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Research Article

Etching of AuNPs Through Superoxide Radical Dismutation by Cu-Zn Superoxide Dismutase Resulted in Remarkable Changes of its Localized Surface Plasmon Resonance

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Background: Superoxide dismutases (SODs) are categorized as antioxidant enzymes that are involved in many processes such as stress signalling responses and cell protection against free radical species. The primary function of SOD is the removal of produced radical species like superoxide ions in different physiological processes. There are various isozymes of SODs which are classified according to the metal cofactor in their active sites into four general types of Fe-SOD, Mn-SOD, Cu/Zn-SOD and Ni-SOD. Among metal nanoparticles, gold nanoparticles (AuNPs) are useful for biological purposes as sensing probe for determining critical analysis based on surface plasmon resonance and colorimetric method. In this study, the human Cu-Zn SOD expressed, purified, and its interaction with AuNPs based on a new colorimetric method was investigated.

Objectives: In this approach, a colorimetric detection method for SOD activity was developed based on the carboxylic stabilized AuNPs.

Material and Methods: The Ni-NTA Sepharose affinity column was performed for the purification process of enzyme. Following SOD purification, the enzyme activity in presence of AuNPs due to the possible etching in the presence of free radicals which are produced by riboflavin, methionine, Na2CO3 and potassium phosphate buffer, have been performed. In addition, Fluorescence spectroscopy analysis toward SOD and gold nanoparticle were performed.

Results: Superoxide radicals generated from the enzymatic reaction would preferentially etch AuNPs and resulted in remarkable changes of localized surface plasmon resonance of AuNPs, which is reduced in the presence of SOD. Under the optimized experimental conditions assay (pH~7.8 and 25 ˚C), better selectivity and sensitivity toward SOD activity was shown.

Conclusions: In this context, an indirect new colorimetric method for determining of SOD activity based on gold nanoparticles (AuNPs) was evaluated. According to the presented result, it may be concluded that by scavenging of free superoxide radicals in the presence of SOD, the amount of AuNP absorbance can be replenished.

Keywords: Etching; Expression; Gold nanoparticles; Superoxide dismutase

1. Background

McCord and Fridorich discovered a copper protein known as Cu-Zn SOD that was dimmer and each of its monomeric units contain an active site, a copper ion, and a zinc ion, which was linked to histidine by imidazole ring. Copper acts as reactive metal and

zinc ion is effective in pH stability (1-4). Superoxide dismutase (SOD, EC 1.15.1.1.) is a metalloenzyme and is considered an oxidoreductase enzyme. SODs have an essential role in the dismutation of superoxide radicals, which are cytotoxic agents to biomolecules such as proteins, DNA, and cells. These enzymes provide the first line of defense against ROS (3, 5). Mutation in the SOD1 gene and subsequent aggregation of mutant proteins has been considered as one of the main influencing factors in the progress of Amyotrophic Lateral Sclerosis (ALS) disease (6-8).

Gold nanoparticles have a long history in biotechnology fields. Understanding the properties of new metal nanoparticles and discovering their potential uses are two main features in the wide variety of nanoscale synthesis(9). Many of the properties of nanomaterials are due to the surface-to-volume ratio and the spatial structure of electrons, photons and electrical fields inside and around particles. The features such as high activity, different elasticity, magnetic properties, increased conductivity or increased light reflection tendency, separate nanomaterials from other materials (5, 9).

Gold nanoparticles have unique physicochemical properties that make them suitable tools for biological sensors, immune assays, targeted drug delivery, marking cells, molecular imaging, and medical applications (10-12). The size and shape of the AuNPs (spherical, rod, cage, cubic, etc.) can adjust their optical properties. Surface variations, shape, and size of spherical and spherical AuNPs have shown different results in terms of stability, bonding, and compatibility in the body (13-15).

The interaction of nanoparticles with biomolecules, especially proteins, is considered as the term of their bioactivity and creates a dynamic system that can have many benefits for both nanoparticles and proteins. The nanoparticle-protein bioconjugation may affect cell infiltration, inflammation, and also accumulation, degradation, and purification of nanoparticles (16). Understanding such interactions can lead to the production of environmentally friendly nanomaterials with surface-controlled properties in a biological environment (17).

The absorption of protein on the surface of nanoparticles is achieved by several forces such as hydrogen bonds, salvation van der Waals, and electrostatic interactions (18). The formation of nanoparticle-protein, also known as corona-protein, is a multistage process that depends not only on the properties of the nanoparticle but also on the interaction of proteins with the surrounding environment (17).

The binding of biochemical molecules to nanoparticles makes them useful for different applications and also makes the nanoparticle-protein complex more stable. In many cases, biological molecules, especially proteins, undergo structural changes in binding to the nanoparticles (17). Research on enzymes and their interaction with nanoparticles has been recently increased. Meanwhile, the interaction of superoxide dismutase enzyme and gold nanoparticle remains unknown. Currently, several methods have been reported for detection of the activity of SOD in association with AuNPs like the spectrofluorimetric method (19), mercury microelectrodes (20), and spectrophotometric method (21). However, these methods have their drawbacks, such as high cost and fluorescence quenching. So studies revealed that gold nanoparticle possesses strong localized surface plasmon resonance (LSPR) from the visible region to near-infrared region and high extinction coefficient (22). These properties are influenced by many factors such as size, shape, composition, and the surrounding media of the reaction.

In the current study, the SOD activity in presence of spherical AuNPs due to the possible etching in the presence of free radicals which are produced by riboflavin, methionine, Na2CO3 and potassium phosphate buffer, have been investigated. The novelty of the work relates to these aspects.

2. Objectives

Herein, after the expression and purification of human Cu-Zn superoxide dismutase, we proposed an effective procedure for colorimetric visualization of the enzyme through the etching of carboxylic-stabilized AuNPs.

3. Materials and Methods

3.1. Materials

K_2HPO_2, KH_2PO_4, $CuSO_4/ZnSO_4$, and Na_2CO_3 purchased from Merck (Germany). Ni-NTA Sepharose affinity column from Novagen, kanamycin, IPTG, Riboflavin, L-methionine, and NBT also from Sigma-Aldrich, was purchased. The water-soluble carboxylic-stabilized gold nanoparticles (AuNPs) with 1.6 nm used in this study were prepared.

3.2. Expression and Purification of Human Cu-Zn Superoxide Dismutase

The E. coli BL21 strain was transformed by human Cu-Zn superoxide dismutase coding gene-containing plasmids (pET28a plasmid) (6). Transformed bacteria were cultured in 10 mL LB medium with 50 $\mu g.mL^{-1}$ kanamycin and incubated overnight at 37 ˚C with

shaking at 150 RPM. Then 1 mL of LB medium was added to 250 mL TB medium with kanamycin and incubated at 37 ˚C with 150 RPM shaking. After almost 3.5-4 h, 0.2 mM $CuSO_4$ and 0.5 mM IPTG were added to TB medium, and incubation at 20 ˚C with150 RPM for 16h was performed. Then after the cell pellets were harvested by centrifugation at 6000 g for 10 min at 4 ˚C. The suspension was sonicated by lysis buffer (50 mM Tris-HCl PH 7.8). Next Centrifugation at 14000 g for 20 min, at 4 ˚C was done to collect the supernatant. Finally, purification with Ni-Sepharose column affinity chromatography was carried out. After that, the dialyzing under 100 mM sodium acetate, 10 mM EDTA and 100 mM NaCl (pH 3.8), 100mM sodium acetate and 200 µm $ZnSo_4$, 200 µm $CuSO_4$ (pH 5.5), 20 mM potassium phosphate (pH 7.4) buffers respectively were carried out. Then the concentration of the dialyzed extract was determined by Bradford assay using coomassie blue and bovine serum albumin as standard.

3.3. SOD Activity Assays

SOD activity was measured by a spectrophotometric method based on nitroblue tetrazolium (NBT) illumination method in 50 mM potassium phosphate buffer (pH 7.8) at 25 ˚C. One unit of SOD activity was defined as the amount of protein required to inhibit the rate of production of formazan by 50% under the assay conditions as described above. For measuring kinetic properties of the enzyme, different dilutions of Riboflavin (0.5, 0.25, 0.12, 0.06, and 0.03 µM) were prepared to determine the Km of SOD1 enzyme (from the original concentration of 20 µM).

L-Methionine (26 mM), Na_2CO_3 (50 mM), NBT (750 µM), and Riboflavin mixed, and each vial exposed to light after 15min and then the absorption at 560nm was read.

3.4. Fluorescence Spectroscopy of the Enzyme in the Presence of Gold Nanoparticles

Intrinsic fluorescence emission spectroscopy of enzymes in the presence of different concentrations of gold nanoparticles was measured by PerkinElmer LS-55. The excitation wavelength of human Cu-Zn SOD was 280 nm and the intrinsic emission spectra were recorded between 290 and 450 nm. All the fluorescence measurements carried out at 25 ˚C in the final volume of 60 microliters.

3.5. Investigating the Activity of the Enzyme in the Presence of Gold Nanoparticles in an Environment Containing Radical Superoxide

The enzyme activity was measured in the presence of gold nanoparticles (c=1×10-7 molar) and NBT. Different concentrations of SOD were prepared and then the equivalent molar of the enzyme was added to each solution. After incubation time (30 min), the activity of each assembly was measured by absorbance reading at 530 nm by PerkinElmer Lambda 25 UV-visible spectroscopy.

3.6. Investigation of the Absorption of Gold and Enzyme Nanoparticles in an Environment Free of Superoxide Radicals

In order to compare and verify the accuracy of our experiments in the previous section, only the different concentrations enzyme in the presence of a concentration of gold nanoparticles with 50 mM phosphate buffer at pH =7.8 evaluated, and the absorption of nanoparticles recorded at 530 nm by a reported Method (23).

4. Results

4.1. Cu-Zn Superoxide Expression

The expressed protein was purified based on the 6His-tagged fusion by affinity (Ni-NTA Sepharose) chromatography. SDS-PAGE analysis of the eluted enzyme showed more than 95% purity of the enzyme (**Fig**. **1**).

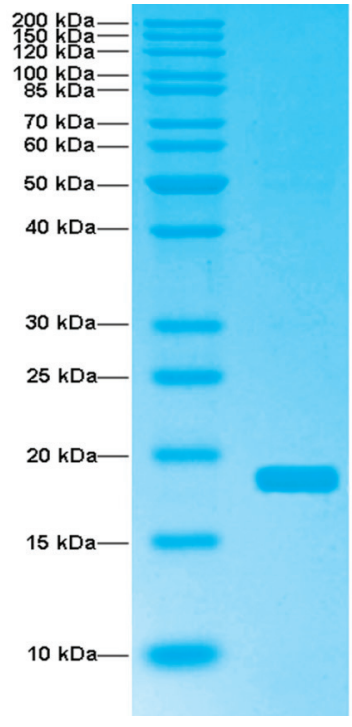

Figure 1. SDS-PAGE of purified human Cu-Zn superoxide dismutase.

4.2. Determination of Kinetic Properties and Specific Activity of the Enzyme

To determine the kinetic properties and specific activity of the enzyme, the substrate was mixed and subjected to irradiation. Absorption, the rate of inhibition, and also its specific activity in the concentration of 0.1 $mg.mL^{-1}$ of the enzyme were reported (**Table 1**). The parameters were determined according to the following formula. The inhibition was 78.8%, and its specific activity was 787 units/mg. Finally the kinetic values of the enzyme after drawing line Weaver-Burk plot (**Fig. 2**) showed that SOD1 enzyme was indicative of a relatively good enzyme tendency toward its substrate (**Table 1**).

$$\text{Inhibition \%} = \frac{\Delta\,(\text{absorbance at 560 nm without enzyme}) - \Delta\,(\text{absorbance at 560 nm with enzyme}) \times 100}{\text{Absorbance (reaction blank)}}$$

$$\text{Specific activity} = \frac{(\,\%\ \text{Inhibition}\,)}{\text{Protein concentration (mg)}}$$

Table 1. Kinetic parameters of purified enzyme

Km (μM)	Inhibition%	Specific activity (units/mg protein)	Vmax (unit/mg)
0.226	78.8	787	69.06

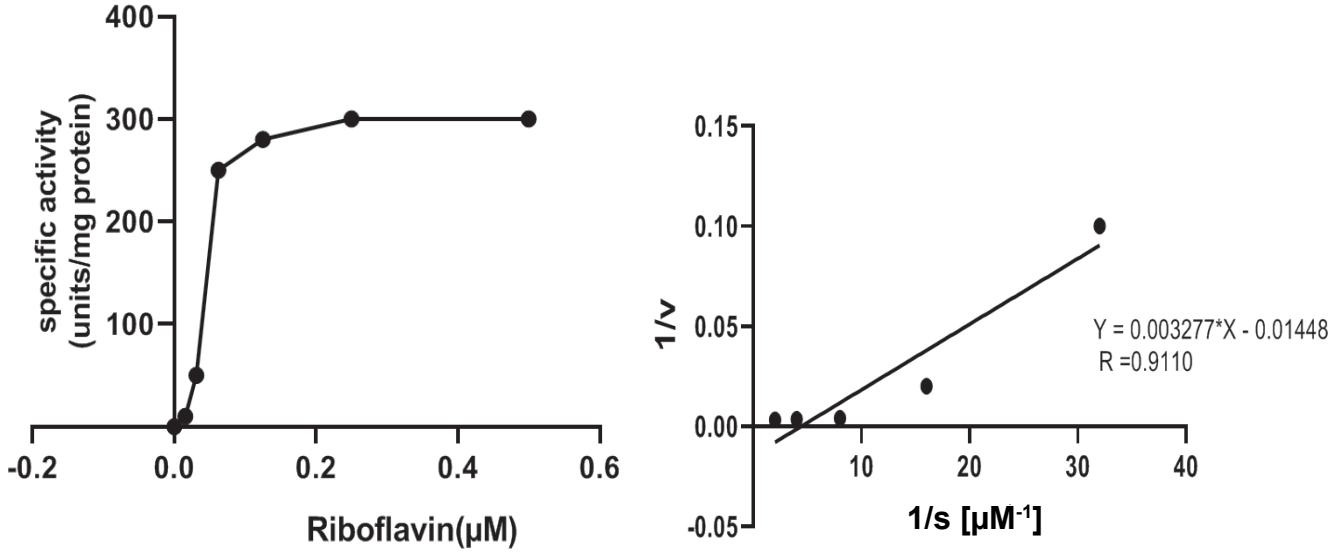

Figure 2. Michaelis Menten and Line Weaver Burke plots for the Kinetic Parameters of the enzyme for the riboflavin substrate

4.3. Fluorescence Spectroscopy

Intrinsic fluorescence measurement of the enzyme in the presence of different concentrations of gold nanoparticles was measured (**Fig. 3**), It was found that how the concentration of nanoparticles may affect the structure of the enzyme. The results showed that none of the nanoparticle concentrations had led to the exposure of inner aromatic residues to the solvent.

4.4. Activity of Enzyme in the Presence of Gold Nanoparticles

The assay of the enzyme activity in the presence of superoxide radicals and nanoparticles was performed (**Fig. 4**). After the production of radical superoxide in the environment, gold nanoparticles began to etch, which occurs for metal nanoparticles in the presence of free radicals which affect the plasmon resonance of the surface. The results showed that the more free radicals in the environment, the higher the etching of AuNPs and thus the more decrease in the absorption.

4.5. AuNp Absorbance Against SOD1 Concentration

The changes in the surface resonance of the gold nanoparticles were recorded, and the difference in their absorption was illustrated by a calibration curve of the various concentrations of superoxide dismutase enzyme. Therefore, given the relatively linear nature of the diagram showed that our results could lead to developing a method for measuring SOD1 enzyme activity (**Fig. 5**).

4.6. Absorption Spectra of Gold Nanoparticles and Enzyme without Superoxide Radical

Different concentrations of the enzyme without superoxide radicals in the environment placed in the vicinity of gold nanoparticles and no significant difference in absorptions were observed, and all of the spectra were overlapped (**Fig. 6**). This data showed that the absorbance reduction has only occurred in the presence of superoxide radical presumably through etching of AuNPs.

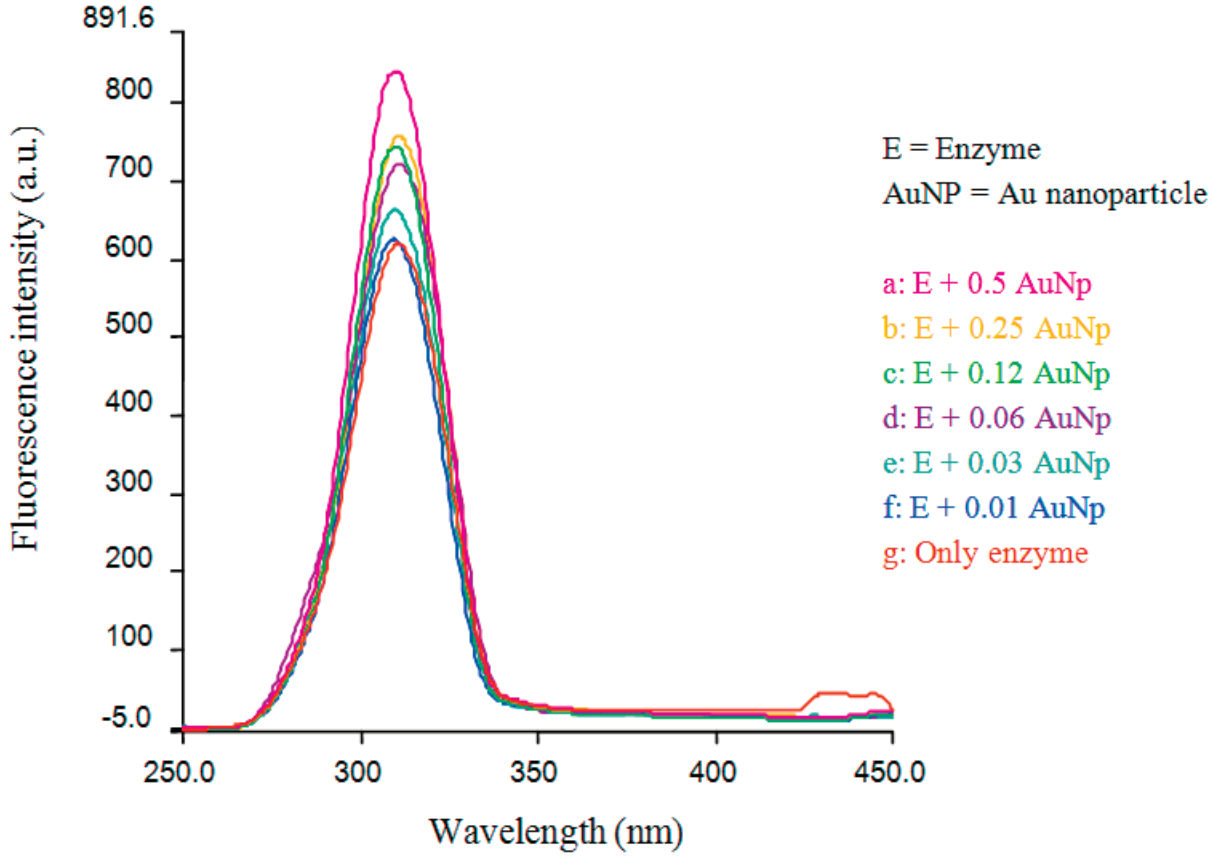

Figure 3. Structural analysis of the enzyme in the presence of different concentrations of Au-nanoparticles using intrinsic fluorescence emission of the enzyme. (a)-(f) shows intrinsic fluorescence of SOD in the presence of 0.5×10^{-7}, 0.25×10^{-7}, 0.12×10^{-7}, 0.06×10^{-7}, 0.03×10^{-7}, 0.01×10^{-7} mol/lit of AuNP, respectively and (g) is the fluorescence emission of the enzyme alone.

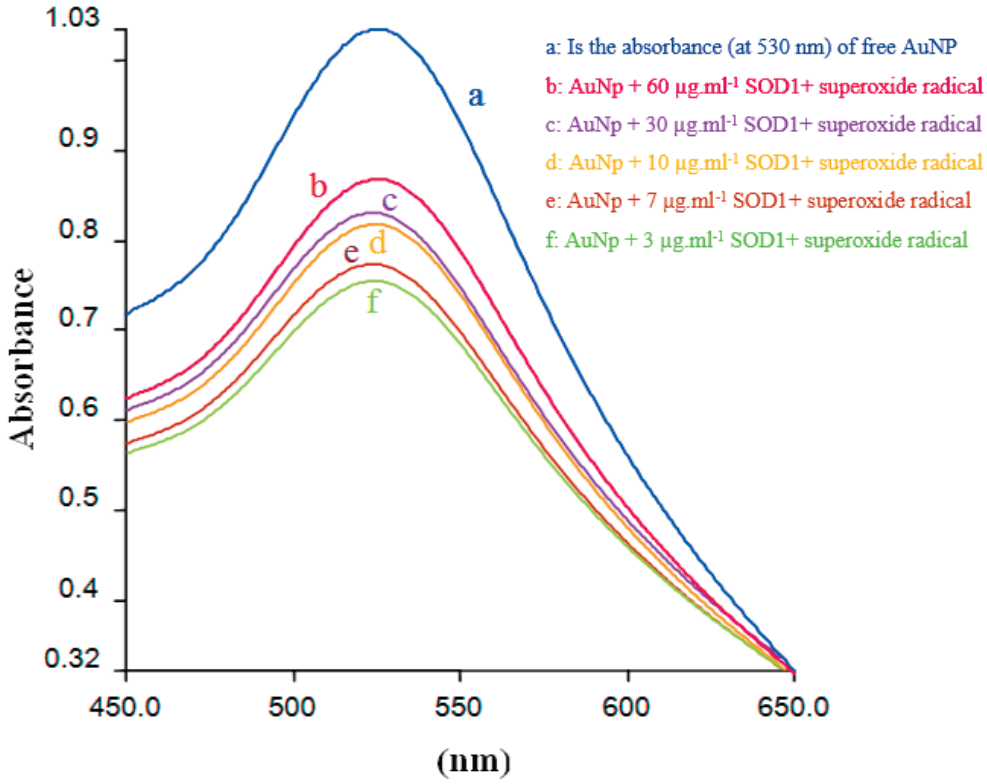

Figure 4. Enzyme activity in the presence of Au-nanoparticles at various concentrations of the enzyme. (a) is the absorbance (at 530 nm) of free AuNP (b)-(f) is the absorption spectra of the same amount of AuNP in presence of constant concentration of superoxide radical generators and various amount of enzyme; 60, 30, 10, 7 and 3 µg/ml^{-1} of SOD1, respectively.

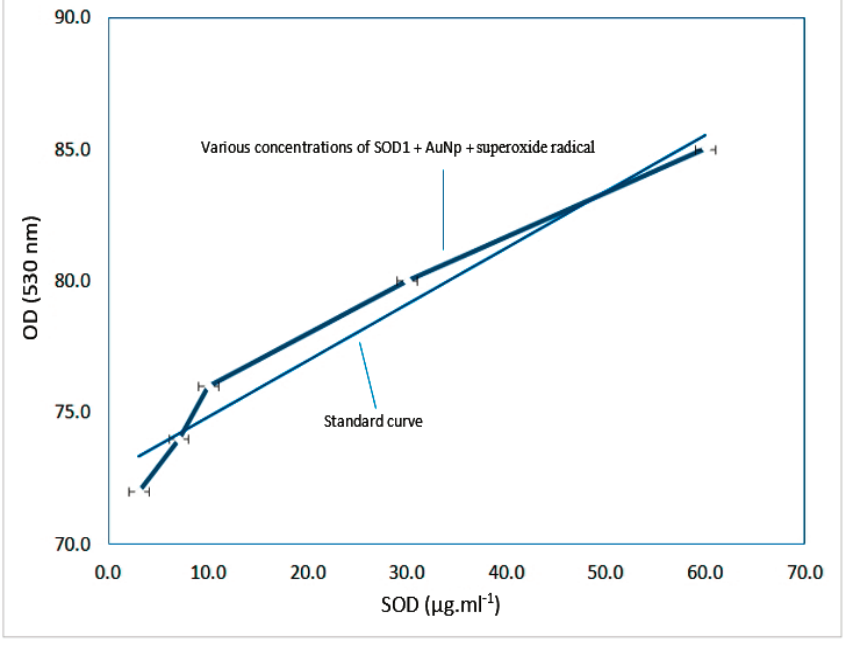

Figure 5. Absorption spectra of AuNP in the presence of superoxide radical source and various concentration of SOD1.

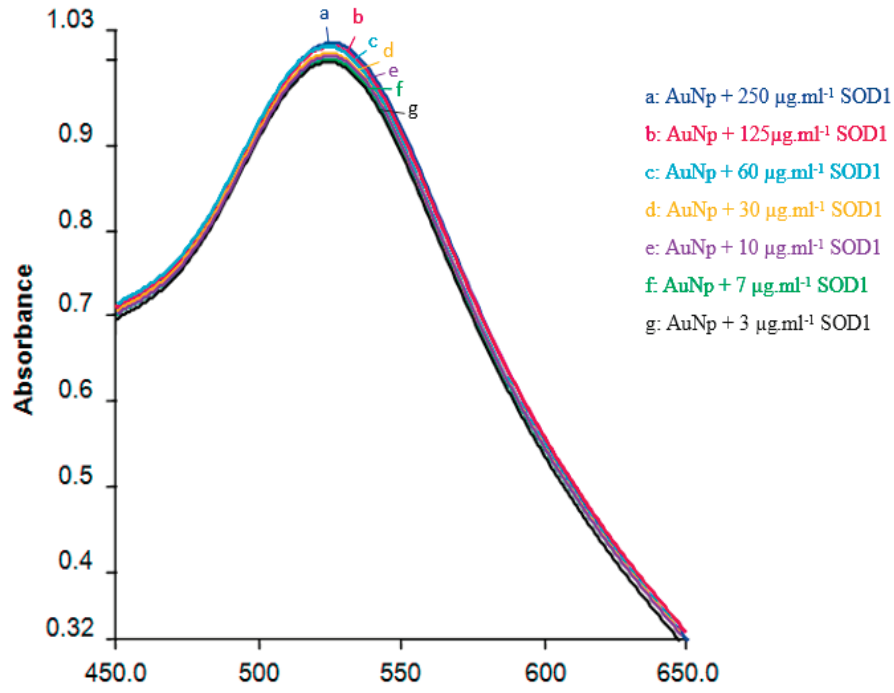

Figure 6. The absorption of AuNP in the presence of various concentrations of enzyme in the absence of superoxide radical with 50 mM potassium phosphate buffer at pH = 7.8.

5. Discussion

The application of nanomaterials in the biomedical sciences has been considered in recent decades. Spherical gold nanoparticles have found many applications because of their unique characteristics rather than bulk materials (24-26).

Gold nanoparticles can be attached to many biomolecules such as proteins, nucleic acids, and lipids for different practical purposes. These particles have unique properties such as high absorption, luminescence, and conductivity and even their usefulness to enhance electromagnetic reactions and provide them for sensing purposes. One of the most attractive aspects of gold nanoparticles is their application in GNPs-based assays by using colorimetric methods (27-30).

Superoxide dismutases are the primary protective barrier against the destructive effect of high concentrations of free radicals, especially superoxide, which cause genital mutations and cancer (31), and it is also accelerated in the aging process (32, 33).

Detection based on colorimetric methods is considered the leading gold method for nanometric assay. Although, existing methods for these assays have still challenges such as nonspecific interactions with biomolecules and difficulties posed by in vivo applications (34, 35). Colorimetric assays have a high potential for high-throughput applications because they are simple, inexpensive, and require minimal instrumentations. Colorimetric assays are very promising for their effectiveness in determining enzyme activity in the biological process. Besides they are excellent candidates for in vitro measurements (29).

Simin Lu *et al.* designed a colorimetric method for superoxide dismutase assay based on gold nanorods and observed that the activity of the enzyme could be kept (29, 36). Zifen Deng *et al.* applied an approach in the direct transfer of electrons from copper and zinc superoxide dismutase to the surface of ZnO nanoparticles by stabilizing the enzyme on nanoparticles. The electrochemical results showed that the enzyme stabilization on the ZnO nanoparticle maintained its activity (37). Surin Hong et al. utilized superoxide dismutase to conjugate on AuNP surface (SOD1-AuNP) for detection of the structural evolution of SOD1 aggregates. The results indicated that significant changes in both surface plasmon resonance spectra and concomitant colors were observed. They showed that the gold nanoparticle (AuNP)-based highly sensitive and colorimetric detection of the temporal evolution of superoxide dismutase (SOD1) aggregates implicated in the pathology of amyotrophic lateral sclerosis (ALS) (38).

In another study by Reynaldo Villalonga *et al*., a bienzymatic supramolecular nanoassembly containing catalase and Cu, Zn-superoxide dismutase was performed. They concluded that Superoxide dismutase was 90-fold more resistant to inactivation by H_2O_2 after bienzymatic immobilization with catalase on metal nanoparticles (39).

Junwei Di *et al*. designed a superoxide dismutase (SOD) based biosensor via a silica sol-gel (SG) thin film and further immobilization of SOD and gold nanoparticles (GNPs) in silica SG network. The resulting biosensor exhibited a fast amperometric response to superoxide anion. Also, the biosensor exhibited high sensitivity and long-term stability (40).

There are not enough researches on the behavior of SOD and AuNPs to estimating of what effect these particles can have on the enzyme, for this purpose the interaction of human Cu-Zn superoxide dismutase with gold nanoparticles was studied through UV-visible spectroscopy and fluorescence techniques and proposed the colorimetric method for detecting the enzyme activity. In this study, the AuNPs aggregation was occurred due to surface attachment to SOD presumably through electrostatic interactions. This issue will reduce the initial tendency of SOD protein for aggregation as an amyloidogenic protein as indicated in recent similar studies on the aggregation of α-synuclein protein (41-44). Kinetic properties of purified SOD are shown in (**Table 1**), which indicates the specific activity of the purified enzyme was 787 unit/mg. The kinetic parameters of the enzyme also showed that the activity of the purified enzyme was suitable for further analysis. Also, the fluorescence properties of enzymes in different concentrations of AuNPs were studied (**Fig. 3**).

In this work, we have developed an indirect new colorimetric method for determining SOD activity based on gold nanoparticles (AuNPs). For this purpose, a constant concentration of nanoparticles was placed adjacent to the produced superoxide radical and various concentrations of the enzyme which indicated that the lower the enzyme concentration, the less absorption of the nanoparticles (**Fig. 5, 6**). So we found that the higher the enzyme concentration will eliminate more free radicals. Therefore, the amount of etching of the nanoparticles is reduced, so the change in the surface plasmon resonance of the nanoparticles is also lower. However, based on the result presented in this manuscript it may be concluded that by scavenging free superoxide radicals in the presence of SOD, the amount of AuNP absorbance can be replenished (**Fig. 7**).

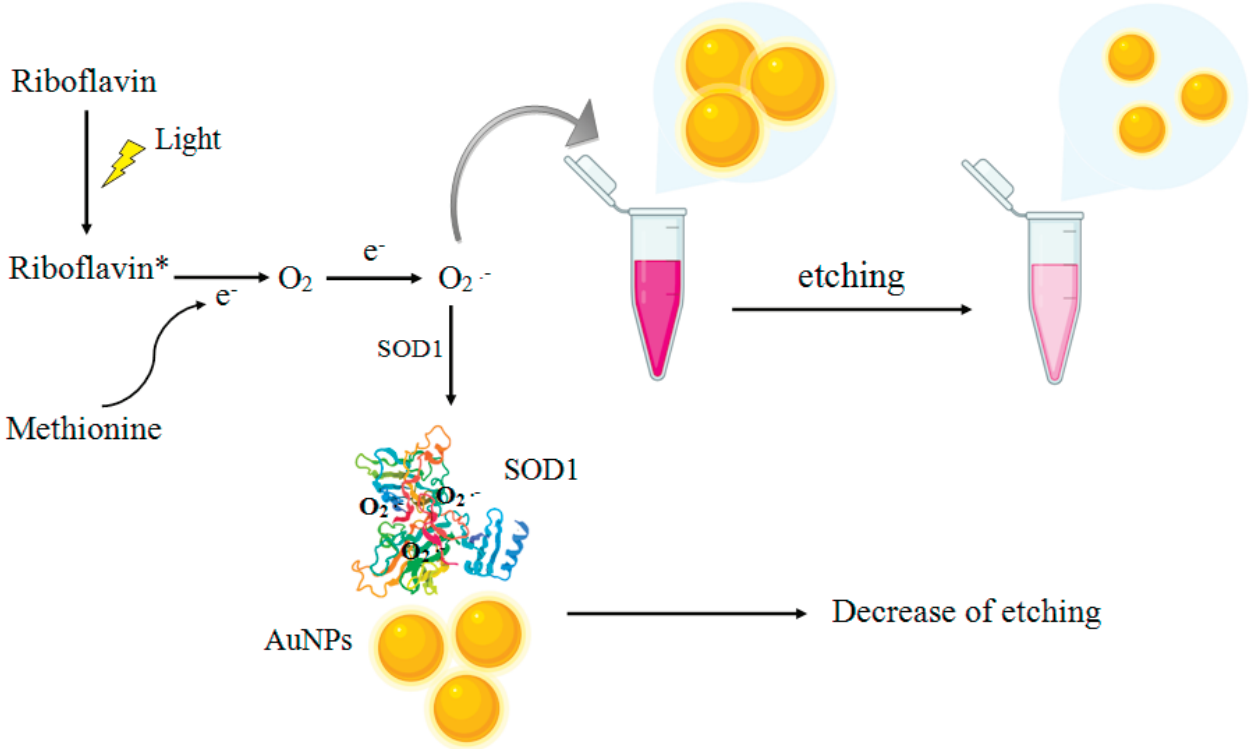

Figure 7. Schematic presentation of inhibition of AuNPs etching in presence of SOD1. PDBe ID for Superoxide dismutase is 5U9M (https://www.ebi.ac.uk/pdbe).

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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