Original research

gold nanoparticles coated with different proteins: cytotoxicity evaluation and related mechanisms

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Abstract

Coated nanoparticles have different surface chemistry, aggregation, and interaction. The aim of this study was to evaluate: 1) the size of aggregation, 2) the quantity and mechanisms of uptake, and 3) the biological impact of gold nanoparticles (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. This research showed that all coated Ag NPs had smaller aggregate size, the more up take, and the less biological impact on Balb/c macrophage cells than naked Ag NPs. Endocytosis mediated pathway was main uptake mechanism in this study. Also, a clathrin-mediated pathway was shown to regulate their uptake.

KEYWORDS: Gold nanoparticle; Coated Ag nanoparticles; Cytotoxicity

Introduction

The nanoparticles quickly adsorb biological components, when enter into a biological medium. Surface chemistry, shape, and aggregation of nanoparticles change after adsorption. This phenomena leads to a new interaction, which is 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 had low aggregation in cell medium because of protein adsorption [3-9]. Importantly, it has also been reported that the important factor of 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 depend 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) don't affect the size of nanoparticle, but Roswell Park Memorial Institute medium (RPMI) supplemented with FBS increases the size of gold nanoparticles [11]. The past study showed nanoparticles adapted the properties of the adsorbed protein [12-14]. For example, gold nanorods and magnetite nanoparticles coated with polyethyleneamine which has a negative charge, switch to positive charge after serum protein adsorption [15, 16]. Several researchers have proposed that adsorbed proteins raised nanoparticle uptake by receptor endocytosis, but the exact role of nanoparticle uptake was not understood [5, 16, 17]. It was shown that the capacity of nanoparticles in protein adsorption affected the nanoparticle-cell association, and did not depend only on the individual characteristics of the adsorbed proteins [18, 19]. In contrast, a reduction of uptake was recorded [17] after protein adsorption of magnetite, carbon, and gold nanoparticles. It has also been observed that there was no significant difference in the internalization of nanoparticles with

Vol. 1, No. 1, 2014, pages 79-99.

changing surface functional groups i.e., COOH and NH2 [20]. Physicochemical characteristic, cellular internalization, and toxicity of nanoparticles were 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) was recorded, when media contained serum [23]. In the other study, Clift et al. (2010) measured tumor necrosis factor-R production and intracellular glutathione (GSH) levels, when polystyrene nanoparticles exposed to J774.A1 macrophage-like cells, and showed that protein coated nanoparticles were less toxic than not-coated 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) plays as a capping agent, and leads to increase uptake [26]. Although there are some study on toxicity of nanoparticles after medium protein adsorption, but there is no comparative study of different proteins including BSA, Serum albumin (HSA), Fetal calf serum (FCS), and Pea nut agglutinin (PNA) after coating on Ag NPs. On the other hand, there is little information on toxicity of coated gold 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.

Material and methods

Material:

Ag NPs were sourced from Lolitech Co., Germany. HSA, BSA, FCS, PNA, HCl, HF, nystatin, sucrose, sodium azide, 2-deoxy-D-glucose3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), Hank's buffered salt solution (HBSS), and RPMI1640 were provided from Sigma, Germany. AlamarBlue 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 gold nitrate and citrate sodium. Ag NPs were ball milled for 5 minutes after centrifugation, washing with distilled water, and drying under atmosphere, and then sterilized at 121 ºC for 20 min by autoclave. In this study, serial concentrations of Ag NPs were prepared distilled water witch included 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 ºC. After incubation, the mixture was centrifuged at 10000 rpm and the supernatant collected. Finally, pellets were resuspended in RPMI1640 and serial concentrations (2000-1000-500-250-125 µg/ml) of protein coated nanoparticle were prepared, same as 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), 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 RPMI1640 and tested by DLS apparatus for particle size distribution at 37 ºC. For protein adsorption confirmation, UV-visible spectrophotometer (ELICO, India) at 280nm and FTIR was used at range of 500-3500 cm^{-1}.

Preparation of peritoneal macrophage cells and measurement of nanoparticle uptake

Ten male BALB/c mice were enrolled in this study. The peritoneal fluid of mice were aspirated and centrifuged. The peritoneal macrophages were suspended in RPMI1640 to reach at final concentration of 5×10^4/ml, and were separately incubated with serial concentrations of coated and naked Ag NPs for 24 hours at 37 ºC with 5% CO2. 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, macrophage cells (5×10^4/ml) were separately exposed to inhibitor treatments for 2 hours at 37 ºC, and then washed with HBSS, as proposed in other study [16]. Inhibitor treatments were 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 ºC

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

Evaluation of cell toxicity

The study of cell metabolic

Macrophage cells incubated with naked and coated Ag NPs for 24 hours at 37 ºC, and then were washed three times with HBSS. Then, 100 μL of RPMI1649 and 25 μL of the AlamarBlue reagent were added to each well and incubated 4 hours at 37 ºC. The cell metabolic was measured by reading optical density (OD) at 590 nm using micro plate reader (Novin gostar, Iran), and compared to control. Macrophage cells which did not expose to nanoparticle were considered as control group.

The study of cell mitochondrial function

First, the cells incubated 24 hours with naked and coated Ag NPs, and washed three times with HBSS. Then, 100 μL of RPMI1649 and 25 μL of MTT at concentration of 5 mg/ml were added to each well, and incubated at 37 ºC for 4 hours. OD of each well was measured at 490 nm by micro plate reader, and compared with control group which did not treat with nanoparticles.

The study of cellular energy

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

Statistical analysis

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

Results

Characterization of coated nanoparticles

Figure 1 shows SEM images of naked Ag NPs (a) and Ag NPs coated with HAS (b), BSA (c), FCS (d), and PNA (e). These images demonstrated that the size of all coated Ag NPs was near 25 nm, but naked Ag NPs was agglomerated. Figure 2 shows the distribution size of naked and coated Ag NPs, obtained by DLS. In consistent with SEM images, DLS results also showed that all coated Ag NPs had smaller aggregate 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-VIS were used, which their results have been shown in Figure 3 and 4, respectively. FTIR spectrum revealed the presences of amide band I (1650 cm^{-1}), amide band II (1550 cm^{-1}), and NH stretching vibrations – amide A and B (3170-3300 cm^{-1})

for all coated Ag NPs. This pattern was not seen in naked Ag NPs. Also, UV-VIS spectrum showed decrease of OD of all protein solution at range of 200-300 nm after incubation with Ag NPs. Both FTIR and UV-VIS results indicated that proteins were adsorbed on Ag NPs surface.

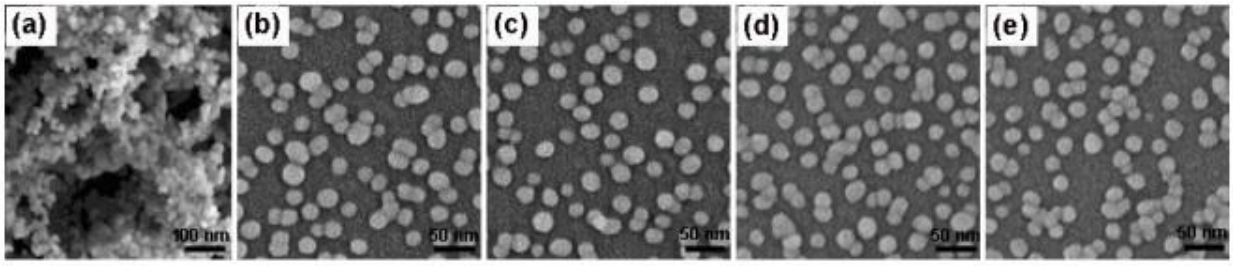

Figure 1. SEM images of naked Ag NPs (a) and Ag NPs coated with HAS (b), BSA (c), FCS (d), and PNA (e).

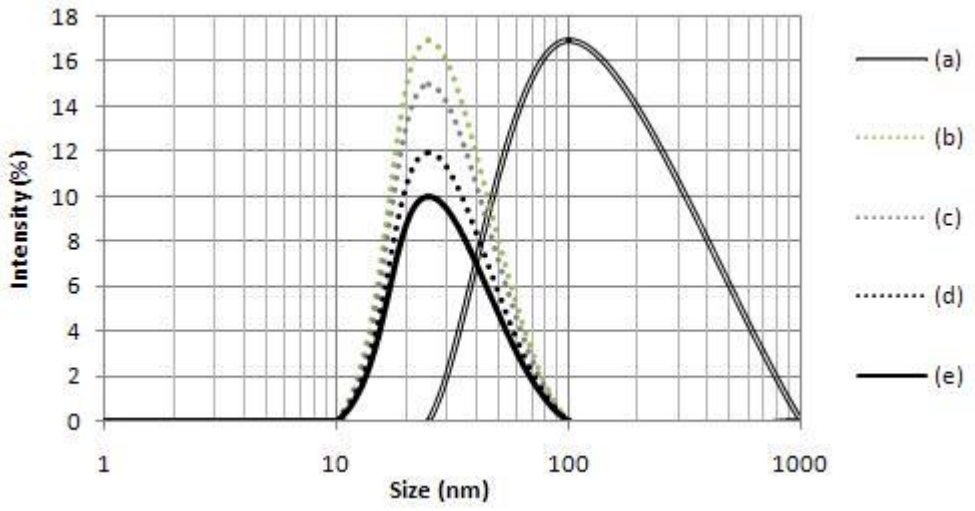

Figure 2. Distribution size of naked Ag NPs (a) and Ag NPs coated with HAS (b), BSA (c), FCS (d), and PNA (e). All nanoparticles were suspended in RPMI1640 and size distribution was carried out by DLS at 37 ºC.

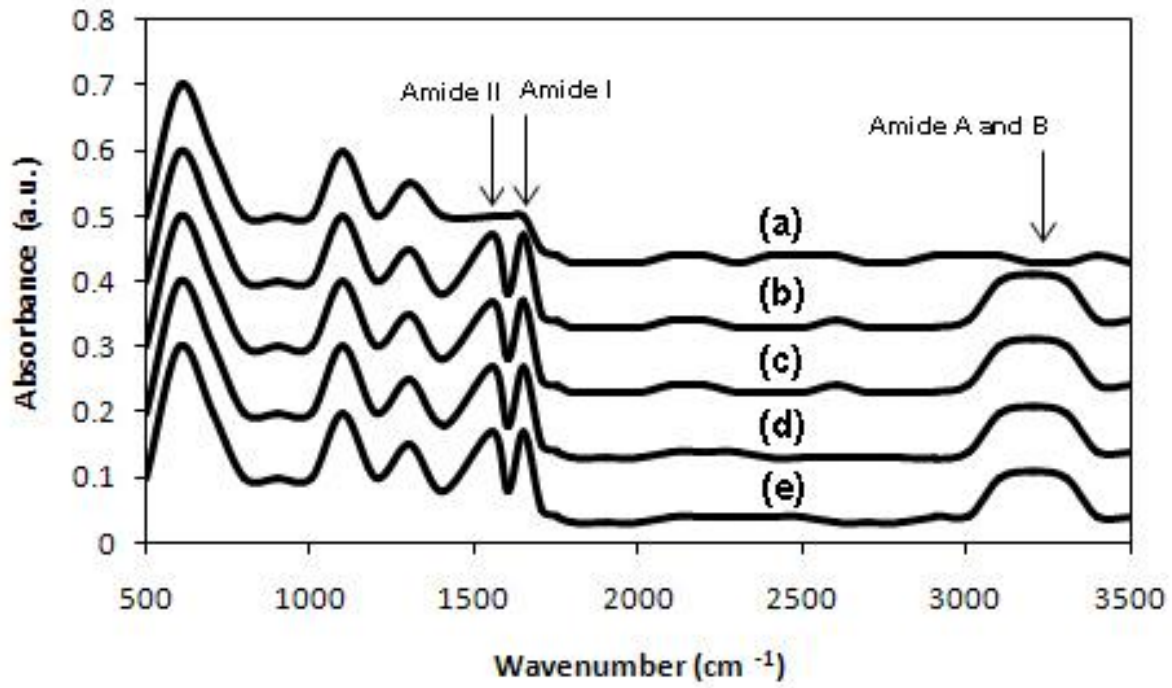

Figure 3. FTIR spectrum of naked Ag NPs (a) and Ag NPs coated with HAS (b), BSA (c), FCS (d), and PNA (e). For FTIR experiment, all nanoparticles were separately dried on KBr and scanned at 500-3500 cm $^{-1}$. Amide band I, amide band II, and amid A&B were seen in all coated Ag NPs.

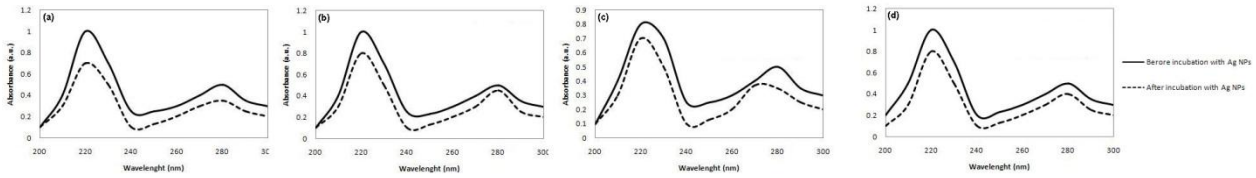

Figure 4. UV-VIS spectrum of protein solutions. A, b, c, and d are HAS, BSA, FCS, and PNA, respectively. For UV-VIS experiment, optical densities of all protein solutions were read after and before incubation with Ag NPs at 200-300 nm.

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

The nanoparticle uptake and the amount of cell proliferation after 24 hours incubation with naked and coated Ag NPs are demonstrated in Figure 5 and Figure 6. Results showed that both parameters were dose dependent. Also, there was a reverse relationship between the nanoparticle uptake and the amount of cells for both naked and coated Ag NPs. It was shown that cells treated with naked Ag NPs had lower quantity of cell count than cells treated with protein coated Ag NPs ($P<0.05$). In contrast, in each concentration, 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 ($P>0.05$). For further investigation, nanoparticle uptake was evaluated at concentration of 500 µg/ml after 6 hour incubation, too. In contrast with prior results related to 24 hours incubation, this experiment showed the high uptake of nanoparticle for naked Ag NPs and 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) The use of incubation at 4 ºC instead of incubation at 37 ºC (2) The use of sodium azide and 2-deoxy-D-glucose, (3) The use of sucrose, and (4) the use of nystatin. As shown in Figure 7, when cells pre-treated at 4 ºC, with sodium azide, and with sucrose, nanoparticle uptake was reduced, compared with cells without pre-treatment ($P<0.05$). It should be mentioned that this difference was observed for all naked and coated Ag NPs. On the other hand, treatment with nystatin led to increase of uptake in both coated and naked Ag NPs, compared with control ($P<0.05$). According to Figure 6, there were no significant differences between different protein

coatings after different pre-treatment (P>0.05). In agreement with uptake evaluation test, results showed that naked Ag NPs also led to lower nanoparticle uptake than protein coated Ag NPs after pre- treatment with inhibitors.

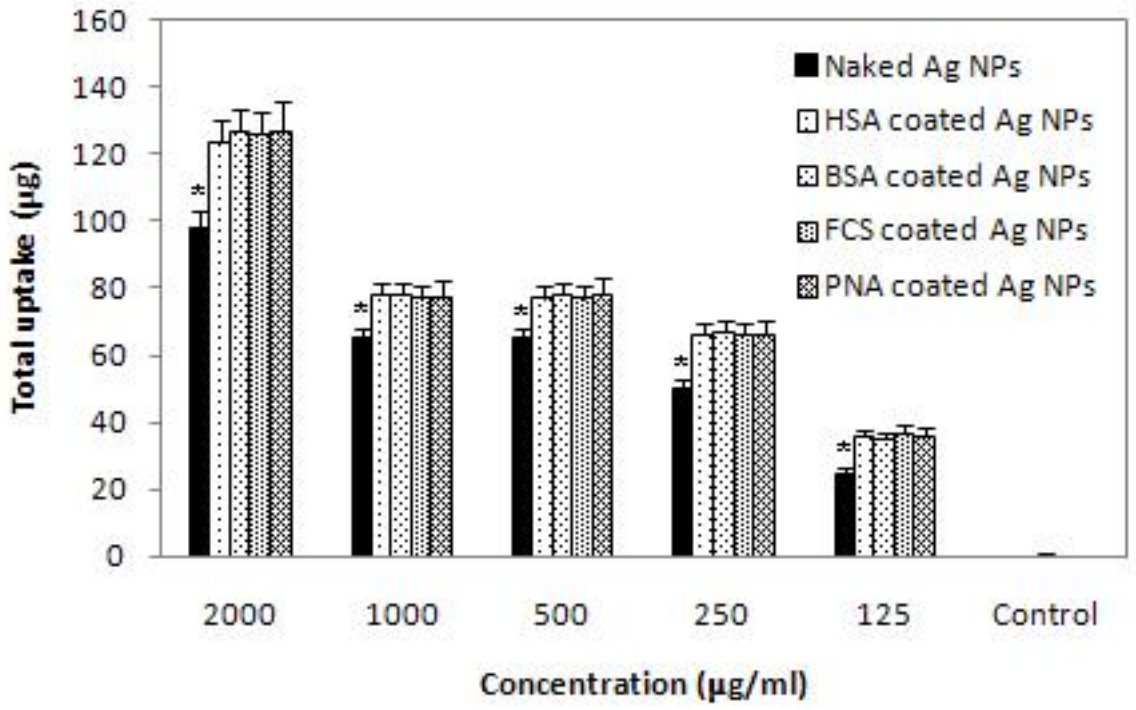

Figure 5. Total nanoparticle uptake. Macrophage cells were incubated with naked and coated Ag NPs for 24 hours at 37 ºC. Then, cell washed with HBSS and treated with HCl and HF for 12 hours, respectively. Finally, Ag quantity was measured by atomic absorption spectrometer. These data were presented as mean ± SD from 10 Balb/c mouse macrophages, and each test was done triplicate. Data analysis was carried out by one-way ANOVA. *P<0.05 compared with all protein coated Ag NPs at same concentration.

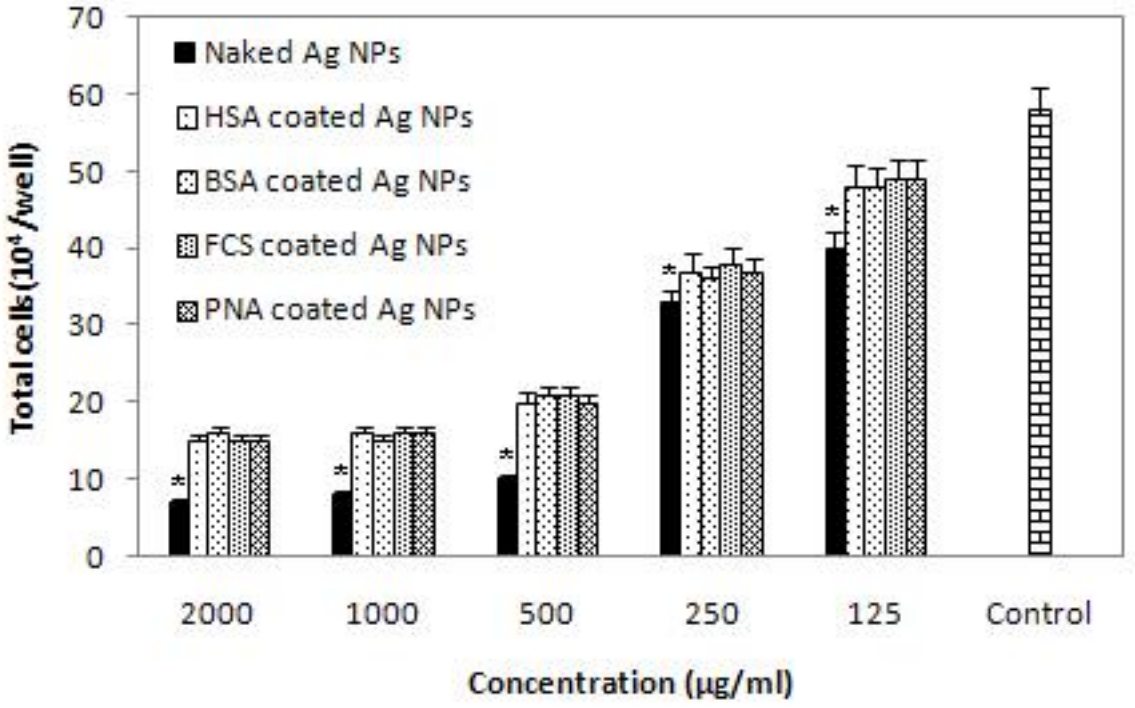

Figure 6. Total cell proliferation. Macrophage cells were incubated with naked and coated Ag NPs for 24 hours at 37 ºC, and then the quantity of cells was counted. This experiment was demonstrated as mean ± SD from 10 Balb/c mouse macrophages, and each test was done triplicate. Data analysis was carried out by one-way ANOVA. *P<0.05 compared with all protein coated Ag NPs at same concentration.

Vol. 1, No. 1, 2014, pages 79-99.

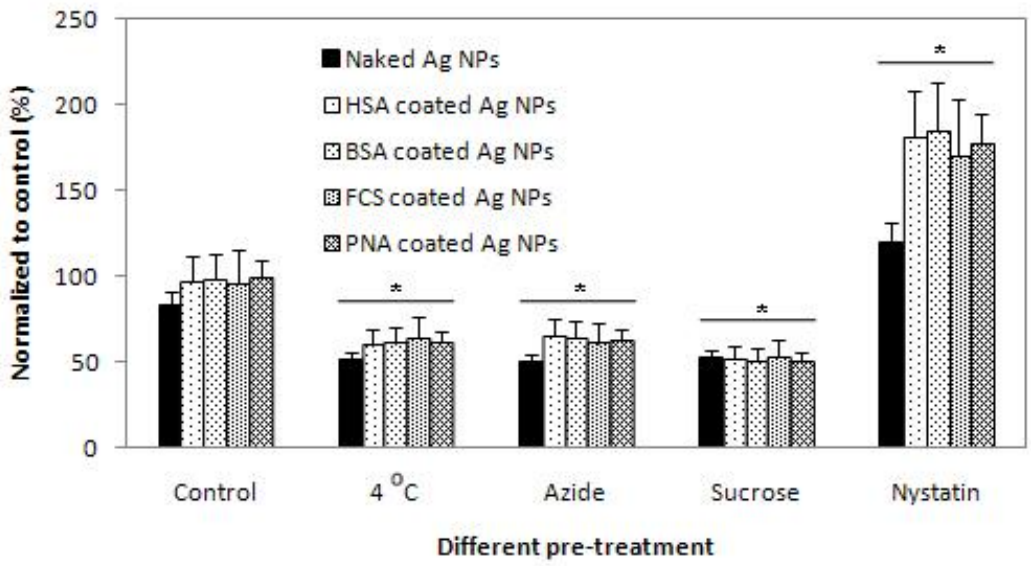

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Figure 7. The effect of different inhibitory treatments on nanoparticle uptake. First, macrophage cells pre-treated with inhibitor included: (1) incubation at 4 ºC, (2) incubation with sodium azide, (3) incubation with sucrose, and (4) incubation with nystatin. Then, cells incubated with naked and coated Ag NPs for 24 hours at 37 ºC, and the quantity of nanoparticle uptake was measured. These data were shown as mean ± SD from 10 Balb/c mouse macrophages, and each test was done triplicate. Data analysis was carried out by one-way ANOVA. *P<0.05 compared with cells without pre-treatment (control which did not treated with nanoparticles).

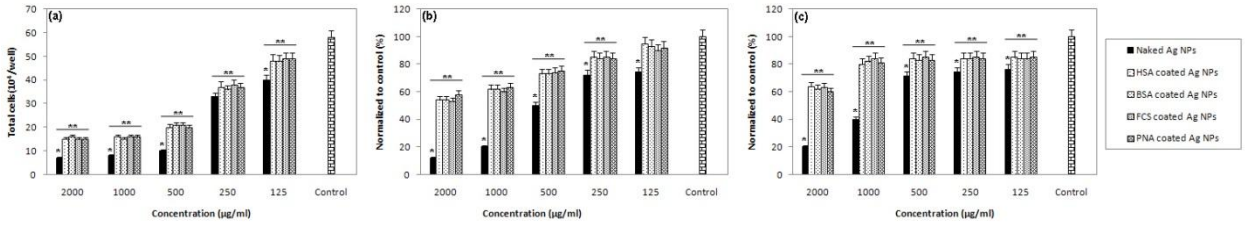

Figure 8. The effect of naked and coated Ag NPs on cell biological parameters which were (a) metabolic activity of macrophage cells, (b) mitochondrial activity of macrophage cells, and (c) cellular ATP levels of macrophage cells. Macrophage cells incubated with naked and coated Ag

NPs for 24 hours at 37 ºC, and then washed with HBSS. Then, these biological parameters were measured by AlamarBlue, MTT, and CellTiter-Glo, respectively. Results presented as mean ± SD from 10 Balb/c mouse macrophages, and each test was done triplicate. Data analysis was carried out by one-way ANOVA. *P<0.05 compared with all protein coated Ag NPs at same concentration, **P<0.05 compared with control, which did not treated with any nanoparticles.

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 included mitochondrial activities, cellular metabolic function, and ATP level were studied after 24 hours exposure. As demonstrated in Figure 8, although there was significant reduction of all three parameters after 24 hours exposure with naked and coated Ag NPs, but 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 high effect was shown at concentration 1000 µg/ml of naked Ag NPs, and in later level 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 biological parameters of different protein coatings (P>0.05).

Discussion

Nanoparticles have a high local charge density and high specific surface area. Nanoparticles interact or react with different components such as protein [2], and lead to changes in their surface chemistry. This effect induce new interaction, which is different from natural or naked

nanoparticles [1]. Although previous study showed different toxicity and uptake of nanoparticles after medium protein adsorption [1, 2], but there is no report on other proteins, which may not contain 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, but HSA, BSA, and PNA are not used as enrichment. This study showed that all coated Ag NPs in RPMI1649 medium were smaller aggregates than naked Ag NPs. Authors suggest that naked Ag NPs when are suspended in RPMI aggregate because of electrostatic forces by medium salt ions such as Na2H2PO4, Na2HPO4 , NaCl, and KCl [27]. In consistent with other study, Tedja et al. showed that TiO2 NPs coated with FBS led 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 by 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 TiO2 NPs [5, 7, 9, 23, 29]. For first time, this study showed influence of different protein on surface of Ag NPs.

This research showed that there was 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 the more nanoparticle uptake with the lower cell counts related to protein coated nanoparticles [28]. On the other hand, this study showed the higher nanoparticle uptake after 6 hours exposure of naked Ag NPs than all protein coated Ag NPs. The higher cellular uptake by naked nanoparticles may be

credit to bigger aggregation size, comparison to coated nanoparticles. It has previously been researched that larger aggregate particles led to rapidly attachment onto the 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. It has been reported previously that exocytosis of nanoparticles with polysaccharide layer carried out in epithelium cells after 1 hour incubation [31]. As shown in present study, in the first stage (6 hours incubation), nanoparticle uptake was low for cells treated with coated nanoparticles, but in later stage (24 hours incubation), nanoparticle uptake was greater than cells treated with naked nanoparticle. This research showed that coated nanoparticle induced an increase of total uptake after 24 hours. Authors pronounced endocytosis, mediated by the binding of proteins, led to the 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 reduced nanoparticle uptake, when the cells were incubated at 4 ºC because of significant reduction of cellular energy at low temperature. This indicated energy dependent endocytosis of nanoparticle uptake. In this study, a reduction of up taken nanoparticles was observed after treatment with sodium azide. This result also confirmed energy dependent endocytosis because sodium azide inhibit production of ATP and block cytochrome c [32, 33]. For further findings, 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 a hypertonic condition led to disrupt clathrin lattice in the cell membrane [34]. This research declared that a decrease of nanoparticles uptake was observed, when cell treated with sucrose. This indicated that the nanoparticle endocytosis carried out by a clathrin-mediated mechanism, but when cell treated with nystatin, high

uptake was observed. As shown in other studies, nystatin damaged caveolae/lipid raft in fibroblasts [35] and endothelial cells [36], and led to macropinocytosis [37] with no effect on clathrin lattice. We speculated that nystatin induced the blocking of caveolae-mediated pathways, and up regulating of macropinocytosis. This result was in agreement with Taylor et al. study, that showed the disruption of caveolae/lipid could subject clathrin-mediated machinery and increase of endocytosis [38].

According to present study, a significant reduction of mitochondrial activities, cellular metabolic function, and ATP level was seen in cell treated with coated and not-coated nanoparticles after 24 hours incubation, and this reduction was dose-dependent. The same results were observed in other research for the biological effect of TiO2 NPs on human cell lines [28].This research demonstrated that all coated Ag NPs had a less biological impact than naked Ag NPs. Other researchers have attributed the less effect of protein adsorbed Au NPs and carbon nanoparticles with a reduction of uptake [23, 39]. The reason of greater effect of naked Ag NPs may be due to structural damage of larger nanoparticle aggregates. Authors hypothesized that different proteins which used in this study made a protective shielding, and limited direct interaction between the nanoparticle and cell organelles, thus led to low cytotoxicity effect. Here, we showed there were no significant differences in nanoparticle uptake and biological parameters between different protein coated nanoparticles. We speculate mouse macrophage can interact and internalize nanoparticle coated with HSA, BSA, FCS, and PNA, lead to more uptake and less toxicity, by 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 was smaller than naked Ag NPs, and affected the nanoparticle cell interactions. Interestingly; nanoparticle uptake was high after exposure with naked Ag NPs in initial period (6 hours), but for all coated nanoparticles high uptake was observed at late stage (24 hours). Endocytosis mediated pathway was the main uptake mechanism in this study, also a clathrin-mediated pathway were 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 were more than naked Ag NPs after 24 hours incubation.

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Conflict of Interest

The authors declare no competing financial interest.

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Vol. 1, No. 1, 2014, pages 79-99.