens the framework for interpreting CD changes.

SDS-PAGE was used to investigate the protein corona and operationally separate free, soft, and hard proteins.

Stepwise separation was performed by centrifugation at different cycles to first collect the supernatant as free protein, then wash and centrifuge again to separate the weaker bound proteins (soft corona) and finally extract the strongly bound proteins (hard corona) with SDS. The use of SDS-PAGE for corona is useful because the band pattern and relative intensity can be an indication of the amount and size range of bound proteins and make the concept of biological identity of the nanoparticle visible at the laboratory level; this logic is consistent with the corona literature, which indicates that the corona determines the subsequent interactions of the nanoparticle with the biological system.

### 2.5. Cytotoxicity analysis and MTT Tests

In the biological section, cytotoxicity was performed using the MTT assay on the PC12 cell line [19, 20], as this line is used as a neuronal/neuroblastoma model in many studies related to oxidative damage and neuronal toxicity. The logic of MTT is that living cells are able to reduce tetrazolium salt to formazan and the intensity of the color is proportional to viability. Therefore, by treating cells with different concentrations of nanoparticles and measuring the absorbance at a standard wavelength, a dose-response curve and, if possible, an IC50 can be estimated.

## 3. Results

The synthesis results showed that all three classes of nanoparticles were produced with acceptable properties for entering the bio-interaction phase (Figure 1), but their physicochemical behavior was not the same and these differences were later reflected in the interaction patterns with SOD. In magnetic iron oxide nanoparticles, simultaneous

control of temperature and pH and prevention of precursor oxidation played a decisive role in the product quality; when iron solutions were exposed to air or heat for a long time, signs of phase change/heterogeneity increased and in successful replicates, preparation close to the time of addition and stabilization with citric acid helped to achieve better distribution.

This observation is consistent with the known behavior of magnetic nanoparticles, as they naturally tend to aggregate and surface modification usually improves colloidal quality. In the UV-Vis study of the nanoparticles before contact with the enzyme, the peaks and shape of the spectra were consistent with the patterns reported for metal oxides and served as initial confirmation of nanoparticle formation (Figure 1). After interaction with SOD, a decrease in absorption intensity was observed for all three nanoparticles, and this decrease was enhanced with contact time; this pattern is generally consistent with one of two scenarios:

either some of the particles have entered aggregation/agglomeration and the effective surface area has decreased, or some of the particles have been involved in corona formation and changed the optical environment near the surface, or a combination of both. Since the corona literature shows that protein binding can change the optical/surface identity of the nanoparticle, the observation of a change in the UV-Vis spectrum after protein contact is quite expected from a surface biophysical perspective. In iron nanoparticles, signs of peak shift towards longer wavelengths (red-shift-like behavior) were observed with increasing time, which is often attributed to an increase in effective size or a change in the dielectric environment around the surface.

In CuO, the rapid and sharp decrease in peak intensity and its broadening at early times indicated that this nanoparticle either enters the aggregation/precipitation phase more quickly or establishes stronger surface interactions with the protein that change the size distribution and surface environment; in ZnO, the overall spectral changes were milder, although the trend of decreasing absorption intensity was still observed. Size and surface charge were quantitatively assessed by DLS and zeta before and after contact.

## Synthesis and Biological Evaluation of Metal Oxide Nanoparticles

**Table 2.** Enzyme assay results of experiments designed based on interaction time, nanoparticle to enzyme ratio, enzyme concentration, and nanoparticle type

Run No	STD	NP type	SOD $\mu\text{g/ml}$	Ratio %	Time min	SOD Activity U/mL	Run No	STD	NP type	SOD $\mu\text{g/ml}$	Ratio %	Time min	SOD Activity U/mL
1	28	CuONPs	25	25	145	64.209	31	19	MONPs	15	15	90	56.014
2	42	ZnONPs	7	7	145	63.898	32	40	CuONPs	15	15	90	60.311
3	35	CuONPs	15	15	90	57.198	33	23	CuONPs	25	25	40	51.448
4	22	CuONPs	7	7	145	72.233	34	15	MONPs	15	15	90	43.276
5	9	MONPs	15	15	5	62.891	35	2	MONPs	7	7	145	70.656
6	38	CuONPs	15	15	90	54.349	36	25	CuONPs	7	7	40	72.995
7	57	ZnONPs	15	15	90	64.152	37	20	MONPs	15	15	90	75.451
8	59	ZnONPs	15	15	90	64.097	38	48	ZnONPs	25	25	145	57.421
9	47	ZnONPs	25	25	40	66.952	39	24	CuONPs	25	25	145	59.941
10	54	ZnONPs	15	15	90	61.395	40	17	MONPs	15	15	90	56.213
11	31	CuONPs	1	1	90	81.934	41	58	ZnONPs	15	15	90	59.818
12	39	CuONPs	15	15	90	57.028	42	43	ZnONPs	25	25	40	63.447
13	44	ZnONPs	25	25	145	55.856	43	32	CuONPs	30	30	90	65.492
14	30	CuONPs	15	15	180	62.556	44	14	MONPs	15	15	90	65.545
15	29	CuONPs	15	15	5	84.491	45	3	MONPs	25	25	40	63.675
16	27	CuONPs	25	25	40	64.736	46	41	ZnONPs	7	7	40	64.537
17	1	MONPs	7	7	40	63.599	47	36	CuONPs	15	15	90	66.606
18	50	ZnONPs	15	15	180	61.881	48	18	MONPs	15	15	90	64.537
19	37	CuONPs	15	15	90	54.396	49	33	CuONPs	15	15	90	47.591
20	21	CuONPs	7	7	40	61.952	50	34	CuONPs	15	15	90	56.096
21	60	ZnONPs	15	15	90	64.308	51	4	MONPs	25	25	145	54.033
22	49	ZnONPs	15	15	5	57.028	52	7	MONPs	25	25	40	68.757
23	16	MONPs	15	15	90	61.881	53	13	MONPs	15	15	90	59.924
24	55	ZnONPs	15	15	90	65.021	54	52	ZnONPs	30	30	90	54.636
25	8	MONPs	25	25	145	63.159	55	45	ZnONPs	7	7	40	47.591
26	10	MONPs	15	15	180	52.309	56	5	MONPs	7	7	40	56.032
27	12	MONPs	30	30	90	44.724	57	53	ZnONPs	15	15	90	75.487
28	51	ZnONPs	1	1	90	53.458	58	46	ZnONPs	7	7	145	59.847
29	11	MONPs	1	1	90	65.328	59	6	MONPs	7	7	145	67.368
30	56	ZnONPs	15	15	90	70.229	60	26	CuONPs	7	7	145	62.855

Before interaction, the size distributions for all three nanoparticles were reported to be in the nanoscale range with acceptable dispersion, and the zeta potential of CuO was higher than that of the other two, indicating higher electrostatic stability (Figure 1D-1F). After contact with the enzyme, in all three cases a significant increase in hydrodynamic size and loss of Gaussian shape of the size distribution occurred; this means that the

system moved from a relatively uniform state towards multiple populations (single particles + clusters).

Mechanistically, this result is consistent with corona formation (increased hydrodynamic diameter) as well as protein bridging between particles (aggregation/agglomeration); corona literature reports exactly such changes as a common outcome of nanoparticle-protein contact.

SEM images also supported this narrative. Before contact, the iron particles were mostly spherical and relatively uniform, CuO tended to have less regular shapes and rough surfaces, and ZnO was more polygonal/polyhedral (Figure 1G-II). After interaction with SOD, all three samples showed signs of aggregation and formation of larger clusters, but the intensity of these changes was not the same. In CuO, larger and more complex clusters and clearer aggregation patterns were observed, which is consistent with a more severe decrease in the UV-Vis peak intensity and an increase in the size dispersion in DLS. In ZnO, growth/cohesion and the formation of two-phase regions were reported, which could be consistent with the surface behavior of ZnO and its sensitivity to environmental conditions. In iron particles, aggregation occurred but a relative preservation of the spherical shape was still observed, which could be related to the role of the surface stabilizer and the magnetic property of the particles. In the experimental design and SOD activity assay section (Table 2), ANOVA analysis (Table 3) showed that the overall model was statistically significant, and no significant lack of fit was reported, which simply means that the model was able to explain the data patterns without large systematic errors (Figure 2). The factors of time, enzyme concentration and nanoparticle type made significant contributions, and some two-factor interactions were also significant. Behaviorally, interaction time was associated with a decrease in SOD activity in almost all nanoparticles, but the magnitude and direction of the effect of nanoparticle to enzyme ratio varied depending on the nanoparticle type; In ZnO and CuO, there was a tendency for activity to decrease with increasing time and ratio, while in magnetic iron, increasing ratio was associated with increased activity (or decreased inhibitory effect) in some regions. This heterogeneity could logically be due to differences in surface area, charge, and aggregation/corona mechanisms, as in one system, increasing ratio may result in more surface adsorption and more disorder and in another system in adsorption of interfering proteins/reduced access to the active surface or even more complex colloidal phenomena. In the structural analysis of SOD by CD (Figure 3), the enzyme's basic spectrum was consistent

with the patterns reported for SOD, and changes in peak intensity and shape were observed after exposure to nanoparticles. Secondary structure content estimation showed that the overall changes were not large and destructive, but there were differences between nanoparticles; CuO caused the largest change, and ZnO and MONPs showed milder changes. From a biochemical perspective, these mild changes are also potentially important, since SOD is a metal-dependent enzyme and the geometry of the active site and the surrounding binding network are critical for catalysis; the metal site is defined by histidines and Asp83, and if a structural change occurs near these regions, it may manifest itself in the functional output under more specific conditions or with longer exposures. In SDS-PAGE (Figure 4), the presence of protein bands in the columns corresponding to the nanoparticle-protein mixture, as well as the separation of the soft and hard corona, confirmed the formation of corona. The band pattern in CuO showed that the hard crown region was brighter and denser, consistent with the stronger binding interpretation; in ZnO, the hard crown bands were weaker and the protein transfer to the soft crown region was more prominent, which could indicate weaker binding or greater exchangeability on the ZnO surface. From the perspective of the corona literature, such differences are natural because the composition and strength of the corona depend on the surface chemistry and the charge and curvature of the nanoparticle, and one would not expect a corona with the same identity in different nanoparticles. In the MTT cytotoxicity assay on PC12 (Figure 5), all three nanoparticles showed a dose-dependent behavior, meaning that viability decreased with increasing nanoparticle concentration. However, the toxicity intensity was not the same among the nanoparticles, with ZnO showing the highest toxicity and CuO the lowest. This result is consistent with a section of the nanotoxicology literature that reports ZnO as riskier in some biological conditions due to its ROS generation potential and surface/electronic properties, although the exact result is always dependent on surface coverage, size and environment. Calculation of the IC50 (where the curve allowed) helped to provide a quantitative index for comparison and showed that the difference

## Synthesis and Biological Evaluation of Metal Oxide Nanoparticles

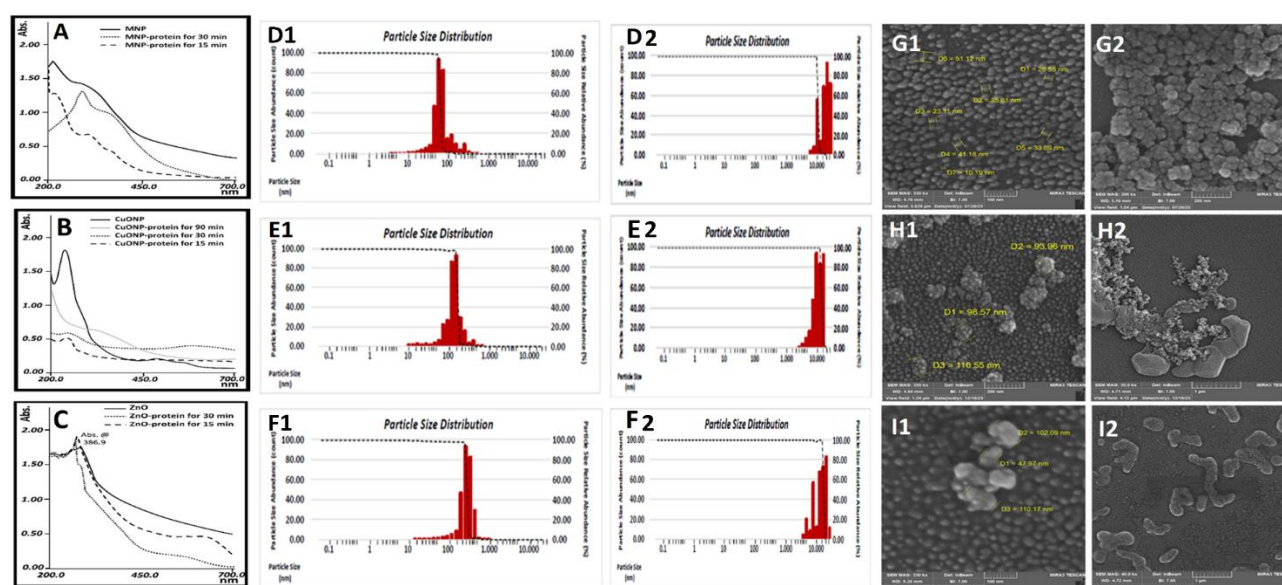
between nanoparticles in terms of dose required to reduce survival by 50% was significant. An exploratory human study also reported a general trend of decreased indices related to antioxidant defense in groups exposed to nanoparticles (Figure 6).

This observation alone is not sufficient to draw causal conclusions, but is important from an environmental/occupational perspective, as in the Alzheimer's and oxidative stress literature, reduced antioxidant capacity could be one of the areas facilitating neurodegeneration.

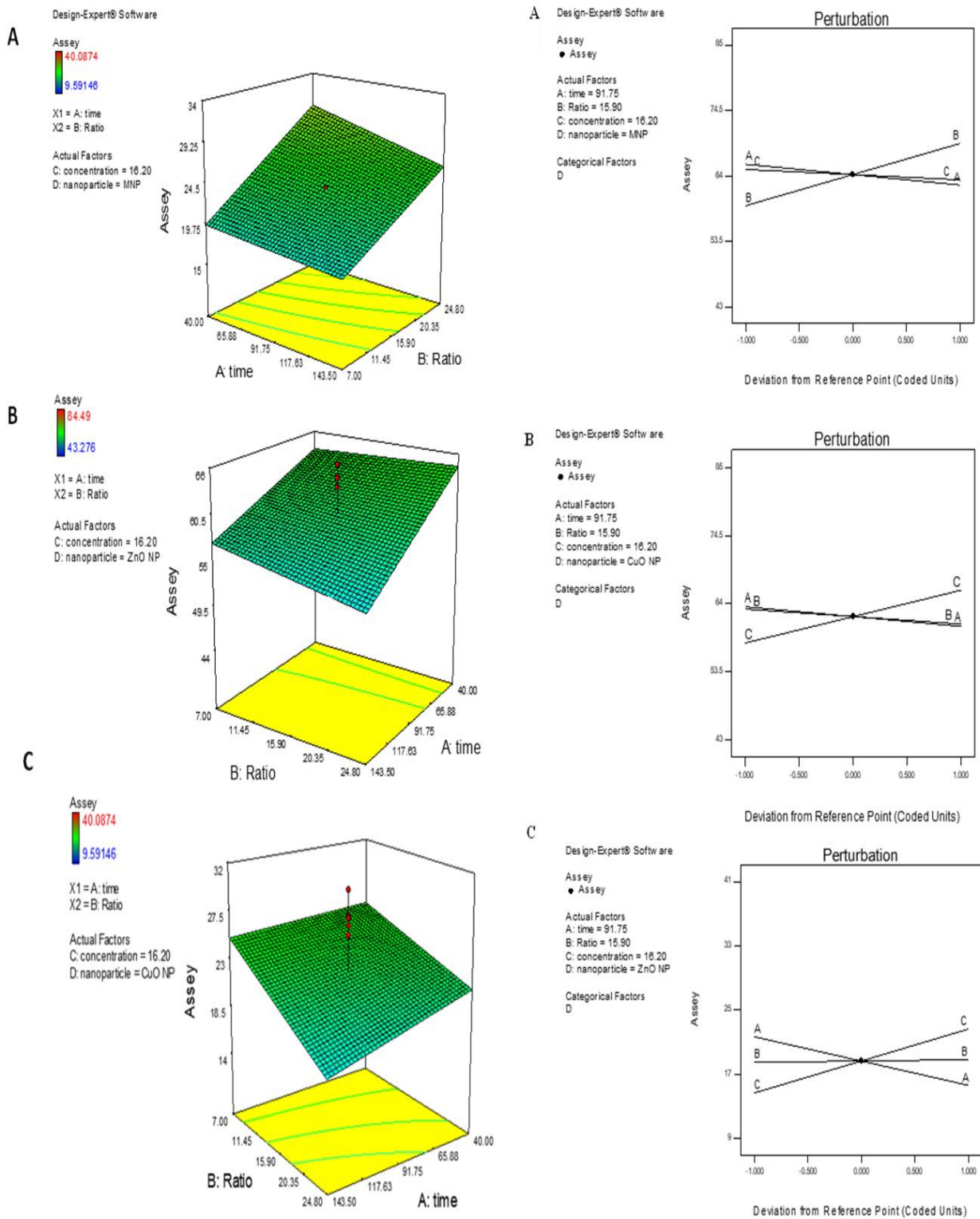
**Table 3.** Analysis Of Variance of data from Superoxidase-MONPs interaction

Source	Sum of Squares	df	Mean Squares	F Value	p-value Prob>F	Definition
Model	2022.15	14	144.44	4.35	< 0.0001	significant
A-time	271.39	1	271.39	8.16	0.0064	
B-ratio	90.07	1	90.07	2.71	0.1067	
C-concentration	387.40	1	387.40	11.66	0.0014	
D-nanoparticle	290.72	2	145.36	4.37	0.0184	
AB	11.82	1	11.82	0.36	0.5540	
AC	263.18	1	263.18	7.92	0.0072	
AD	79.77	2	39.89	1.20	0.3106	
BC	18.34	1	18.34	0.55	0.4615	
BD	272.09	2	136.04	4.09	0.0233	
CD	337.36	2	168.68	5.07	0.0103	
Residual	1495.75	45	33.24			
Lack of Fit	1166.23	30	38.87	1.77	0.1217	not significant
Pure Error	329.51	15	21.97		R-squared	0.5748
Cor Total	3517.89	59	144.44		Adj R-squared	0.4425

(SOD Assay) = +61.92 - 2.57 × A + 1.48 × B + 3.08 × C + 2.63 × D [1] + 0.1 × D [2] - 0.70 × AB - 3.31 × AC + 0.91 × AD [1] + 1.06 × AD [2] + 0.87 × BC + 3.50 × BD [1] - 2.64 × BD [2] - 3.90 × CD [1] + 0.99 × CD [2]

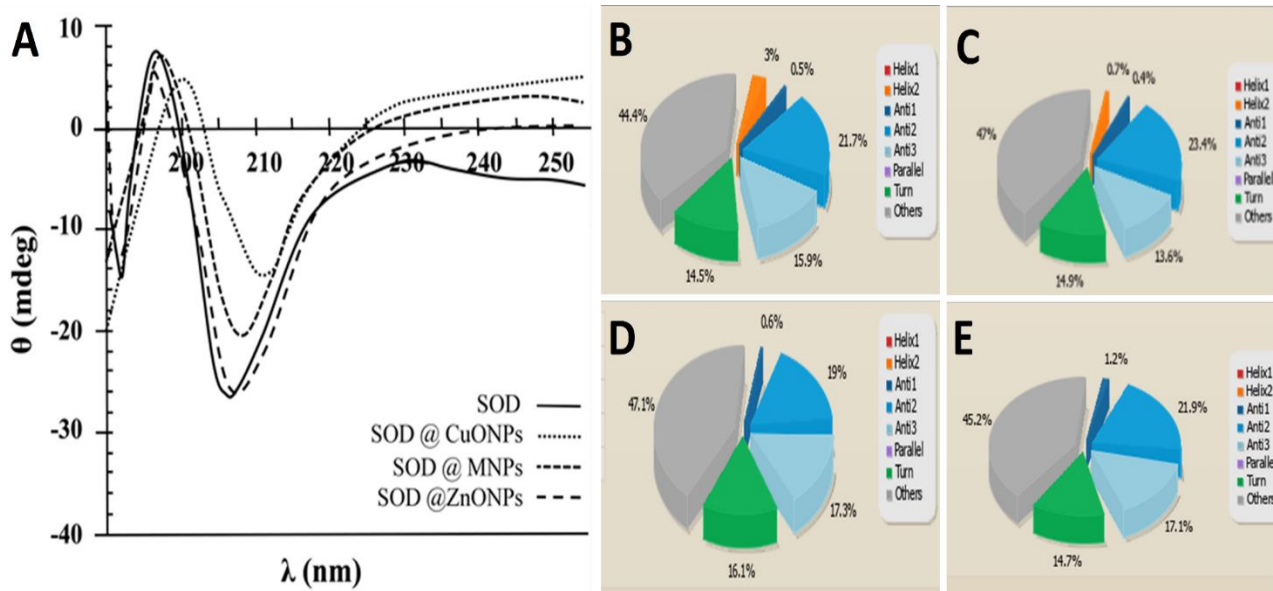


**Figure 1.** UV-Visible absorption spectra of A: MONPs, B: CuO nanoparticles and C: ZnO nanoparticles before and after interaction to Superoxide dismutase. Size distribution via DLS in case of D: MONPs, E: CuO nanoparticles and F: ZnO nanoparticles (1: before and 2: after) interaction to Superoxide dismutase. SEM images of G: MONPs, H: CuO nanoparticles & I: ZnO nanoparticles (1: before and 2: after) interaction to Superoxide dismutase

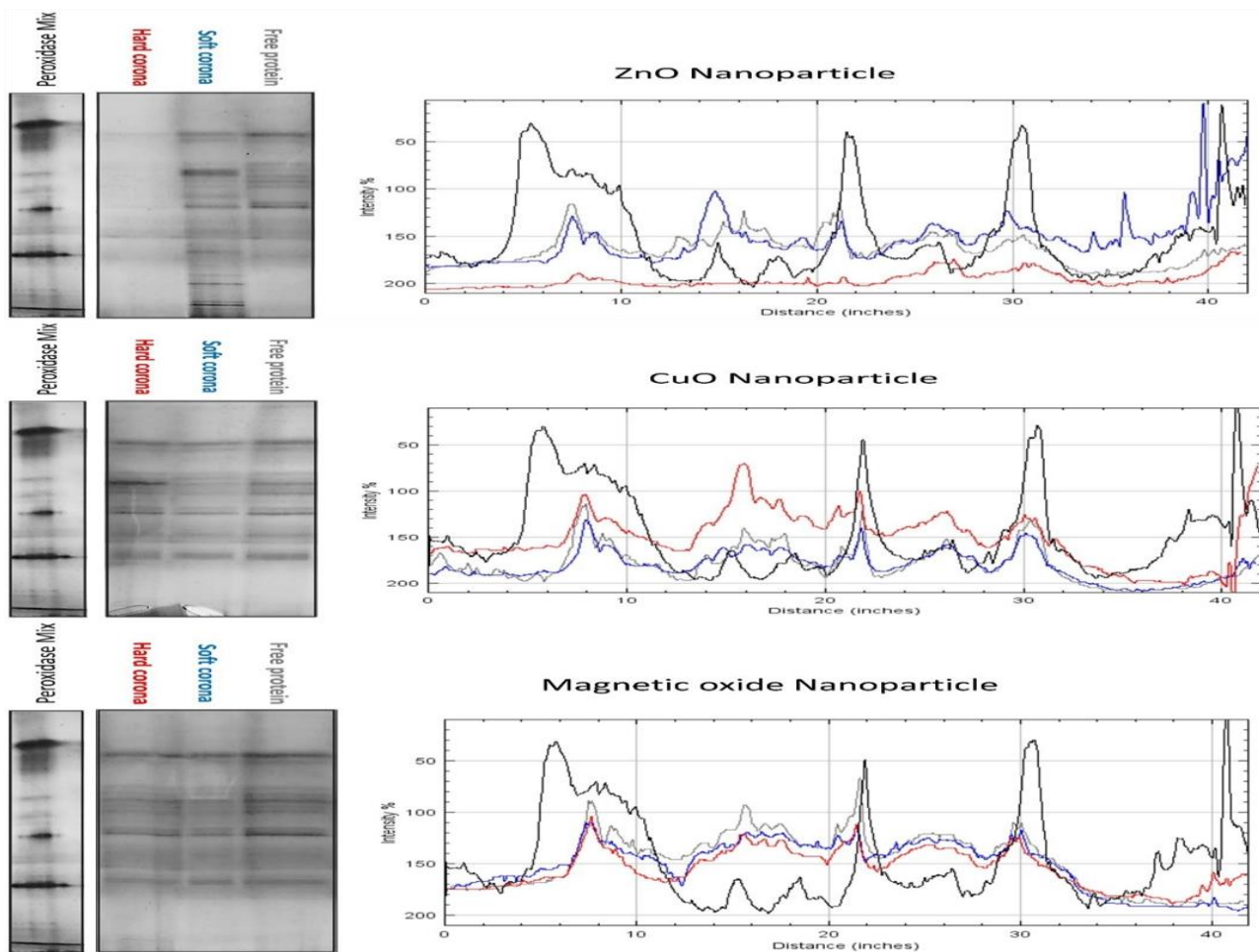


**Figure 2.** response surface diagrams illustrating the correlation between Superoxidase assay and independent variables; time, Superoxidase concentration, Superoxidase to MONPs ratio, and MONPs size. 3D diagrams demonstrate the effect of two quantitative variables on Superoxidase assay, while 2D diagrams display the relationship of a quantitative variable and MONPs size with Superoxidase assay

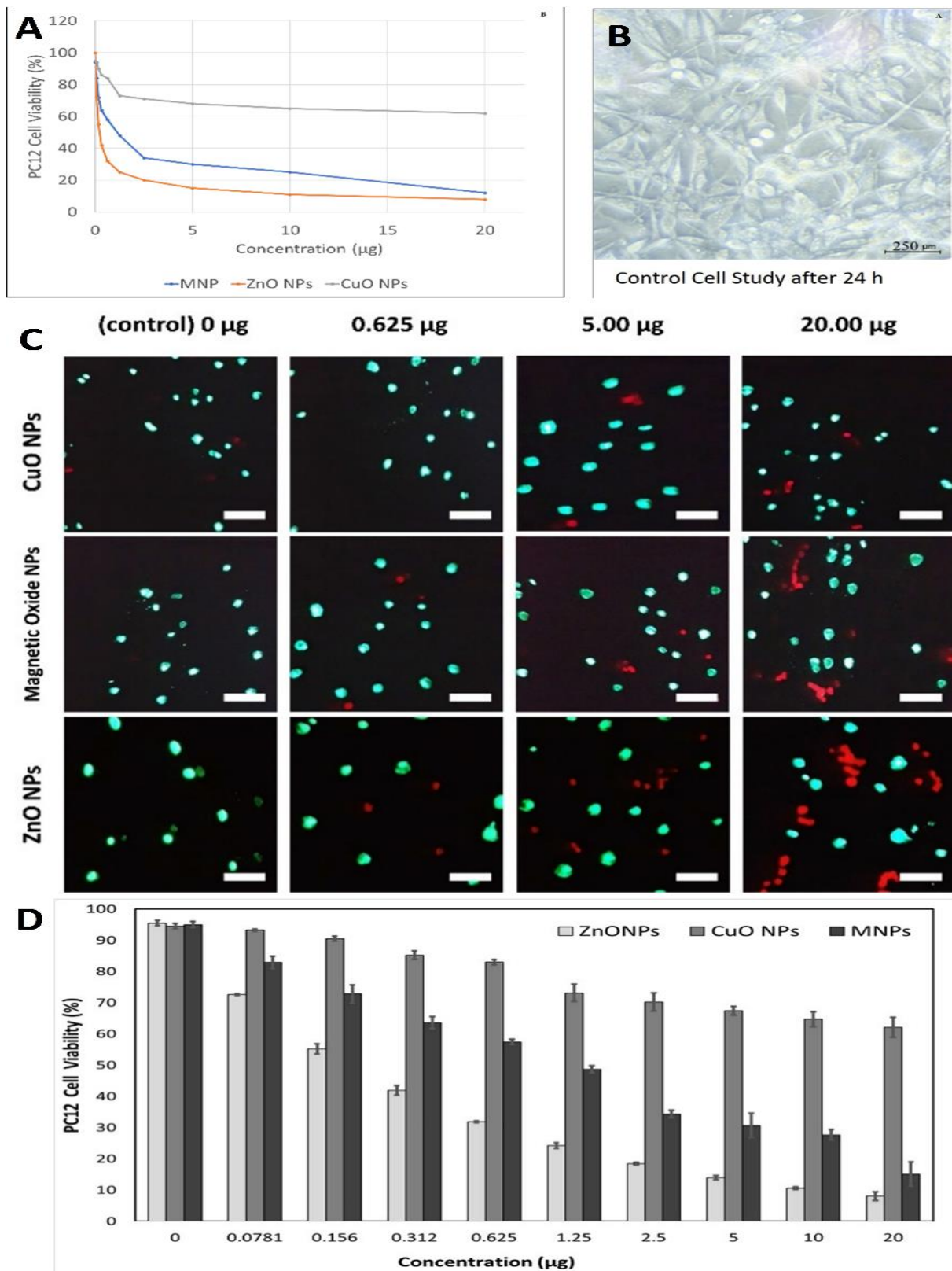
# Synthesis and Biological Evaluation of Metal Oxide Nanoparticles



**Figure 3.** Circular dichroism spectra (A) and secondary structure content of Superoxidase, before (B) and after interaction with C: ZnO nanoparticles D: MONPs and E: CuO nanoparticles



**Figure 4.** SDS PAGE results for interaction samples to A: ZnO nanoparticles B: CuO nanoparticles and C: MONPs. Silver Nitrate Staining are performed for gels visualization. Band intensity to migration distance is also determined



**Figure 5.** Results of A: the Dose-response cytotoxicity relationship of nanoparticles in the MTT test of CuO nanoparticles, MONPs, and ZnO nanoparticles. B: Control cell viability image. C: fluorescence microscopy images of live (green) and dead (red) cells' nuclei, 24 hours after being treated and D: the rate of PC12 cells' viability against various nano treatment in different concentrations

## Synthesis and Biological Evaluation of Metal Oxide Nanoparticles

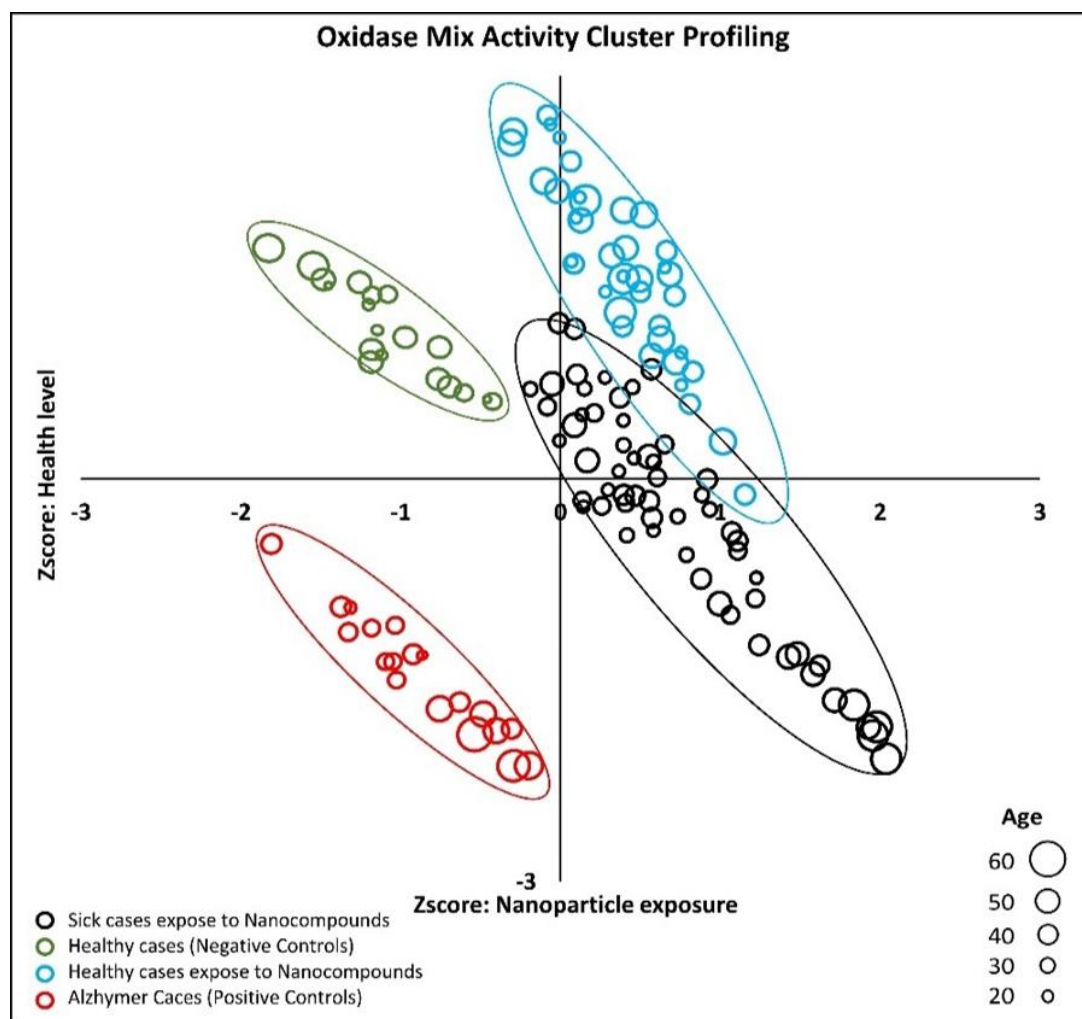


Figure 6. IBMSPSS cluster profiling of participants based on plasma Superoxidase levels and CuONP/MONPs/ZnONP exposure

### 4. Discussions

The discussion of this research can be built on three main axes: the first axis behavior of the nanoparticle after entering the protein environment, the second axis structural/functional response of SOD, and the third axis linking these findings to oxidative stress and the context of Alzheimer's [5, 21]. In the first axis, converging data from UV-Vis, DLS and SEM showed that contact with SOD causes an increase in hydrodynamic size and cluster formation; that is, the nanoparticle enters from a relatively stable state to a more dynamic state in which corona and aggregation occur simultaneously. This is exactly what the corona literature predicts: in the biological environment, proteins sit on the surface of the nanoparticle and, in addition to changing the

biological identity, can enhance agglomeration by bridging between particles. Therefore, even if the primary goal of the study is the effect of the nanoparticle on the enzyme, a more important conclusion is that the enzyme also affects the nanoparticle and this effect can change the path of biological exposure; for example, a nanoparticle that is initially small and tissue-permeable may, after corona formation and aggregation, acquire sedimentation/uptake behavior by macrophages or other interactions [22].

The difference between CuO, ZnO and MONPs in the intensity of post-exposure changes is most likely rooted in surface chemistry and colloidal stability. CuO in your data showed signs of more intense change in UV-Vis and more pronounced aggregation in SEM, while at the same time having a more intense hard crown in SDS-PAGE.

This convergence suggests a consistent interpretation: the CuO surface probably provides more active sites for strong protein binding (or specific groups on the protein), resulting in proteins being immobilized with higher affinity in the hard corona, and this immobilization could lead to subtle structural changes in the protein as well as more drastic changes in colloidal behavior. Such a result is consistent with the concept of bio-identity, since a nanoparticle that forms a harder and richer corona is likely to behave differently in the body [23].

In contrast, ZnO had a weaker hard corona in the gel and more protein was found in the soft corona, which could mean weaker binding or faster exchange of proteins. This is interesting because on the one hand, ZnO showed the highest toxicity in the MTT assay, but on the other hand, SOD binding to its surface seems to be less tight. This apparent contradiction raises an important methodological point: Cytotoxicity does not necessarily equate to direct destruction of a single protein [24]. ZnO can promote cell death through other pathways, such as increased ROS, membrane disruption, or disruption of the cell's ionic/oxidative balance; expert reviews show that metal oxides can generate ROS through surface pathways and environment-dependent reactions, and that this production is dose- and physiologically dependent. Thus, ZnO may collapse the cellular system through overall oxidative stress without keeping SOD hard on the surface, and in such cases SOD, as part of the defense network, is also stressed.

The second focus of discussion is the SOD response. The activity data show that under most conditions, SOD activity does not undergo a drastic decay after interaction. This result is consistent with some literature reports that some metal nanoparticles (such as gold/silver in some studies) do not induce large changes in SOD activity, while other enzymes such as catalase may be more sensitive [10]. Logically, as a highly evolutionarily stable enzyme, SOD is likely to be somewhat resistant to environmental changes; but the key point is that overall activity is an aggregate indicator and may mask local changes in structure or changes in protein subpopulations. This is where the CD data becomes important: you saw that CuO induces the largest secondary structure

changes. Given that the active site of SOD1 depends on the precise geometry of histidines and the presence of copper/zinc ions, even small structural changes can produce nonlinear effects over longer periods or in more complex biological environments.

In the Alzheimer's literature, SOD is not just a simple enzyme, but part of a network that is involved with mitochondria, inflammation, and amyloid [25, 26]. The review by Butterfield and Halliwell emphasizes that oxidative stress forms feedback loops with energy pathways and protein damage in Alzheimer's. Experimental evidence has also shown that manipulation of SOD2 can alter the severity of Alzheimer's-like pathology. Thus, if chronic exposure to nanoparticles reduces antioxidant capacity or increases ROS load, even without complete destruction of SOD, the network may be pushed towards a more vulnerable state. Your human study, while exploratory, can be interpreted in this way: the observation of reduced oxidative stress markers in exposed individuals is consistent with the idea that exposure can lower defense capacity or increase antioxidant intake [26]. Of course, caution is needed here: human data are affected by diet, stress, sleep, other exposures, and measurement errors, and we need longitudinal and more controlled designs to draw causal conclusions.

The third axis is to connect the findings to the concept of real risk. One of the most difficult parts of nanotoxicology is that the actual dose of human exposure and the actual form of the nanoparticle in the body are not easily reconstructed. Expert reviews on ROS and nanotoxicology emphasize that the physiological environment, proteins, ions and surface modifications can change the reactivity path of the nanoparticle. Your findings show exactly this: the nanoparticle is one thing before contact, it becomes something else after contact with the protein.

So if someone wants to draw conclusions about biological behavior only with the pure dry nanoparticle data, they are likely to make a serious mistake. In this respect, your SDS-PAGE section has an important added value: it shows that the corona is formed and even the difference between soft/hard corona is visible in different nanoparticles; that is, the biological identity is different at the very first step.

## Synthesis and Biological Evaluation of Metal Oxide Nanoparticles

Finally, a unified interpretation of the entire data is that the nanoparticle–SOD interaction is a two-way phenomenon: the nanoparticle can slightly alter the structure/activity of SOD, and the SOD/protein environment can dramatically alter the behavior of the nanoparticle (aggregation, spectral shift, size change). In such a situation, even if the enzyme activity remains stable in the short term, subtle structural changes in SOD or changes in the protein network adsorbed on the nanoparticle can become important in long-term exposures or in susceptible individuals (elderly, individuals with genetic/inflammatory background); as the Alzheimer's literature shows, the nervous system is sensitive to oxidative stress and redox perturbations.

### 5. Conclusion

This study began with a specific goal: to investigate how three widely used metal oxide nanoparticles (magnetic iron oxide, copper oxide, and zinc oxide) can interact with the enzyme superoxide dismutase, one of the pillars of antioxidant defense, and whether this interaction remains only a numerical change in activity or also leaves structural and physicochemical imprints. The overall outcome showed that the answer to this question is multifaceted: the enzyme activity does not undergo a drastic collapse under many conditions, but at the same time there is strong evidence that the nanoparticle and the protein acquire different identities and behaviors upon contact, and these differences can be important in more complex biological encounters.

In terms of synthesis and characterization, all three nanoparticles were produced with sufficient instrumental evidence and their properties were suitable for entering the biological interaction phase. UV-Vis, DLS/Zeta, SEM and XRD data together showed that particles were formed and that the morphology and colloidal properties could be assessed. The key point was that after contact with SOD, the colloidal behavior changed: the hydrodynamic size increased, the size distribution was no longer simple and nearly uniform, and more aggregation and clustering were observed in the microscopic images. These changes can be logically interpreted by corona formation as well as protein bridging between particles. Therefore,

one of the fundamental results of this project is that protein contact is a decisive step in the fate of nanoparticles and should be considered as part of the biological effect, not just a side effect.

In the enzymatic activity section, the use of a CCD statistical design helped to simultaneously investigate the effects of time, nanoparticle-to-enzyme ratio and enzyme concentration, as well as their interactions. The statistical results showed that the system is not linear and simple and some interactions are significant; That is, changing one factor can have a different outcome depending on the level of another factor. This result is important for nanoscale studies, as it suggests that single-factor methods may provide an incomplete picture. In terms of the overall trend, increasing contact time was generally associated with a decrease in SOD activity, but the magnitude of this decrease and its dependence on ratio and concentration varied between nanoparticles; these differences are likely related to surface properties, colloidal stability, and corona type, which are also observed in the physicochemical data. Structural and corona tests provided the second layer of evidence. The CD data showed that changes in the secondary structure of SOD occur after contact with nanoparticles, although these changes are not large or destructive in most cases. However, the magnitude of the changes was not uniform among nanoparticles, with more pronounced changes observed upon contact with CuO. At the same time, SDS-PAGE and relative separation of free protein, soft corona, and hard corona showed that coronas form and that there are differences in the strength of binding between nanoparticles; in particular, CuO tends to form a harder/bolder corona. Together, these two lines of evidence (CD and SDS-PAGE) provide an important message: even if the enzymatic activity is apparently stable, the structure and binding environment of the protein may have changed, and these changes could potentially be affected over longer periods of time or in the presence of other biological components. In terms of biological consequences, the MTT assay on PC12 showed that nanoparticles have a dose-dependent effect on survival and the severity of toxicity varies among nanoparticles; in your data, ZnO showed the most toxicity and CuO the least toxicity. This result is consistent with the notion that the mechanisms of toxicity can be

different and are not necessarily limited to tighter binding to a specific protein. ZnO may stress the cell more through general oxidative stress or surface/ionic effects, even if its hard corona pattern is not as pronounced as CuO. Therefore, the relationship between corona, protein structural changes, and cellular outcomes is not a linear relationship and requires a network view.

Finally, the most important practical conclusion is that the assessment of the effect of nanoparticles on biological systems should cover three layers simultaneously: pre-contact physicochemical properties, post-contact changes (especially corona and aggregation), and functional/biological outcomes (enzyme activity and cellular response). The present study demonstrated that if only one of these layers is observed, an incomplete or even misleading impression may be formed. A natural suggestion for further research is to replicate this framework in more complex environments (e.g., whole serum, multicomponent protein mixtures, or 3D neural models), to investigate longer times and doses closer to real-world exposures, and to measure oxidative/inflammatory indices in addition to enzymatic activity. Overall, this work suggests that nanoparticles can create conditions that, in terms of colloidal behavior and protein interactions, predispose a biological system to increased susceptibility to oxidative stress without causing immediate severe SOD degradation; and this is precisely the point that is worth pursuing seriously for the fields related to neurohealth and occupational exposures.

### Declaration

All procedures were approved by biological research committees at Shahid Beheshti University under the ethical code: IR.SBU.REC.1404.104-Nasiri. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Authors' contributions

FM: Conceptualization, Supervision, Resources, Formal analysis, Writing and editing the original draft, NaPa: Methodology, Data curation, PN: Ethical attention and Human study, TN: Writing

the original draft, PK: Data curation, formal analysis, NaPo: Investigation and Data curation.

### Acknowledgements

We thank the Shahid Beheshti University Council for their instrumentation and support.

### Availability of data and materials

Data will be made available on request.

### References

- [1] Nikolova MP, Joshi PB, and Chavali MS. Updates on Biogenic Metallic and Metal Oxide Nanoparticles: Therapy, Drug Delivery and Cytotoxicity. Vol. 15, Pharmaceutics. Multidisciplinary Digital Publishing Institute (MDPI) (2023).
- [2] Nowak-Jary J and Machnicka B. Pharmacokinetics of magnetic iron oxide nanoparticles for medical applications. *J. Nanobiotechnology* 2022 201 [serial online] 2022 June [cited 2023 Dec 30], 20: 1–30. Available from: URL: <https://jnanobiotechnology.biomedcentral.com/articles/10.1186/s12951-022-01510-w>
- [3] Li J, Zhang T, Pan M, Xue F, Lv F, Ke Q, et al. Nanofiber/hydrogel core-shell scaffolds with three-dimensional multilayer patterned structure for accelerating diabetic wound healing. *J. Nanobiotechnology* [serial online] 2022 December [cited 2025 Jul 6], 20. Available from: URL: <https://pubmed.ncbi.nlm.nih.gov/34998407/>
- [4] Bashiri G, Padilla MS, Swingle KL, Shepherd SJ, Mitchell MJ, and Wang K. Nanoparticle protein corona: from structure and function to therapeutic targeting. *Lab Chip* [serial online] 2023 March [cited 2023 Dec 30], 23: 1432–1466. Available from: URL: <https://pubs.rsc.org/en/content/articlehtml/2023/lc/d2lc00799a>
- [5] Song N, Sun S, Chen K, Wang Y, Wang H, Meng J, et al. Emerging nanotechnology for Alzheimer's disease: From detection to treatment. *J. Control. Release* (2023) 360: 392–417.
- [6] Dhahi TS, Yousif Dafhalla AK, Al-Mufti AW, Elobaid ME, Adam T, and Gopinath SCB.

## Synthesis and Biological Evaluation of Metal Oxide Nanoparticles

- Application of Nanobiosensor engineering in the diagnosis of neurodegenerative disorders. *Results Eng.* [serial online] 2024 December [cited 2025 Sep 20], 24: 102790. Available from: URL: [https://www.sciencedirect.com/science/article/pii/S2590123024010454?utm\\_source=chatgpt.com](https://www.sciencedirect.com/science/article/pii/S2590123024010454?utm_source=chatgpt.com)
- [7] Bhattacharyya A, Chattopadhyay R, Mitra S, and Crowe SE. Oxidative Stress: An Essential Factor in the Pathogenesis of Gastrointestinal Mucosal Diseases. *Physiol. Rev.* [serial online] 2014 [cited 2025 Jul 11], 94: 329. Available from: URL: <https://pmc.ncbi.nlm.nih.gov/articles/PMC4044300/>
- [8] Shao G, Chen M, Wang W, Mou R, and Zhang G. Iron nutrition affects cadmium accumulation and toxicity in rice plants. *Plant Growth Regul.* [serial online] 2007 September [cited 2020 Dec 15], 53: 33–42. Available from: URL: <https://link.springer.com/article/10.1007/s10725-007-9201-3>
- [9] Zhao H, Wu L, Chai T, Zhang Y, Tan J, and Ma S. The effects of copper, manganese and zinc on plant growth and elemental accumulation in the manganese-hyperaccumulator *Phytolacca americana*. *J. Plant Physiol.* (2012) 169: 1243–1252.
- [10] Elsner M, Gurgul-Convey E, and Lenzen S. Relative importance of cellular uptake and reactive oxygen species for the toxicity of alloxan and dialuric acid to insulin-producing cells. *Free Radic. Biol. Med.* (2006) 41: 825–834.
- [11] Abdel Aal SM, Ahmed SM, Abdelrahman SA, Abdelrahman AA, and Samy W. Duration-dependent effects induced by titanium dioxide nanoparticles on pancreas of adult male albino rats (histological and biochemical study). *Ultrastruct. Pathol.* [serial online] 2020 [cited 2023 Dec 30], 44: 342–358. Available from: URL: <https://pubmed.ncbi.nlm.nih.gov/32600082/>
- [12] Iborra M, Moret I, Rausell F, Bastida G, Aguas M, Cerrillo E, et al. Role of oxidative stress and antioxidant enzymes in Crohn's disease. *Biochem. Soc. Trans.* (2011) 39: 1102–1106.
- [13] Charbgo F, Nejabat M, Abnous K, Soltani F, Taghdisi SM, Alibolandi M, et al. Gold nanoparticle should understand protein corona for being a clinical nanomaterial. *J Control Release* [serial online] 2018 272: 39–53. Available from: URL: <https://www.ncbi.nlm.nih.gov/pubmed/29305922>
- [14] Mishra RK, Ahmad A, Vyawahare A, Alam P, Khan TH, and Khan R. Biological effects of formation of protein corona onto nanoparticles. *Int. J. Biol. Macromol.* [serial online] 2021 175: 1–18. Available from: URL: <https://doi.org/10.1016/j.ijbiomac.2021.01.152>
- [15] Wang H, Shang L, Maffre P, Hohmann S, Kirschhöfer F, Brenner-Weiß G, et al. The Nature of a Hard Protein Corona Forming on Quantum Dots Exposed to Human Blood Serum. *Small* [serial online] 2016 November [cited 2023 Dec 4], 12: 5836–5844. Available from: URL: <https://onlinelibrary.wiley.com/doi/full/10.1002/sml.201602283>
- [16] Nejabat S, Siadat SOR, Tahmasian Z, Mirzajani F, Fatemi F, Hosseinkhani S, et al. How Co-Precipitation Reaction Parameters Control the Characteristics and Features of Iron Oxide Nanoparticles. Vol. 9, *Physical Chemistry Research. Physical Chemistry Research* (2021) 242–252. Available from: URL: [https://www.physchemres.org/article\\_125061.html](https://www.physchemres.org/article_125061.html)
- [17] Singh P, Ali SW, and Kale RD. Antimicrobial Nanomaterials as Advanced Coatings for Self-Sanitizing of Textile Clothing and Personal Protective Equipment. Vol. 8, *ACS Omega. American Chemical Society* (2023) 8159–8171.
- [18] Guo Z, Zhang P, Luo Y, Xie HQ, Chakraborty S, Monikh FA, et al. Intranasal exposure to ZnO nanoparticles induces alterations in cholinergic neurotransmission in rat brain. *Nano Today* (2020) 35.
- [19] Tang Y, Cui Y, Luo F, Liu X, Wang X, Wu A, et al. Cell viability and dopamine secretion of 6-hydroxydopamine-treated PC12 cells cocultured with bone marrow-derived mesenchymal stem cells. *Neural Regen. Res.* [serial online] 2012 May [cited 2024 Jul 14], 7: 1101. Available from: URL:

/pmc/articles/PMC4340024/

<https://doi.org/10.1016/j.apsb.2021.05.004>

- [20] Pascua-Maestro R, Corraliza-Gomez M, Diez-Hermano S, Perez-Segurado C, Ganformina MD, and Sanchez D. The MTT-formazan assay: Complementary technical approaches and in vivo validation in *Drosophila* larvae. *Acta Histochem.* (2018) 120: 179–186.
- [21] Fischer R and Maier O. Interrelation of Oxidative Stress and Inflammation in Neurodegenerative Disease: Role of TNF. *Oxid. Med. Cell. Longev.* [serial online] 2015 January [cited 2025 May 3], 2015: 610813. Available from: URL: [/doi/pdf/10.1155/2015/610813](https://doi.org/10.1155/2015/610813)
- [22] Eisazadeh B, Mirzajani F, and Sefidbakht Y. How is the Effect of Silver Nanoparticles and Lipase/Cellulase Enzymes on Each Other? *Iran. J. Sci. Technol. Trans. A Sci.* (2020) 44.
- [23] Tang Z, Xiao Y, Kong N, Liu C, Chen W, Huang X, et al. Nano-bio interfaces effect of two-dimensional nanomaterials and their applications in cancer immunotherapy. *Acta Pharm. Sin. B* [serial online] 2021 11: 3447–3464. Available from: URL: <https://doi.org/10.1016/j.apsb.2021.05.004>
- [24] Gupta SD, Agarwal A, and Pradhan S. Phytostimulatory effect of silver nanoparticles (AgNPs) on rice seedling growth: An insight from antioxidative enzyme activities and gene expression patterns. *Ecotoxicol. Env. Saf* [serial online] 2018 161: 624–633. Available from: URL: <https://www.ncbi.nlm.nih.gov/pubmed/29933132>
- [25] Mani R, Ezhumalai D, Muthusamy G, and Namasivayam E. Neuroprotective effect of biogenically synthesized ZnO nanoparticles against oxidative stress and  $\beta$ -amyloid toxicity in transgenic *Caenorhabditis elegans*. *Biotechnol. Appl. Biochem.* [serial online] 2024 February [cited 2025 Aug 23], 71: 132–146. Available from: URL: [/doi/pdf/10.1002/bab.2527](https://doi.org/10.1002/bab.2527)
- [26] Cheignon C, Tomas M, Bonnefont-Rousselot D, Faller P, Hureau C, and Collin F. Oxidative stress and the amyloid beta peptide in Alzheimer's disease. *Redox Biol.* (2018) 14: 450–464.