RESEARCH ARTICLE



Interaction of Silver Nanoparticles with Human Alpha-2-macroglobulin: Biochemical and Biophysical Investigation

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Background: Silver nanoparticles (AgNPs) have a broad spectrum of applications in nanoscience and nanomedicine due to their flexible properties, such as antibacterial, antifungal, anti-inflammatory and anti-angiogenic. Present study investigated the interaction of chemically synthesized AgNPs with human major antiproteinase alpha-2-macroglobulin (α_n M).

Materials and methods: The first step of the study involved the synthesis and characterization of AgNPs using various biochemical and biophysical techniques, such as UV-visible spectroscopy, fluorescence quenching spectroscopy, synchronous fluorescence, and circular dichroism (CD). Different methods were used to explore the primary and secondary structural changes induced in $\alpha_s M$ by the binding of AgNPs.

Results: The UV-visible spectroscopy revealed hyperchromicity in the absorption spectra of $\alpha_2 M$. The presence of a static quenching mechanism was indicated by the temperature-dependent fluorescence spectroscopy. The synchronous fluorescence revealed a change in the microenvironment of the tryptophan residues in $\alpha_2 M$. The CD results showed the reduction in β -helical content of $\alpha_2 M$. The activity of $\alpha_2 M$ decreased significantly with the increase of AgNPs concentration. **Conclusion:** Our result suggests that AgNPs cause modifications in the structure and functional activity of $\alpha_2 M$. The interaction of nanoparticles with proteins is important for understanding their potential risks to human health.

Keywords: alpha-2-macroglobulin, antiproteinase, silver nanoparticles, fluorescence quenching, FTIR, TEM

Introduction

Nanotechnology is one of the most rapidly developing fields of science and technology.¹ The nanoparticles (NPs) have potential applications in nanotechnology and biomedical applications, such as drug transport, molecular imaging, and therapy.^{2,3} Our understanding of the biocompatibility

and hazards of nanomaterial exposure has been limited thus far. As a result, there is a pressing need to comprehend the chemical mechanisms behind NPs-biological system interactions. In the biological medium, NPs may interact with proteins, nucleic acids, and lipids due to their nanosize and large surface-to-mass ratio.⁴ Understanding the interaction of nanoparticles with proteins is helpful

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in evaluating their possible hazards to human health and environment.

Due to their unique and different properties, such as antibacterial, antifungal, antiviral, anti-inflammatory, anticancer, and anti-angiogenic⁵, silver nanoparticles (AgNPs) are the most critical and appealing type of NPs.^{6,7} AgNPs are NPs with a diameter of less than 100 nm and a silver content of 20-15,000 atoms.8 Since AgNPs are likely to interact with serum/plasma cellular proteins, understanding the interaction between AgNP and plasma protein is critical. Alpha-2-macroglobulin (α, M) is a proteinase inhibitor with broad specificity found in abundance in human plasma. It has the ability to inhibit all types of proteinase and is involved in the binding, targeting, and transportation of a variety of molecules.9 The human α₂M is a 720 kDa soluble tetrameric glycoprotein made up of two identical 180 kD subunits linked through disulphide bonds and the dimers are joined by non-covalent interactions to form a tetrameric protein molecule.¹⁰ The bait area, internal thiol ester, receptor-binding site, transglutaminase reactive site and metalloprotein are the five reactive sites found in $\alpha_2 M_{\odot}^{-11}$

The protein-NPs interactions could have a big impact on the structure and function of proteins. 12 The aim of this study was to investigate how AgNPs interact with human antiproteinase-α₂M and factors that influence functional activity, structure, and conformation of $\alpha_a M$. The nature of the subsequent interaction, binding affinity, interaction forces involved, and stoichiometry of binding sites were also examined, which is important for designing nanomaterials for future biological applications.⁵ AgNPs were produced using a chemical technique, and the synthesized AgNPs were characterized using a combination of UV-Visible spectrophotometry, Fourier transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), X-ray diffraction (XRD). Multi-spectroscopic and such as UV absorption spectroscopy, fluorescence spectroscopy, FTIR, and circular dichroism (CD) investigations were used to monitor structural and conformational changes in $\alpha_a M$ as a result of interactions with AgNP. Fluorescence quenching investigations at various temperatures were used to measure the binding affinity constant, mode of binding and subsequent binding energetics. The present study on the synthesis and binding of silver nanoparticles to $\alpha_2 M$ is significant because most of the previous research used aurum nanoparticles and serum proteins for various applications, especially drug delivery systems.

Materials and methods

Synthesis and Characterization of AgNPs

AgNPs were synthesized with the chemical methods using a metal precursor (AgNO₂, silver nitrate), a reducing agent (NaBH₄, sodium borohydride) and a stabilizing/capping agent (sodium citrate, Na,C,H,O,). 13,14 The results of the reaction were yellowish-brown AgNPs. 15,16 The optical density of the AgNPs suspension was measured at room temperature at a resolution of 1 nm using a Lambda 25 double beam UV-visible spectrophotometer (PerkinElmer, Waltham, MA, USA). The reduction mechanism of Ag⁺ into AgNPs in the solution was investigated using UVvisible spectra. The presence of putative biomolecules and functional groups was detected by using FTIR spectroscopy.¹⁷ The transmittance mode of the FTIR spectrum was acquired using a JASCO FT/IR-6300 spectrometer (JASCO, Easton, MD, USA) with a resolution of 4 cm⁻¹. ¹⁸ To further evaluate the formation, crystalline behavior, and quality of AgNPs powder, XRD was performed. The surface morphology of AgNPs was examined under a TEM.

Purification of a,M and Sample Preparation

Purification of $\alpha_2 M$ from human blood was carried out based on previous work^{19,20} that had been standardized in our facility. To create a homogenous suspension, AgNPs solution was suspended in phosphate buffer and sonicated. Before completing any spectroscopic experiments, purified human $\alpha_2 M$ (10 μM) was incubated for 1 h at 37°C with different concentrations (5, 10, 15 and 20 μM) of AgNPs. Experiments were carried out in duplicate.

a,M Antiproteinase Activity Assay

Activity assay involves a technique for determining the antiproteinase activity of $\alpha_2 M$ pre-incubated with AgNPs. The technique primarily utilizes the capacity of $\alpha_2 M$ to protect trypsin's amidolytic action from degradation. Initially, $10~\mu M~\alpha_2 M$ was incubated for 15 min at 37°C with a threefold molar excess of trypsin (Sigma-Aldrich Chemical Co., St. Louis, MO, USA). Following the trypsin incubation, 100~mL of soya bean trypsin inhibitor (STI) (Sigma-Aldrich Chemical Co.) was added and incubated for another 15 min at 37°C, followed by the addition of 2 mL N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Sigma-Aldrich Chemical Co.), the trypsin substrate, and incubation for another 30 minutes. The absorbance was measured at 410 nm. The activity of untreated $\alpha_2 M$ was used as a control.

UV-visible Spectroscopy

Lambda 25 double beam UV-visible spectrophotometer was used to measure the UV/visible spectra. In a cuvette with a cell length of 10 mm, the absorbance was measured in the wavelength range of 200-400 nm at a scanning speed of 1000 nm/min. NPs solution without $\alpha_2 M$ were used as blank and subtracted.

Intrinsic Fluorescence Measurements

The protein was excited at a low wavelength (280 nm) and the fluorescence emission spectra were recorded at a higher wavelength range (300–400 nm) at three temperatures, *i.e.* 298, 303, and 310 K. To adjust the background fluorescence, appropriate blanks corresponding to the AgNPs concentration were subtracted. The Stern-Volmer equation was used to derive the Stern–Volmer quenching constant (K_{sv}), the binding constant (K_{b}) and the stoichiometry (n) of binding sites. Change in free energy (ΔG°) was calculated with Gibbs-Helmholtz equation.

Synchronous Fluorescence Measurements

The aim of the synchronous fluorescence measurements was to see how AgNPs affect the microenvironment of protein fluorophores. Fluorescence properties of a molecule are determined by both the molecule and its surroundings. Changes in fluorophore surroundings cause shifts in emission maxima, which predict polarity changes around tryptophan and tyrosine residues. In synchronous fluorescence, the excitation and emission monochromators were scanned concurrently. The synchronous fluorescence spectra describes about the perturbation in the microenvironment of tryptophan residues when the wavelength interval between excitation and emission ($\Delta\lambda$) is set at 60 nm, and change in the microenvironment of tyrosine residues when $\Delta\lambda$ is set at 15 nm. 24,25

Circular Dichroism Spectroscopy

Quartz cuvette with a 1 mm path length was used to record CD spectra in far-UV region (190-260 nm) on JASCO CD J-815 spectropolarimeter (JASCO). AgNP concentrations used were 10 μM and 20 μM . CD spectra analysis was used to investigate changes in the secondary structure of $\alpha_2 M^{26}$ as a result of interactions with AgNPs.

Statistical Analysis

The data were presented as mean and standard deviation (SD) values and each experiment was performed in triplicate.

Results

Characterization of AgNPs

The results of AgNPs synthesis from colorless AgNO, was a yellowish-dark brown compound. The UV-visible absorption spectroscopy at the visible range (400-500 nm) demonstrated that AgNPs showed a surface plasmon resonance (SPR) band caused by free electron excitation. FTIR analysis was used to investigate the functional groups responsible for the reduction and stabilization of AgNPs. The particle size and surface morphology of the AgNPs were evaluated using TEM. The AgNPs were found to be almost spherical, well dispersed, and scattered in nature. The average particle size was 15 nm. XRD revealed a narrow particle size distribution with an average diameter of 15 nm. The resulting XRD spectrum, combined with the TEM image, clearly indicated that the AgNPs were crystalline in form. The synthesized AgNPs in the present study had similar results and characteristics with previous studies.^{27,28}

Effect of AgNPs on a,M Antiproteinase Activity

To assess the inhibitory activity of human $\alpha_2 M$ when it interacted with AgNPs, a trypsin inhibitory test was used. Native $\alpha_2 M$ had the highest activity (100%) and was used as a reference. The activity of $\alpha_2 M$ decreased significantly with the increase of AgNPs concentration (5-25 μM) (Figure 1). Antiproteolytic activity in the protein was 77% at 10 μM AgNPs, and when the AgNPs concentration was increased to 20 μM , protein activity decreased to 58% of its original level. There was no loss of trypsin activity in the control group. This could be related to structural and conformational changes in $\alpha_2 M$ caused by AgNPs binding. The binding activity of $\alpha_2 M$ decreased as it was converted from a native to a non-native form.

UV-visible Absorption Spectroscopy of a,M

UV-visible absorption spectroscopy is a common tool for tracking changes that occur when NPs attach to proteins, and these changes are suggestive of conformational alterations. The aromatic amino acids (tryptophan, phenylalanine, and tyrosine) in proteins are responsible for their unique absorption at 280 nm. After one-hour incubation period, the UV spectrum of $\alpha_2 M$ was examined in the absence and presence of increasing quantities of AgNPs. The SPR band of AgNPs was affected by its interactions with biomolecules. Figure 2 showed that the maximum absorption was at 280 nm, and when the concentration of AgNPs (5-20 μM)

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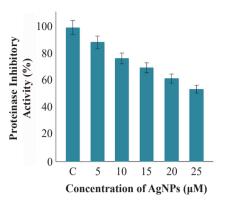


Figure 1. The effect of AgNPs concentrations on proteinase inhibitory activity of α ,M. C: Control.

increased, the absorption intensity increased consistently with no shift in peak location. This indicated a structural change in $\alpha_2 M$, confirming the formation of the $\alpha_2 M$ -AgNPs complex. As a result, the increase in absorption intensity could be attributable to the creation of a ground state complex between $\alpha_2 M$ and AgNPs. The background spectra of AgNPs solution were removed from the spectra of protein-NPs. A similar explanation has been reported by another study on the interaction of CuO NPs with galactosidase. 14

Intrinsic Fluorescence of a,M

Intrinsic fluorescence spectroscopy measures the total fluorescence of the protein's fluorophores: tyrosine, tryptophan, and phenylalanine. The conformational changes within the protein can be determined by intrinsic fluorescence intensity measurement.^{32,33} In the absence and presence of increasing quantities (5-20 µM) of AgNPs, the

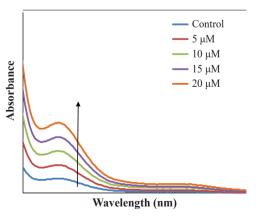


Figure 2. Absorption spectra of native $\alpha_2 M$ in the presence of AgNPs and incubated at 37°C for 1 h.

intrinsic fluorescence of $\alpha_2 M$ was evaluated. As shown in Figure 3A, $\alpha_2 M$ had a high fluorescence emission peak at 340 nm that gradually decreased with the increase of AgNPs concentration. This showed that AgNPs have caused a structural shift in $\alpha_2 M$. Fluorescence properties of AgNPs were also investigated, and it was discovered that they had no sensitivity to fluorescence.

The Stern–Volmer equation was used to investigate the quenching of fluorescence intensity at three distinct temperatures: 298, 303, and 310 K. Figure 3B showed a linear relationship between F_0/F and AgNPs molar concentrations (1:1), indicating that a single quenching mechanism could be dynamic or static. The ground state complex between the fluorophore and ligand leads to static quenching, whereas collision between the fluorophore and ligand leads to dynamic quenching.³³ Stern-Volmer K_{sv} and bimolecular quenching constant (K_q) were calculated using Stern-Volmer equation to determine the sort of quenching that occured in the AgNPs- α_2 M system. K_q was found to be 100 times greater in the AgNPs- α_2 M interaction (Table 1).

The highest scatter collision K_a of various quenchers with biopolymers (2×10¹⁰ M⁻¹s⁻¹) was found to be 100 times greater than the value of K_α for AgNPs-α₂M interaction, indicating that the static quenching was the most likely quenching mechanism. The interaction of AgNPs with bovine serum albumin (BSA) has been documented to have a similar static mechanism of fluorescence quenching. 34,35 Table 1 showed that the K_{sy} value decreased with the increase of temperature, indicating that the quenching process was static rather than dynamic.35 Furthermore, the K_b value decreased with the increase of temperature, implying that higher temperatures led to less stable complex formation. Figures 3C and Figure 3D showed a modified Stern-Volmer plot at three different temperatures, as well as a van't Hoff plot for K_b's temperature dependency. The stoichiometry of binding (n) was nearly equal to unity, indicating that AgNPs had a single distinct class of binding site on α_2 M. The free energy change (ΔG) had a negative value (Table 1), indicating that the reaction occurred spontaneously.

Synchronous Fluorescence of a₂M

Synchronous fluorescence investigation provides information on the microenvironmental alterations that occur around the protein's aromatic moieties. Changes in polarity around tryptophan and tyrosine residues are predicted by shifts in emission maxima. Figure 4 showed synchronized fluorescence spectra of $\alpha_3 M$ after interaction

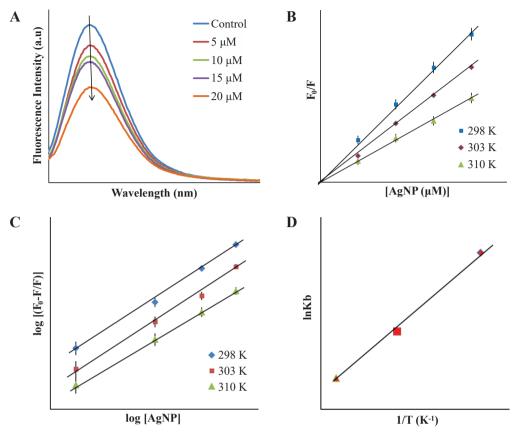


Figure 3. Intrinsic fluorescence spectra, Stern-Volmer plot and van't Hoff plot of α_2 M. A: Intrinsic fluorescence spectra of α_2 M in absence and presence of AgNPs; B: The Stern-Volmer plot; C: The modified Stern-Volmer plot of fluorescence quenching of α_3 M by AgNPs at different temperatures; D: The van't Hoff plot for temperature dependence of K_b .

with different AgNPs concentrations (5-20 μ M). When $\Delta\lambda$ =60 nm, a minor red shift in the peak (from 340 to 346 nm) was observed, accompanied with a decrease in fluorescence intensity, implying a change in the microenvironment around tryptophan residues. This change indicated that the interaction of AgNPs with tryptophan residues caused them to move from a non-polar to a polar environment, lowering the hydrophobicity around them.³⁰ When $\Delta\lambda$ =15 nm, AgNPs did not produce any shift, indicating that the microenvironment around tyrosine residues had not changed.

It can be concluded that tryptophan played a key role in the quenching of α_2M fluorescence, and AgNPs approached tryptophan residues more than tyrosine residues.

CD of $\alpha_{n}M$

CD is commonly used to determine changes in protein secondary structure during protein-ligand interactions. The interaction of ligand with protein leads to the alteration in protein structure, which corresponds to the changes in CD spectra. The content of secondary structure in native $\alpha_2 M$

Table 1. Binding parameters of α_2 M-AgNPs interaction obtained from fluorescence quenching experiment at different temperatures.

Temperature (K)	K _{sv} (M ⁻¹)	K _q (M ⁻¹ s ⁻¹)	K _b (M ⁻¹)	n	ΔG (kcal mol ⁻¹)
298	2.69×10 ⁴	2.69×10 ¹²	3.47×10 ⁴	1.15	-7.24
303	2.12×10^4	2.12×10^{12}	2.82×10^4	1.14	-6.03
310	1.83×10^4	1.83×10 ¹²	2.07×10 ⁴	1.15	-4.19

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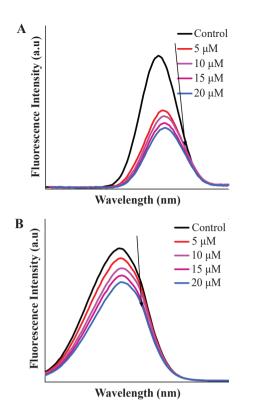


Figure 4. Synchronous fluorescence spectra of α_2 M. A: $\Delta\lambda$ =60 nm; B: $\Delta\lambda$ =15 nm.

was mostly β -helical (70%)³⁰ and it exhibited a pronounced negative peak at 215 nm (Figure 5). However, $\alpha_2 M$ spectra indicated a decrease in negative ellipticity with no shift in negative peak position after incubation with 10 μM AgNPs for 1 h at 37°C. Negative ellipticity diminished further after subsequent incubation with 20 μM AgNPs. Our finding suggested that AgNPs triggered structural changes in $\alpha_2 M$. The β -helicity of proteins was measured using the K2D2 software.³⁶ The β -helicity of $\alpha_2 M$ decreased

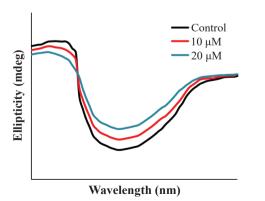


Figure 5. Far-UV CD spectra of native and AgNPs-treated $\alpha_{3}M$.

from 66.19±1.03% to 64.05±1.55% at 1:1 $\alpha_2 M$ to AgNPs molar ratio, and from 66.19±1.03% to 62.60±1.38% at 1:2 $\alpha_2 M$ to AgNPs molar ratio. This suggested that interaction of AgNPs with $\alpha_2 M$ reduced the β -helical content of $\alpha_2 M$ secondary structure.

Discussion

AgNPs, due to their unique properties⁵ are increasingly used in different sectors^{2,3}, since they are likely to interact with serum/plasma cellular proteins. Application of NPs in the biomedical field relies on their interactions with proteins, cells and tissues.⁴ It is important to investigate any cellular alterations caused by NPs in human serum proteins such as $\alpha_2 M$. To the best of our knowledge, this was the first attempt to investigate the structural and functional alterations in $\alpha_2 M$ induced by AgNPs through various biochemical/biophysical and multi-spectroscopic techniques.

Absorption intensity of α₂M increased in UV-visible spectra, indicating a structural alteration in α₂M. Steadystate fluorescence measurement unveiled concentrationdependent quenching in α_2 M spectra, and the mechanism of quenching was static in nature, due to ground-state complex formation. At different temperatures, intrinsic fluorescence experiments demonstrated that quenching of α₂M spectra occurred due to the formation of ground state complexes with static quenching mechanism.²⁶ K_{sv} , K_{a} and K_{b} values decreased with the increase of temperature, implying the presence of a static quenching mechanism. Synchronous fluorescence spectroscopy determines the alteration in the local environment of tryptophan residues of $\alpha_n M^{29}$ on AgNP interaction. AgNP drived tryptophan residues toward a more polar environment.³⁷ CD spectra revealed a decrease in the β-helical content of α₂M, indicating secondary structural alteration.38

Conclusion

Our study was the first to reveal that $\alpha_2 M$ incubation with AgNPs results in loss of antiproteolytic potential of $\alpha_2 M$. Results of multi-spectroscopic techniques suggest that AgNPs cause modifications in structure and conformation of $\alpha_2 M$. Interaction of nanoparticles with proteins is important for understanding their potential risks to human health.

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