Published in final edited form as:

Small. 2013 January 28; 9(2): 300–305. doi:10.1002/sml.201201129.

Surface functionality of nanoparticles determines cellular uptake mechanisms in mammalian cells

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Keywords

Gold nanoparticles; surface monolayer; uptake mechanisms; scavenger receptor; dynamin-dependent endocytosis

Nanoparticles (NPs) are key tools for biology and medicine, offering new strategies for biomedical applications including drug delivery^[1] and gene therapy.^[2] However, development of NPs with improved therapeutic efficacy requires a thorough knowledge of their interactions with cells to enhance delivery efficiency.^[3] In particular, the endocytic mechanisms by which NPs are transported into the cell control uptake and can potentially provide delivery strategies featuring enhanced targeting and minimal cytotoxicity.^[4]

NPs can be taken up by cells via two major mechanisms: phagocytosis and pinocytosis.^[5] Phagocytosis occurs for particles larger than 0.5 μm in a limited number of mammalian cell types such as macrophages and monocytes. Pinocytosis is a far more general process that can be further classified into two subcategories: macropinocytosis and micropinocytosis. Macropinocytosis involves non-selective uptake of solute macromolecules of more than 0.2 μm diameter,^[6] whereas micropinocytosis (clathrin-mediated, caveolae/lipid raft-mediated, and clathrin/caveolae-independent) occurs for smaller particles in all cell types (Scheme 1a).^[7] Given the size regime of NPs commonly used for therapeutic purposes (10–200 nm), NPs are expected to enter cells predominantly via micropinocytosis.

Recent investigations have revealed that size,^[8] shape,^[9] and surface charge^[10] of NPs govern entry and subsequent cytosolic access of NPs into living cells.^[11] For example, cationic NPs have better cell membrane penetration efficiency than anionic NPs and are shown to enter mammalian cells via a different pinocytic mechanism.^[12] Likewise, particles with diameters of <200 nm were taken up via clathrin-coated pits, whereas caveolae-mediated internalization became predominant with increasing particle size.^[13] In addition to size and charge, NP surface functionality also plays an important role in terms of cellular uptake^[14] and eliciting cellular responses.^[15] Recently, Maysinger and coworkers have

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demonstrated that quantum dots bearing non-specific ligands on the surface were predominantly taken up by a lipid raft-mediated endocytosis in human kidney and liver cells.^[16] However, it is not clear how changes on the NP surface (e.g., hydrophobicity and aromaticity) affect cellular internalization processes.

Herein, we report a mechanistic study of cellular uptake pathways of four cationic gold NPs (~2 nm core) featuring cationic head groups differing in structure and hydrophobicity (Scheme 1b). Experiments were performed in the presence of different endocytic inhibitors using healthy and cancerous cells to determine the specific entry route of the gold NP in human cells. The results indicate that the mode of cellular uptake is strongly dependent on the subtle changes of the NP surface monolayer. Importantly, these gold NPs possess different uptake mechanisms in normal and cancer cells, providing a potential strategy for selective delivery of drugs to tumor tissues.

To probe the effect of NP surface functionality on the uptake mechanisms, we synthesized four structurally related gold NPs (**NP1-NP4**) that feature cationic ligands with diverse headgroups presented on a non-interacting scaffold (Scheme 1b). All particles were synthesized from a single batch of pentanethiol-capped gold NPs (~2 nm core) via place exchange reactions^[17] and showed substantial peak broadening in ¹H NMR spectra with no sign of free ligands after the reaction (see Supporting Information (SI)). As expected, all of the particles have similar hydrodynamic diameters and positive zeta potentials (SI). We studied NP uptake in two human cell lines: HeLa (a cervical carcinoma cell line) and MCF10A (a non-tumorigenic mammary epithelial cell line), providing a platform to examine the uptake mechanisms of gold NPs in both malignant (HeLa) and non-malignant cells (MCF10A).

NP uptake in cells was quantified using inductively coupled plasma mass spectrometry (ICP-MS).^[18] After 1 h incubation of HeLa and MCF10A cells with **NP1-NP4** (100 nM each) in serum free media, the cells were analyzed for intracellular gold content. The uptake amount increases from **NP1** to **NP4** in HeLa cells (Figure 1a) while remained similar in the case of MCF10A cells (Figure 1b). No significant cytotoxicity was observed for any of the NPs (100 nM each) in either cell type (see SI).

Next, we investigated the effect of endocytic inhibitors on the cellular uptake mechanisms for the NPs. For all experiments, HeLa or MCF10A cells were pretreated with an inhibitor for 1 h followed by 1 h of incubation with the NPs in the presence of the inhibitor. Experiments were performed in serum free condition to exclude the effect of protein adsorption on NP surfaces that can potentially alter the endocytic pathways.^[19] We focused first on the three major pinocytic pathways of cellular entry: macropinocytosis, clathrin-dependent, and caveolae/lipid raft-dependent micropinocytosis. No uptake inhibition was observed in either cell type for **NP1-NP4** using wortmannin (WMN) (Figure 2a), an inhibitor that blocks the action of phosphoinositide 3-kinase, a key regulator in macropinocytosis.^[20] This determination was further confirmed using sodium azide (NaN₃)/2-deoxyglucose (DOG), a system that depletes cellular ATP levels required for macropinocytosis (see SI).^[21]

Membrane invagination during micropinocytosis requires microtubule and actin filament reorganization,^[22] a process observed in NP uptake.^[23] To evaluate the effect of cytoskeletal rearrangement on NP uptake, we used two inhibitors: cytochalasin D (CytD)^[24] and nocodazole^[25] that disrupt F-actin polymerization and microtubule formation, respectively. In the case of CytD treated cells, the uptake of the NPs remained unaffected in both cell types except for **NP3** that showed ~20 % inhibition in HeLa cells relative to the control (Figure 2b). Likewise, treatment with nocodazole did not show any significant inhibition for any of the particles in either HeLa or MCF10A cells (see SI), demonstrating cytoskeletal rearrangement is not critical for uptake of monolayer-protected NPs in either cell type.

Previous reports have demonstrated that cationic NPs (diameters ~90 nm) can enter HeLa cells via clathrin-dependent pathways.^[26] However, we did not observe any strong inhibition in either HeLa or MCF10A cells using chlorpromazine (CPM),^[27] an inhibitor of clathrin-mediated endocytosis (Figure 2c). In contrast, strong uptake inhibition (~50 %) was observed for **NP2** in the cells pretreated with the cholesterol-depletion agent methyl- β -cyclodextrin (MBCD),^[28] demonstrating the possible involvement of caveolae/lipid raft-mediated endocytosis for **NP2** in HeLa cells (Figure 2d). In MCF10A cells, however, only **NP4** showed significant uptake inhibition using MBCD. Therefore, subtle changes in the NP monolayer can switch the endocytic pathways in malignant and non-malignant cells, offering the potential for selective targeting strategies.^[29] The other particles studied did not show any significant reduction of cellular uptake in the presence of any of the above inhibitors, indicating that the broader classes of endocytosis including macropinocytosis and clathrin-dependent endocytosis are not the major routes of entry of these monolayer-protected gold NPs into HeLa and MCF10A cells.

Recently, the dynamin-dependent endocytosis pathway has been extensively studied as a possible clathrin-independent transport mechanism into the cell.^[30] We observed a significant inhibition of **NP2** uptake (~60 %) in HeLa cells using dynasore (Figure 3a), an endocytic inhibitor that interferes with the dynamin-dependent pathways by blocking dynamin-GTPase.^[31] Therefore, **NP2** uptake is governed by both caveolae/lipid raft (Figure 2d) and dynamin-dependent pathways, a distinctly different feature from the other NPs studied. Surprisingly, in the case of MCF10A cells, uptake of all NPs was strongly inhibited by dynasore (Figure 3a) with **NP2** being the most strongly inhibited (~80 %). Therefore, the dynamin-dependent pathway plays a key role in **NP1-NP4** endocytosis in MCF10A cells, a striking difference from HeLa cells where only **NP2** showed significant inhibition.

To investigate whether a more specific endocytic pathway is involved for the other NPs, we studied the involvement of scavenger receptors and membrane-bound G-protein coupled receptor (GPCR)^[32]-mediated uptake pathways in both of the cell types. Scavenger receptors bind to a variety of ligands including low density lipoproteins and polysaccharides^[33] and have been shown to be involved in gold NP uptake.^[34] Poly I, a well-known inhibitor of scavenger receptors,^[35] inhibited **NP1** and **NP3** uptake in both HeLa and MCF10A cells (Figure 3b), confirming the involvement of scavenger receptors for NP uptake in different mammalian cells. Significant uptake inhibition was observed for **NP2** in MCF10A cells in the presence of poly I, however, this effect was not pronounced in HeLa

cells. Conversely, Gq α -protein (sub-family of GPCR) inhibitor pertussis toxin (PTX)^[36] and phospholipase C inhibitor U-73122^[37] did not show any reduction in uptake for any of the particles (see SI), demonstrating that GPCRs play no role in **NP1-NP4** uptake in HeLa and MCF10A cells.

Taken together, the broader classes of endocytic pathways including macropinocytosis and clathrin-mediated endocytosis were not the predominant uptake mechanisms for **NP1-NP4** in both malignant and non-malignant cells. However, highly regulated uptake was observed via caveolae and dynamin-dependent micropinocytosis as well as through specific membrane-bound receptors in both of the cell types. For example, **NP1** and **NP3** showed scavenger receptor-mediated uptake while **NP2** showed both caveolae and dynamin-dependent uptake in HeLa cells, demonstrating multifaceted internalization mechanisms of NPs into cells.^[38] **NP4** did not show strong uptake inhibition in HeLa cells for any of the classes of inhibitors tested, demonstrating the role of clathrin-independent/dynamin-independent mechanisms in mammalian cells.^[30] In MCF10A cells, both dynamin and scavenger receptor-mediated uptake was predominant for all of the particles except **NP4** that also showed caveolae/lipid raft-mediated uptake (Table 1 and Scheme 2).

In summary, we have demonstrated that the mechanisms of small (~10 nm hydrodynamic diameter) cationic gold NP uptake in both malignant and non-malignant cells are strongly dependent on the gold NP monolayer structures and mostly rely on dynamin, scavenger receptors, and caveolae/lipid raft-mediated pathways. Significantly, the existence of differential uptake pathways for the same NP in cancer and normal cells provides an opportunity to design nanocarriers of specific therapeutic action and reduced cytotoxicity. Taken together, these studies indicate the importance of NP surface monolayer structures in endocytic pathways and signify the role of particle design in nanomedicine.

Experimental Section

Cell culture

HeLa cells were grown in low glucose Dulbecco's Modified Eagle's Medium (GIBCO, Catalog# 10567) supplemented with 10% fetal bovine serum (Thermo Scientific, SH3007103) and 1% antibiotics (Cellgro, 30-004-CI). The cells were maintained in the above media and subcultured once every four days. MCF10A cells were grown in mammary epithelium growth medium (Lonza, CC-3051A) supplemented with bovine pituitary extract (Lonza, CC-4009). Media were replaced every two days and cells were subcultured every seven days.

NP uptake and inhibition studies

HeLa or MCF10A cells were seeded in a 48 well plate at a density of $\sim 2 \times 10^4$ cells/well 24 h prior to the experiment. On the following day, cells were washed one time with phosphate buffered saline (PBS) and incubated with following endocytic inhibitors in serum free media for 1 h at 37 °C: cytochalasin D (10 μ g/mL), methyl- β -cyclodextrin (5 mg/mL), nocodazole (10 μ g/mL), 3 mg/mL NaN₃/50 mM 2-deoxyglucose, wortmannin (100 ng/mL), pertussis toxin (100 ng/mL), U-73122 (4 μ g/mL), polyinosinic acid (10 μ g/mL), chlorpromazine (10 μ g/mL) and dynasore (80 μ M). The concentrations of the inhibitors were used as described

in previous reports.^[39] After 1 h, media were replaced with fresh media containing the inhibitors with NPs (100 nM) and further incubated for 1 h at 37 °C. Untreated cells and cells treated with only NPs (no inhibitor) were used as negative and positive controls, respectively. After incubation, cells were washed three times with PBS and lysis buffer was added to each well. All lysed cell samples were then further processed for ICP-MS analysis (*vide infra*) to determine the intracellular amount of gold. All inhibitors were purchased from Sigma except for dynasore, chlorpromazine, and pertusis toxin that were obtained from Fisher Scientific. Particle uptake (%) was calculated based on the following equation:

$$\text{NP uptake (\%)} = (\text{NP uptake in presence of inhibitors} / \text{NP uptake in absence of inhibitors}) \times 100$$

Sample preparation for ICP-MS and ICP-MS instrumentation

After cellular uptake, the lysed cells were digested with 0.5 mL of fresh aqua regia (highly corrosive and must be used with extreme caution!) for 10 minutes. The digested samples were diluted to 10 mL with deionized water. A series of gold standard solutions (20, 10, 5, 2, 1, 0.5, 0.2, and 0 ppb) were prepared before each experiment. Each gold standard solution also contained 5% *aqua regia*. The gold standard solutions and cellular uptake sample solutions were measured on an Elan 6100 ICP mass spectrometer (PerkinElmer SCIEX, Waltham, MA). The instrument was operated with 1550 W RF power and the nebulizer Ar flow rate was optimized at 0.96 L/min.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a grant from the National Institutes of Health (grants EB014277 and GM077173). NSF support is acknowledged for NSEC Center for Hierarchical Manufacturing (CMMI-1025020, BY) and MRSEC facilities (DMR-0820506). We thank Prof. J. F. Tyson for the access to ICP-MS instrumentation.

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Scheme 1.

(a) Schematic representation of major pinocytic pathways of NPs in mammalian cells. (b) The gold NPs used in the present study.

Scheme 2.

Summary of endocytic pathways of gold NPs in HeLa and MCF10A cells.

Figure 1. ICP-MS measurements of intracellular uptake of **NP1-NP4** in (a) HeLa and (b) MCF10A cells after 1 h of NP incubation in serum free media. Error bars represent standard deviation.

Figure 2.

Uptake % of **NP1-NP4** (compared to the positive controls) in the presence of different endocytic inhibitors in HeLa and MCF10A cells, (a) wortmannin (WMN), (b) cytochalasin D (CytD), (c) chlorpromazine (CPM), (d) methyl- β -cyclodextrin (MBCD). Error bars represent standard deviation. * $p<0.05$, ** $p<0.01$ compared to the control.

Figure 3.

Uptake % of **NP1-NP4** (compared to the positive controls) in HeLa and MCF10A cells in the presence of (a) dynasore and (b) poly I. Error bars represent standard deviation.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the control.

Table 1

Summary of uptake inhibition of NPs in presence of endocytic inhibitors

Inhibitor	Function	NP1		NP2		NP3		NP4	
		HeLa	MCF10A	HeLa	MCF10A	HeLa	MCF10A	HeLa	MCF10A
CytD	Inhibits F-actin polymerization	-	-	-	-	++	-	-	-
MBCD	Cholesterol depletion/caveolae	-	-	++	-	-	-	-	++
Nocodazole	Disruption of microtubules	-	-	-	-	-	-	-	-
NaN ₃ /DOG	ATP depletion	-	-	-	-	-	-	-	+
WMN	Inhibits phosphoinositide 3-kinase	-	-	-	-	-	-	-	-
PTX	Inhibits G α i protein	-	-	-	-	-	-	-	-
U-73122	Inhibits phospholipase C (GPCR)	-	-	-	-	-	-	-	-
Poly I	Scavenger receptor inhibitor	++	++	+	+++	++	+++	-	-
CPM	Inhibits Rho GTPase (Clathrin)	-	-	-	-	-	+	+	-
Dynasore	Inhibits Dynamin-GTPase	-	+++	+++	+++	+	+++	-	++

+p<0.05, ++p<0.01, +++p<0.001 through unpaired t-test between control and inhibitor-treated groups

- no significant inhibition