Regular Article

PHYSICAL CHEMISTRY RESEARCH
Published by the
Iranian Chemical Society
www.physchemres.org
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Phys. Chem. Res., Vol. 8, No. 2, 241-250, June 2020
DOI: 10.22036/pcr.2020.213260.1713

Silver Nanoparticle Trypsin Corona Formation and the Impacts on Enzymatic Potency

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(Received 25 December 2019, Accepted 22 January 2020)

Herein, the protein corona formation on the spherical metal nanoparticles is studied to investigate the possible effects of silver nanoparticles (AgNPs) on the protein activity and conformation. The digestion capability of trypsin was monitored on the human serum albumin (HSA) at standard enzymatic hydrolysis conditions in the absence and presence of different concentrations of AgNPs. So, the ratio of enzyme:HSA, the duration and the temperature of nanoparticle treatment were evaluated. The activity of treated trypsin molecules, in the form of hard (HC) and soft corona (SC) was studied using sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE), and nano liquid chromatography electrospray (ion trap) mass spectrometry (nano LC-ESI/MS, LC-ESI/MS). In addition, the characteristics of silver nanoparticles and the formation of HSA corona on the nanoparticle surface were monitored using ultra-violet/visible spectroscopy (UV-Vis), Dynamic light scattering (DLS) and fluorescence spectrophotometry. The results demonstrated that not only the corona formation but also the AgNPs/Trypsin interaction decreases the hydrolysis potency of trypsin. Furthermore, interaction of AgNPs/HSA could influence both nanoparticles and HSA molecule features, companied with fluorescence study, that the HSA secondary structure. Also, LC-ESI/MS data revealed the most affected HSA triptics have *α-helix* structure.

Keywords: Human serum albumin, Protein corona, Silver nanoparticles, Trypsin, Enzymatic potency

INTRODUCTION

Recently, nano-materials have gained great attention because of their increasing applications in various fields such as textiles, electronics, cosmetics, drug delivery systems, therapeutics, biosensors and the environmental pollution remediation [1]. Due to the concerns about their environmental and human health impacts the investigation of nano-materials toxicity is needed [2]. Silver nanoparticles (AgNPs) are one of the most populated metallic nanoparrticles, (NPs), especially because of their strong antimicrobial activity [3].

It's progressively believed that once NPs come into a biological system their surfaces are immediately covered by the bio-molecules such as proteins resulted in a structure known as "protein corona" [4]. The adsorbed protein molecules on NPs surfaces make some complexes stable in solution, along with making some changes on the physical, biological, and chemical properties of both NPs and proteins [5]. Hard corona (HC) layer consist of proteins absorbed and strongly bond on the NP surfaces and having low exchange rate with the environment [6]. The formation of corona is dynamic and time dependent equilibrated process;

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so that, at equilibrium time, HC has stable composition and contains only a few number of proteins. Those adsorbed proteins weakly bonded by the protein-protein interactions form the outer layer on NPs surfaces, with high exchange rate with the fluid, namely *SC* [4,7].

The question raised is that what happened to proteins while encountering NPs, regardless the SC and/or HC location; for example their possible enzymatic activity?" Previous studies verified that gold NPs disrupt the enzyme structure and subsequently had interrupting effects on their activity [4,8,9].

The aim of this study is to investigate the protein features after interaction with the NPs and corona formation. For this purpose, trypsin and spherical AgNPs were targeted to investigate the protein-protein interactions. The digestion capability of trypsin was assessed by human serum albumin (HSA) as substrate. Moreover, the characteristics of AgNPs and HSA were studied using different analytical techniques.

EXPERIMENTAL

Materials

Human serum albumin (HSA, MW: 66478 Da) and Trypsin were purchased from Bio test Co. (Germany) and Rush Co. (USA), respectively. *Staphylococcus aureus* PTCC1431 and *Escherichia coli* PTCC1399 were purchased from the Iranian Research Organization for Science and Technology (Iran). In throughout the experiments Milli-Q HPLC grade water was used. HPLC grade Acetonitrile and phosphate buffer saline (PBS) were obtained from Chem-lab NV Co. (Belgium) and cytomatin gel Co. (Iran), respectively. Ammonium bicarbonate, silver nitrate, three sodium citrate salt, polyvinyl alcohol (PVA MW: 22000 Da), acrylamide, N-methylene bisacrylamide, sodium dodecyl sulfateand other chemicals used in SDS-PAGE were obtained from Sigma Co. (USA).

Silver NP Synthesis and Characterization

The homemade AgNP colloid (100 μg ml^{-1}) was prepared by a chemically reduction procedure introduced previously [13]. Briefly, 0.4 M silver nitrate solution was added to aqueous solution contained 0.1% PVA, as surfactant, at stirring and heating condition to achieve 0.001 M silver nitrate solution. At boiling point of 97 °C ± 2, sodium citrate solution was added to the solution in a drop-wise manner. After 15 min, a yellowish gray colloid was acquired.

The optical properties of spherical AgNPs in the presence and absence of HSA were evaluated using a 2501PC Shimadzu Co. (Kyoto, Japan) UV-Vis spectrophotometer, to evaluate the spectra having a λ_{max} 418-430 nm.

Hydrodynamic diameters of polyvinyl alcohol coated AgNPs in the presence and absence of HSA were characterized using a Nanophox DLS equipped with 632.8 nm He-Ne laser from Sympatec Co. (Clausthal-Zellerfeld, Germany), in the range of 0.1-100000 nm.

For biological activity evaluation, minimum inhibitory concentration (MIC) of the synthesized AgNPs was assessed against *Staphylococcus aureus* ATCC25923, and *Escherichia coli ATCC25922* as described previously [10]. The variations causing by the AgNPs treatment with HSA, were monitored using DLS and UV-Vis, in the case of size, homogeneity and spectrum.

Evaluation the Treatment of HSA with AgNPs

First of all, HSA sample was purified using a cellulose ester tube membrane with a MWCO (molecular weight cutoff) of 3000 Da against 40 mM ammonium bicarbonate at pH 8 for removing any probable interfering substances. In order to examine the AgNPs stability in treatment condition (1 mM HCl, pH 3), AgNPs colloid were prepared at different concentrations in same solution and incubated at 37 °C for 6 h, and the stability was examined by evaluation of their hydrodynamic size using DLS instrument. HSA at the concentration of 10 μg μl^{-1}, treated with different concentrations of AgNPs colloid, was prepared and incubated for 6 h in 37 ºC. The incubation influences on AgNPs stability were examined comparind the controlling conditions (experiment in the absence of HAS) (1 mM HCl, pH 3, 37 °C, 6 h).

Secondary structure changes in HSA were examined using tryptophan (Trp^{214}) Fluorescence spectroscopy Perkin Co. (USA) instrument. Excitation, emission wavelengths and slits were functioned at 295, 350 and 10 nm, respectively.

Treatment of Trypsin with AgNPs

Trypsin solution (0.1 μg μl^{-1}, 40 μl) was mixed with 30 μl AgNP colloid and 30 μl of 1mM HCl, pH 3, incubated at 37 °C for 6 h.

Separation of hard and soft corona. A mixture of *HC*, *SC* and unbound proteins was separated and kept for further analysis. For doing so, the remaining solution was centrifuged at 8000 rpm, 4 °C, for 10 min; so that NP-trypsin complexes were pelleted. The supernatant containing unbound proteins was separated and the pellet washed three times with 20 μl of PBS at 10000 rpm, 4 °C, for 10 min. For this, the *HC* and *SC* were separated. To each one, 10 μl HSA solution was added and each sample was incubated at 37 °C for 18 h for digestion of HSA. Then, the samples were analyzed using 12% SDS-PAGE and Nano LC-ESI/MS.

Sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE). To examine the HSA tryptics activity, the SDS-PAGE analysis was performed according to standard protocol of lammeli. The above mentioned samples were loaded onto 12% acryl amide gel. Silver nitrate staining was performed as illustrated elsewhere.

Nano LC-ESI/MS. Nano LC-ESI-MS experiments were performed on *Platin Blue* Knauer Co. (Germany) equipped with a binary gradient pump, auto sampler, vacuum degasser and temperature controlled column compartment conjucated with a Finnigan TMLCQTMDECA mass spectrometer ion trap system Thermo fisher Scientific (USA). For separation, the C_{18} column (250 mm, 1.5 mm, 5 μm, 100 Å) of Thermo scientific Co. (USA) was used. The injection volume was 5 μl. The nano LC-ESI/MS data were acquired for 35 min, in gradient manner of 0-100 acetonitrile with 5 μl min^{-1} flow rate. In order to identification of peptides produced by trypsin, 5 μl samples were injected to the LC-MS system, Knauer Co. (Germany). For shortening distance between the column terminuses and capillary of MS, capillary of nano column, attached to MS, directly. Peptides obtained from HSA proteins were analyzed by *Xcalibor*2SR.2 program.

RESULTS AND DISCUSSION

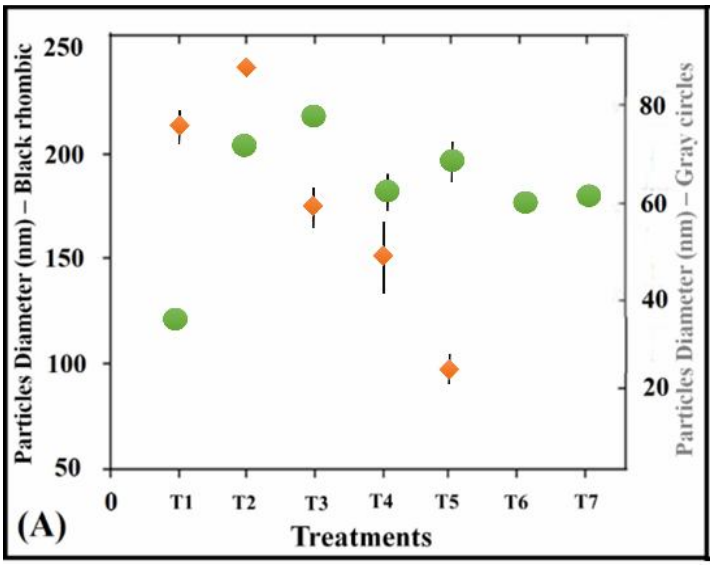
AgNPs Characterization

In our recent study [10], the characteristic of home-made spherical AgNPs has been discussed. Briefly, the hydrodynamic size was recorded in the range of 20-40 nm with a narrow size distribution at DLS; 420 nm λ_{max} in UV-Vis absorbance. The X-ray powder diffraction patterns showed crystalline planes of cubic Ag crystals with the highest peak intensity related to 111 [10]. The MIC for the synthesized AgNPs against *Staphylococcus aureus*, and *Escherichia coli* were ~3 and ~2 μg ml^{-1}, respectively, in comparison with cefixime. Based on our previous studies, among the antibiotics, cefixime has a pit formation mechanism similar to that of silver nanoparticles [10].

AgNPs-HSA Interaction

DLS studies of AgNPs. The influences of AgNPs on HSA have been evaluated by DLS analysis. The DLS results (T_1 to T_5) demonstrate that by decreasing the AgNPs/HSA ratio, hydrodynamic diameter is reduced and by increasing the ratio, this effect is increased (Fig. 1A, Orange Rhomb). In addition, T_1 and T_5 show maximum and minimum diameter, respectively. It revealed that the increase in protein/copolymer NPs ratio is resulted in reduction of the corona size [5]. The NPs aggregation can occur by NPs cross-linking assisting by proteins. Proteins adsorbed and denatured on the NPs surfaces [6]. It has been shown that with the increase in plasma concentration, silica NPs zeta potential was decreased, which may be due to the particle stability in plasma. These were confirmed by DLS measurements. Moreover, goy-Lopez *et al.* showed that when NPs enter the cell medium, prior to NPs aggregation, steric repulsion rapidly rises, so that, NPs meet proteins before they face each other [9]. Our findings are in agreement with these recent publications.

AgNPs colloid stability surface plasmon resonance in the presence of HSA. Spherical NPs have an individual plasmon band along one dimension (radius) [9]. The NPs-complex UV band broadening and the red shift correspond to the size and aggregation states. For spherical rough AgNPs, surface plasmon resonance (SPR) occurred at near 400-450 nm. The SPR adsorption intensity gives information about the particle concentration, and the SPR peak position presents size and shape of particles. To investigate the amount of AgNPs SPR stability in the presence of HSA, different concentrations of them were



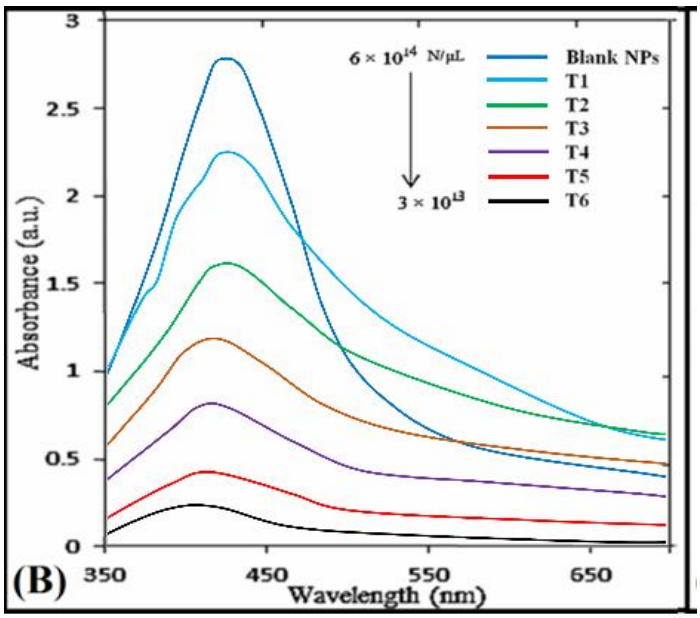


Fig. 1. (A) Green circle: DLS measurement for different concentrations of AgNPs treated with HCl 1 mM, in the HCl: AgNPs ratios of T1-0:100, T2-50:50, T3-60:40, T4-70:30, T5-80:20, T6-90:10, T7-95:5, (v/v), Orange Rhomb: Recorded hydrodynamic diameters for different concentrations of AgNPs colloid used for treatment of constant amount of HSA. With increasing in HSA/AgNPs ratio ($T_1 \rightarrow T_5$), AgNPs size was decreased, maybe because of corona formation which resulted in particle aggregation reduction. (B) The positions of AgNPs SPR bands, as particle count in the presence of HAS solution, are reduced. It shows a blue shift in λ_{max}, which could be attributed to increase in creation of shell of proteins around AgNPs and monomeric like distribution. (C) Fluorescence emission spectra of HSA- AgNPs in bicarbonate buffer, pH 8.5, and λ_{max} = 340 nm.

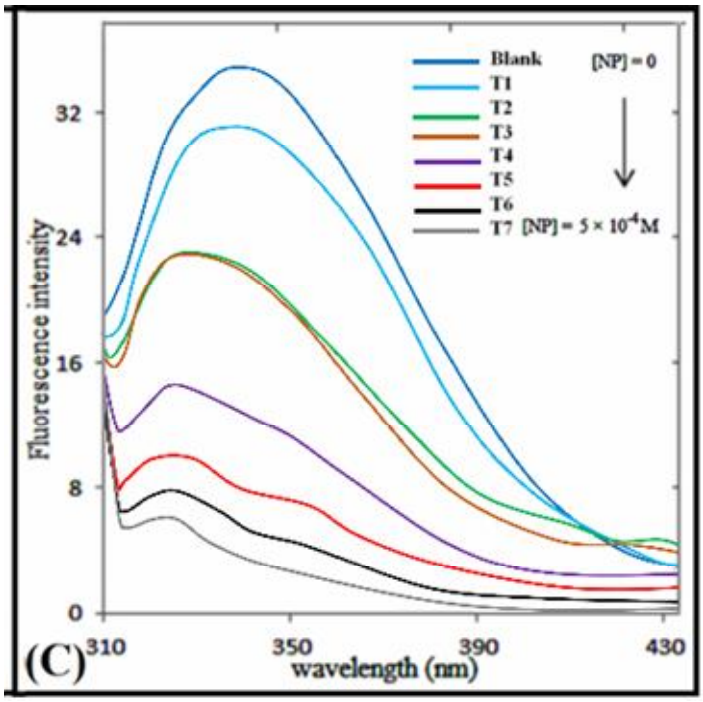


Fig. 1. Continued.

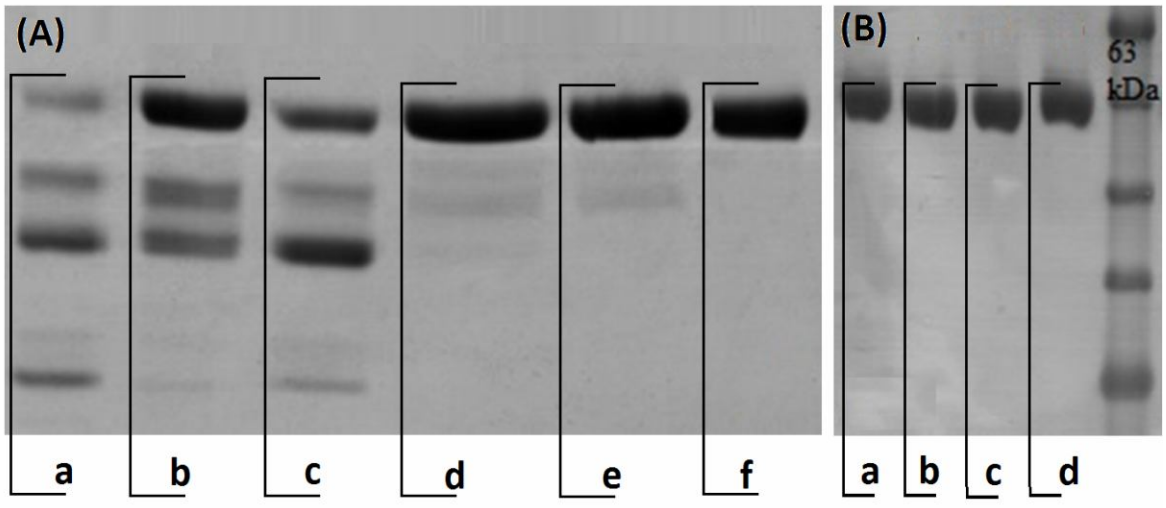


Fig. 2. (A): The SDS-PAGE analysis of HSA hydrolysis with AgNPs treated trypsin; this figure shows digested band resulted from HSA by a: untreated trypsin (as control), b: HAS digested by AgNPs treated trypsins, c: HSA digested by supernatant of AgNPs treated trypsin, d: HSA digested by soft corona and e: HSA digested by hard corona in comparison with f: non digested HSA. (B) The SDS-PAGE analysis of HSA in the presence of 3 different concentrations of AgNPs which shows no digestion has been occurred *via* AgNPs itself; a) untreated HSA and b: with 1/1, b: 40/100 and d: 10/100 v/v of NPs/HSA ratio.

treated with a constant concentration of HSA. Based on the previous results, protein corona along with different interaction times could stabilize the nanoparticles and shifts the resonance wavelength to a longer wavelength [11]. It was expected that in the presence of proteins, AgNPs aggregation decreased while their stability increased. Moreover, with an increase in protein concentration, the λ_{max} is shifted to a longer wavelength [12,13].

The results revealed that by increase in the NPs content, T_6 to T_1, the λ_{max} intensity was increased (Fig. 1B). Red shifts in SPR bands are witness on the increments in NP size and aggregation. Moreover, the increase in AgNPs, is followed by a growth in accumulation and red shift in λ_{max} [14]. Addison and Brolo showed that increasing the deposited gold NPs is accompanied by the red shift in λ_{max} and integrity adsorption coefficient, due to NPs affinity for surface energy minimizing, under "Ostwald ripening" [9]. Therefore, red shift in λ_{max} decrement is due to the decrease in AgNPs amount and particle aggregation.

Now, the next question is "what is the HSA role"? NPs colloidal stability rises with rising the protein/ NPs ratio. It showed that shifts to the shorter wavelength (blue shift) happened by decrease in AgNPs number ($T_1 \rightarrow T_6$) and increase in the HSA/AgNPs ratio. With reduction in particle content, the λ_{max} blue shifts were not significant (~2-3 nm), this mild effect was attributed to HSA role and rise in AgNPs stability because of decrease in aggregation due to corona forming, followed by the red shifts in UV-Vis spectra. This phenomenon and last discussions are witness on corona shaping on AgNPs surfaces.

Fluorescence studies of human serum albumin. HSA in the presence and absence of NPs has the maximum emission wavelength around 342 nm [15]. Fluorescence quenching (FQ) spectra for HSA solution in blank and AgNPs treatments were monitored at 300-450 nm.

With the increase in AgNPs concentration, the fluorescence intensity (FI) has decreased, (Fig. 1C). HSA has a tryptophan residue (Trp^{214}) in sub domain IIA ligands site, which has an important physiological role and it makes protein, sensitive to the environmental condition. It was mentioned that Trp^{214} is responsible for FI [10]. The FQ is related to the distance between chromosphere and quenching agent [16]. The FQ data confirmed that changes in HAS conformation by the *α-helix* content was about 57.2%, while after adsorption on cholesterol NPs were reduced. They showed that the maximum HSA emission was decreased along interaction with NPs, due to reduction in *α-helix* content. Besides, surface hydrophobicity has higher effect than treatment time on the FQ. Thus, interacting forces among chromosphere and NPs, is crucial in the FQ. For example, electrostatic interaction of Ag(I) *via* negative coating of AgNPs and positively charged residues besides hydrogen interfaces with amines and carboxylic groups are mentioned. Observed reduction in FI was related to the conformational changes in HSA and interaction of foreigner molecules and finally distance shortening between them. Also, increase in NPs was accompanied by a rise in quenching effects ($T_7 \rightarrow T_1$) [Fig. 1C] [2,17].

Study of the Digestion of HSA Using AgNPs Treated Trypsin

AgNPs stability in electrolyte solution. It was revealed that the proteins adsorption on the NPs surfaces, due to the change in NPs Brownian motion, can increase their size, and over the electrostatic or steric effects, prevent their aggregation [3]. On the other hand, trypsin acidic hydrolysis condition, with a high electrolyte content, will influence on AgNPs stability that may lead to a level of aggregation. Therefore, the AgNPs colloidal stability should be studied in 1 mM HCl solution, pH 3, according to the preparation instructions. In order to study the NPs maximum impact on trypsin structure at different states, the DLS study was performed. Regarding to DLS, (Fig. 1A, Green circles), increasing the AgNPs size at T_1, T_2, T_4 treatments those containing nearly 1.806×10^{14} number/µl AgNPs. This increase was more than the increase in the case of T_3, T_5, T_6, T_3 condition (containing nearly 9.87×10^{11} number/µl trypsin).

SDS-PAGE analysis of trypsin digested HAS. The investigation of AgNPs toxicity on human enzymes has not been so extensive. In this report, trypsin activity in strong and weak binding to NPs, was studied. The question was that "Is enzymatic activity of trypsin influenced by its interaction with AgNPs?" Initially, SDS-PAGE was used to monitor the impacts of AgNPs on the trypsin digestion potency. The results (Fig. 2a) showed that only native trypsin could create four HSA peptide species with different intensities. In Fig. 2B, it is obvious that AgNPs at different concentrations in the HSA solution have no role in HSA cleavage, on the other hand, the digestion activity was only

attributed to trypsin.

NanoLC-ESI/MS of tryptic species. One of the most important purposes of this research was to investigate the AgNPs effect on trypsin at molecular scale, thus nano LC-MS was used, (Fig. 2A). It was observed that under hydrolysis condition by native or different trypsins that treated with varied concentrations of AgNPs, the intensity of bands varied. Results indicated that tryptic species and their intensities in hard and soft trypsin corona, in comparison to native trypsin, show some decrements. Moreover, initial amount of trypsin was very small, in a way that we could not determine the adsorbed proteins quantitatively. Not only numbers of these lanes are reduced by treated trypsin, but also their intensities were reduced significantly. Trypsin treated by AgNPs, create specie varied molecular mass peptides. Results shows that the studied peptides had no binding to AgNPs surfaces.

Albumin has 609 amino acids, average molecular weight of 69633.68 Da and isoelectric point, pI = 5.92 (http://www.uniprot.org/uniprot/P02768). $[M+H]^{+}$ and $[M+H]^{+2}$ of tryptic HSA were taken from http://web.expasy.org/peptide_mass/, miss cleavage = 3, in 0-2000 Da. The most HSA cleavage sites are present in 187-243, 439-469 and 566-597 Da range, which their secondary structures are shown in Table 1.

Thorough peptide sequences identified, QRLKCASLQKFGER, YICENQDSISSKLK, CCKHPEAKR and RDAHKSEVAHR were available in overall treated trypsin tryptics activity. On the other hand, its catalytic triad has hydrolysis capability. ETCFAEEGK, AAFTECCQAADKAACLLPK and EQLKAVMDDFAAFVEK were present in hard corona solution, but QRLKCASLQK peptide is at soft corona only (Fig. 3). About peptides with 588 and 831 Da molecular weight, nominated with star in Table 1, interesting annotations are observable that verify some assumptions.

The peptide having 588 Da molecular weight is similar but shorter than peptide having 831 Da MW. This residue (hydrolyz along with arginine residue) observable in all treated trypsin. However, 831 Da fragment, hydrolyzed along with Lysine residue, was emitted in hard corona. The other peptide couples were 757 and 963 Da that indicated the same comment. Peptide by 760 Da was only in the mixture solution, both cleavage sites are hydrolyzed along with lysine residue. The sequence having 653 Da MW, that hydrolyzed along with Arg, observable in both in whole digesting tests [9]. This observation confirms the interaction of AgNPs with Lys residue in HSA cleavage site. The AgNPs have a preferential interaction with Lys local pair electron compared to Arg, but Arg's pair electron is unlocal involving in resonance structure. On the basis of density functional theory (DFT) calculations, three basic amino acids, arginine, lysine and histidine are the strongest binder for Ag(I); in agreement with other DFT-based studies [26]. It should be noted, in regarding to trypsin pI = 8.0 and HSA pI = 5.92, at experiment condition of pH ~ 7.0, trypsin is positively charged but HSA is negatively charged, so, a favored interaction with trypsin molecules exists for AgNPs. Besides, since the initial amounts of AgNPs have been excess at ratio to trypsin molecules, still numerous free or even aggregated AgNPs in hard trypsin solution were remained which can interact with additive HSA for digestion test. These interactions can create steric repulsion for targeting interfaces [4,7,9]. Binding of over the remaining non-bounded AgNPs with amine group of amino acids involving in cleavage site in target protein creates a steric repulsion for the same or other cleavage sites in HSA molecules.

5.

6. CONCLUSIONS

Results demonstrated that trypsin bioactivity is considerably decreased through the interaction with AgNPs; this effect in hard state is much more than that in soft corona. This research confirmed that partial binding to NPs surface in soft state is accompanied by diminishing activity. AgNPs can interact with basic residues and those are present in catalytic site in trypsin, moreover their effects on HSA lysine residues are verified by means of mass spectrometry. Human serum albumin has a physiologically importance in blood, for example, since having flexible conformation, it contains ligand sites transferring diverse molecules such as oleic acid. On the basis of DLS measurements, its interaction with AgNPs causes albumin corona formation on AgNPs surfaces. The UV-Vis spectra showed the HSA influence in reduction of AgNPs aggregation and decrease the particle stability and steric repulsion. The AgNPs FQ analysis confirm an *α-helix*

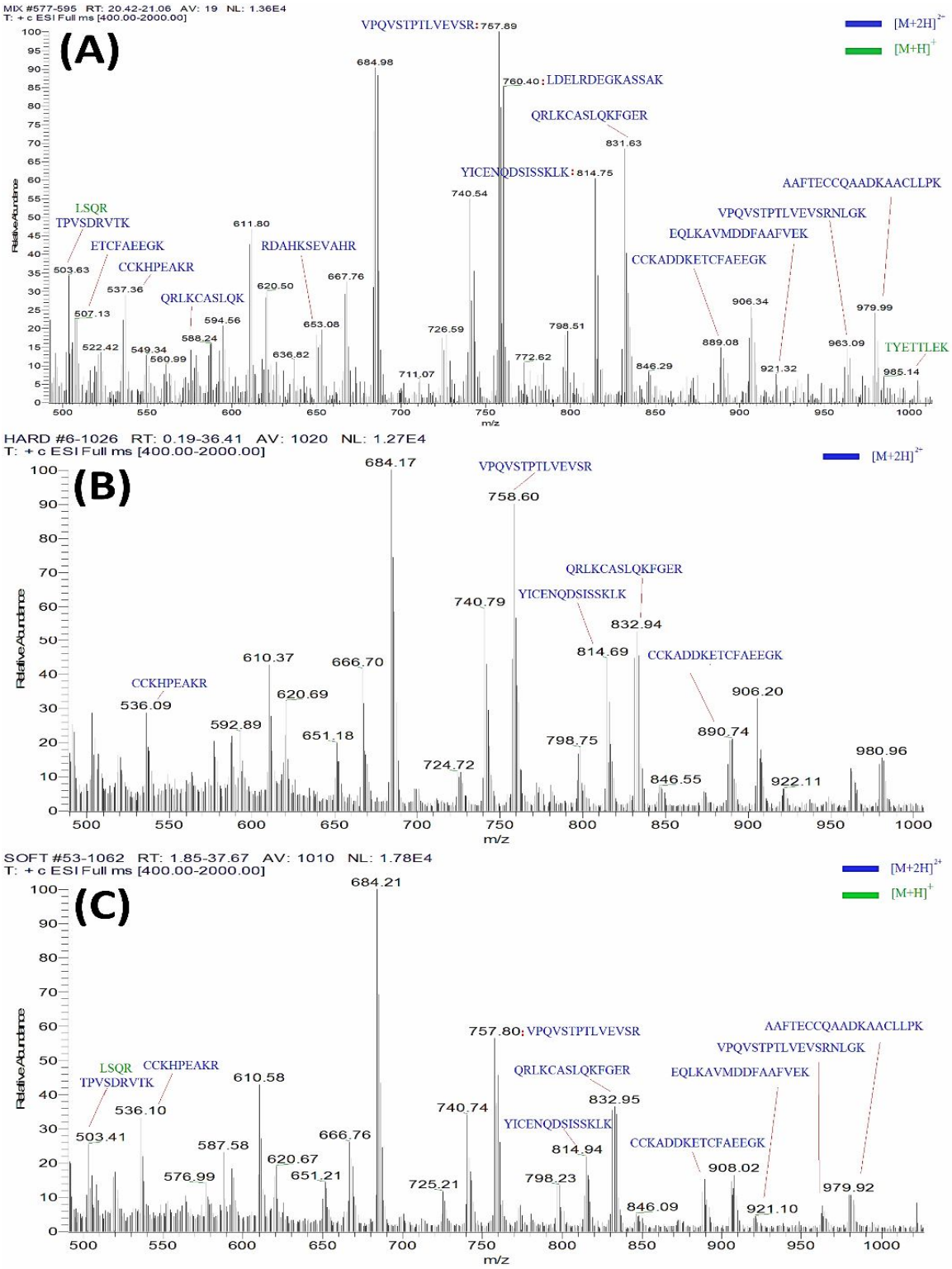
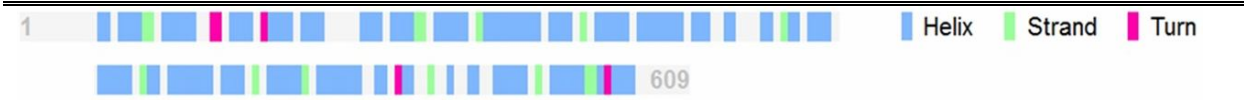


Fig. 3. Nano LC-ESI-MS analysis of (A): not treated HSA and (B): Hard corona and (C) soft corona of HAS after AgNPs treatment.

Table 1. Identified Created Species in HSA by Trypsin



Mass weight	Amino acid sequence	Position	Secondary structure	Graphical view
503	TPVSDRVTK	491-499	-	
507	ETCFAEEGK	588-597	Turn	
537	CCKHPEAKR	461-469	-	
588	QRLKCASLQK*	220-229	Helix	
653	RDAHKSEVAHR	24-34	Helix	
757	VPQVSTPTLVEVSR*	438-502	-	
760	LDELRDEGKASSAK	206-219	Helix	
814	YICENQDSISKLK	287-300	Helix	
831	QRLKCASLQKFGER*	220-233	Helix	
889	CCKADDKETCFAEEGK	582-597	βstrand, Helix, Turn	
921	EQLKAVMDDFAAFVEK	566-581	Helix	
963	VPQVSTPTLVEVSRNLGK*	438-456	-	
979	AAFTECCQAADKAACLLPK	187-205	~ Helix	

Bioinformatic results were study on UniProt (http://www.uniprot.org/uniprot/P02768) with F88FF61DD242E818 as the protein code. *: was mention in discussion.

content reduction and interval shortening between chromophore and AgNPs surfaces.

ACKNOWLEDGEMENTS

Financial support from the Research Council of Shahid Beheshti Universityand is grate-fully acknowledged. This publication represents a section of the MSc thesis of Khorshid Darvishi-Ganji at the Department of Chemistry, Faculty of Chemistry of Shahid Beheshti University, Tehran, Iran.

CONFLICT OF INTERESTS

The Authors mentioned that there is no conflict of interests.

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