Effects of physicochemical properties of zinc oxide nanoparticles on cellular uptake

J Yu, M Baek, HE Chung and SJ Choi

Department of Food Science and Technology, Seoul Women’s University, 126 Gongneung 2-Dong, Nowon-Gu, Seoul 139-774, South Korea

E-mail: sjchoi@swu.ac.kr

Abstract. Zinc oxide (ZnO) nanoparticles have been used as a source of zinc, an essential trace element in food industry and also widely applied to various cosmetic products. However, there are few researches demonstrating that the cellular uptake behaviours of ZnO with respect to the physicochemical characteristics such as particle size and surface charge in human cells. In this study, we evaluated the cellular uptake of ZnO with two different sizes (20 and 70 nm) and different charges (positive and negative). Human lung epithelial cells were exposed to ZnO for a given time, and then the uptake amount of ZnO was measured with inductively coupled plasma-atomic emission spectroscopy (ICP-AES). The results showed that the smaller sized ZnO could more easily enter the cells than the larger sized ZnO. In terms of surface charge, positively charged ZnO showed high cellular uptake compared to ZnO with negative charge. The internalization pathway of positively charged ZnO nanoparticles was determined to be primarily related to the energy-dependent endocytosis. It is, therefore, concluded that the particle size and surface charge of ZnO nanoparticles are critical factors influencing on their cellular uptake. Understanding the cellular uptake behaviours of nanoparticles with respect to physicochemical properties may be important to predict their toxicity potential on human.

1. Introduction

ZnO has been used as a source of zinc, an essential trace element in food industry. ZnO has been also widely applied to cosmetics such as personal care and sunscreen products, since it acts as an invisible barrier that scatters UV radiation away from the skin rather than allowing its harmful energy to be absorbed [1-4]. But, absorption rate of zinc is low via oral ingestion and bulk-sized ZnO has poor dispersion property, resulting in low UV blocking capacity, low transparency on skin, and hard agglomeration compared to small-sized ZnO. Thus, nano-sized ZnO has recently attracted much attention in order to enhance the uptake of zinc and increase UV filtering efficiency with cosmetic clarity. It is now generally accepted that nanoparticles efficiently penetrate the cell membrane compared to micro-sized particles. However, few researches were performed to demonstrate the effects of physicochemical parameters of ZnO nanoparticles on cellular uptake, which may provide critical information on their toxicity potential [5-7]. In this study, we evaluated cellular uptake behaviours of ZnO nanoparticles with different sizes (20 and 70 nm) and different charge (positive: +, and negative: -) in human lung epithelial cells. Moreover, the uptake mechanism of each nanoparticle was further investigated to understand its uptake behaviours.

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2. Experimental methods

2.1. Materials
ZnO nanoparticles of two different sizes (20 and 70 nm) were purchased from Sumitomo (Japan) and American Elements (U.S.A.), respectively. For surface modification of ZnO nanoparticles with negative charge, ZnO (10 g) was suspended in 20 mM HEPES buffer (pH 7.0) containing 1% sodium citrate [8-10]. The particle size and surface charge (zeta potential) of ZnO nanoparticles were determined by transmission electron microscopy (TEM: JEM-1010, JEOL) and a zeta potentiometer (Zetasizer Nano ZS system, Malvern Instruments), respectively.

2.2. Cell culture
Human lung epithelial cells (A549) were purchased from the Korean Cell Line Bank and cultured in RPMI1640 medium supplemented with 10% heat inactivated fetal bovine serum (Welgene, Ltd., South Korea), 100 units/ml penicillin, and 100 µg/ml streptomycin, under a humidified atmosphere (5% CO₂ plus 95% air).

2.3. Cellular uptake
The cells (5 × 10⁵/ml) were incubated overnight at 37ºC under a 5% CO₂ atmosphere and the medium in the wells was then replaced with fresh medium containing nanoparticles. After incubation for a given time, the cells were washed twice with phosphate buffered saline (PBS), harvested by scraping, and then re-suspended in deionized and distilled water. The cell pellets were then digested in 3 ml of ultrapure nitric acid. After adding 0.5 ml of H₂O₂, each mixture was heated at about 160 ºC until the cell pellets were completely digested. Then, the remaining nitric acid was removed by heating until the solutions were colorless and clear. The solutions were finally diluted to 3 ml with ultrapure water and ICP-AES (Optima 3300DV) was used to analyze Zn concentration in the samples. The cells incubated in the absence of particles were used as control.

2.4. Uptake mechanism
Energy-dependent endocytic mechanism of ZnO nanoparticles was evaluated by incubating the cells (5 × 10⁵/ml) overnight at 37ºC under a 5% CO₂ atmosphere, and replacing the medium in the wells with fresh medium containing nanoparticles. The cells were incubated with 125 µg/ml at 37ºC and 4ºC, respectively, for 1 h. And the cells were washed twice with PBS, harvested by scraping, and then the pellets were digested for ICP-AES analysis as described above.

2.5. Statistical analysis
Statistical analyses were performed using Student’s t test for unpaired data and p values of less than 0.05 were considered significant. All data are presented as mean ± standard error of the mean (S.E.M.).

3. Results and discussion

3.1. Characterization of ZnO nanoparticles
Particle size and surface charge of ZnO nanoparticles were measured by TEM and zeta potentiometer, respectively. As shown in Table 1, net charge of ZnO nanoparticles in aqueous solution at pH 7.0 was determined to be positive, about 25 mV, thus surface modification to obtain negatively charged ZnO nanoparticles was performed with citrates. Citrates are widely used as capping agents for inorganic nanoparticles, giving rise to negative surface coating. This is based on the fact that citrates are important biological ligands for metal ions to form strong metal complexes. The measured particle size of 20 nm was well distinguished from that of 70 nm (Figure 1) and the surface charge for
positively or negatively charged ZnO was well prepared for comparative cytotoxicity study in the next step.

**Table 1.** Particle size and surface charge of ZnO nanoparticles as measured by TEM and zeta potentiometer, respectively.

| Sample  | Particle size [nm] | Zeta potential [mV] |
|---------|--------------------|---------------------|
| 20 nm   | Positive charge    | 24 ± 5.57           | 25.0 ± 0.5\(^\text{b}\) |
|         | Negative charge    |                     | -44.6 ± 0.7\(^\text{c}\) |
| 70 nm   | Positive charge    | 67 ± 12.41          | 29.1 ± 0.6\(^\text{b}\) |
|         | Negative charge    |                     | -45.2 ± 0.8\(^\text{c}\) |

\(^\text{a}\) Average particle size was measured by randomly selecting 500 particles from TEM images.

\(^\text{b}\) Zeta potential was measured in distilled water (pH 7.0).

\(^\text{c}\) Zeta potential was measured in 20 mM HEPES buffer (pH 7.0) containing 1% sodium citrate.

**Figure 1.** Histogram of particle size distribution for ZnO nanoparticles.
3.2. Uptake behaviors of ZnO nanoparticles: effect of incubation time

A549 cells were exposed to different ZnO nanoparticles with a concentration of 125 μg/ml for a given time and their intracellular uptake amount was evaluated by measuring Zn concentration with ICP-AES. Figure 2 showed a clear difference between nanoparticles tested; higher uptake was found in the cells exposed to positively charged nanoparticles than negatively charged ones. Moreover, size-dependent uptake behaviours of ZnO nanoparticles were remarkable; 20 nm was determined to largely enter the cells compared to 70 nm. Thus, ZnO with 20 nm (+) was the most efficient one in terms of high cellular uptake. The cellular uptake of different ZnO nanoparticles reached a maximum level at 4 h post-incubation and decreased as incubation time increased, suggesting the possible uptake saturation of ZnO nanoparticles in the cells.

![Figure 2. Cellular uptake of ZnO nanoparticles with respect to incubation time.](image)

3.3. Uptake behaviors of ZnO nanoparticles: effect of ZnO concentration

When the cellular uptake of each ZnO nanoparticle was evaluated with respect to nanoparticle concentration after 4 h incubation, charge- and size-dependent uptake behaviours were also remarkable (Figure 3). Smaller size, 20 nm, and positively charged ZnO nanoparticles showed higher cellular uptake than larger size, 70 nm, and negatively charged ones, which was well consistent with the result presented in Figure 2. The cellular uptake of ZnO nanoparticles reached a plateau at 500 μg/ml, indicating that their cellular uptake was probably saturated. According the results as shown in Figure 2 and Figure 3, it seems that surface charge of ZnO nanoparticles is more important factor affecting their cellular uptake rather than their particle size. It can be explained by high interaction of positively charged ZnO nanoparticles with negatively charge plasma membrane, resulting in high and easy cellular uptake.
3.4. Uptake mechanism of ZnO nanoparticles

Energy-dependent endocytic mechanism of different ZnO nanoparticles was evaluated by comparing cellular uptake amount of ZnO after incubation at 37°C with that at 4°C. This is based on the fact that endocytic uptake mechanism is a typical energy-driven, in other words, ATP-dependent process, thus can be blocked when ATP synthesis is inhibited [11]. Therefore, the effect of energy depletion on cellular uptake process of ZnO nanoparticles was examined by incubating the cells with nanoparticles at 37°C and at 4°C, respectively, followed by comparison of each uptake level. Figure 4 demonstrated that the uptake amount of positively charged ZnO nanoparticles significantly decreased after incubation at 4°C compared to 37°C, while the uptake of negatively charged ZnO nanoparticles was not considerably affected by energy depletion, suggesting that energy-dependent endocytosis is the primary uptake mechanism for the internalization of positively charged ZnO nanoparticles. It seems that uptake amount of negatively charged ZnO nanoparticles at 37°C is too low to be distinguished from that at 4°C. It is worth noting that particle size did not influence on the cellular uptake mechanism of ZnO nanoparticles. It is, therefore, concluded that high cellular uptake of positively charge ZnO nanoparticles is strongly associated with their energy-dependent endocytic pathway.
Figure 4. Effect of energy depletion on cellular uptake of ZnO nanoparticles in A549 cells after 1 h. Uptake amount of each ZnO at 37ºC was calculated as 100%.

4. Conclusion
The effects of physicochemical properties of ZnO nanoparticles such as particle size and surface charge were evaluated in human lung cells. As a result, small-sized ZnO nanoparticles, 20 nm, with positive charge showed the highest cellular uptake with a following order of 70 (+), 20 (-), and 70 (-). It seems that surface charge of ZnO nanoparticles plays a critical role in their internalization, probably due to strong attractive force between positive surface charge of ZnO nanoparticles and the plasma membrane with negative charge. Moreover, the cellular uptake of positively charged ZnO nanoparticles decreased remarkably by inhibition of ATP synthesis, suggesting the main energy-dependent uptake mechanism for their internalization. It is, therefore, concluded that the particle size and surface charge of ZnO nanoparticles are critical factors affecting their cellular uptake. Understanding the cellular uptake behaviour of nanoparticles with respect to physicochemical properties may be important to predict their toxicity potential on human.

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