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Protein binding modulates the cellular uptake of silver nanoparticles into human cells: Implications for in vitro to in vivo extrapolations?

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Abstract

Nanoparticles (NP) absorbed in the body will come in contact with blood proteins and form NP/protein complexes termed protein coronas, which may modulate NP cellular uptake. This study quantitated human epidermal keratinocyte (HEK) uptake of silver (Ag) NP complexed to different human serum proteins. Prior to HEK dosing, AgNP (20 nm and 110 nm citrate BioPure™; 40 nm and 120 nm silica-coated) were preincubated for 2 h at 37 °C without (control) or with physiological levels of albumin (44 mg/ml), IgG (14.5 mg/ml) or transferrin (3 mg/ml) to form protein-complexed NP. HEK were exposed to the protein incubated AgNP for 3 h, rinsed and incubated for 24 h, rinsed in buffer and lysed. Ag was assayed by inductively-coupled plasma optical emission spectrometry. Uptake of Ag in HEK was <4.1% of applied dose with proteins suppressing citrate, but not silica coated Ag uptake. IgG exposure dramatically reduced 110 nm citrate AgNP uptake. In contrast, greatest uptake of 20 nm silica AgNP was seen with IgG, while 110 nm silica AgNP showed minimal protein effects. Electron microscopy confirmed cellular uptake of all NP but showed differences in the appearance and agglomeration state of the NP within HEK vacuoles. This work suggests that NP association with different serum proteins, purportedly forming different protein coronas, significantly modulates Ag uptake into HEK compared to native NP uptake, suggesting caution in extrapolating in vitro uptake data to predict behavior in vivo where the nature of the protein corona may determine patterns of cellular uptake, and thus biodistribution, biological activity and toxicity.

Keywords

Protein corona; Protein binding of nanoparticles; Cellular uptake of silver nanoparticles; Albumin cell uptake; Transferrin cell uptake; IgG cell uptake

1. Introduction

Extensive research has demonstrated that nanoparticles (NP) in a biological environment interact with proteins and other biomolecules to form a nanoparticle-protein corona complex (Lynch et al., 2007; Lundqvist et al., 2008; Nel et al., 2009; Monopoli et al., 2012). This is a dynamic process and undergoes various stages of protein–nanoparticle exchanges until a stable or so-called hardcorona is established (Cedervall et al., 2007; Dell'Orco et al., 2010; Monopoli et al., 2011). The formation of a NP-protein corona is believed to be a primary factor that influences its pattern of in vivo biodistribution and thus its pharmacokinetic profile (Riviere, 2009; Walkey and Chan, 2012). Studies have also suggested that the nature of the corona determines the extent and mechanism of NP uptake into cells (Chithrani and Chan, 2007; Lunov et al., 2011; Safi et al., 2011; Lesniak et al., 2012). This phenomenon has implications for the design of rational targeted nanomedicines and in the risk assessment of manufactured nanomaterials.

Assessment of biological activity and toxicity of nanomaterials is most often accomplished using efficient in vitro cell culture studies (Monteiro-Riviere et al., 2005; Monteiro-Riviere and Inman, 2006; Ryman-Rasmussen et al., 2007a,b; Zhang et al., 2007, 2008; Monteiro-Riviere et al., 2009; Monteiro-Riviere and Riviere, 2009; Samberg et al., 2010; Monteiro-Riviere et al., 2010; Saathoff et al., 2011). Workers have often associated NP incubation with serum proteins with reduced NP uptake into cells (Bajaj et al., 2009; Zhu et al., 2009), usually discussed in terms of colloidal stability and aggregation in media. In contrast, complexation with specific proteins such as transferrin or apolipoprotein has been described in nanomedicine literature to target NP for cellular uptake (Chithrani and Chan, 2007; Kim et al., 2007). The formation of a NP-protein corona has recently been shown to occur with in vitro cell culture media proteins (Shannahan et al., 2013). The nature and composition of the corona would also evolve over time as it transitions from conditions seen in vitro compared to in vivo (Monopoli et al., 2011). The existence of this phenomenon raises the question as to whether in vitro systems should be assessing the behavior and activity of NPs based on the physiochemical properties of the actual NP, upon the behavior of NP–protein corona complex that would be encountered in vivo, or rather the behavior of NP–protein complexes that form with cell culture media proteins to achieve colloidal stability. Similarly, if NPs in vivo form stable hard-corona complexes with proteins encountered in vivo, how do in vitro assessments where these in vivo proteins are not present, relate to in vivo behavior where coronas of different compositions would be formed?

The purpose of this study was to assess this phenomenon using a well-studied and characterized human epidermal keratinocyte (HEK) cell culture model with which our group has had extensive experience. The effect of pre-exposure incubation with three different proteins on the HEK uptake of four different silver nanoparticles (AgNP) (two sizes, two surface coatings) was evaluated. Proteins selected have often been identified as primary components of NP protein coronas in vivo, the so-called adsorbome (Lundqvist et al., 2008; Walkey and Chan, 2012) which includes human serum albumin, the dominant protein found in human serum; IgG as the primary plasma opsonin; and transferrin, a protein often studied for NP tissue targeting applications (Chithrani and Chan, 2007; Salvati et al., 2013).

2. Materials and methods

2.1. Nanoparticles

Citrate BioPure™ silver NP (20 nm and 110 nm) and silica-coated AgNP (40 nm and 120 nm) (nanoComposix Inc., San Diego, CA) were fully characterized (Table 1). Particle size and surface characterization was determined by dynamic light scattering (DLS) and transmission electron microscopy (TEM) to confirm the manufacturer-identified diameters

and surface characterization. To measure the hydrodynamic diameters and the zeta potential, samples of AgNPs were suspended in deionized water, immediately placed in a disposable cuvette and analyzed using a Zetasizer Nano-ZS (Malvern Instruments, Inc., Worcestershire, UK). The DLS readings were performed at the standard characterization temperature of 25 °C. Each measurement was repeated five times, with 10–20 runs as optimized by the instrument. Data was culled based on the correlogram, size quality report, and expert advice rendered by the Dispersion Technology Software (5.03). Additionally, TEM was utilized to characterize the AgNP's structure, shape and size uniformity. Samples were prepared by placing a drop of homogeneous suspension of each AgNP solution in deionized water onto a formvar-coated copper grid and air dried. Samples were examined on an FEI/Philips EM 208S TEM operating at an accelerating voltage of 80 kV. Using ImagePro software, AgNP diameters were measured on 100 randomly selected particles from each individual TEM image using the magnification recorded during TEM viewing.

2.2. Synthesis and characterization of Ag-nps

The 20 nm Ag-NPs (1.00 mg/ml) were obtained from nanoComposix (San Diego, CA, USA). AgNPs were synthesized by ammonium hydroxide catalyzed growth of Ag onto 5 nm gold seed particles while in the presence of citric acid. According to the manufacturer, following synthesis the AgNPs were concentrated via tangential flow filtration, serially washed and suspended in deionized water and 2 mM citrate buffer. Upon arrival, the AgNPs were stored at 4°C in the dark. Silica coated AgNP were fabricated by transferring the AgNP into isopropanol followed by the condensation of tetraethoxysilane onto the surface. The thickness of the silica shell is controlled by varying the relative concentration of the tetraethoxysilane and the AgNP.

2.3. Cell culture and protein binding

HEK were seeded at 4×10^5 and grown for three days in 25 cm² flasks to ~70% confluency. Immediately prior to cellular exposure, the AgNP were incubated for 2 h at 37 °C without (control, in either citrate or silica stock solution) or with physiological concentrations of human serum proteins [albumin, 44 mg/ml (Sigma–Aldrich); IgG, 14.5 mg/ml (Sigma–Aldrich); transferrin, 3 mg/ml (Sigma–Aldrich)]. Then HEK were exposed to control citrate AgNP (25 µg/ml) or silica-coated AgNP (100 µg/ml) or the AgNP/protein complexes ($n = 2$ flasks/treatment) for 3 h, rinsed with Hank's Balanced Salt Solution (HBSS), and grown with fresh medium for 2, 4, and 24 h. As a control for the AgNP/protein complexes, HEK were grown for 24 h with each protein alone. At the end of the growth period, the HEK were rinsed in HBSS and lysed with lysis buffer (0.9% Triton X in HBSS). All media, HBSS rinses, lysis buffers, and HEK were stored in the dark at 4°C until prepared and analyzed for Ag using a Model 2000DV Perkin Elmer inductively-coupled plasma optical emission spectrometer (ICP-OES).

2.4. Quantification of Ag

Samples (500 µl) were pipetted into 15 ml polypropylene tubes and 1.5 ml of 15.8 M nitric acid was added to each sample. Samples were digested at room temperature for at least 6 h, after which silica-coated samples received an additional 0.2 ml of 29 M hydrofluoric acid (HF) to digest their coating. Samples were subsequently heated in a water bath at 95° C for 30 min and then cooled to room temperature. The samples then received 0.5 ml of 12.1 M hydrochloric acid (HCl), and brought to a final volume of 5 ml by the addition of 2% HCl. Samples were shaken and analyzed for Ag at a wavelength of 328.069 nm by ICP-OES utilizing a Ryton Scott spray chamber and a perfluoroalkoxy opal mist nebulizer. A laboratory standard (Scandium) was analyzed every 12 samples, and if the value varied by more than 5% the standard curve was rerun. No carryover was observed in the sample transport system. An internal standard was run with the samples adjoined to the sample

transport line to correct for minor differences in sample transport and background matrices. If the internal standard varied more than 20%, the analysis was stopped and the sample torch cleaned. Method blanks were prepared in the same manner as samples and analyzed for background.

2.5. Transmission electron microscopy (TEM)

HEK were grown to 70% confluency in 25 cm² flasks and exposed to the NP treatments as described above for 24 h. Following exposure, HEK were harvested with 0.05% trypsin/EDTA, rinsed in HBSS, and fixed for 24 h in Trump's fixative (McDowell and Trump, 1976). Cells were rinsed in 0.1 M phosphate buffer, embedded as a pellet in 3% agar, post-fixed in 2% osmium tetroxide, dehydrated through an ascending series of ethanol, cleared in acetone, infiltrated and embedded in Spurr's resin. Thin sections (800 Å) were mounted on copper grids and examined unstained on a FEI/Philips EM208S TEM operating at an accelerating voltage of 80 kV. Cells were not stained to allow for better visualization of the AgNPs and to ensure the absence of stain artifacts resulting from the lead citrate and uranyl acetate.

2.6. Statistical analysis

The mean for each treatment was calculated and significant differences were determined using Tukey's test followed by the Dunnett's test at a $p < 0.05$ level of statistical significance for particle uptake across protein-treatment incubations and control for each NP type and time point. Statistical analysis was performed using the PROC GLM Procedure (SAS 9.3 Windows; SAS Institute, Cary, NC).

3. Results

3.1. Nanoparticles

The characterization of the NP is summarized in Table 1. By TEM, the native citrate AgNP appear as homogeneous electron-dense spheres while the native silica-coated silver appear as electron-dense spheres encapsulated by a thick electron-lucent coating.

3.2. Cell culture and protein binding

The HEK dosed on day 3 had reached ~70% confluency in the 25 cm² flasks. The cells were ~95% confluent, thus not proliferating, by the end of the experiment. The physical appearance of some AgNP/protein complexes changed during the 2 h preincubation. After protein incubation, the 20 nm citrate AgNP solutions became more amber in color with albumin, transferrin and IgG, the 110 nm citrate AgNP solution became dark-green and flocculent with IgG incubation, and the 120 nm silica-coated AgNP became more flocculent with IgG incubation. The Ag recovery was greater for citrate compared to silica coatings and greater for AgNP incubated with the three proteins. Overall, the majority of the AgNP (pooled from protein-bound and control NP) remained in the 3 h dosing medium with HEK uptake of AgNP less than 4.1% of the applied dose across all dosing conditions.

Fig. 1 depicts cellular uptake as reflected by ICP-OES assayed Ag concentrations in cytoplasm for all NP, while Fig. 2 depicts Ag concentrations within the particulate membrane HEK fractions. For the 20 nm and 110 nm citrate AgNP, protein incubation significantly ($p < 0.05$) modulated AgNP uptake. Incubation with albumin and IgG, and to a lesser extent with transferrin, significantly ($p < 0.05$) reduced the amount of Ag uptake into the cells (Fig. 1a,b). The IgG exposure dramatically reduced the 110 nm citrate Ag uptake. For the silica coated AgNP (Fig. 1c,d), statistically significant ($p < 0.05$) protein effects were also seen. IgG showed the greatest uptake with the 20 nm silica coated AgNP, while protein effects on the silica coated 120 nm AgNP at 24 h appeared mixed (Fig. 1d). Similar patterns

were also seen with the AgNP membrane fraction concentrations with protein incubation often significantly altering particle membrane association (Fig. 2).

3.3. Transmission electron microscopy

All AgNP, regardless of type, size, and nature of protein exposure, were found within the cytoplasmic vacuoles of the HEK. The 20 nm citrate AgNP bound to albumin were more dispersed and appeared to break down or disintegrate within the vacuoles (Fig. 3a), while NP bound to IgG (Fig. 3b), transferrin (Fig. 3c), and native control (Fig. 3d) showed slight agglomeration.

The 110 nm citrate AgNP without protein were numerous and present in many vacuoles throughout the cells, but the IgG treated AgNP were less obvious and not as dispersed as the native AgNP. This is also consistent with the cellular concentration profiles in Fig. 1 that depicts a decrease concentration. The agglomerates that were present within the vacuoles were relatively large, and the presence of particulates may indicate the breakdown of the NP adjacent to the protein-bound (Fig. 4a–c) and control (Fig. 4d) NP. Fig. 4a is an electron micrograph of 110 nm citrate AgNP with albumin depicting large spherical black particles in the vacuoles. Fig. 4b is with IgG protein, which depicts these AgNP complexes that have lost the internal black core and now possess only a rim of black with a central gray core that appears to have disintegrated. The transferrin 110 nm citrate AgNP complexes (Fig. 4c) had a different appearance in the vacuoles that showed black spherical AgNP with smaller particles possessing a gray core and many times a central black core with some precipitation or disintegration of the NP that resembled some of the native citrate AgNP (Fig. 4d).

Fig. 5 shows the 40 nm silica-coated AgNP that were present in many of the cell's vacuoles as very tight spherical agglomerates with numerous nanoparticulates often adjacent to the AgNP. The silica shells often appeared empty and devoid of Ag (black core) in all of the protein-bound (Fig. 5a–c) and in the native control (Fig. 5d). The appearance of the 120 nm silica-coated AgNP with albumin, IgG or transferrin (Fig. 6a–d) was similar to the 40 nm silica-coated AgNP, and readily found in many cells although the number of 120 nm silica coated AgNP were less frequently present in the IgG and albumin/AgNP complexes. Unlike the 40 nm silica-coated AgNP, fewer empty silica shells were noted. Fig. 7, is a transmission electron micrograph at a low magnification that has captured an HEK cell in the process of phagocytosis of several 120 nm silica-coated AgNP.

4. Discussion

These studies clearly demonstrate that incubation of AgNP with three common serum proteins associated with the NP adsorbome; albumin, IgG and transferrin, significantly modulated both the amount and pattern of HEK Ag cellular uptake compared to native NP for all four particle types (citrate Ag 20 nm, 110 nm, and silica-coated Ag, 40 nm and 120 nm diameters) studied. For the citrate coated AgNP, protein incubation reduced cellular concentrations, especially for the 20 nm NP. In the larger 110 nm citrate AgNP, incubation with IgG dramatically reduced the uptake of AgNP. In contrast for the silica-coated AgNP, IgG showed the greatest uptake for the 20 nm size while for the 120 nm NP, the protein coating had a mixed effect.

The exact pattern of protein modulation of AgNP uptake is not as important as the observation that different proteins unequivocally modify AgNP uptake into HEK differently. A close examination of differences seen between the cytoplasmic data in Fig. 1 and the membrane bound fraction data in Fig. 2, especially relative to the larger particles, is in itself interesting. Membrane bound data is more difficult to interpret since it contains NP that are

both bound to the surface of cells as well as a fraction within the cells. In contrast, cytoplasmic data reflects on NP that unequivocally entered the cellular domain.

A similar phenomenon of natural complex protein corona effects on uptake of silica NP into other cell types has been described (Lesniak et al., 2012). Similarly, corona formation also eliminated the targeting ability of transferrin-functionalized silicon dioxide NPs into A549 human lung carcinoma epithelial cells (Salvati et al., 2013). Assessing the mechanism and kinetics of these protein-specific effects relative to specific cellular trafficking pathways require additional studies.

AgNP were found individually and as agglomerates within the cytoplasmic vacuoles of HEK by TEM, regardless of the protein treatment, confirming the cytoplasmic analytical data. The AgNP in most cells appeared to be breaking down, as evidenced by particulates located adjacent to the NP. This is consistent with AgNP used in other studies in our laboratory (Samberg et al., 2010, 2011, 2012). We were not able to discern a definitive protein corona around any of the NP due to this apparent breakdown of the AgNP. An interesting observation was the presence of empty silica shells within many of the cells treated with silica-coated Ag. It is possible that the medium, the microenvironment within the cell vacuoles, or both may have caused dissolution of the Ag. The pH for lysosomes is an acidic 5.0 and is most likely responsible for dissolution of the Ag cores, therefore explaining the appearance the AgNP (Liu et al., 2012). The toxicological stress that this amount of Ag dissolution places on cells has not been assessed.

These studies were conducted in HEK which are skin cells and may not reflect the specific pathways seen with other non-epithelial cell types where surface markers and transport pathways would be expected to be different (Lunov et al., 2011; Bajaj et al., 2009; Kim et al., 2007; Lesniak et al., 2012). However, skin is a sentinel organ relative to NP exposure in the environment, in many occupational scenarios and after topical application from cosmetics or drug formulations (Monteiro-Riviere and Riviere, 2009).

It is also the target tissue for Ag accumulation as seen in perfused tissue studies after AgNP arterial infusion (Leavens et al., 2012). Systemic silver exposure has been associated with argyria, a condition in humans where in vivo deposition of silver in skin occurs after oral exposure to primarily colloidal Ag preparations. It has been determined that this condition is not due to Ag deposits from translocated intact AgNP, but rather are secondary particles formed by the dissolution in the gastrointestinal tract of AgNPs followed by ion uptake, circulation of Ag complexes and immobilization as zerovalent AgNP by photoreduction in light affected skin regions (Liu et al., 2012). The roll of actual skin deposition of systemically exposed AgNP in the formation of argyria has not been assessed.

It must be stressed that these studies were not designed to determine the composition of any protein corona surrounding the particles studied. It is a reasonable assumption that incubation of the NPs with individual proteins would form coronas with the incubated proteins. The differential cellular uptake pathways seen confirm this assumption. Since corona formation and persistence is a dynamic process, we do not anticipate that in vivo, similar proteins would compose the coronas for these NPs. Determination of what proteins constitute a corona is a function of the surface properties of the NP relative to the physiochemical properties of the proteins or other macromolecules encountered, relative concentrations, as well as the NPs geometry and size (Lundqvist et al., 2008; Xia et al., 2010; Walkey and Chan, 2012; Deng et al., 2012; Shannahan et al., 2013). Based on the NP protein corona formation kinetic data available, a 2 h incubation period would result in relatively stable protein association (Cedervall et al., 2007; Dell'Orco et al., 2010; Walkey and Chan, 2012). A 3 h exposure window was provided to insure sufficient cellular uptake

of NPs based on previous native AgNP studies (Samberg et al., 2010). This time frame is also consistent with the time one would expect a AgNP to be circulating in the blood stream and be available for interaction with serum proteins with subsequent uptake into tissues, specifically skin (Leavens et al., 2012; Riviere, 2009). We have begun to define simpler modeling approaches to define the precise kinetics of media protein dynamic exchange with NP adsorbome proteins to design appropriate in vitro testing strategies (Sahneh et al., 2013).

These studies clearly demonstrate that NPs with different protein coronas, formed by preferential incubation before exposure with specific proteins, influenced uptake into human keratinocytes. What is the impact of this phenomenon on interpretation of in vitro cell bioassays where native, non-protein complexed NPs are exposed? It is well known that NP–protein interactions provide colloidal stability to many types of NP, and that differential patterns of cell uptake occur in protein free versus protein rich media because of precipitation and solubility issues intrinsic to these colloidal suspensions (Safi et al., 2011; Bajaj et al., 2009; Walkey and Chan, 2012). The same occurs in vivo where colloidal stability in plasma is achieved through protein corona formation with serum proteins, and with opsonization and resultant targeting to reticuloendothelial cells and removal from the systemic circulation being a well-known example. It has long been postulated that the surface of a NP that living cells “see” is a function of the protein corona that exists in vivo (Lynch et al., 2007).

This hypothesis suggests that in vitro cell bioassay systems intending to model in vivo biodistribution and uptake into specific tissues, should assess NP in the form that they occur in vivo, that is with their “natural” protein corona. The nature of an AgNP traversing the lipoidal pathways of the stratum corneum would also be different than seen from particles reaching the viable keratinocytes from the systemic circulation where coronas formed from serum proteins would be expected. How should NPs be exposed to HEKs when systemic or topical exposure are the routes being studied?

The present experiments strongly suggest that the same NP, possessing different protein coronas, behave differently relative to HEK uptake compared to control NP not aged with serum proteins. Effects seen were both particle size and surface coating dependent; that is for the same small set of serum proteins, all AgNP do not behave the same. These results bring into serious questions as to how in vitro assays using native NP should be interpreted relative to what would be expected to occur in vivo.

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Highlights

- Nanoparticles with different protein coronas influence cell uptake.
- Albumin, IgG and transferrin blood proteins modulate amount of cell uptake of silver nanoparticles.
- Findings have major implication to the design and interpretation of toxicology screens.

Fig. 1. Significant effect of Ag uptake into HEK cytoplasm by incubating AgNP with albumin, transferrin and IgG proteins prior to exposure. (a) 20 nm citrate Ag, (b) 110 nm citrate Ag, (c) 20 nm silica-coated Ag, (d) 120 nm silica-coated Ag. * denotes statistical significance ($p < 0.05$) of protein incubation compared to control.

Fig. 2. Effect of Ag uptake into HEK membranes by incubating AgNP with albumin, transferrin and IgG proteins prior to exposure. (a) 20 nm citrate Ag, (b) 110 nm citrate Ag, (c) 20 nm silica-coated Ag, (d) 120 nm silica-coated Ag. * denotes statistical significance ($p < 0.05$) of protein incubation compared to control.

Fig. 3. Transmission electron micrographs of 20 nm citrate AgNP within the cytoplasmic vacuoles of HEK. (a) Albumin/AgNP complexes; (b) IgG/AgNP complexes; (c) transferrin/AgNP complexes; (d) control AgNP. Arrow denotes breakdown of AgNP. Bars= 100 nm.

Fig. 4. Transmission electron micrographs of 110 nm citrate AgNP within the cytoplasmic vacuoles of HEK. (a) Albumin/AgNP complexes; (b) IgG/AgNP complexes; (c) transferrin/AgNP complexes; (d) control AgNP. Arrows denote breakdown of AgNP. Bars= 100 nm.

Fig. 5. Transmission electron micrographs of 40 nm silica-coated AgNP within the cytoplasmic vacuoles of HEK. (a) Albumin/AgNP complexes; (b) IgG/AgNP complexes; (c) transferrin/AgNP complexes; (d) control AgNP. Arrows denote breakdown of AgNP. Bars= 100 nm.

Fig. 6. Transmission electron micrographs of 120 nm silica-coated AgNP within the cytoplasmic vacuoles of HEK. (a) Albumin/AgNP complexes; (b) IgG/AgNP complexes; (c) transferrin/AgNP complexes; (d) control AgNP. Arrows denote breakdown of AgNP. Bars= 100 nm.

Fig. 7. Transmission electron micrograph of an HEK engulfing 120 nm silica-coated AgNP. Arrow denotes AgNP within invagination. Bar= 2 μ m.

Table 1

Characterization of AgNP.

Nanomaterial	State	Concentration (mg/ml)	Diameter (by TEM) (nm)	Diameter (by DLS) (nm)	Zeta potential (mV)
Citrate silver	Solution	1.0	19.2	27.4	-36.8
Citrate silver	Solution	1.0	108.5	106.0	-44.4
Silica-coated silver	Solution	4.7	40.5 ^a	146.7	-25.4
Silica-coated silver	Solution	3.2	118.5 ^b	175.7	-31.7

^aCore diameter; does not include 24.6 nm thick shell.^bCore diameter; does not include 22.8 nm thick shell.