

Original Article

Cytotoxicity of Silver Nanoparticles Coated with Different Proteins on Balb/c Mouse Macrophage

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ABSTRACT

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Background and Aims: Coated nanoparticles have different surface chemistry, aggregation, and interaction properties. The aim of this study was to investigate the cytotoxicity of silver nanoparticles AgNP_s coated with different proteins on Balb/c macrophages.

Materials and Methods: In this study these items were evaluated: 1) the size of aggregation, 2) the quantity and mechanisms of uptake, and 3) the biological impact of Ag NPs and protein coated Ag NPs. Human serum albumin, bovine serum albumin, fetal calf serum, and pea nut agglutinin were used as coating agents.

Results: This research showed that all coated Ag NPs had smaller aggregate size, more uptake, and less biological impact on Balb/c macrophages than naked Ag NPs. Endocytosis mediated pathway was the main uptake mechanism. Also, a clathrin-mediated pathway was shown to regulate their uptake.

Conclusion: It can be concluded that mitochondrial activities, cellular metabolic function, and ATP level of cells treated with protein coated Ag NPs are higher than naked Ag NPs after 24-hour incubation.

Introduction

Nanoparticles way quickly adsorb varios components when entered into a biological medium. Surface chemistry, shape, and aggregation of nanoparticles change after adsorption of such materials. This phenomenon leads to new interactions, which are different from natural or naked nanoparticles [1]. Nanoparticles have a high local charge density with high specific surface area, and can interact or react with different components [2].

It was shown that nanoparticles of ceria oxide, fullerenes, polystyrene, magnetite, titanium dioxide, tungsten carbide, and zirconia have low aggregation in cell medium because of protein adsorption [3-9]. More importantly, it has also been reported that the most important factor in protein adsorption is the eventual size of nanoparticles [6,10]. On the other hand, it has been demonstrated that the formation of protein corona or layer on the surface of nanoparticles depends not only on the size of nanoparticles but also on the medium ingredients. Interestingly, Dulbecco modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS) do not affect the size of nanoparticle, but Roswell Park Memorial Institute medium (RPMI) supplemented with FBS increases the size of gold nanoparticles [11].

Previous studies have shown that nanoparticles adapt the properties of the adsorbed protein [12-14]. For example, gold nanorods and magnetite nanoparticles coated with polyethyleneamine which have a negative

charge, switch to positive charge after serum protein adsorption [15, 16].

Several researchers have proposed that adsorbed proteins raise nanoparticle uptake by receptor endocytosis, but the exact mechanism of nanoparticle uptake has not yet been understood [5, 16, 17]. It has been shown that the capacity of nanoparticles in protein adsorption affects the nanoparticle-cell association, but does not depend only on the individual characteristics of the adsorbed proteins [18, 19]. In contrast, a reduction of uptake has been recorded after protein adsorption of magnetite, carbon, and gold nanoparticles [17]. It has also been observed that there is no significant difference in the internalization of nanoparticles after changing surface functional groups i.e. COOH and NH₂ [20].

Physicochemical characteristics, cellular internalization, and toxicity of nanoparticles are changed by adsorption. For example, Poloxamer surfactant (Pluronic F127) which has been used for dispersion of single-walled carbon nanotubes (CNT) and silica nanoparticles, leads to significant protein adsorption and low toxicity for macrophage-like cell line [21].

Toxicity of CdS nanoparticles decline significantly on different cell lines in serum-free medium [22]. The lower toxicity of gold nanorods for human cervical cancer cells (HeLa cells) has been recorded when media contains serum [23]. In another study, Clift et al. measured tumor necrosis factor-R

production and intracellular glutathione (GSH) levels when polystyrene nanoparticles were added to J774.A1 macrophage-like cells, and indicated that protein coated nanoparticles are less toxic than naked nanoparticles. This effect was not shown in smaller nanoparticles [24]. Other molecules can change nanoparticle interaction and toxicity, too. Polyethylene glycol (PEG) and dextran can be coated on iron oxide nanoparticle, and induce a reduction of cytotoxicity [25].

Bovine serum albumin (BSA) can act as a capping agent and contributes to increase uptake [26]. Although there are some studies on toxicity of nanoparticles after medium protein adsorption, but there is no comparative study of different proteins including BSA, Serum albumin (HSA), fetal calf human (FCS), and pea nut agglutinin (PNA) after coating on Ag NPs. On the other hand, there is little information on toxicity of coated silver nanoparticles (Ag NPs). Thus, the aim of this study was to evaluate the aggregation size, uptake quantity, and biological impact of naked and coated Ag NPs with different proteins including BSA, HSA, FCS, and PNA.

Materials and Methods

Ag NPs were sourced from Lolitech Co. Germany. HSA, BSA, FCS, PNA, HCl, HF, nystatin, sucrose, sodium azide, 2-deoxy-D-glucose 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Hank's balanced salt solution (HBSS), and RPMI-1640 were provided from Sigma, Germany. Alamar Blue reagent and CellTiter-Glo Luminescent cell viability reagent were

purchased from Invitrogen, UK, and ten male BALB/c mice were provided from Pasteur Institute, Tehran, Iran.

Preparation of naked and protein coated Ag NPs

Ag NPs with average size of 25 nm were used in this research. Ag NPs were manufactured by reacting silver nitrate and sodium citrate. Ag NPs were ball milled for 5 minutes after centrifugation, washed with distilled water, dried in the atmosphere, and then sterilized at 121 °C for 20 minutes by autoclave. In this study, serial concentrations of Ag NPs were prepared in distilled water to final concentrations 2000, 1000, 500, 250, 125 µg/ml. In the next step, the nanoparticle suspension at concentration of 2000 µg/ml was incubated with different proteins (HSA, BSA, FCS, and PNA) at concentration of 2 mg/ml for 2 hours at 37 °C. After incubation, the mixture was centrifuged at 10000 rpm and the supernatant collected. Finally, pellets were resuspended in RPMI-1640 and serial concentrations (2000-1000-500-250-125 µg/ml) of protein coated nanoparticle were prepared similar to the method for naked Ag NPs.

Characterization of nanoparticles

The structure, size, and composition of naked and protein coated Ag NPs were characterized by scanning electron microscopy (SEM) (Hitachi S-2400, Japan), dynamic light scattering (DLS) (Malvern Instruments, Italy), and Fourier transform infrared spectroscopy (FTIR) (ELICO, India). For preparation of SEM images, nanoparticles were dried on a copper holder and coated with a thin layer of

gold by sputtering. For DLS assay, naked and protein coated Ag NPs were dispersed in the RPMI-1640 and were tested by DLS apparatus for determination of particle size distribution at 37 °C. For protein adsorption confirmation, UV-visible spectrophotometer (ELICO, India) at 280 nm and FTIR were used at the range of 500-3500 cm^{-1} .

Preparation of peritoneal macrophages and measurement of nanoparticle uptake

To obtain peritoneal macrophages, 5 mL of RPMI-1640 was injected to peritoneal cavity, and after 30 minutes, all the fluid in peritoneal cavity was aspirated and centrifuged. The peritoneal macrophages were suspended in RPMI-1640 to reach the final concentration of $5 \times 10^4/\text{mL}$ and were separately incubated with serial concentrations of coated and naked Ag NPs for 24 hours at 37 °C with 5% CO_2 . Next, treated cells were washed three times with HBSS to remove non-bonded nanoparticles. Cells were treated with 10 M HCl for 12 hours and were then reacted with 60% (w/w) HF for 12 hours at room temperature. The entire content of each sample was diluted 10 times and the Ag element was quantified by atomic absorption spectrometer (Model 603, Perkins-Elmer, USA).

The study of uptake inhibition

In order to determine uptake mechanism, macrophages ($5 \times 10^4/\text{mL}$) were separately exposed to inhibitor treatments for 2 hours at 37 °C, and then washed with HBSS, as proposed in another study [16]. Inhibitor treatments included:

- (1) The use of 60 μM nystatin
- (2) The use of 0.5 M sucrose

(3) The use of 60 mM sodium azide and 2-deoxy-D-glucose

(4) The use of incubation at 4 °C

After treatment with inhibitor, cells were exposed to serial concentrations of naked and coated Ag NPs. Then, cells were washed with HBSS and the quantity of nanoparticle uptake was measured as described previously.

The study of cell metabolic status

Macrophages were incubated with naked and coated Ag NPs for 24 hours at 37 °C, and then were washed three times with HBSS. Then, 100 μL of RPMI-1649 and 25 μL of the AlamarBlue reagent were added to each well and incubated 4 hours at 37 °C.

The cell metabolic activity was measured by reading optical density (OD) at 590 nm, induced by reduction of AlamarBlue using micro plate reader (Novin gostar, Iran); this, however, was not done with the control group. Macrophages which were not exposed to nanoparticles were considered as the control group.

Assessment of cell mitochondrial function

At First, the cells were incubated 24 hours with naked and coated Ag NPs, and were washed three times with HBSS. Then, 100 μL of RPMI-1640 and 25 μL of MTT at concentration of 5 mg/mL were added to each well and incubated at 37 °C for 4 hours. Later, 100 μL isopropanol 70% was added to each well and was incubated for 30 minutes. After incubation, OD of each well was measured at 490 nm by micro plate reader, and was compared with the control group which was not treated with nanoparticles.

Evaluation of cellular energy

After 24-hours incubation with naked and coated Ag NPs and washing with HBSS, 100 μ l of CellTiter-Glo Luminescent cell viability reagent was added to cells and was incubated 10 minutes until stabilized luminescence signals appeared. Then ATP level was quantified by luminometer (Turner Biosystems, model 9100-102, USA), and was compared with the control group.

The study design was approved by the Ethics Committee of Shahid Beheshti University of Medical Sciences.

Statistical analysis

The means and standard deviation of each group was calculated. Statistical comparison of multiple groups was done using one-way ANOVA. The statistical tests were carried out using SPSS software (V.16.0 for Windows; SPSS Inc, USA.), and statistical significance value of differences was set at $P < 0.05$.

Results

Characterization of coated nanoparticles

Figure 1 shows SEM images of naked Ag NPs (a) and Ag NPs coated with HSA (b), BSA (c),

FCS (d), and PNA (e). These images demonstrated that the size of all coated Ag NPs was approximately 25 nm, but naked Ag NPs was agglomerated. Figure 2 displays the distribution size of naked and coated Ag NPs, obtained by DLS. Consistent with SEM images, DLS results also indicated that all coated Ag NPs had smaller aggregates in RPMI1649 medium than naked Ag NPs. Conversely, naked Ag NPs had mixtures of both micrometer and nanometer aggregates in cell culture medium. To confirm protein adsorption on Ag NPs surface, FTIR and UV-visible were used, the results of which are shown in Figures 3 and 4, respectively. FTIR spectrum revealed the presence of amide band I (1650 cm^{-1}), amide band II (1550 cm^{-1}), and NH stretching vibrations – amide A and B ($3170\text{--}3300\text{ cm}^{-1}$) for all coated Ag NPs. This pattern was not seen in the naked Ag NPs. Also, UV- visible spectrum showed a reduction of OD in the protein solution at range of 200-300 nm after incubation with Ag NPs. Both FTIR and UV-visible results indicated that proteins were adsorbed on Ag NPs surface.

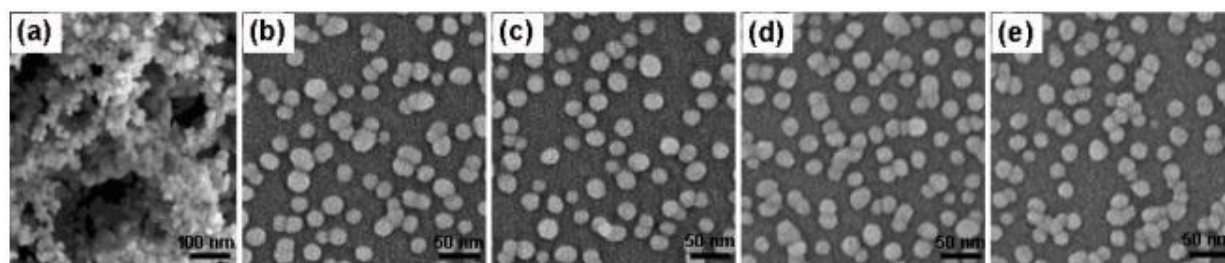


Fig. 1. SEM images of naked Ag NPs (a) and Ag NPs coated with HAS (b), BSA (c), FCS (d), and PNA (e).

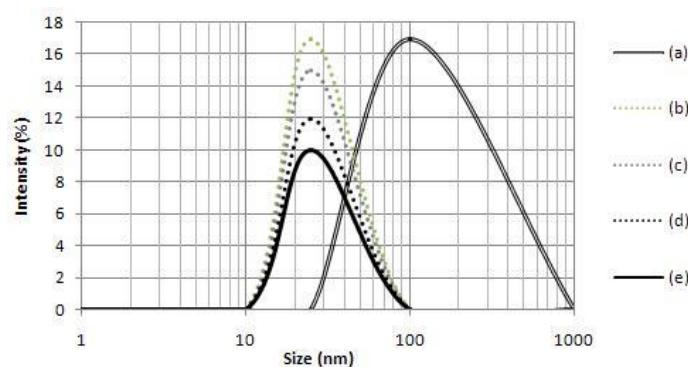


Fig. 2. Size distribution of naked Ag NPs (a) and Ag NPs coated with HAS (b), BSA (c), FCS (d), and PNA (e). All nanoparticles were suspended in RPMI-1640 and size distribution was carried out by DLS at 37 °C.

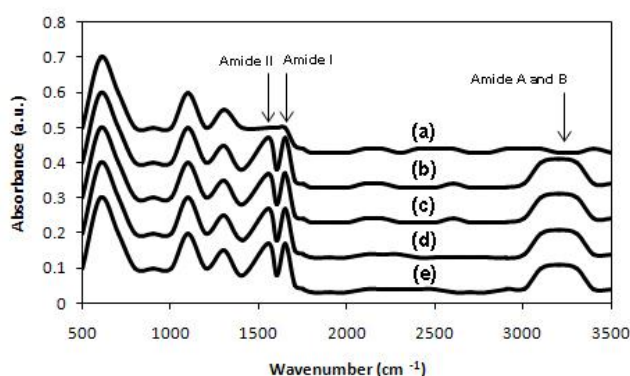


Fig. 3. FTIR spectrum of naked Ag NPs (a) and Ag NPs coated with HAS (b), BSA (c), FCS (d), and PNA (e). For FTIR experiment, all nanoparticles were separately dried on KBr and scanned at 500-3500 cm^{-1} . Amide band I, amide band II, and amid A&B were seen in all coated Ag NPs.

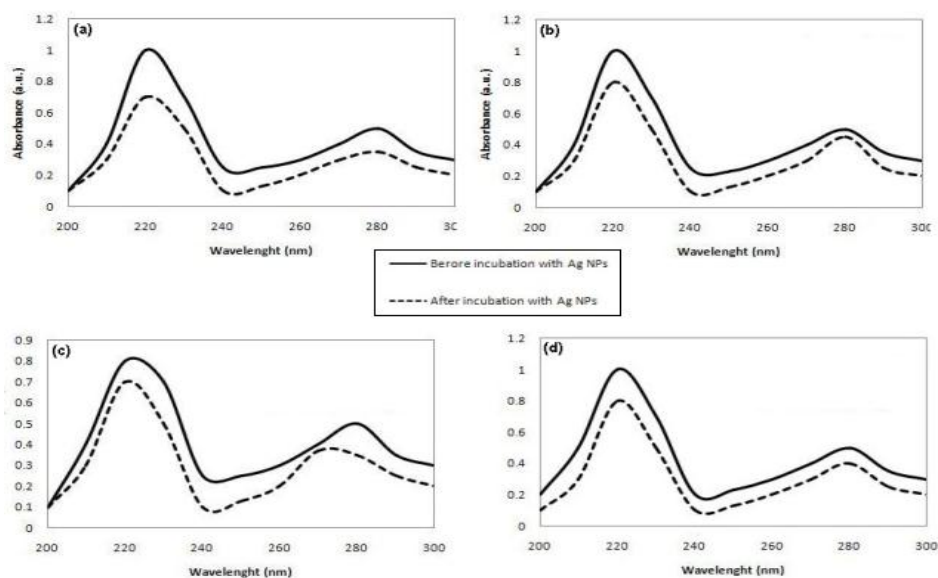


Fig. 4. UV- visible spectrum of protein solutions. The a, b, c, and d are HAS, BSA, FCS, and PNA, respectively. For UV- visible experiment, optical densities of all protein solutions were read after and before incubation with Ag NPs at 200-300 nm.

The effect of naked and coated Ag NPs on cell count and nanoparticle uptake

The nanoparticle uptake and the amount of cell proliferation after a 24-hour incubation with

naked and coated Ag NPs are demonstrated in Figure 5 and Figure 6, respectively. Results showed that both parameters are dose dependent. Also, there was a reverse relationship between the nanoparticle uptake and the number of cells for both naked and coated Ag NPs. It was shown that the cells treated with naked Ag NPs had lower cell count than cells treated with protein coated Ag NPs ($p < 0.05$). In contrast, in each concentration, the cells treated with naked Ag NPs had higher nanoparticle uptake than protein coated Ag NPs ($p < 0.05$).

Interestingly, this study showed that different protein coating did not lead to different cell count and nanoparticle uptake. No significant differences were observed between different protein coated Ag NPs in cell count and uptake ($p = 0.1$). For further investigation, nanoparticle uptake was evaluated at concentration of 500 $\mu\text{g/ml}$ after 6-hour incubation, too. In contrast to the prior results related to 24-hour incubation, this experiment revealed a high uptake of nanoparticles for naked Ag NPs and a low uptake for all coated Ag NPs.

The study of nanoparticle uptake

Mechanism

The cellular uptake mechanisms were studied by cell treatment with specific inhibitory materials [16], that included: (1) incubation at 4 °C instead of incubation at 37 °C (2) sodium azide and 2-deoxy-D-glucose, (3) sucrose, and (4) nystatin. As is shown in Figure 7, when cells were pre-treated at 4 °C with sodium azide and with sucrose, nanoparticle uptake was significantly reduced compared with the cells without pre-treatment ($p < 0.05$). It should be explained that this difference was observed for all naked and coated Ag NPs. On the other hand, treatment with nystatin led to significant increase of uptake in both coated and naked Ag NPs ($p < 0.05$). According to Figure 6, there were no significant differences between different protein coatings after different pre-treatments ($p = 0.2$). In agreement with uptake evaluation test, our results showed that naked Ag NPs also lead to lower nanoparticle uptake than protein coated Ag NPs after pre-treatment with inhibitors.

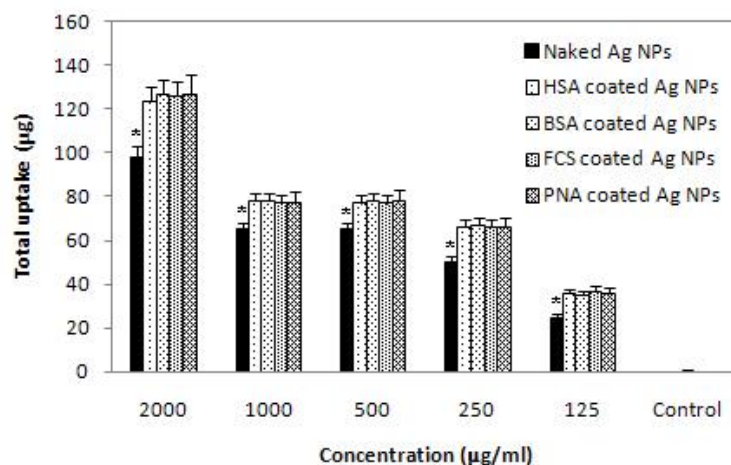


Fig. 5. Total nanoparticle uptake. Macrophages were incubated with naked and coated Ag NPs for 24 hours at 37 °C. Then, the cells were washed with HBSS and treated with HCl and HF for 12 hours, respectively. Finally, Ag quantity was measured by atomic absorption spectrometer. These data are presented as mean $\pm$ SD from 10

Balb/c mouse macrophages, and each test was performed triplicate. Data analysis was carried out by one-way ANOVA. (P-value <0.05 was set with all protein coated Ag NPs at the same concentration.)

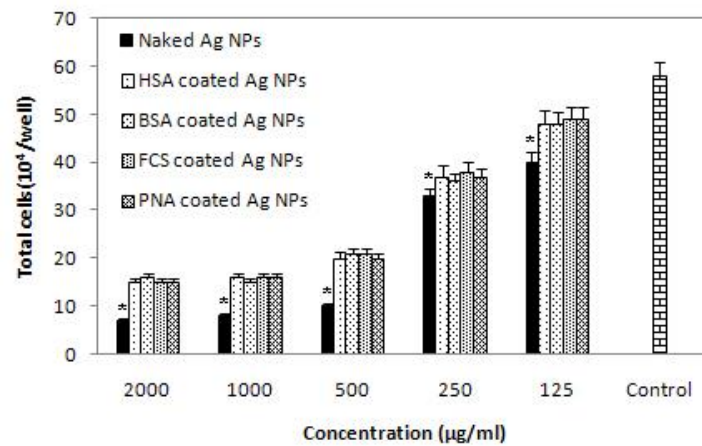


Fig. 6. Total cell proliferation. Macrophages were incubated with naked and coated Ag NPs for 24 hours at 37 °C, and then the quantity of cells was counted. Data are demonstrated as mean $\pm$ SD from 10 Balb/c mouse macrophages, and each test was done triplicate. Data analysis was carried out by one-way ANOVA. (P-value <0.05 was set with all protein coated Ag NPs at the same concentration.)

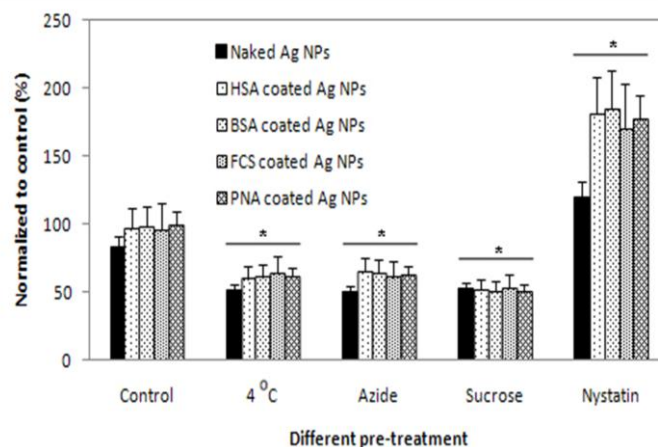


Fig. 7. The effect of different inhibitory treatments on nanoparticle uptake.

At first, macrophages were pre-treated with inhibitors including: (1) incubation at 4°C, (2) incubation with sodium azide, (3) incubation with sucrose, and (4) incubation with nystatin. Then, the cells were incubated with naked and coated Ag NPs for 24 hours at 37°C, and the quantity of nanoparticle uptake was measured.

These data are shown as mean $\pm$ SD from 10 Balb/c mouse macrophages, and each test was performed triplicate. Data analysis was carried

out by one-way ANOVA. (P-value <0.05 was set with all protein coated Ag NPs at the same concentration.)

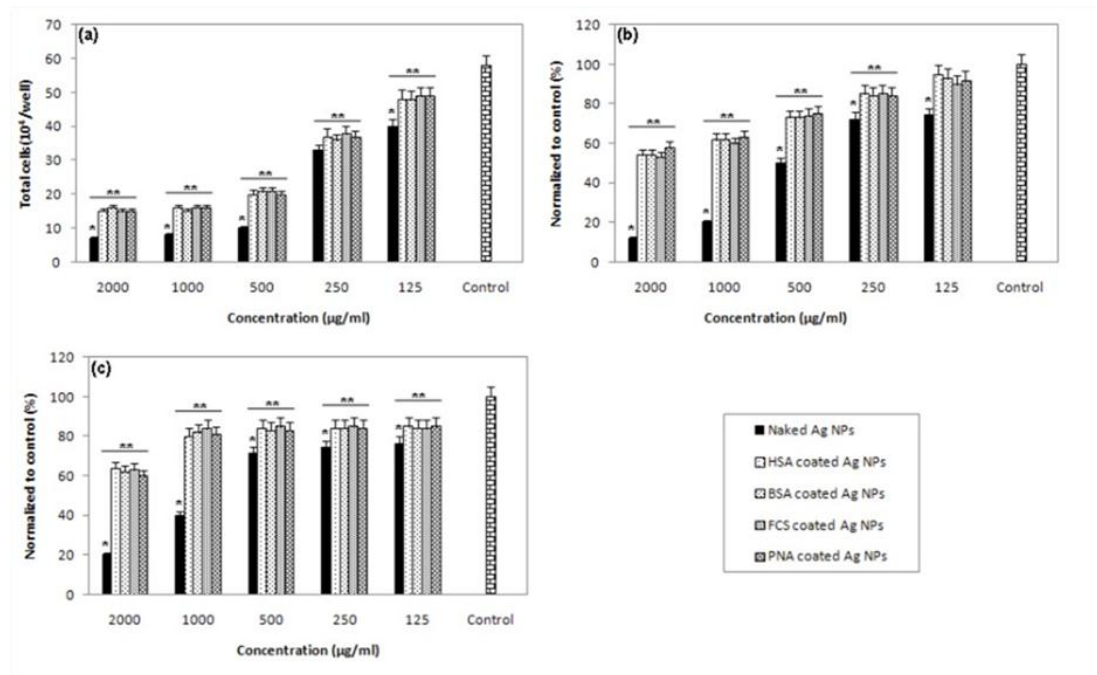


Fig. 8. The effect of naked and coated Ag NPs on cell biological parameters which were (a) metabolic activity of macrophages, (b) mitochondrial activity of macrophages, and (c) cellular ATP levels of macrophages.

Macrophages were incubated with naked and coated Ag NPs for 24 hours at 37 °C and then were washed with HBSS. Later, these biological parameters were measured by Alamar Blue, MTT, and CellTiter-Glo, respectively.

Results are presented as mean $\pm$ SD from 10 Balb/c mouse macrophages, and each test was done triplicate. Data analysis was carried out by one-way ANOVA. P-value <0.05 was set with all protein-coated Ag NPs at the same concentration.

The effects of naked and coated Ag NPs on the cell biological parameters

To evaluate toxicity of different concentrations of coated and naked Ag NPs, different parameters including mitochondrial activities, cellular metabolic function, and ATP level were studied after 24 hours of exposure. As is demonstrated in Figure 8, although there was significant reduction of all three parameters after 24 hours of exposure with naked and coated Ag NPs, these parameters were more reduced by naked Ag NPs ($p=0.05$). In

general, the pattern of reduction of all parameters was dose-dependent. The highest effect was shown at concentration 1000 µg/ml of naked Ag NPs and also for protein-coated NPs at concentration of 1000 µg/ml. interestingly, ATP level was more affected by both naked and coated nanoparticles than mitochondrial activities and cellular metabolic function. Like cell count and uptake experiments, there was no significant difference between different protein coatings regarding biological parameters ($p=0.2$).

Discussion

Nanoparticles have a high local charge density and high specific surface area. Nanoparticles interact or react with different components such as proteins [2], which leads to changes in

their surface chemistry. This induces new features which are different from natural or naked nanoparticles [1]. Although previous studies show altered toxicity and uptake of nanoparticles after medium protein adsorption [1, 2], there is no report on other proteins which may not be contained in medium. Also, there is no report on cytotoxicity of protein-coated Ag NPs. Indeed, the aim of this study was to evaluate the aggregation size of nanoparticle in RPMI medium, the quantity of uptake by macrophages, the mechanisms of uptake, and the biological impact of both naked and protein coated Ag NPs. In this study, different protein coating agents were used, which included HSA, BSA, FCS, and PNA. It must be mentioned that although FCS is a medium enrichment additive, but HSA, BSA, and PNA are not used as enrichment factor. This study showed that all coated Ag NPs in RPMI-1640 medium had smaller aggregates than naked Ag NPs. The authors suggest that naked Ag NPs when suspended in RPMI, aggregate because of electrostatic forces of medium salt ions such as $\text{Na}_2\text{H}_2\text{PO}_4$, Na_2HPO_4 , NaCl , and KCl [27]. In consistent with other studies, Tedja et al. showed that TiO_2 NPs coated with FBS leads to reduction of nanoparticle aggregation size by protein corona layer [28]. Also, Wiogo et al. demonstrated that adsorbed protein could induce steric hindrance [7]. In this study, all proteins were adsorbed on Ag NPs, and the same stabilization mechanisms were suggested. The effect of protein adsorption or coating on nanoparticles has been investigated previously for gold, fullerenes, magnetite,

polystyrene, and TiO_2 NPs [5, 7, 9, 23, 29]. For the first time, this study showed the influence of different proteins on the surface of Ag NPs.

This research showed that there is a higher cell count and less uptake for cells treated with coated nanoparticles than cells treated with naked nanoparticles. In contrast, Tedja et al. indicated that more nanoparticle uptake with lower cell counts are obtained by protein coated nanoparticles [28]. On the other hand, this study showed a higher nanoparticle uptake after 6 hours of exposure to naked Ag NPs than all protein-coated Ag NPs. The higher cellular uptake by naked nanoparticles may be owing to bigger aggregation size compared to coated nanoparticles. It has previously been investigated that larger aggregated particles lead to rapid attachment onto different cells [28, 30]. Interestingly, after high nanoparticle uptake at initial exposure, the next step was steady stage because of exocytosis, a dynamic process for exiting foreign material with recycling of the cell membrane. Exocytosis of nanoparticles with polysaccharide layer carried out in epithelium cells after 1 hour incubation has been reported [31]. As is shown in this study, in the first stage (6 hour-incubation), nanoparticles uptake was low for cells treated with coated nanoparticle, but in the later stage (24 hour-incubation), their nanoparticle uptake was greater than cells treated with naked nanoparticles. This study showed that coated nanoparticles induced an increase in total uptake after 24 hours. The authors put forth that endocytosis mediated by the binding to proteins leads to a high nanoparticle uptake

with naked and coated Ag NPs in the late phase.

This research demonstrated that both cells (treated with naked and coated Ag NPs) reduce nanoparticle uptake when the cells are incubated at 4°C because of significant reduction of cellular energy at low temperature. This indicates energy-dependent endocytosis of nanoparticle uptake. In this study, a reduction of engulfed nanoparticles was observed after treatment with sodium azide. This result also confirmed energy-dependent endocytosis because sodium azide inhibits production of ATP and blocks cytochrome c [32, 33]. For further elucidation, the cells were exposed to sucrose for inducing a hypertonic environment and also were treated with nystatin which is a cholesterol sequestration agent. It has been shown that a hypertonic condition can disrupt clathrin lattice in the cell membrane [34]. This research reveals that a decrease in nanoparticle uptake is observed when cells are treated with sucrose. This indicates that the nanoparticle endocytosis is carried out by a clathrin-mediated mechanism, and when cells are treated with nystatin, high uptake is observed. As is shown in other studies, nystatin damages caveolae/lipid raft in fibroblasts [35] and endothelial cells [36], and thus leads to macropinocytosis [36] with no effect on clathrin lattice. We speculate that nystatin induces blocking of caveolae-mediated pathways and upregulation of macropinocytosis. This result is in agreement with Taylor's et al. study that showed disruption of caveolae/lipid could affect

clathrin-mediated machinery and increase endocytosis [37].

According to the present study, a significant reduction of mitochondrial activities, cellular metabolic function, and ATP level was seen in cells treated with coated and naked nanoparticles after a 24-hour incubation and this reduction was dose-dependent. The same results were observed in other researches on biological effect of TiO₂ NPs on human cell lines [28]. This research demonstrated that all coated Ag NPs have lower biological impact compared with the naked Ag NPs. Other researchers have attributed the less effect of protein adsorbed Au NPs and carbon nanoparticles to a reduction of uptake [23, 38]. The reason for greater effect of naked Ag NPs may be due to structural damage of larger nanoparticle aggregates. The authors hypothesize that different proteins which were used in this study make a protective shielding and a limited direct interaction between the nanoparticle and cell organelles thus leading to low cytotoxicity effect. Here, we showed that there is no significant difference in nanoparticle uptake and biological parameters between different protein coated nanoparticles. We speculate that mouse macrophage can interact with and internalize nanoparticle coated with HSA, BSA, FCS, and PNA thus leading to more uptake and less toxicity through shielding or covering nanoparticles.

Conclusion

This research showed that the aggregate size of Ag NPs coated with HSA, BSA, FCS, and PNA in RPMI medium is smaller than the

naked Ag NPs which affects the nanoparticle-cell interactions. Interestingly, nanoparticle uptake was high after exposure to naked Ag NPs in initial period (6 hours), but for all coated nanoparticles high uptake was observed at a late stage (24 hours). Endocytosis-mediated pathway was the main uptake mechanism in this study, but a clathrin-mediated pathway was shown to regulate the uptake. This study showed that mitochondrial activities, cellular metabolic function, and ATP level of cells treated with protein-coated Ag NPs are higher than naked Ag NPs after 24 hours of incubation.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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