Quasi-spherical silver nanoparticles with high dispersity and uniform sizes: preparation, characterization and bioactivity in their interaction with bovine serum albumin

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ABSTRACT: A stepwise seeded growth route for the preparation of silver nanoparticles (AgNPs) is reported. In the process, silver nitrate was used as a precursor, with sodium borohydride as a reducing agent and trisodium citrate as both a reductant and stabilizer. The AgNPs were characterized using several methods, including UV–vis spectroscopy, X-ray diffraction and transmission electron microscopy. The prepared AgNPs were quasi-spherical and crystalline, with an average diameter of 21 nm. Interactions between the AgNPs and bovine serum albumin (BSA) were investigated using UV–vis, fluorescence spectroscopy and synchronous fluorescence spectroscopy (SFS). It was proved that the quenching mechanism is a static process. The binding constants and number of binding sites were calculated. The thermodynamic parameters implied that the binding process was spontaneous and the main driving force of the interaction was electrostatic. The results of the SFS indicated that the conformational change of BSA was induced by AgNPs. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: silver nanoparticles; stepwise seeded growth; bovine serum albumin; transmission electron microscopy; spectroscopic method

Introduction

Because of their novel physicochemical properties, which are based on their size, composition and morphology, noble metal nanoparticles are used in a large number of fields, with applications in catalysis, optics, biomedicine and consumer products (1–4). Among areas of potential research, the synthesis and characterization of silver nanoparticles (AgNPs) has attracted wide interest around the world because of the prospect of further applications (5–7). However, it is hard to avoid contact between AgNPs and organisms, particularly because of their applications in biomedicine and consumer products. For this reason, studies have been undertaken to explore the potential toxicity mechanisms of AgNPs. Kittler et al. reported that the release of silver led to considerably increased toxicity of AgNPs toward human mesenchymal stem cells (8); Yang et al. reported that the surface coating influenced AgNPs toxicity (9); and Pratsinis et al. reported that the cytotoxicity of large AgNPs (>10 nm) decreased as their size increased (10). Despite some progress being made in toxicity research, there have been many difficulties due to the complexity of biological systems and the various properties of AgNPs. Therefore, further research is needed to build a more comprehensive and clear theoretical system.

Serum albumins are the main component of blood plasma in vertebrates and have attracted a great deal of interest because of their vital physiological properties; functions such as anticoagulation, acting as carrier proteins for numerous endogenous and exogenous ligands, and maintaining the osmotic pressure of blood, etc. (11,12). Studies on the binding of drugs to proteins within a physiological environment are of increased importance due to a significant need to understand the absorption, transportation, metabolism and elimination of drugs in the human body (13–15). It has been reported that bovine serum albumin (BSA) and human serum albumin (HSA) are homologous proteins (16). In this study, BSA was used as a model protein because of its clear structure, important physiological function, high structural homology with HSA and low cost. Hence, investigation into the binding interaction between AgNPs and BSA is essential to illustrate the mechanism of interaction between AgNPs and serum albumin, and to explore the biological activities and toxicity of AgNPs.

In this work, quasi-spherical AgNPs were prepared and characterized by UV–vis spectroscopy, X-ray diffraction (XRD) and transmission electron microscopy (TEM). The interaction of AgNPs with BSA was also investigated using UV–vis, fluorescence spectroscopy and synchronous fluorescence spectroscopy (SFS).

Experimental

Materials

Silver nitrate (AgNO₃, ≥ 99.8%), trisodium citrate dihydrate (Na₃C₆H₅O₇·2H₂O, ≥ 99.0%), sodium borohydride (NaBH₄, ≥ 98.0%),

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sodium chloride (NaCl, ≥ 99.5%) and hydrochloric acid (HCl, ~ 36%–38%) all of analytical grade were used without further purification. BSA and Tris with a purity of no less than 99.0% were purchased from Sigma (St. Louis, MO, USA). The BSA solution was dissolved in Tris/HCl buffer solution (0.05 mol/L Tris, 0.1 mol/L NaCl, pH 7.40) and stored at 4 °C. Ultrapure water was used throughout.

Methods

The morphology of the AgNPs was characterized using a JEM-2100 TEM (JEOL, Tokyo, Japan) at an accelerating voltage of 200 kV, and fitted with a CCD camera.

The crystal structure of the AgNPs was confirmed by recording the crystallograms in a D8 Advance XRD (Bruker, Bremen, Germany) using CuKα (k = 1.5406 Å) as an X-ray source.

A UV-9000S spectrophotometer (Metash, Shanghai, China) was used to measure UV–vis spectra, with 1.0 cm quartz cells over a wavelength range of 190–900 nm. Fluorescence spectra were recorded using a LS-55 fluorescence spectrophotometer (Perkin-Elmer, Waltham, MA, USA) equipped with 1.0 cm quartz cells. The excitation and emission slit widths were maintained at 12.0 and 7.5 nm, respectively.

The concentration of the AgNPs was confirmed by using an AA-6200 atomic absorption spectrophotometer (Shimadzu, Kyoto, Japan).

Results and discussion

Synthesis of AgNPs

AgNPs were prepared by stepwise seeded growth, as described in the literature (17). The first step was the preparation of citrate-capped 4 nm silver seeds. In a 100 mL three-necked round-bottom flask, 10 mL of a 1% citrate solution and 37.5 mL of water were mixed and brought to 70 °C in a water-bath for 15 min. Then, 0.85 mL of a 1% AgNO₃ solution was introduced into the mixture, followed by the quick addition of 1 mL of a 0.1% freshly prepared NaBH₄ solution. The reaction solution was kept under stirring at a speed of 550 rpm at 70 °C for 1 h and then cooled to room temperature. The second step was the preparation of AgNPs with an average size of 21 nm using starter seeds. One milliliter of a 1% citrate solution was mixed with 40 mL of water and brought to the boil using a heating mantle for 15 min. Next, 5 mL of starter seeds solution was added under a stirring speed of 550 rpm, followed by the addition of 0.85 mL of a 1% AgNO₃ solution. Stirring continued for 1 h while refluxing and the mixture was then cooled to room temperature.

Characterization

UV–vis spectra analysis. UV–vis is widely used to identify the formation of nanoparticles. Nano-structured metals, such as AgNPs, exhibit a characteristic absorption band due to surface plasmon resonance, which arises from a coherent oscillation of the free electrons of a metallic particle in resonance with a light wave (18). The UV–vis spectra of the prepared silver seeds and the AgNPs are shown in Fig. 1 (curves a and b). The characteristic absorbance band at 391 and 393 nm indicates the formation of nanoparticles. In addition, the change in the color of the reaction solution from colorless to yellow was also proof of the formation of AgNPs (19).

Crystal structure analysis. The phase and crystal structure of the AgNPs were confirmed by XRD and are shown in Fig. 2. For both the silver seeds (Fig. 2A) and the AgNPs (Fig. 2B), five diffraction peaks were observed over a 2θ range of 20–80°, which were consistent with the (111), (200), (220), (311), (222) lattice planes of the face-centered cubic (fcc) structure of metallic silver (JCPDS, file no. PDF#65–2871) (20). Additionally, no impurity peaks were observed in the crystallograms. The results indicated that the prepared AgNPs are pure crystalline silver. The XRD analysis results are in good agreement with the UV–vis adsorption spectra and demonstrate the formation of AgNPs.
TEM characterization. It is well known that TEM characterization is the most visual and effective method to investigate the morphology, shape and size of nanoparticles. The prepared silver seeds and AgNPs were characterized using TEM and the results are shown in Fig. 3(A) and Fig. 3(B,C), respectively. The TEM images revealed that both the silver seeds and the AgNPs exhibited good dispersion and were quasi-spherical in shape. The particle size distribution of the silver seeds and the AgNPs, as shown in Fig. 3(D,E), was obtained from the TEM images by measuring 900 particles. The results demonstrated that the average diameter calculated from the images was 4 nm for silver seeds and 21 nm for the AgNPs.

Mechanism studies of AgNPs with BSA

Emission spectra. Fluorescence emission spectral measurement is an effective and vital method to explore the binding of small molecules to protein, and was used in this study to investigate the interaction of AgNPs with BSA. The emission spectra acquired for BSA at pH 7.40 with various concentrations of AgNPs are shown in Fig. 4. It can be observed that the maximum emission wavelength of BSA in the absence of AgNPs was observed at 348 nm when excited at 295 nm. An excitation wavelength of 295 nm was selected because the intrinsic fluorescence of BSA was attributed to its tryptophan residues and the interference of AgNPs was negligible (curve I) (21). It was observed that the fluorescence intensity of BSA decreased gradually (Fig. 4) when the concentration of AgNPs increased. The results indicated that AgNPs can bind to BSA and quench its intrinsic fluorescence. Meanwhile, a significant blue shift of 8 nm from 348 to 340 nm suggested that the fluorophores of BSA moved to a more hydrophobic environment, most likely due to the aggregation of protein molecules on the addition of AgNPs (22).

Quenching mechanism

The two fluorescence-quenching mechanisms, dynamic quenching and static quenching, can usually be distinguished by their differing dependence on viscosity and temperature. The dynamic quenching constant increases when the temperature increases because higher temperatures lead to larger values of the diffusion coefficient. By contrast, the static quenching constant decreases as the temperature increases because the stability of the ground-state complex weakens (23).

To obtain a more specific mechanism for the fluorescence quenching, UV–vis was used to investigate the structural change in BSA on addition of AgNPs. As shown in Fig. 5(A), a characteristic adsorption peak at 278 nm was observed with UV–vis for BSA in the absence of AgNPs. It can be seen that the intensity of the characteristic BSA peak at 278 nm and the characteristic absorbance band of AgNPs at around 400 nm increased with the addition of AgNPs (Fig. 5A, inset). The results indicated the formation of a AgNPs–BSA complex. In general, dynamic quenching affects only the excited state of fluorophores, and will not change the absorption spectra. Nevertheless, the formation of a complex will induce a change in the absorption spectrum of proteins (24). The UV–vis spectra of BSA only (curve a), AgNPs–BSA complex (curve b), AgNPs only (curve c) and the difference between the AgNPs–BSA complex and the AgNPs (curve d), which come from subtracting the absorption spectrum of curve c from that of curve b at the same concentration, are shown in Fig. 5(B). It is obvious that curves d and a could not be superposed within the experimental error,
this result indicated that the quenching mechanism was probably a static quenching process.

For further confirmation of the quenching mechanism, the changing trends in the fluorescence quenching constant ($K_{SV}$) of the interaction of AgNPs and BSA can be evaluated with the help of the Stern–Volmer equation (25):

$$F_0/F = 1 + K_{SV}[Q]$$

(1)

where $F_0$ and $F$ are the steady-state fluorescence intensities in the absence and presence of quencher (AgNPs), respectively, $K_{SV}$ is the Stern–Volmer quenching constant, and $[Q]$ is the concentration of the quencher. According to equation (1), the linear regression for the $(F_0 - F)/F$ against $[Q]$ plots of AgNPs with BSA at different temperatures were applied to calculate the values of $K_{SV}$ by the slopes and the results are presented in Table 1. The values of $K_{SV}$ decreased when the temperature increased within the investigated temperature range, suggesting that the quenching process resulted from the formation of a AgNPs–BSA complex and that the quenching mechanism was a static quenching process rather than a dynamic mechanism.

### Binding parameters

For a static quenching process, the binding constants ($K_a$) were analyzed according to the modified Stern–Volmer equation (26):

$$F_0/\Delta F = 1/K_a + 1/F$$

(2)

where $\Delta F$ is the difference in fluorescence in the absence and presence of the quencher at a concentration $[Q]$, $f$ is the fraction of accessible fluorescence, and $K_a$ is the effective quenching constant for the accessible fluorophores, which are analogous to the associative binding constants for the quencher–acceptor system. According to equation (2), the binding constants $K_a$ can be obtained by the $F_0/\Delta F$ vs. $[Q]^{-1}$ plots and the results are displayed in Table 2.

To obtain the number of AgNPs binding with BSA, the Scatchard equation was used to calculate the binding constants and number of binding sites (27):

$$r/D_r = nK_f - rK_b$$

(3)

where $r$ is the moles of ligand bound per mole of protein, $D_r$ is the molar concentration of free ligand, $n$ is the binding site
multiplicity per class of binding site, and \( K_b \) is the equilibrium binding constant. According to equation (3), the binding constant \( K_b \) and the number of binding sites can be calculated from the plot of \( r/D_f vs. r \) and the results are shown in Table 2.

It can be seen from Table 2 that the binding constants (\( K_a \) and \( K_b \)) obtained using the two methods decreased with increases in temperature, in agreement with the trend in the dependence of \( K_{SV} \) on temperature, as presented in Table 1. The results indicated that the stability of the complex might be damaged when the temperature increases during a static process. In addition, the number of binding sites, \( n \), is ~1, showing that there was only one binding site on the BSA for AgNPs during their interaction.

**Thermodynamic parameters and binding mode**

Typical interactions have vital roles during the process of a drug binding to a protein, including hydrophobic interaction forces, hydrogen bonds, van der Waals interactions and electrostatic interactions (28). In order to investigate the driving force of the interaction between the AgNPs and BSA, the thermodynamic parameters that depend on temperature were observed. If the enthalpy change (\( \Delta H \)) does not vary significantly over the temperature range studied, then \( \Delta H \) and the entropy change (\( \Delta S \)) can be estimated using the van’t Hoff equation (29):

\[
\ln K_a = \frac{\Delta H}{RT} + \frac{\Delta S}{R}
\]

where \( R \) is the gas constant and \( K_a \) is the binding constant at the corresponding temperatures. \( \Delta H \) was calculated from the slope and intercept of the plot of \( \ln K_a \) vs. \( 1/T \). The free energy change (\( \Delta G \)) can be obtained from the following relationship:

\[
\Delta G = -RT \ln K_a
\]

According to Ross and Subramanian, \( \Delta H < 0 \) and \( \Delta S < 0 \) correspond to van der Waals forces or hydrogen bond formation, \( \Delta H > 0 \) and \( \Delta S > 0 \) imply a hydrophobic interaction, and \( \Delta H < 0 \) and \( \Delta S > 0 \) suggest an electrostatic force (28). The thermodynamic parameters were calculated and are shown in Table 2. The negative \( \Delta G \) value revealed that the binding process was spontaneous. The negative \( \Delta H \) value and positive \( \Delta S \) value indicated that the main driving force of the interaction between the AgNPs and BSA was an electrostatic force.

**Conformational change in BSA induced by AgNPs**

SFS was introduced by Lloyd and is an effective method to investigate the protein conformations that can give information about the microenvironment in the vicinity of the chromospheres (30). When the intervals (\( \Delta \lambda \)) between the excitation and emission wavelengths were stabilized at 60 and 15 nm, the fluorescence due to tryptophan and tyrosine residues, respectively, was observed on the SFS. To investigate the effect of AgNPs on the conformational change in BSA, the emission spectra for the tryptophan and tyrosine residues were obtained and are shown in Fig. 6. The decreasing trend in the fluorescence intensities for the tryptophan and tyrosine residues on addition of AgNPs was in accordance with the fluorescence emission spectrum of BSA, as shown in Fig. 4. Furthermore, a slight blue shift in the maximum

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**Table 2. Binding constants and thermodynamic parameters for AgNPs with BSA at different temperatures (pH = 7.40)**

<table>
<thead>
<tr>
<th>( T ) (K)</th>
<th>Modified Stern–Volmer method</th>
<th>Scatchard method</th>
<th>( \Delta H ) (kJ/mol)</th>
<th>( \Delta G ) (kJ/mol)</th>
<th>( \Delta S ) (J/mol/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( 10^{-3} K_a ) (L/mol)</td>
<td>( R )</td>
<td>( 10^{-3} K_b ) (L/mol(^{-1}))</td>
<td>( n )</td>
<td></td>
</tr>
<tr>
<td>292</td>
<td>9.52</td>
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<td>8.43</td>
<td>1.12</td>
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</tr>
<tr>
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<td>7.38</td>
<td>1.17</td>
<td>-22.52</td>
</tr>
<tr>
<td>304</td>
<td>8.11</td>
<td>0.9999</td>
<td>6.76</td>
<td>1.20</td>
<td>-22.75</td>
</tr>
<tr>
<td>310</td>
<td>7.46</td>
<td>0.9998</td>
<td>6.15</td>
<td>1.20</td>
<td>-22.98</td>
</tr>
</tbody>
</table>

\( R \) is the correlation coefficient.

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Figure 6. Synchronous fluorescence spectra of BSA in the absence and presence of AgNPs. \( c(BSA) = 2.0 \times 10^{-6} \text{ mol/L}; c(\text{AgNPs})/10^{-5} \text{ mol/L}. \) (A–k) From 0.0 to 15.0 in increments of 1.5. (A) \( \Delta \lambda = 60 \text{ nm}; \) (B) \( \Delta \lambda = 15 \text{ nm}. \)
emission wavelengths of the tryptophan residues was observed, whereas the maximum emission wavelength of the tyrosine residues did not shift. The blue shift indicated that the polarity around the tryptophan residues decreased and hydrophobicity increased (31). The results revealed that the conformational change in BSA induced by AgNPs might be due to an alteration in the microenvironment surrounding the tryptophan residues, whereas the characteristics of the tyrosine residues remained undisturbed.

Conclusions

Citrate-coated AgNPs were prepared using stepwise seeded growth and were fully characterized, and the bioactivity was investigated. UV–vis analysis indicated the formation of AgNPs due to the characteristic absorbance band observed at 393 nm. The results of XRD analysis indicated that the prepared AgNPs were pure crystalline silver with a fcc structure. The TEM images revealed that AgNPs exhibited good dispersion and were quasi-spherical in shape, with an average diameter of 21 nm. It was proven that the fluorescence quenching of BSA by AgNPs was a static quenching process due to complex formation. The binding constants and number of binding sites during the static quenching process were obtained. The thermodynamic parameters revealed that the binding process was spontaneous and the main driving force of the interaction was an electrostatic interaction. SFS results indicated that the slight conformational change in BSA induced by AgNPs can be attributed to an alteration in the microenvironment surrounding the tryptophan residues. These results should be able to evaluate the biocompatibility of AgNPs primarily and provide a theoretical and practical foundation for the application of nanomaterials.

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References

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