Iranian Journal of Microbiology
Volume 15 Number 5 (October 2023) 697-704

ORIGINAL ARTICLE

A new nanobiotic: synthesis and characterization of an albumin nanoparticle with intrinsic antibiotic activity

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Received: January 2023**, Accepted:** September 2023

ABSTRACT

Background and Objectives: With entering the "post-antibiotic era", antibiotic resistance is one of the most important problems in food security, health and medicine. Invention of nanoparticles with intrinsic antimicrobial activity has been provided a new tool to combat the problem, including some metal nanoparticles. But protein nanoparticles have been often used as nano-carrier for antibiotic drugs, not for their own antibiotic activity. In this article we have fabricated a very small BSA-NP without any chemical modification on BSA molecules showing antibacterial activity.

Materials and Methods: Bovine serum albumin nanoparticle (BSA-NP) was synthesized using botton-up approach, by dissolution of BSA in urea-containing Tris buffer for 60 min at 60°C. Then, the BSA solution was dialyzed against distilled water in order to nanoparticle formation. The resulted BSA-NP has been characterized by dynamic light scattering (DLS), field emission surface electron microscopy (FESEM), SDS-PAGE, Fourier transform infrared spectroscopy (FTIR) and UV-spectrophotometery. Minimum inhibitory concentration (MIC) method was used for evaluation of antibacterial activity of BSA-NP against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

Results: The results obtained by DLS technique indicated that BSA molecules were self-assembled into small aggregates with a hydrodynamic diameter of 23.23 ± 2.1 nm. With a small polydispersity index (PDI=0.522), the nanoparticles had good spherical uniformity. The nanoparticles made from a single type of protein molecule (BSA) and have a relatively transparent appearance. The BSA-NPs caused a decrease in cell growth of both *P. aeruginosa* and *S. aureus*. Moreover, they had a bacteriostatic effect on *P. aeruginosa* (MIC=112×10^{-5} μM).

Conclusion: In this study, using a green synthesis method, we succeeded in synthesizing a very small uniform BSA nanoparticles without any chemical modification on BSA molecules. It also has bacteriostatic properties against *P. aeruginosa*. Therefore, it is hypothesized that our BSA-NPs may be effective as a new approach to combat antibiotic resistance.

Keywords: Bovine serum albumin; Nanoparticles; Antibacterial activity; Antibiotic resistance; Green synthesis

INTRODUCTION

With entering the "post-antibiotic era", antibiotic resistance is one of the most important problems in food security, health and medicine. It leads to prolonged hospitalization, increased medical costs and increased mortality. Treatment of infectious disease are becoming harder due to less effectiveness of antibiotics. Therefore, the world urgently needs to provide new ways to fight bacterial infections. Recently, nanoparticle (NP) technology have made accessible new tools to combat deadly bacterial infections (1,

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2). Antimicrobial nanotherapeutics can overcome the antibiotic resistance problems faced by traditional antimicrobials (3-6).

Nanoparticles are a broad class of materials containing particulate matter with at least one dimension less than 100 nm (7). NPs are of great interest for various applications due to their high surface-to-volume ratio and unique chemical and physical properties. These NPs have promising properties such as biodegradability, non-antigenicity, metabolization, surface area modifiers, in vivo storage stability, relative ease of manufacture and particle size monitoring. These particles have the ability to covalently bind drugs and ligands (8-10).

A type of NP is protein nanoparticles which has applications in a variety of targeted therapies, including pulmonary delivery, cancer therapy, tumor therapy, and vaccines (11).

Such NPs which derived from natural proteins, are biodegradable and metabolizable (12). One of the most common proteins for the preparation of protein nanoparticles is albumin. Serum albumins are the most abundant (52-62%), water-soluble proteins in blood plasma (13).

Bovine serum albumin (BSA) is a heart-shaped protein with a molecular weight of approximately 66.8 kDa. BSA contains three homologous domains I-II-III and have unique binding properties (14). Seventeen disulfide bridges of amino acid cysteine (Cys) stabilize the structure of its domains so that it can be heated to 60°C for 10 hours without showing any denaturation effect on structural domains (15). In addition, the presence of several binding sites in its hydrophobic clefts improves BSA ability to bind to various active molecules including physiological and pharmaceutical ligands, so that this protein is one of the most important biological macromolecules, which acts as a carrier of various molecules in the serum, such as ions, fatty acids, as well as small molecules of drugs (16). Indeed, albumin has gained attention as an effective drug carrier with multiple intrinsic properties, firstly because it is an endogenous protein, it is non-toxic, non-immunogenic, degradable and biocompatible (17). The non-immunogenic property of albumin is very important because it prevents toxicity due to lack of immune reactions. Secondly, albumin has a high binding capacity. Thirdly, albumin with good water solubility can carry insoluble drugs through aqueous compartments to reach its medicinal target (17). In addition, albumin has the unique potential of active targeting that enhances cellular uptake, by endothelial (18) and cancer cells (19). Moreover, albumin can undergo structural changes to escape from lysosomes and low pH endosomes and cause effective release of therapeutic agents into the cellular environment while accompanied by proteolytic degradation of albumin (20). Finally albumin is an extraordinary extracellular antioxidant (21) and provides a strong protection against free radical attack (22). In general, all these properties have made albumin a desirable carrier in drug-carrying nanotechnology.

In this article, the method of synthesizing bovine serum albumin nanoparticles with a very small size in a green and simple way is reported without any chemical modification on BSA molecules. This nanoparticle showed a considerable intrinsic antibiotic property without loading any antibiotic drugs.

MATERIALS AND METHODS

Bovine Serum Albumin (BSA), Urea, Tris, Acrylamide, Bis Acrylamide, Sodium Dodecyl Sulfate (SDS), Ammonium Persulfate (APS), Tetramethylethylenediamine (TEMED), Nutrient broth, Nutrient agar, Coomassie Brilliant Blue (R250), Agar, Bromophenol Blue, β-mercaptoetanol, Glacial Acetic Acid, Isopropanol, Glycerol, Methanol, were purchased from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, USA). All chemicals used were of analytical grade.

Synthesis of BSA nanoparticles. In this research, albumin nanoparticles were synthesized by the bottom-up dissolvation method. For this purpose, BSA with a concentration of 10 mg/ml was dissolved in a solution containing 0.1 M Tris buffer (pH 8), 1 M urea and incubated at 60°C for 1 hour. Then, the solution was dialyzed against distilled water by dialysis tube (12-15 kDa MWCO) for 72 hours at 4°C. The resulted dialysate is BSA-NP suspension.

Dynamic light scattering (DLS). To determine the size distribution of the BSA-NPs, Zetasizer Nano device was used (ZEN3600, Malvern Co.) to examine BSA-NP suspension.

FESEM analysis. The morphology of BSA-NPs was imaged using field emission surface electron

microscopy (FESEM) (FEI NOVA NANOSEM 451). BSA-NP solutions were aliquoted on FESEM stubs and allowed to dry at room temperature. The samples were coated with 10 nm gold layer using a sputter coater. ImageJ software was used for analyzing the particle size distribution (23).

SDS-PAGE. In this study, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight and purity of the produced BSA proteins. The electrophoresis was carried out in a vertical slab gel unit. The Separation and stacking gels were prepared by Laemmli method (24) containing 12% polyacrylamide gel and 4% acrylamide gel, respectively. A volume of 22 microliters of the sample were dissolved in 8 microliters of sample buffer 5× (pH6.8) and heated at 70°C for 5 minutes. Then the sample was centrifuged at 13400 rpm for 30 minutes at 4°C. The supernatant (15 µL) was loaded into each gel well. A protein marker with molecular weights between 5 and 240 kDa were used. Electrophoresis was performed at a voltage of 100 V for 150 minutes.

Fourier transform infrared spectroscopy (FTIR). The BSA-NP chemical structure was recorded using Tensor 27 spectrophotometer in wavelength range of 500 to 4000 cm-1 and resolution of 0.1%T (Brucker Co.) by KBr pellet technique.

UV spectroscopy. UV/vis spectroscopy was performed using UV-spectrophotometer (SHIMADZU, Tokyo, Japan) at wavelengths of 200 to 900 nm.

Minimum inhibitory concentration (MIC). The antibacterial activity of the BSA-NPs was investigated by MIC method against two typical standard strains, Gram-positive (G+) *Staphylococcus aureus* (ATCC 1431) and Gram-negative (G-) *Pseudomonas aeruginosa* (ATCC 27843). Different concentration of synthesized BSA-NP including 225×10^{-5}, 112×10^{-5}, 56×10^{-5}, 28×10^{-5} and 14×10^{-5} µM were used to evaluate the antibacterial activity of BSA-NP against *S. aureus* and *P. aeruginosa*. For this purpose, the fresh culture of bacteria was prepared in nutrient broth medium. Bacterial suspensions were prepared equal to 0.5 McFarland turbidity at OD_{625}. This turbidity is equivalent to OD 0.08-0.013 and 1.5×10^{8} CFU/ml). In a 96-well plate, each well contained culture medium (100 µl) and 50 µl BSA-NP colloidal solution with 150 $\mu g.ml^{-1}$ concentration. Each well was inoculated with 50 µl bacterial suspension at 0.5 McFarland concentration. The plate was incubated for 24 hours at 35°C and the OD of the well content was read at 600nm by ELISA reader (Epoch). As a positive control, the antibiotic gentamicin with the concentration approved by CLSI (16 µg/ml) was used, which has antibacterial activity for both Gram positive and negative strains used in this study. The lowest concentration of BSA-NP, which was detected no visible bacterial growth, taken as the MIC value.

Minimum bactericidal concentration (MBC). MBC is the lowest concentration of antimicrobials that kills upper 99.9% of the initial bacteria. After determining the MIC of the synthesized BSA-NPs, from the wells without growth, has been cultured on nutrient agar. Then, Plates were incubated for 24 hours at 37°C.

RESULTS

Determination of size distribution of BSA-NPs. The most reliable method for proving the formation of a nanoparticle in a solution is dynamic Light scattering (DLS) even if the colloidal solution of nanoparticles would be apparently clear. DLS analysis of the dialysate showed a relatively sharp single peak indicating BSA-NPs (Fig. 1). The most popular BSA-NPs have 23.23±2.1 nm hydrodynamic diameter. Polydispersity index (PDI) was 0.522. Size analysis of BSA-NP using FESEM micrograph of dried BSA-NP and histogram of their size distribution showed similar diameter (22-24 nm).

Surface morphology of BSA-NPs. Surface morphology of BSA-NPs was shown in the FESEM image in Fig. 2. The BSA molecules is aggregated as very small spherical particles in nanometer dimension size and formed BSA-NPs (Fig. 2A). Each large sphere shown in the Fig. 2B, contains several albumin nanoparticles that have agglomerated and formed a larger sphere.

SDS-PAGE. SDS-PAGE was used to confirm the protein identity of the nanoparticles. Using the standard curve that was obtained according to this gel, the molecular weight of the protein of these nanoparticles was calculated to be 66.96 kDa (Fig. 3).

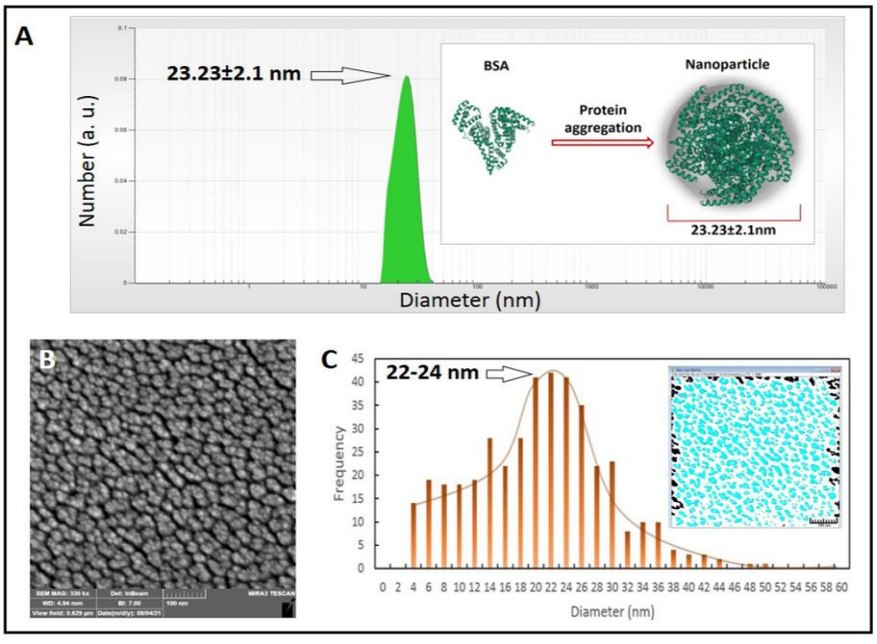


Fig. 1. Size analysis of BSA-NP. A) Size distribution in colloidal solution by DLS analysis, B) FESEM micrograph of dried BSA-NP and histogram of their size distribution (C) analyzed by imageJ software.

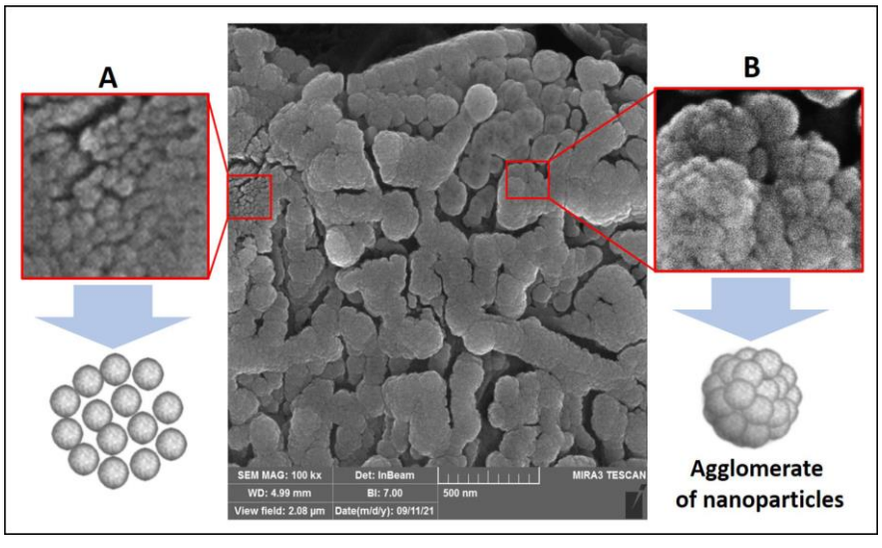


Fig. 2. Morphological analysis of BSA-NPs using FESEM. A) Small aggregation of NPs. B) Larger agglomerated of BSA-NPs.

FTIR. The FTIR spectrum of BSA-NPs showed three spectral regions specific to protein secondary structures, which indicates the protein nature of the nanoparticles: amide I, II and III at wave numbers 1651, 1540 and 1236 cm^{-1}, respectively (Fig. 4). The presence of a peak at 3296 cm^{-1} and 3000 cm^{-1} are signs of N-H stretching, named Amide A and Amide B, respectively. The peak observed in wavenumber 519 cm^{-1} indicates the presence of S-S band in any disulfide-rich proteins and nanoparticles.

UV/vis spectroscopy. As shown in Fig. 5B, BSA-NPs have a relatively transparent appearance, with very low turbidity and without any sediment. The decrease in transmitted UV/vis light, which occurs due to light scattering by nanoparticles in the colloidal solution of BSA-NPs, was monitored as a raised spectrum after nanoparticle formation (BSA-NP) (Fig. 5).

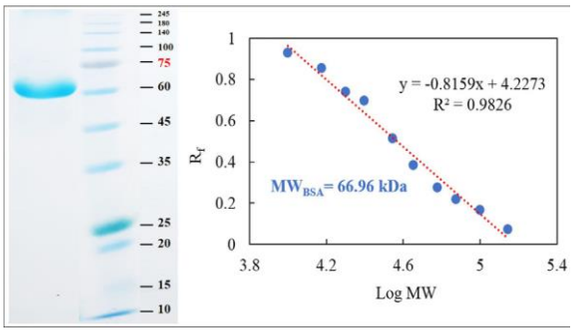


Fig. 3. Confirmation of BSA component of nanoparticles by SDS-PAGE. A) Electrophoretic pattern of nanoparticle. B) Standard curve of the SDS-PAGE for estimation of BSA molecular weight.

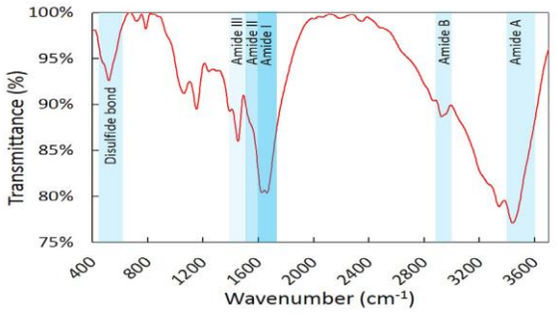


Fig. 4. Confirmation of protein characteristics of BSA-NPs by FTIR. Blue regions are indices of proteins FTIR spectra.

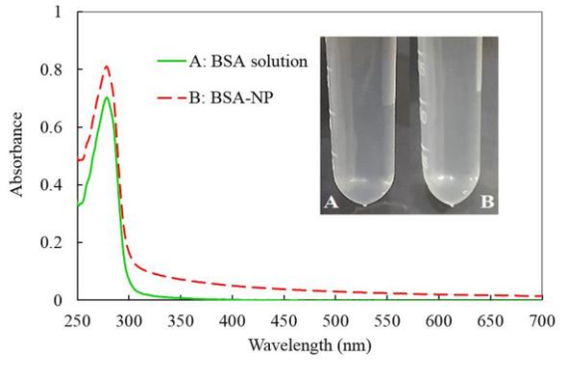


Fig. 5. UV-visible spectra and appearance of BSA solution, before (A) and after (B) nanoparticle formation.

Both BSA solution (A) and BSA-NP colloidal solution (B) had the same concentration.

The result of MIC and MBC. In our study, the MIC of BSA-NPs was determined against *P. aeruginosa* and *S. aureus*. The MIC of BSA-NP against *P. aeruginosa* ATCC27843 was evaluated to be 112×10^{-5} µM (Fig. 6A), and the BSA-NP did not show

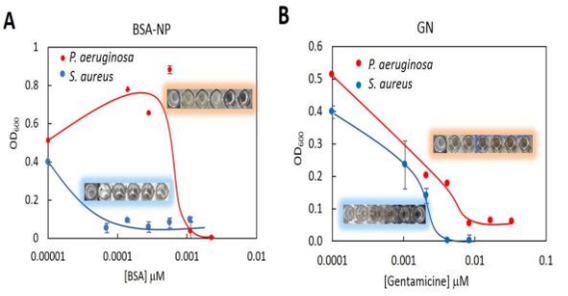
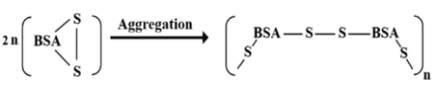


Fig. 6. Dose response curves from MIC experiments. A) Comparison of antibacterial activity of BSA-NP between *P. aeruginosa* and *S. aureus*. B) antibacterial activity of gentamicin on *P. aeruginosa* and *S. aureus* as control.

any antibacterial activity against *S. aureus* ATCC1431. Furthermore, after performing the MBC test, the result showed that the BSA-NP had not any bactericidal activity against *P. aeruginosa*.

DISCUSSION

Our hypothesis was to consider three important principles for the synthesis of BSA-NPs: 1- During the synthesis of BSA-NPs, the chemical and biochemical structure of BSA should be maintained as much as possible. One of the most important features of BSA is its functionality, i.e. the extraordinary ability of BSA in ligand binding, which is often used for drug delivery (17). 2- We considered ourselves committed to use a green and ecofriendly synthetic method: we did not use highly toxic chemicals such as 2-mercaptoethanol or glutaraldehyde, which are commonly used to form protein aggregates and nanoparticles (25). Additionally, urea and Tris which were used to dissolve BSA are natural or biocompatible ingredients. 3- The synthesis method of BSA-NPs should be cheap with available materials and equipment. In these regards, urea might have destroyed intra-molecular hydrogen bonds and hydrophobic interactions of BSA molecules to make possible inter-molecular exchange of disulfide (S-S) bonds between neighboring BSA molecules (26), resulting to their self-assembling and aggregation (Equation 1).



Equation 1

When urea molecules were removed by dialysis, new intra- and inter-molecular hydrophobic interactions as well as disulfide and hydrogen bonds were formed in BSA molecules. Finally, each BSA molecule is refolded and self-assembled in form of BSA-NP aggregates. In other reports, some methods including cross-linking (27), emulsification (28), nano-spray (29), and coacervation (30), have been used for synthesis of BSA-NPs. Using these methods, BSA-NPs have diameters more than 70 nm up to about 700 nm. Nanoparticles larger than 200 nm can activates the complement system, resulting in rapid clearance of the NPs from the bloodstream, but accumulates them in liver and spleen (31, 32). Furthermore, the methods have some additional disadvantages: The synthesis of nanoparticle due to cross-linking by glutaraldehyde (25) is a general method for any protein or molecules which contain amine group (-NH2), regardless of other intrinsic properties of the protein. This lead to eliminate the amine groups as potentially important functional groups for the protein function or structure. In addition, glutaraldehyde is a very toxic substance and even a small amount of its residue cannot be ignored in medical applications.

In this article, a very small BSA-NP with a diameter of 23.23±2.1 nm is reported (Fig. 1B) without any chemical modification on BSA molecules. The DLS spectrums of proteins are usually wide and numerous due to the presence of various reactive groups that cause progressive inter-molecular connections (33). But our nanoparticles showed a single and sharp peak (Fig. 1B). The micrograph of our nanoparticles showed their ability to make larger agglomerates when they were condensed during sample preparation for SEM (Fig. 2), because increasing the concentration of any solution during their drying causes an increase in inter-molecular interactions, especially in the case of proteins that have various functional groups on their surface.

Our BSA-NP has been synthesized based on the chemical properties of the BSA protein molecules themselves. To keep inherent structural and functional properties of BSA molecules we tried to minimize chemical modification and limited them to some disulfide rearrangement. Chemical integrity of BSA was confirmed by SDS-PAGE (Fig. 3) and FTIR spectrum (Fig. 4). The blue regions in FTIR spectrum represent the characteristic peaks of secondary structures of BSA molecule (34). The integrity of BSA molecules is important, because if nanoparticles are composed of pure and intact molecular populations of proteins, uniformity and well-defined surface morphology will obtain. This is a key factor in increasing the efficiency of nanoparticle application especially in medical applications (35, 36). Considering that BSA is a disulfide-rich protein, the peak of disulfide bonds confirms reformation of disulfide bonds in BSA-NPs (Fig. 4).

As a general rule, the amount of light scattered by particles depends on the size of the particles and the wavelength of the incident light (37). In UV-visible spectrophotometry, light scattering results in reduced transmission of light with a wavelength-dependent manner. According to this, as a result of nanoparticle formation (Fig. 5), the apparent absorption increased in all wavelengths, without increase in albumin concentration, which is a characteristic of nanoparticle production. Meanwhile, a relatively clear appearance of the BSA-NP colloidal solution is related to very small size of the nanoparticles, which has good compatibility with the results obtained by DLS.

As a rule, the smaller the nanoparticle, the greater the possibility of penetrating the cell membrane. Therefore, we investigated the possible antimicrobial effect of BSA-NPs on a Gram-positive (G+) bacterium and a Gram-negative (G-) bacterium as models. The MIC and MBC of the synthesized BSA-NP were studied against *S. aureus* and *P. aeruginosa* (Fig. 6) and the results show the bacteriostatic effect of the BSA-NP against *P. aeruginosa*. As can be seen in the Fig. 6A, the BSA-NP not only had not any antibacterial effect on *P. aeruginosa* at low concentration (56×10^{-5} μM), but also it was supported the growth of these bacterial cells. Furthermore, other components of the culture media in BSA-NP suspension can supply the possibility of *P. aeruginosa* growth too. At the concentration of 112×10^{-5} μM, with sharp slope, it reduced the *P. aeruginosa* growth significantly, and finally the bacterial growth was inhibited at a concentration of 225×10^{-5} μM. The cells of G- bacterial are encircled by a tiny peptidoglycan and an outer membrane (OM) containing lipopolysaccharide. In G+, the OM is absent, instead bacterial cells are surrounded by a thick layer of peptidoglycan. *Pseudomonas aeruginosa*, an opportunistic pathogen, has particular OM and outer membrane vesicles (OMVs). It was shown OM and OMVs a crucial role in bacterial virulence as well as its resistance to antibiotics (38). The differences of susceptibility in *Staphylococcus aureus* and *Pseudomonas aeruginosa* to the

BSA-NP can be attributed to the differences of their cell wall structures.

Additionally, the results showed that gentamicin reduced the growth of *P. aeruginosa* by 88.3% and the BSA-NP had a greater effect on *P. aeruginosa* strain than gentamicin. Albumin can induce immunomodulatory effects through binding to various microbial products (39), although there is not enough information about antibacterial activity of BSA-NPs. Arzumanian et al. studied the effect of different types of albumin on microbial strains, and confirmed its antimicrobial activity. They stated that albumin in concentration of 10 mg/ml can only disrupted the microbial cell membranes *in vitro*, whereas with 50 mg/ml concentration in serum, can disrupted the cell membranes (40). To the best of our knowledge, there is not any report related to BSA-NP on microbial cells. We guess that the BSA-NP can not only change the permeability of the cell membrane of bacteria, but destroy the cell wall peptidoglycans in bacteria and inhibit bacterial cell replication and translation processes. Although the BSA-NP showed bacteriostatic activity, they may be used in the treatment of infectious diseases in combination with other drugs. Furthermore, combination of the BSA-NP with other antibiotics, can reduce the dose of these antibiotics, used as treatment. Finally, it may be a novel approach to use less chemical drugs, which results reduction of antibiotic resistant bacteria. Antibiotic resistance is one of the biggest and worrying challenges of the century.

CONCLUSION

In this research, using the green synthesis method, we succeeded in synthesizing a very small BSA-based nanoparticle with a diameter of 23.23±2.1 nm without any chemical modification on BSA molecules. With a small polydispersity index (PDI=0.522), the nanoparticles had good spherical uniformity. The nanoparticles made from a single type of protein molecule (BSA) and have a relatively transparent appearance. Surprisingly, it also has bacteriostatic property against *P. aeruginosa*. Therefore, it is suggested that our BSA-NPs can be a new generation of antibiotics (protein-based nanobiotics) to combat antibiotic resistance, which may have wide application in medical, pharmaceutical and cosmetic industries in future.

ACKNOWLEDGEMENTS

Alzahra University is acknowledged for providing funds and facilities to carry out this research.

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