Mechanisms of cell uptake, inflammatory potential and protein corona effects with gold nanoparticles

Aim: To assess inflammation, cellular uptake and endocytic mechanisms of gold nanoparticles (AuNP) in human epidermal keratinocytes with and without a protein corona. **Materials & methods:** Human epidermal keratinocytes were exposed to 40 and 80 nm AuNP with lipoic acid, polyethylene glycol (PEG) and branched polyethyleneimine (BPEI) coatings with and without a protein corona up to 48 h. Inhibitors were selected to characterize endocytosis. **Results & conclusion:** BPEI-AuNP showed the greatest uptake, while PEG-AuNP had the least. Protein coronas decreased uptake and affected their mechanism. AuNP uptake was energy-dependent, except for 40 nm lipoic-AuNP. Most AuNP were internalized by clathrin and lipid raft-mediated endocytosis, except for 40 nm PEG was by raft/noncaveolae mediated endocytosis. Coronas inhibited caveolae-mediated-endocytosis with lipoic acid and BPEI-AuNP and altered 40 nm PEG-AuNP from raft/noncaveolae to clathrin. Inflammatory responses decreased with a plasma corona. Results suggest protein coronas significantly affect cellular uptake and inflammatory responses of AuNP.

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Engineered nanoparticles (NP) such as gold (Au) NP have unique physicochemical properties making them beneficial for biomedical applications such as near-infrared imaging [1], drug delivery [2] and cancer therapy [3]. It is these physicochemical properties such as size and surface charge that have an effect upon biological systems [4]. Several studies reported that the biodistribution and cellular uptake of AuNP were size-dependent [5–7] but the surface properties also affected cell uptake [8–12]. AuNP with different cationic surface coatings showed diverse mechanisms of cellular uptake in HeLa and MCF10A epithelial cells [13]. Others have reported that the cell uptake mechanisms of 2, 4 and 6 nm AuNP were dependent on size and surface functional groups [14]. It is well-known that the interac-

tive surface and fate of an NP in a biological system is determined by the biocorona. Complex proteins, ions, lipids and other macromolecules will readily adsorb onto an NP and these biomolecules will form the biocorona [15,16]. Therefore, cell uptake of NP may also be modulated by its biomolecular surface [17]. Our laboratory showed that with different protein coronas at physiological concentrations of albumin (44 mg/ml), IgG (14.5 mg/ml) or transferrin (3 mg/ml) form NP protein complexes that significantly affected NP uptake in human epidermal keratinocytes (HEK) [18]. Particle size and surface ligand grafting density can also affect the adsorption of serum proteins and cell uptake in macrophages [19]. In the presence or absence of a protein corona, silica NP showed different uptake efficiencies [20].

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Yet, it is unclear how the protein corona of AuNP affects the cell uptake and its mechanisms.

The presence of serum in cell cultures may confound NP uptake by forming a serum protein corona [20]. In order to avoid this disadvantage, cryopreserved primary neonatal HEK were used as a suitable model system because they are grown in serum-free medium. HEK are epithelial cells that are an important component of innate immunity. This study provides a better understanding on how epithelial cells are involved in AuNP cell uptake and their interactions with the innate immune system thereby, providing key information on the design of smart NP to avoid being trapped within the innate immune system.

The uptake mechanisms of NP are not well-understood, however, endocytosis of NP encompasses a series of processes that depend on the size, shape and functional surface properties of the NP [6,11,21–23] which make it a more diverse and difficult process to comprehend. Although the precise mechanisms of endocytosis are unclear, the process of endocytosis occurs through multiple mechanisms and is often defined as phagocytosis (cell eating) or pinocytosis (cell drinking) [23]. Macropinocytosis is receptor independent and fluid-based, while clathrin-mediated endocytosis and nonclathrin-mediated endocytosis are considered as receptor dependent endocytosis [22–24].

The objectives of this study were to investigate cell uptake in HEK from 15 min to 48 h with 40 and 80 nm AuNP with lipoic acid (negatively charged), polyethylene glycol (PEG, neutral charged) and branched polyethyleneimine (BPEI, positively charged) surface coatings in the presence or absence of a human plasma (HP) or human serum albumin (HSA) protein corona and to study the inflammatory potential. In addition, different cellular uptake inhibitors were selected to characterize the mechanism of endocytosis and to gain insight on the role of the protein corona on the mechanism of cell uptake.

Materials & methods

AuNP synthesis

Biopure™ 40 and 80 nm lipoic, PEG and BPEI-coated AuNP were purchased from nanoComposix (CA, USA) and the synthesis was conducted by using the reduction of hydrogen tetrachloroaurate (III) in a basic solution of potassium carbonate. Tangential flow filtration was used to concentrate and wash the NP. The lipoic acid functionalized surfaces were synthesized by adding 0.2 mg lipoic acid with 1 mg Au. PEG

functionalization was performed by adding 0.5 mg of 5 kDa thiol-methoxy-terminated PEG for each 1 mg of Au. Carboxylic acid of the lipoic and the free amines on the BPEI were linked by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide coupling chemistry to synthesize the BPEI functionalized surface. After each functional group was synthesized, AuNP were washed by tangential flow filtration and filtered under sterile conditions. Then BPEI-AuNP were centrifuged to remove any residual BPEI. Endotoxin, an important biological contaminant was also evaluated in all samples and confirmed to be less than 2.5 EU/ml [25,26]. All NP were stored at 4°C in the dark.

Preparation of the protein corona on NP

Human blood was collected from healthy donors ($n = 5$) and was obtained from the Biological Specialty Corporation (PA, USA) and shipped the same day and stored at -80°C. Plasma was obtained by centrifugation of the anti-coagulated blood for 10 min at 2500 $\times g$ at room temperature. The collected HP was pooled together. AuNP were incubated with 55% v/v human pooled plasma (HP) or 44 mg/ml HSA (Sigma-Aldrich, MO, USA) for 1 h at 37°C in an orbital shaking incubator at 250 rpm. After incubation, the unbound and weakly associated proteins (known as soft protein corona) were removed by three repeated phosphate-buffered saline (PBS) washes, centrifuged at 16,000 $\times g$ at 20°C for 15 min.

Nanoparticle characterization

AuNP with and without a protein corona were fully characterized: by transmission electron microscopy (TEM) on an FEI Tecnai G2 Spirit BioTWIN TEM (FEI, OR, USA) with energy-dispersive x-ray spectroscopy (EDX) at an accelerating voltage of 80 kV. The hydrodynamic diameter and ζ -potential with the Zetasizer Nano ZS (Malvern Instruments, Worcester, UK); and ultraviolet-visible (UV-VIS) spectrophotometry on a BioTek Hybrid reader (BioTek Instruments, Inc., VT, USA). The size distribution and ζ -potential were obtained with four separate measurements and each measurement counts at least ten-times. The characterization of AuNP in PBS is presented in **Figure 1 & Table 1**, and the characterization of AuNP in HEK medium (serum-free condition) is presented in **Supplementary Table 1**.

Cytotoxicity

Cryopreserved primary neonatal HEK (Lonza, MD, USA) were cultured in KGM-Gold™ keratinocyte

Figure 1. Transmission electron microscopy and ultraviolet-visible spectra of gold nanoparticles (see facing page).

Upper panel is representative of 40 and 80 nm AuNP; lower panel represents the size distribution profiles of 40 and 80 nm Lipoic, PEG and BPEI-AuNP.

AuNP: Gold nanoparticles; BPEI: Branched polyethyleneimine; PEG: Polyethylene glycol.

Table 1. Characterization of gold nanoparticles with and without human plasma and human serum albumin corona.

AuNP	Bare			Human plasma			Human serum albumin		
	Hydrodynamic diameter (nm)	Pdl	ζ -potential (mV)	Hydrodynamic diameter (nm)	Pdl	ζ -potential (mV)	Hydrodynamic diameter (nm)	Pdl	ζ -potential (mV)
40 Lipoic	47.08	0.071	-44.00	74.29	0.094	-11.50	79.25	0.236	-15.30
40 PEG	61.10	0.066	-4.60	72.70	0.086	-9.87	61.77	0.070	-6.37
40 BPEI	56.43	0.186	39.20	296.50	0.305	-14.00	127.10	0.356	-12.30
80 Lipoic	91.67	0.053	-53.60	131.10	0.083	-12.10	111.90	0.119	-14.50
80 PEG	109.80	0.057	-3.44	113.50	0.078	-9.73	112.60	0.019	-7.96
80 BPEI	105.40	0.112	46.20	266.40	0.303	-9.26	188.10	0.295	-9.81

AuNP: Gold nanoparticle; BPEI: Branched polyethyleneimine; Pdl: Polydispersity index; PEG: Polyethylene glycol.

growth medium and expanded in T-75 flasks to 80% confluency. Cells were detached with trypsin and seeded in 96-well black microplates (0.32 cm² growth area) with 10,000 cells/well and grown overnight at 37°C in a humidified environment of 5% CO₂. To minimize evaporation of the culture medium, the peripheral 96-well plates were filled with PBS. Medium was replaced with fresh medium (control) or medium with different concentrations of AuNP (12.5, 25, 50 and 100 µg/ml; n = 6). After 24 or 48 h, HEK were washed three-times with PBS at pH 7.4 and alamar blue contained medium (1:10 volume ratio) was added to each well and cultured for 3 h. Fluorescence was quantitated with the BioTek Hybrid reader (BioTek Instruments, Inc.) at excitation/emission of 540/590 nm. Cell viability, proportional to an increase in fluorescence, was subtracted from the fluorescence background and normalized to the controls and expressed as percent viability [27].

AuNP cellular uptake in HEK

HEK were seeded in six-well plates at a density of 2.5×10^5 cells/ml and cultured overnight at 37°C in a humidified environment of 5% CO₂. Lipoic, PEG and BPEI-AuNP with or without HP or HSA protein coronas were added to HEK at 25 µg/ml and incubated for 0.25, 0.5, 1, 3, 6, 12, 24 and 48 h at 37°C. After incubation, the cells were washed three-times with PBS and then subjected to an etching process with 1 ml iodine and potassium iodide (I₂/KI: 0.34 mM/2.04 mM) for 3 min [10]. After etching, cells were washed with PBS for three-times and collected for inductively coupled plasma mass spectrometry (ICP-MS).

Cellular uptake mechanisms

HEK were seeded in 12-well plates at a density of 2×10^5 cells/ml and cultured overnight. Different endocytic inhibitors were first preincubated with HEK for 1 h in [NaN₃ (sodium azide, 10 mM, Sigma-Aldrich), 2-deox-

yglucose (DOG, 50 mM, Sigma-Aldrich), sucrose (450 mM, Sigma-Aldrich), chlorpromazine hydrochloride (CPZ, 28 nM, Sigma-Aldrich), nystatin (180 nM, Sigma-Aldrich), or methyl- β -cyclodextrin (M β CD, 5 mM, Sigma-Aldrich)]. The concentrations used were based on previous studies [14,28,29], and the treatment with different inhibitors did not induce cytotoxicity. After preincubation with the different inhibitors, the medium was replaced, and lipoic, PEG and BPEI-AuNP with or without HP protein corona were added to HEK at 25 µg/ml in the presence of different inhibitors. All AuNP and AuNP with HP protein corona without inhibitors served as the control. After 3 h of incubation, HEK were washed with PBS and etching was conducted for 3 min, followed by another three PBS washes and then HEK were collected for ICP-MS analysis.

Temperature & energy-dependent uptake pathways

HEK were seeded as mentioned above and pretreated with/without 10 mM NaN₃ and 50 mM DOG at 4°C or 37°C for 1 h. HEK were incubated with 25 µg/ml with all AuNP or AuNP with a HP protein corona for 3 h with and without NaN₃/DOG at 4°C or 37°C. AuNP or AuNP with a HP protein corona without inhibitors were cultured at 37°C to serve as the control. After 3 h of incubation, HEK were washed with PBS and etched for 3 min, then washed three-times with PBS and collected for ICP-MS.

ICP-MS analysis

Cells were collected and dried using a heat block at 90°C for 5 h. Samples were digested by hydrochloric acid and nitric acid (volume ratio 3:1) at 95°C overnight. After digestion, each sample was diluted to 5 ml with 1% nitric acid and 2% hydrochloric acid solution. A series of Au standards (0, 1, 10, 25, 50 and 100 ppb) were prepared each time with the samples. Samples and fresh Au standards were analyzed using the Nex-

ION 350X ICP-MS spectrometer (Perkin Elmer, MA, USA). The Au ion concentration of the samples was quantitated relative to the Au standard curve.

Cell internalization amount of AuNP was quantified with the mass of intracellular gold that converted to the AuNP number using a reported method [30]. The internalization rate of AuNP was calculated by AuNP internalization amount that divided by incubation period. Cell uptake efficiency (%) was normalized to cells without inhibitors.

Transmission electron microscopy

For TEM characterization of the AuNP, a drop of each type of AuNP in deionized water were placed on formvar-coated carbon grids and allowed to air dry. All AuNP were viewed on FEI Tecnai G2 Spirit BioTWIN TEM at an accelerating voltage of 120 kV.

For the uptake study, HEK were grown to 80% confluency and treated with 25 µg/ml of all three surface coatings of AuNP for 1 and 24 h. Following exposure, HEK were harvested with 0.05% trypsin/EDTA, rinsed in buffer and fixed for 24 h in 4% formaldehyde and 1% glutaraldehyde in phosphate buffer. Cells were rinsed in 0.1 M phosphate buffer, embedded as a pellet in 3% agar and postfixed in 1% osmium tetroxide for 1 h, washed in buffer and centrifuged for 5 min. All pellets were dehydrated in a graded ethanol series, cleared in acetone, infiltrated and embedded in Spurr's resin (Polysciences, Inc. PA, USA). Thin sections (800Å) were mounted on copper grids and examined unstained on a FEI Tecnai G2 Spirit BioTWIN TEM at an accelerating voltage of 80 kV. Cells were not stained to allow for better visualization of the AuNP within the vacuoles and to ensure the absence of stain artifacts resulting from the lead citrate and uranyl acetate. TEM and scanning transmission electron microscopy (STEM) images were taken and EDX was conducted on an Oxford detector at <135 eV.

Cytokine detection

HEK were treated with 25 µg/ml of each type of AuNP for 24 and 48 h. Supernatants were collected and measured for human cytokines IL-1α, IL-1β and IL-8 and quantitated by ELISA. The ELISA kits were purchased from R&D Systems (MN, USA), and performed according to the manufacturers' instructions. The raw data were quantitated relative to the standard curve. The cytokine detection limits were: IL-1α (7.8), IL-1β (3.9), IL-8 (31.3) pg/ml.

Statistical analysis

Mean values for each treatment were calculated and the significant differences ($p < 0.05$) determined with the F -test and Student's t -test. The cell viability data

are expressed as mean $\pm$ SD ($n = 5$ wells/treatment) and the cytokine and cell uptake data as mean $\pm$ SD ($n = 3$ wells/treatment).

Results & discussion

Characterization of AuNP

All 40 and 80 nm lipoic acid, PEG and BPEI-coated AuNP were characterized by TEM, UV-VIS spectrophotometry and Zetasizer analysis (Figure 1 & Table 1). After a 1-h incubation with HP or HSA, the protein corona-coated AuNP were recharacterized and showed an increase in size with all three surface coatings (Table 1). A protein corona surrounding a AuNP was observed by TEM (Supplementary Figure 1) similar to what was reported by our laboratory [31]. The hydrodynamic diameters of AuNP were generally larger than the core particle size noted by TEM. After the AuNP were exposed to HP and HSA protein coronas, the hydrodynamic diameters increased and the Z-potential had a slight negative charge (Table 1). The hydrodynamic diameters of AuNP exposed to the cell culture medium can be seen in Supplementary Table 1. All NP were screened for endotoxins because it is a common biological contaminant that can interfere with the biological effects of NP [25,26,32].

Cytotoxicity of AuNP

To assess the cytotoxicity of AuNP in HEK, the HEK were exposed to various concentrations ranging from 12.5, 25, 50 and 100 µg/ml of AuNP for 24 and 48 h. Figure 2A–D depicts the toxicity of 40 and 80 nm lipoic, PEG and BPEI-coated AuNP which showed no toxicity with most coatings at 12.5 µg/ml and 25 µg/ml. However, the 50 µg/ml and 100 µg/ml of 40 and 80 nm BPEI-AuNP showed a dose dependent toxicity at 24 h; while at 48 h the 40 nm lipoic and BPEI-AuNP and 80 nm PEG and BPEI-AuNP showed a decrease in cell viability. These results were not surprising because most NP will show a decrease in cell viability at high concentrations at 24 h and a more exacerbated response by 48 h. Therefore, the nontoxic concentration of 25 µg/ml was selected to study cell uptake.

Cell uptake of AuNP in HEK

HEK were exposed to the nontoxic dose of 25 µg/ml of bare 40 and 80 nm lipoic, PEG and BPEI-coated AuNP from 15 min to 48 h. Cells were washed and etched with 1 ml I_2/KI (0.34 mM/2.04 mM) for 3 min to remove the surface bound AuNP [10]. This procedure ensures that all Au ions are released from the intracytoplasmic vacuoles of the HEK and measured by ICP-MS. Two different methods were used to show the cell uptake results: the uptake rate (uptake amount per hour) seen in Figure 3 by profiling the

Figure 2. Cell viability of 40 and 80 nm lipoic acid, polyethylene glycol and branched polyethyleneimine-coated gold nanoparticles in human epidermal keratinocytes at 12.5, 25, 50 and 100 µg/ml for 24 and 48 h. 40 nm AuNP at 24 h (A) and 48 h (C); 80 nm AuNP at 24 h (B) and 48 h (D). Data represent as mean $\pm$ SD (n = 5 wells/treatment). *p < 0.05, **p < 0.01, ***p < 0.001 as AuNP versus control.

AuNP: Gold nanoparticles; BPEI: Branched polyethyleneimine; Lipoic: Lipoic acid; PEG: Polyethylene glycol.

characteristics of the uptake of 40 and 80 nm NP and the ability of HP and HSA protein coronas on cell uptake profiles. The uptake accumulative amounts overtime seen in Figure 4, which compares the different uptake amounts of bare, HP and HSA corona-coated AuNP. Figure 3A–C & Figure 4 (black square) show the internalization rate and amount of the lipoic, PEG and BPEI-AuNP in HEK. Not surprisingly, PEG-AuNP showed the least uptake in HEK compared with the lipoic and BPEI coatings. This may be due to the neutral surface charge that minimized the cell uptake [8,33]. As expected, the BPEI-AuNP had the greatest cell uptake presumably due to its positive charge which increases the interaction between

the BPEI-AuNP and the HEK's membrane, thereby promoting an increase in cell uptake [10]. The 40 nm lipoic and BPEI-AuNP with charged surfaces showed an increase in uptake compared with the 80 nm lipoic and BPEI-AuNP (Figure 3A & C; Figure 4B, C, E & F). In contrast, the 40 nm PEG-AuNP with a neutral charge showed less internalization than the 80 nm PEG-AuNP (Figure 3B, Figure 4A & D). The internalization rate of AuNP showed a decrease with lipoic and BPEI coatings over time, while PEG showed an increase after 1 h (Figure 3A–C). This suggests that if AuNP are used for drug delivery or cancer therapy by direct injection into the target tissue, charged AuNP and especially the BPEI-coated NP will be more effective. However,

if PEG-AuNP are used, then the route and time of administration becomes important since a rapid influx of AuNP was noted at later time points.

Ultrastructural localization of AuNP

TEM depicted the intracellular localization of bare AuNP within cytoplasmic vacuoles in HEK (Figure 5). At 1 h, most 40 and 80 nm AuNP were localized within the endocytic/cytoplasmic vesicles close to the periphery of the cell membrane with the exception of 40 and 80 nm PEG-AuNP. It was very difficult to view PEG-AuNP within HEK but after searching several cells, only one cell in both the 40 and 80 nm AuNP had AuNP at 1 h. At 24 h, most AuNP were localized adjacent to the nucleus but were never noted within the nucleus. In Figure 5, the TEM micrographs showed 40 and 80 nm BPEI-AuNP in contact with the outer cell membrane at 1 h but less was noted with the 80 nm BPEI-AuNP compared with the 40 nm BPEI-AuNP. Both 40 and 80 nm PEG-AuNP were rarely located within the cytoplasm at 1 h, but more AuNP were present at 24 h. The amount of 40 nm PEG-AuNP noted by TEM was less when compared to the 40 and 80 nm lipoic, BPEI and 80 nm PEG-AuNP at 24 h, which is consistent with the ICP-MS data. Our STEM image allowed us to get an overall intracellular view of the location of the AuNP within the cell (Supplementary Figure 2). The 40 nm BPEI-AuNP were in close proximity to the outer cell membrane at 1 h. Over time, endocytosis of the AuNP showed several 40 nm BPEI-AuNP agglomerates surrounding the nucleus at 24 h (Supplementary Figure 2). Using EDX, 40 nm BPEI-AuNP (Supplementary Figure 2) showed an increase in AuNP within the cytoplasm of HEK and EDX verified the element to be Au.

Protein corona can inhibit AuNP uptake in HEK

Pooled sodium citrate human blood plasma (HP) (55%v/v) and physiological concentrations of HSA (44 mg/ml) were added to all AuNP in an orbital shaking incubator for 1 h to aid in the formation of the corona over the AuNP. Protein corona effects on AuNP cell uptake are illustrated in Figure 4. In all cases, HP protein corona reduced AuNP uptake with all three surface coatings. These results were similar to that reported by others and in our laboratory with silver NP [18] which showed a decrease in NP cell uptake in the presence of plasma or serum [9,14,34]. Figure 4, showed a similar and consistent amount of AuNP cell uptake in HEK over time with HP-AuNP, except for 40 and 80 nm BPEI-AuNP which showed an increase immediately but remained relatively consistent over time. Also, the 80 nm PEG-AuNP with an HP corona showed an increase in uptake at 12 h.

HSA has been reported to be one of the most adsorbed proteins to NP [35,36]. The HSA corona in Figure 4 showed a decreased in AuNP cell uptake similar to the HP corona, in particular with the 40 and 80 nm BPEI-AuNP which showed a consistent but minimal cell uptake over time (Figure 4C & F). However, HSA caused a slight increase in uptake at 24 and 48 h for all lipoic-AuNP and PEG-AuNP. Figure 3D–I, shows the internalization rate of AuNP with HP and HSA protein coronas in which similar patterns were noted. However, comparing the internalization rate of both the HP and HSA protein coronas and bare NP, the coronas on the lipoic-AuNP altered the internalization rate of 40 and 80 nm AuNP. The 80 nm corona-coated lipoic-AuNP showed greater internalization compared to the 40 nm corona-coated lipoic-AuNP (Figure 3A, D & G). In all cases, the internalization rate of the protein coronas of AuNP showed a decrease in cellular uptake compared to the bare NP, except for HSA-coated PEG-AuNP.

HP protein corona alters the endocytic uptake

It has been reported that the surface properties can modulate the protein corona and alter the cellular uptake efficiency [18–20]. Therefore, it is logical to suspect that in the presence of a protein corona, the cell uptake pathways selected may be affected. Therefore, AuNP endocytic pathways were investigated with HEK in the absence or presence of an HP protein corona.

Energy-dependent endocytic pathway of AuNP in the absence or presence of a protein corona

Energy-dependent endocytic pathways may be suppressed at low temperatures (low metabolism) and ATP depletion with sodium azide and 2-deoxyglucose as inhibitors [28]. In Figure 6A & C, all bare AuNP showed a reduction in cell uptake in the presence of ATP depletion agents or at low temperatures (4°C) except for the 40 nm lipoic-AuNP. The surface coatings of 40 nm AuNP significantly affected the energy-dependent endocytic pathways. These results suggest that BPEI-AuNP with a positive charge was energy-dependent, while PEG-AuNP with a neutral charged surface was less energy dependent because it showed less of a decrease in cell uptake compared to BPEI-AuNP. However, the negatively charged lipoic-AuNP was energy nondependent. The 40 nm lipoic-AuNP may be internalized by macropinocytosis which is a nonspecific endocytotic pathway [23], or another possibility would be that it could directly traverse through the cell membrane and into the cytoplasm of the cell due to its small size and same surface charge as the cell membrane. In contrast, the 80 nm AuNP with negative, neutral and positive surfaces all showed an energy-dependent endocytosis.

Figure 3. The internalization rate of 40 and 80 nm gold nanoparticles in human epidermal keratinocytes over time. Human epidermal keratinocytes were exposed to 25 $\mu\text{g}/\text{ml}$ of 40 and 80 nm AuNP in the presence or absence of HP corona or HSA corona from 0.25 to 48 h. Cell uptake was analyzed by ICP-MS and the internalization rate (uptake number/h) was calculated. (A–C) Bare Lipoic, PEG and BPEI-AuNP; (D–F) HP-coated Lipoic, PEG and BPEI-AuNP; (G–I) HSA-coated Lipoic, PEG and BPEI-AuNP. Data represent as mean $\pm$ SD ($n = 3$ wells/treatment).

AuNP: Gold nanoparticles; BPEI: Branched polyethyleneimine; HP: Human plasma; HSA: Human serum albumin; Lipoic: Lipoic acid; NP: Nanoparticle; PEG: Polyethylene glycol.

The HP protein corona of AuNP significantly affected the uptake ratio and mechanisms in HEK. Although BPEI-AuNP with a HP corona was still mediated by an energy-dependent endocytosis, the uptake ratio was less compared to bare AuNP, which signifies that BPEI-AuNP with a HP corona (Figure 6B & D) can be internalized with less energy. The effect was similar to the

40 and 80 nm PEG-AuNP and 80 nm lipoic-AuNP. However, the bare 40 nm lipoic-AuNP was nonenergy-dependent, and it is not surprising to find that the HP corona could alter the 40 nm lipoic-AuNP to become energy-dependent. These results showed that the uptake of the HP protein corona of 40 nm lipoic-AuNP could be partially blocked by ATP depletion.

Figure 4. Cell uptake of gold nanoparticles in the presence or absence of a protein corona. Human epidermal keratinocytes were exposed to 25 $\mu\text{g}/\text{ml}$ of 40 nm (left panel) and 80 nm (right panel) AuNP in the presence or absence of HP corona or HSA corona ranging from 0.25 to 48 h. (A–C) 40 nm PEG, Lipoic and BPEI-AuNP; (D–F) 80 nm PEG, Lipoic and BPEI-AuNP. Data represent as mean $\pm$ SD ($n = 3$ wells/treatment). AuNP: Gold nanoparticles; BPEI: Branched polyethyleneimine; HP: Human plasma; HSA: Human serum albumin; Lipoic: Lipoic acid; NP: Nanoparticle.

Mechanisms of receptor-mediated endocytosis of AuNP in the absence or presence of a protein corona

Clathrin and nonclathrin-mediated endocytosis all fall into a receptor-mediated internalization pathway. In order to understand how AuNP become internalized

into the cell and how the protein corona affects these uptake pathways, a set of endocytic inhibitors were used to determine the mechanisms of endocytosis of AuNP in the absence or presence of an HP protein corona.

Clathrin is a protein and clathrin-coated pits are found in regions of the cell membrane specialized in receptor-

Figure 5. Transmission electron micrographs depicting Lipoic acid, polyethylene glycol and branched polyethyleneimine-coated gold nanoparticles in human epidermal keratinocytes at 1 and 24 h. Human epidermal keratinocytes were exposed to 25 $\mu\text{g}/\text{ml}$ of 40 and 80 nm Lipoic, PEG and BPEI-gold nanoparticles (AuNP) for 1 and 24 h. The inset in the 40 and 80 nm PEG at 1 h depicts a higher magnification of a single AuNP (Scale bar = 2 μm). BPEI: Branched polyethyleneimine; Lipoic: Lipoic acid; PEG: Polyethylene glycol.

mediated endocytosis. They are formed by invaginations of the cell membrane within minutes which then gets pinched off to form a clathrin-coated vesicle which can fuse with early endosomes [23]. Clathrin-mediated endocytosis has been studied with CPZ and sucrose as inhibitors in HEK, HeLa and other cells [29,37–39]. Sucrose provides a hypertonic condition preventing the interaction of clathrin and adaptors by inhibiting the formation of shallow-coated pits (type I-coated pits) or receptor clustering; while CPZ can specifically inhibit clathrin mediated pathways by inhibiting the progression from type II-coated pits to type III-coated pits [40]. **Figure 7A & C** shows 40 and 80 nm BPEI-AuNP were inhibited significantly in the presence of clathrin inhibi-

tors without size dependency. A slight inhibitory effect was observed with 40 nm lipoic-AuNP but the presence of these clathrin inhibitors significantly inhibited 80 nm lipoic-AuNP. PEG-AuNP showed similar effects to lipoic-AuNP, which depicted that 80 nm PEG-AuNP were inhibited by clathrin inhibitors, but no effects were detected with the 40 nm PEG-AuNP.

The mechanisms of clathrin independence internalization are considered to be through nonclassic endocytic pathways which are very sensitive to cholesterol depletion [24]. This is commonly known as lipid raft-mediated endocytosis which can be divided further as caveolae-dependent and caveolae-independent [41]. Lipid rafts provide a platform and several types of receptors,

adaptors, regulators and other proteins can act as signaling complexes to mediate endocytosis [29]. Caveolae (50–80 nm) are flask-shaped invaginations of the cell membrane that are conferred by caveolin and are an integral membrane protein [29]. Caveolin is a dimeric protein that binds cholesterol, inserts into the plasma membrane as a loop and self-associates to form a caveolin coat on the membrane invaginations [23,42]. Caveolae-independent endocytosis is not fully understood, however, it requires a ‘raft’ for internalization and may be defined as raft-/noncaveolae-mediated endocytosis [23].

Cholesterol depletion agents, nystatin and M β CD, were used to investigate lipid raft-mediated pathways of AuNP [23,43–45]. In the presence of nystatin, caveolae-mediated endocytosis was inhibited which significantly decreased cell uptake of 40 and 80 nm lipoic and BPEI-AuNP but had no effect on the 40 and 80 nm PEG-AuNP. Since M β CD alters cholesterol-

rich domains of the cell membrane, treatment with M β CD in HEK can inhibit the lipid raft-mediated endocytosis pathways (both caveolae and noncaveolae pathways), which decreases the cell uptake of all AuNP (Figure 7A & C). Therefore, the uptake of 40 and 80 nm PEG-AuNP had no effect with caveolae inhibitors but caused a reduction in lipid raft inhibitors. The 40 and 80 nm PEG-AuNP were primarily internalized by caveolae-independent raft-mediated endocytosis.

In addition, the endocytic mechanisms were investigated in the presence of HP protein corona (Figure 7B & D) which showed alterations in the internalization pathway of 40 nm PEG-AuNP from a raft/noncaveolae pathway to a clathrin-mediated pathway, even though there was only a 30% decrease of cell uptake in the presence of inhibitors. Also, it caused inhibition of the raft-/noncaveolae-mediated endocytic pathway with 80 nm PEG-AuNP. For the 40 and 80 nm lipoic and BPEI-AuNP,

Figure 6. Energy-dependent endocytosis of 40 and 80 nm Lipoic, polyethylene glycol and branched polyethyleneimine-coated gold nanoparticles in the absence or presence of a human plasma protein corona.

Human epidermal keratinocytes were treated with 25 μ g/ml of 40 nm (A & B) and 80 nm (C & D) AuNP in the absence (A & C) or presence (B & D) of human plasma corona. Cell uptake efficiency (%) was normalized to cells without inhibitors. Data are presented as mean $\pm$ SD (n = 3 wells/treatment).

*p < 0.05, **p < 0.01, ***p < 0.001 inhibitor treatment versus normalized controls.

#p < 0.05, ##p < 0.01, ###p < 0.001 with a protein corona AuNP versus bare AuNP without a protein corona.

AuNP: Gold nanoparticles; BPEI: Branched polyethyleneimine; Lipoic: Lipoic acid; PEG: Polyethylene glycol.

Figure 7. Endocytic mechanisms of gold nanoparticles in the absence or presence of a human plasma protein corona. Human epidermal keratinocytes were exposed to endocytic inhibitors and treated with 25 $\mu\text{g}/\text{ml}$ of 40 nm (A & B) and 80 nm (C & D) AuNP in the absence (A & C) or presence (B & D) of a plasma protein corona. Cellular uptake efficiency (%) was normalized to cells without inhibitors. Data are presented as mean $\pm$ SD ($n = 3$ wells/treatment).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ with inhibitors versus normalized controls.

$p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ with a protein corona AuNP versus bare AuNP without a protein corona.

AuNP: Gold nanoparticles; BPEI: Branched polyethyleneimine; Lipoic: Lipoic acid; MβCD: Methyl- β -cyclodextrin; PEG: Polyethylene glycol.

the caveolae-mediated endocytosis was eliminated in the presence of a HP protein corona and only a raft-/noncaveolae-mediated endocytosis existed. However, clathrin-mediated endocytosis usually are not affected in the presence of a HP protein corona (some inhibition effects were noted; Figure 7) with the exception of the 80 nm BPEI-AuNP which showed a nonclathrin-dependent endocytosis with the HP corona. The HP corona of 80 nm BPEI-AuNP significantly reduced the clathrin and caveolae-mediated endocytosis, while the raft-/noncaveolae-mediated endocytosis increased. HP corona of 40 nm BPEI-AuNP had little effect on the cell uptake pathways. Clathrin-mediated endocytosis was still the primary cell uptake pathway for the 40 nm BPEI-AuNP with a HP protein corona, while caveolae-mediated endocytosis was inhibited in the presence of a HP corona. The 40 nm lipoic-AuNP with a HP corona was mediated by clathrin and raft/noncaveolae medi-

ated internalization, while the HP corona for the 80 nm lipoic-AuNP decreased clathrin and caveolae-mediated internalization which suggests that raft-/noncaveolae-mediated endocytosis was the primary pathway.

Summary of cellular endocytic mechanisms

In summary, Table 2 provides a list of inhibitors used to dose the HEK in order to determine the AuNP primary endocytic cell uptake pathways. Figure 8 is a schematic diagram depicting all lipoic, PEG and BPEI-AuNP internalization pathways. The 40 and 80 nm lipoic and BPEI-AuNP were internalized by clathrin or lipid raft-mediated endocytosis. Moreover, the 40 nm lipoic-AuNP can also be internalized by energy-independent fluid-based pathway, directly penetrating into the cells or through macropinocytosis. HP protein corona of these AuNP caused some effect on the clathrin-mediated endocytosis because the inhibitors par-

tially blocked the pathway, except for the 80 nm BPEI-AuNP which showed that the HP corona inhibited the clathrin pathway. Moreover, the HP corona inhibited the caveolae-mediated internalization with all lipoic and BPEI-AuNP. However, the 40 and 80 nm PEG-AuNP showed a different uptake mechanism. The raft-/noncaveolae-mediated endocytosis dominated the 40 nm PEG-AuNP internalization. It showed that the cell uptake can vary to clathrin-mediated endocytosis in the presence of a protein corona. The 80 nm PEG-AuNP was internalized by clathrin and raft-/noncaveolae-mediated endocytosis and the HP corona had no effect on the uptake pathways.

Proinflammatory effects of HEK with AuNP

The inflammatory effects of AuNP were also evaluated with the 'nontoxic' concentration of 25 µg/ml so that the response will not cause cell death but may interfere with other physiological functions and induce additional cell responses [46,47]. In the innate defensive response, the release of chemokines and cytokines is a key step for its role in recruiting neutrophil chemotaxis and as a chemoattractant for T cells and basophils which all play a role in the development of inflammation but does not necessary lead to cell death. Figure 9 shows only the 40 and 80 nm BPEI-AuNP-induced IL-1α at 24 and 48 h and some IL-1β secretion.

IL-1α is a dual-function cytokine that acts as an alarmin and is constitutively expressed in the cytoplasm of epithelial cells [48]. The N-terminal of pro-IL-1α contains a nuclear localization sequence which can translocate to the nucleus of cells to perform regulatory functions [49]. However, it can be released into the microenvironment upon danger and recruit other innate immune cells such as monocytes and neutrophils to the inflamed tissue to initiate an inflammatory process [50,51,52]. The 40 and 80 nm BPEI-AuNP induced a proinflammatory effect in HEK (Figure 9A). This might be due to the increase in cell uptake of BPEI-AuNP in HEK at 24 and 48 h leading to HEK being stressed thus causing some membrane damage thereby, releasing pro-inflammatory mediators. The presence of a HP corona reduced the uptake of BPEI-AuNP significantly, thereby causing IL-1α and IL-1β secretion to be dramatically reduced compared to bare AuNP (Figure 9B & D). IL-1β secretion indicates that HEK had entered an activation state in response to these NP. The same effect was seen with IL-8 which is a chemokine that can recruit neutrophils to the infection site. IL-8 was highly secreted by HEK after incubation with all AuNP at 48 h, while 40 and 80 nm BPEI-AuNP triggered IL-8 secretion at 24 h (Figure 9E). IL-8 has been seen in response to injury or irritation and in hyperproliferative disorders such as acute contact dermatitis

Table 2. Summary of the mechanisms of cellular uptake with endocytic inhibitors.

Inhibitors	Functions	40 nm						80 nm					
		Lipoic			BPEI			Lipoic			BPEI		
		Bare	HP	Bare	Bare	HP	Bare	Bare	HP	PEG	Bare	HP	HP
NaN ₃ +DOG	ATP depletion	-	++	-	++	+++	++	++	++	+++	+++	+	++
4°C	Low metabolism	-	-	+	++	+++	++	+	+	++	++	++	++
NaN ₃ +DOG+4°C	ATP depletion + low metabolism	-	-	++	++	+++	++	+	+	+++	+++	+	++
Sucrose	Clathrin-mediated endocytosis inhibition	+	++	-	+++	+	+++	-	-	++	++	++	-
CPZ	Rho GTPase inhibition (clathrin)	-	-	-	++	++	+++	+	+	-	-	-	-
Nystatin	Caveolae-mediated endocytosis inhibition	++	-	-	++	-	+++	-	-	-	-	-	-
MβCD	Lipid raft-mediated endocytosis inhibition	++	++	+	++	++	+++	+++	+++	++	++	+	++

-: No significance; +p < 0.05; ++p < 0.01; +++p < 0.001.

BPEI: Branched polyethyleneimine; CPZ: Chlorpromazine hydrochloride; HP: Human plasma corona; MβCD: Methyl-β-cyclodextrin; NaN₃: Sodium azide; +DOG: Deoxyglucose; PEG: Polyethylene glycol; Lipoic acid: Lipoic.

Figure 8. Schematic diagram of gold nanoparticles cellular uptake pathways. Clathrin and lipid raft-mediated endocytic pathways. Lipid raft endocytosis is subdivided as caveolin and raft/noncaveolin-mediated endocytic pathways. AuNP: Gold nanoparticles; BPEI: Branched polyethylenimine; Lipoic: Lipoic acid; PEG: Polyethylene glycol.

Figure 9. Proinflammatory cytokines IL-1α, IL-1β and chemokine IL-8 secretion in human epidermal keratinocytes after treatment with gold nanoparticles. Human epidermal keratinocytes were exposed to 25 μg/ml of 40 and 80 nm lipoic-, PEG- and BPEI-gold nanoparticles (AuNP) in the absence (A, C & E) or presence (B, D & F) of human plasma protein corona for 24 and 48 h. Data represent mean ± SD (n = 3 wells/treatment).

*p < 0.05, **p < 0.01, ***p < 0.001 AuNP versus control.

##p < 0.01, ###p < 0.001 of human plasma versus bare AuNP.

BPEI: Branched polyethyleneimine; Lipoic: Lipoic acid; PEG: Polyethylene glycol.

and psoriasis [52,53]. HEK release TNF- α and IL-1 β that are capable of stimulating the production of IL-8 in a time-dependent manner [54,55,56]. IL-8 is not found in normal skin but rather plays a significant role in dermal injuries and in inflammatory skin diseases [57]. These cytokines are produced in a wide variety of chemicals, irritants, sensitizers, allergens and jet fuels [58,59,60]. IL-8 expression in HEK have been reported with several types of NP including multi-walled carbon nanotubes (MWCNT) [54], single walled carbon nanotubes [61], quantum dots [62], functionalized C₆₀ [63], silver NP [64], aluminum [65] and others [66]. In the presence of a HP corona, IL-8 secretion was eliminated (Figure 9F). This is similar to other studies with MWCNT treated with the ionic surfactant Pluronic F-127 which coated the MWCNT thereby, decreasing the IL-8 expression [56]. All of these results suggest that the presence of a HP protein corona with AuNP could significantly suppress the inflammatory effects making it more biocompatible for biomedical applications. Since protein coronas decreased the uptake of AuNP, this could also reduce oxidative stress within the cells but it might also decrease the surface reactivity by providing a more biocompatible surface.

Conclusion

This research shows that HP and HSA protein coronas can significantly reduce the cellular uptake of 40 and 80 nm lipoic, PEG and BPEI-AuNP in HEK. Also, HP protein coronas on AuNP can reduce the pro-inflammatory responses. The mechanistic cell uptake study suggests that AuNP were internalized by clathrin and lipid raft-mediated endocytosis, except for the 40 nm PEG-AuNP which showed cell uptake by raft/noncaveolae-mediated endocytosis. HP coronas may inhibit the caveolae-mediated endocytosis in lipoic and BPEI-AuNP

and alter the 40 nm PEG-AuNP route from raft-/non-caveolae-mediated to the clathrin-mediated pathways. However, in the presence of HP coronas, 80 nm PEG-AuNP showed no effect. This study stresses the important interrelationships between size, surface coating and protein coronas of AuNP on the mechanisms of cellular uptake and inflammatory potential, thereby, providing a better understanding of the role of protein coronas have on cellular uptake pathways and inflammatory effects.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/nnm-2016-0303

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Executive summary

- The physicochemical properties of gold nanoparticles (AuNP) play an extremely important role on the formation of a protein corona, mechanisms of cell uptake, and inflammation.
- Cellular uptake for 40 and 80 nm lipoic acid, polyethylene glycol (PEG), and branched polyethyleneimine (BPEI)-AuNP from 15 min to 48 h in human epidermal keratinocytes (HEK) showed different effects in the absence or presence of a protein corona.
- BPEI-AuNP showed the greatest cellular uptake in HEK, whereas the PEG-AuNP had the least. Protein coronas significantly decreased AuNP cell uptake.
- The rate of cell uptake and not just the amount of uptake are important factors to profile NP cellular internalization.
- Most AuNP uptake was energy-dependent, except for 40 nm lipoic acid-AuNP. Almost all AuNP were internalized by clathrin and lipid raft/noncaveolae mediated endocytosis, except for 40 nm PEG-AuNP which was by raft/noncaveolae mediated endocytosis.
- Protein coronas inhibited caveolae-mediated endocytosis with lipoic acid and BPEI-AuNP and altered the 40 nm PEG-AuNP pathway from raft/noncaveolae to clathrin-mediated endocytosis.
- Protein coronas did not alter the 80nm PEG-AuNP endocytic pathway.
- Protein coronas on AuNP can significantly affect cell uptake and proinflammatory responses in HEK.

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•• of considerable interest

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