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Simple Analysis for Interaction between Nanoparticles and Fluorescence Vesicle as a Biomimetic Cell for Toxicological Studies

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With continuing progress of nanotechnologies and various applications of nanoparticles, one needs to develop a quick and fairly standard assessment tool to evaluate cytotoxicity of nanoparticles. However, much cytotoxicity studies on the interpretation of the interaction between nanoparticles and cells are non-mechanistic and time-consuming. Here, we propose a simple screening method for the analysis of the interaction between several AgNPs (5.3 to 64 nm) and fluorescence-dye containing vesicles (12 μm) acting as a biomimetic cell-membrane. Fluorescence-dye containing vesicle was prepared using a fluorescence probe (1,6-diphenyl-1,3,5-hexatryene), which was intercalated into the lipid bilayer due to their hydrophobicity. Zeta potential of all materials except for bare-AgNPs (+32.8 mV) was negative (−26 to −54 mV). The morphological change (*i.e.*, rupture and fusion of vesicle, and release of dye) after mixing of the vesicle and AgNPs was observed by fluorescence microscopy, and fluorescence image were different with coating materials and surface charge of x-AgNPs. In the results, we found that the surface charge of nanoparticles is the key factor for vesicle rupture and fusion. This proposed method might be useful for analyzing the cytotoxicity of nanoparticles with cell-membranes instead of *in vitro* or *in vivo* cytotoxicity tests.

Key Words : Silver nanoparticles, Toxicology, Biomimetic, Vesicle, Fluorescence

Introduction

Because of the plasmonic and antimicrobial properties of silver nanoparticles (AgNPs), these nanoparticles are already finding applications in wide fields, such as environmental and biomedical sensors, and chemical and electrical applications. With increasing utilization of AgNPs in consumer products, the potential release of AgNPs into the environment and their impacts on the ecosystem and human health have been the issues of concern.[1,2] Over the last decade, there have been a great amount of studies regarding toxic effects of AgNPs on cells and micro-organisms.[3,4]

Although the cytotoxicity mechanisms behind the activity of AgNPs on cells and bacteria are still not well understood, the three most common mechanisms have been proposed:[5] (1) uptake of free silver ions to disrupt ATP production, (2) generation of reactive oxygen species by Ag^+ and AgNPs and (3) direct damage to cell membrane by AgNPs attack. To well elucidate these cytotoxicity mechanisms, *in vivo* and *in vitro* test using micro-organisms and mammalian cells should be carried out. However, these cytotoxicity testing methodologies are time-consuming and are not suitable for rapid screening of various nanoparticles used in industrial applications.[6] A common factor in cytotoxicity mechanisms is that adsorption and rupturing features are based on the interaction between cells and nanoparticles.[7] Therefore, a method for analyzing the interaction between cells and nanoparticles is required as a non-biological or biological screening method for a quick and simple investigation of the cytotoxicity mechanisms.[8,9]

Spontaneously formed unilamellar vesicles have great potential for applications as vehicles for drug delivery and colorimetric sensors. In particular, phospholipids are the main constituents of the cell membrane, making them a promising group of materials suitable for engineering biocompatible systems.[10] Therefore, lipid vesicles are used as well-defined models for cell surface and for investigating molecular events in the membrane.[11] In recent, Shiraki group suggested a simple test method for adsorption and disruption of lipid bilayers by nanoscale protein aggregation.[12] Frank and coworkers investigated on what happens when POPC (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) vesicles are exposed to the amphipathic helix peptide. Therefore, mimetic lipid vesicles could be used as a well-designed testing tool for understanding the interaction between nanoparticles and the cell-membrane and for rapidly screening the direct damage of cells by adsorption of nanoparticles or cell rupturing.

Herein, fluorescence-dye contained vesicle was prepared and fluorescence microscopy was used to directly observe the adsorption or rupturing of the vesicle by the attack of AgNPs. Generally, the cell-membrane has a negative charge and thus fluorescence-dye contained DMPG (1,2-dimyristoyl-*sn*-glycero-3-[phosphor-*rac*-glycerol]) was selected as the biomimetic cell-membrane. Herein, four different AgNPs were selected as target nanoparticles; i) AgNP powder, ii) bare-AgNPs without any additive, iii) citrate-AgNPs with citrate coating, and iv) PVP-AgNPs with PVP (polyvinyl-pyrrolidone) coating.

Experimental

Preparation of Fluorescence-dye Contained Vesicle.

DMPG vesicle was prepared with the chloroform dispersion method.[13] The sodium salt of the phospholipid DMPG was purchased from Avanti Polar Lipids. Chloroform lipid solution was spread on the rough side of a Teflon disk, which stayed under reduced pressure overnight to remove all traces of organic solvent. In the prehydration stage, the bottle was left in a humid atmosphere for 2 h at 37 °C. For the florescence analysis, fluorescent probe DPH (1,6-diphenyl-1,3,5-hexatryene) was used. 4 mL of the mixture solution of 1xPBS and 0.5 mol % of DPH was gently poured inside the bottle. The bottle was closed with a cap and left at 37 °C for 2 days. After centrifugation at 4000 rpm for 4 min, the supernatant was discarded to remove the rest of the dye and then the final material was re-dispersed in PBS solution.

Preparation of Bare-AgNPs. A nano-silver powder (Sigam-Aldrich, 99%, < 100 nm) without suspension additives was prepared as a suspension of AgNPs in an aqueous phase *via* the THF (tetrahydrofuran) method,[14] which can be readily exchanged with water and easily removed by evaporation. Ag power was added to THF and the resulting solution was treated with sonication, followed by stirring at approximately 300 rpm until THF had completely evaporated (1-2 days). Deionized (DI) water was then added to replace THF. The resulting sample was then filtered through a polycarbonate membrane filter (50 nm isopoere, Adventec).

Preparation of Citrate-AgNPs. With our reporting method,[15] citrate coated AgNPs were prepared by a dropwise method that added 0.6 mL of 7 mM $NaBH_4$ (Sigma-Aldrich) solution to 1 mL of 26 mM $AgNO_3$ (Sigma-Aldrich) in 19 mL of 1 mM trisodium citrate (Sigma-Aldrich) under vigorous stirring. The color of the solution immediately changed to dark yellow after $NaBH_4$ was added, which indicated particle formation. After 2 h, this solution was centrifugated at 13000 rpm for 15 min.

Preparation of PVP-AgNPs. PVP-AgNPs was prepared by the seed-mediated method.[16] 9 mL of 0.1 M silver nitrate was added to 280 mL of 5.36 g/L aqueous PVP suspension while stirring. After stirring for 5 min, 11 mL of 0.08 M sodium borohydride was then added to the solution, and stirred for 10 min. The PVP-AgNPs were ultracentrifuged at 14,000 rpm, then re-suspended in water.

Characterizations. Particle size and zeta potential were measured by electrophoretic light scattering (ELS-Z, Otsuka). TEM images of the vesicle and AgNPs were analyzed with transmission electron microscopy (JEM 1010, JEOL). Fluorescence images were obtained by confocal laser scanning microscope (LSM 5 Exciter, Carl-Zeiss).

Results and Discussion

SPR and UV spectroscopy can be used to investigate the interaction between the cell-membrane (*i.e.* vesicles) and nanoparticles.[17] In our previous results,[18] the change of reflectance and resonance angle of the vesicle loaded on the gold chip in SPR spectroscopy revealed the effect of AgNPs on the vesicle and the possibility of vesicle rupture or fusion after the injection of AgNPs. In UV spectroscopy, the absorbance deviation between spectra A for mixing of vesicle/x-AgNPs and spectra B for simple summation of vesicle and x-AgNPs showed information for the attraction energy between vesicles and AgNPs. Namely, we could be indirectly defining the interaction between AgNPs and vesicles based on the change of reflectance in SPR spectra and that of refractance in UV spectra. However, these methods are indirect measuring tools to define particle interactions, and thus an additional direct analyzing tool is required, such as microscopic pictures.

TEM is mainly used to directly observe the adsorption of AgNPs on the cell-membrane or cell rupture by the attack of AgNPs. However, during sample preparation, drying the colloidal sample on TEM Cu-grid, colloidal particles were easily aggregated between each other and then the drying process would have changed the morphology of vesicle/x-AgNPs. As an alternative microscopy, DFM (dark-field microscopy) was suggested to obtain the light scattering images of vesicles and AgNPs in liquid media. Metallic nanoparticles scatter light intensely, and they are much brighter than chemicals in the aqueous phase.[20] The vesicles had no bright color intensity in DFM image, as compared to the AgNPs, and vesicles after mixing with bare-AgNPs showed bright color at their bilayer zones.

Therefore, fluorescence microscopy was suggested as another alternative tool for observing the interaction between AgNPs and vesicles. Fluorescence probe molecule was intercalated in the phospholipid bilayer and the fluorescence-dye containing vesicle was used to directly investigate the interaction between vesicles and AgNPs. In preparation of the fluorescence vesicle, dye residue was removed by centrifugation and washing. As shown in Figure 1, AgNPs were absorbed or separated from the vesicles after exposure, depending on the intensity of the electrostatic charge repulsion. When the attraction force is dominant between the vesicles and AgNPs, AgNPs are readily adsorbed on the outer surface of the vesicle and eventually induce vesicle rupture or fusion. This cytotoxicity of AgNPs on the cell-membrane is concerned with direct damage of cells. There-

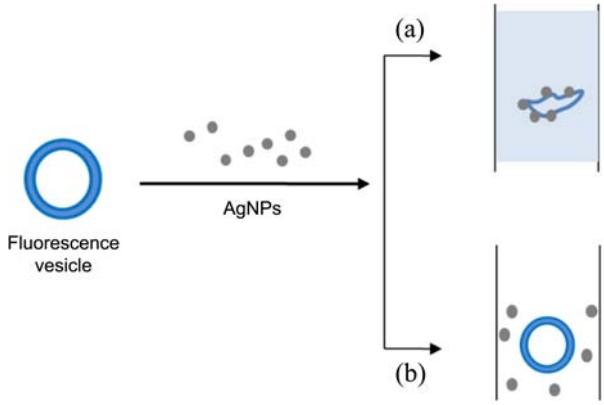

Figure 1. Scheme of cytotoxicity screening method using fluorescence vesicle. (a) Vesicle rupture and release fluorescence-dye from vesicle by attack of AgNPs, and (b) separation between neighboring particles.

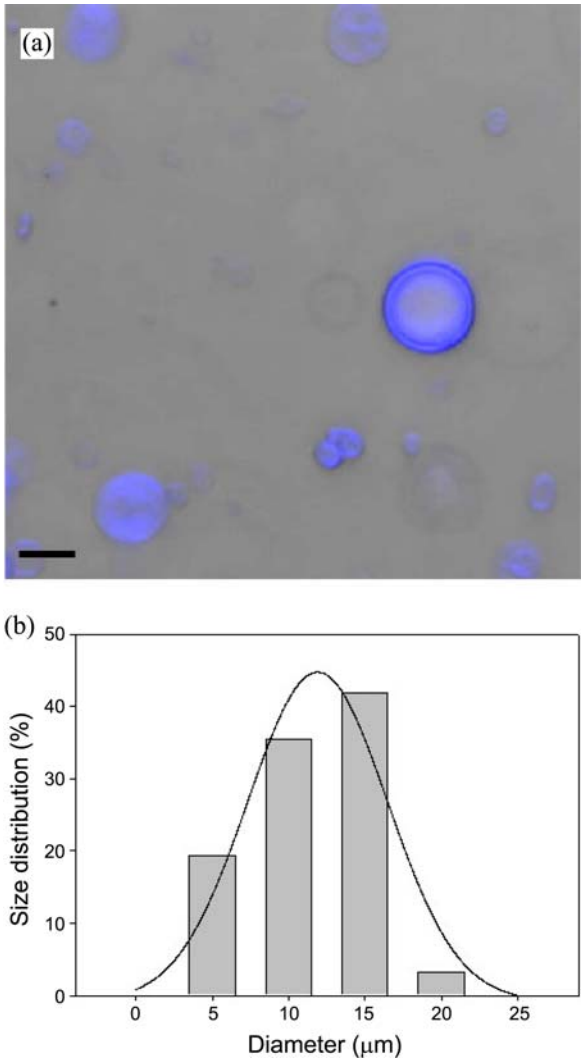

Figure 2. (a) Florescence image of florescence-dye contained vesicle (scale bar = 10 μm) and (b) size distribution of florescence vesicle analyzed by ImageJ software.

fore, a fluorescence probe gets released into the solvent after the vesicle rupture.

As shown in Figure 2, the fluorescence vesicle showed ca. 12 μm of size, which has size range of 5 and 20 μm. We can confirm from the fluorescence image that the fluorescence probe was only intercalated in the lipid bilayer, not contained in the inner space of the vesicle. In general, bilayer thickness of DMPG giant vesicle is of several nanometers, but that of fluorescence vesicle is hundreds of nanometers which can be clearly observed with the naked eye in Figure 2(a). Because DPH with two benzene groups is hydrophobic, fluorescence probe was only located in the bilayer and thus its thickness was expanded. Fluorescence wavelength of DPH is known as 350 nm.

Size and zeta potential of four different AgNPs were measured by TEM and ELS, and summarized in Table 1. These AgNPs had different surface charges, and thus showed different interactions with the dye-containing vesicles. Different coating groups on their surface make the material

Table 1. Diameter and zeta potential of vesicle and x-AgNPs

Materials	Diameter (nm)	Zeta potential (mV)
Fluorescence vesicle	12 μm	−39.8
AgNPs powder	64.4	−26.5
Bare-AgNPs	5.3	+32.8
Citrate-AgNPs	33.9	−54.1
PVP-AgNPs	35.0	−35.2

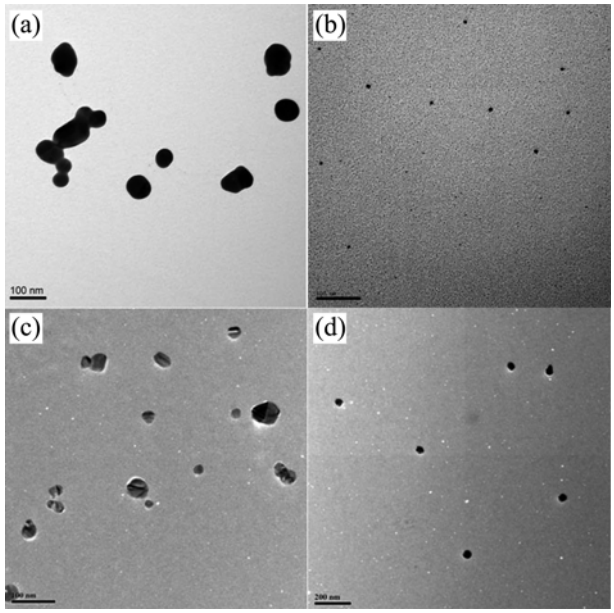

Figure 3. TEM images of (a) AgNPs powder, (b) bare-AgNPs, (c) citrate-AgNPs, and (d) PVP-AgNPs. Scale bar is 100 nm for (a) to (c), and 200 nm for (d).

less or more toxic depending on the reactivity of the functional groups within the living cells. AgNPs powder purchased from Sigma-Aldrich had 64 nm in TEM (Fig. 3(a)), but this sample was unstable in the aqueous phase and was easily aggregated, followed by sedimentation. The dispersion stability of nanoparticles in the parent solvent was the key factor in the cell test.

Herein, we used the THF/water phase exchange method to disperse AgNPs in the water phase. Bare-AgNPs were ten times smaller in size (5.3 nm) as shown in Figure 3(b), due to the removal of aggregated larger AgNPs by centrifugation. Citrate- and PVP-AgNPs had a diameter of 35 nm (Fig. 3(c) and 3(d)). Surface charge of the fluorescence vesicle was −39.8 mV, which showed larger electrostatic repulsion, and thus separation distance between the neighboring vesicles was large.[16] Based on the simple spherical model,[10] the interparticle distance can be estimated. The concentrations of the vesicles and AgNPs were quite diluted, and thus, the intervesicular distance between vesicles was large (> 50 μm) as shown in Figure 2(a). While the interparticle distance of the bare-AgNPs was about 100 nm, citrate- and PVP-AgNPs showed the interparticle distance of less than 150 nm. Therefore, the interparticle distance of individual particles is enough for them to fully separate from each other in a stable

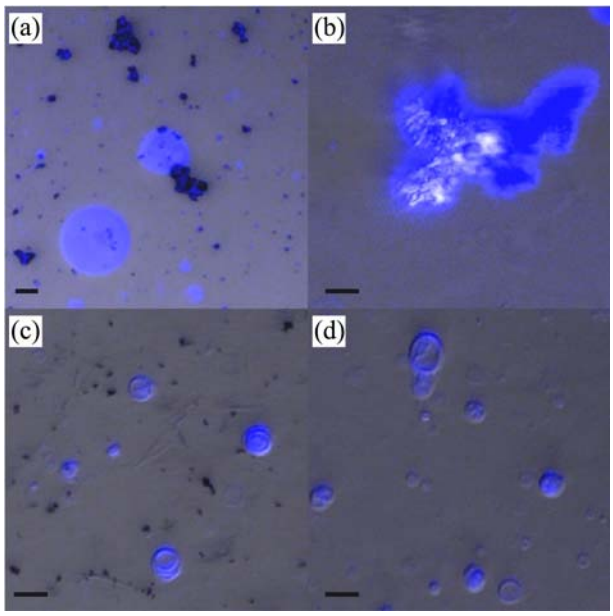

Figure 4. Florescence images after addition of (a) AgNP powder, (b) bare-AgNPs, (c) citrate-AgNPs, and (d) PVP-AgNPs in a florescence vesicle containing solution (scale bar = 10 μm).

manner in solutions before mixing with vesicle and x-AgNPs.

All AgNPs except bare-AgNPs showed a negative charge between −26 and −54 mV, which were closed values indicating moderate stability by the ASTM (American Society for Testing and Materials) standard. This feature for a positive charge of bare-AgNPs might be due to the co-existence of dispersed small AgNPs and silver ions. Namely, Ag+ ion was attached on the AgNPs and revealed the screening effect of the surface charge. For ISE (ion selective electrode) measuring, a significant amount of Ag+ ions for bare-AgNPs was detected, compared to the citrate- and PVP-AgNPs.

When x-AgNPs were exposed into the solvent with fluorescence vesicle, fluorescence images were different depending on the type of AgNPs, as shown in Figure 4. AgNP powder was less dispersed in the aqueous phase and showed some aggregation or agglomeration (Fig. 4(a)). Even though AgNP powder had a negative charge without any stabilizer, spontaneously aggregation could have occurred due to frequent collisions between neighboring particles.[19] In addition, the bilayer of vesicle was not clearly observed and fluorescence dye was found in aggregates. It is noted that AgNP powder was directly attacked the vesicle by aggregation of AgNPs and induced the vesicle rupture and co-aggregation with the vesicle and AgNPs. Fluorescence dye was released into the solution and thus fluorescence iamges (Fig. 4(a)) appeared blue as a whole.

The positive charge of bare-AgNPs allow for a high attractive force with fluorescence vesicles with a negative charge. Thus, vesicle rupture is likely to occur by adsorption of bare-AgNPs on the outer surface of the vesicle. As shown in Figure 4(b), mixing solutions of vesicle and bare-AgNPs showed the aggressive rupture and flocculation of vesicles. High attraction between the vesicle and bare-AgNPs can reduce the stability of vesicles and eventually induce vesicle rupture and fusion. On the other hand, citrate-AgNPs with a negative charge was stabilized with carboxyl groups and thus high repulsion revealed between the vesicle and citrate-AgNPs. In Figure 4(c), citrate-AgNPs was fully separated with the fluorescence vesicle and the lipid bilayer of the vesicle is observed clearly, which was identical to that of Figure 2(a). PVP-AgNPs with a negative charge was shown to display similar phenomena for the citrate-AgNPs. It is noted that the surface charge of nanoparticles is the key factor for vesicle rupture and fusion.

These findings were coherent with the results of *in vitro* and *in vivo* cytotoxicity tests of nanoparticles. AgNPs coated with polysaccharide diminished the toxic effects of silver, indicating that coating and surface chemistry play important roles in inducing adverse effects.[21] AgNPs coated with stabilizers are released into the environment and toxicity may be sustained when they have improved suspension stability.[14] Namely, Surface charge plays a role in toxicity with cationic surface being more toxic than anionic and neutral surfaces which are most biocompatible, due to the affinity of cationic particles to the negatively charged cell-membrane.[3] Therefore, high zeta potential of nanoparticles is the key factor to stabilize nanoparticles in solutions and is likely to reduce the cytotoxicity. However, we should think about this; high stability will make nanoparticles to reside in the environment for a long time.

Conclusions

Herein, we tried to investigate the cytotoxicity of metallic nanoparticles in a rapid and direct manner through analyzing the interaction between the biomimetic cell-membrane and various AgNPs. Various AgNPs with different surface charges were exposed into the solution of fluorescence-dye containing vesicles, which was prepared using a fluorescence probe (DPH). Morphological changes (*i.e.*, rupture and fusion of vesicle, and release of dye) were then observed by fluorescence microscopy. Fluorescence probe was intercalated into the lipid bilayer due to their hydrophobicity, and thus it was released into the solution when the vesicle was directly damaged by attack of AgNPs. Morphological changes of the vesicle after addition of x-AgNPs was different depending on the coating materials and surface charge of x-AgNPs. Even though the surface charge of AgNP powder without any additives was the same as that of the vesicle, spontaneous aggregation occurred due to frequent collisions between neighboring particles. Following this, vesicle rupture was induced indirectly and formed co-aggregates with the vesicle and AgNPs. When the surface charge of AgNPs was the opposite to that of the vesicle, the high attraction between the vesicle and AgNPs reduced the stability of vesicles and eventually induced vesicle rupture and fusion. This finding was similar with the results of *in vitro* and *in vivo* cytotoxicity tests of nanoparticles. Therefore, this proof-of-concept test will open the possibility of mimicking cell-membranes

(*i.e*. vesicle) to rapidly and conveniently screen for nanotoxicity in other metallic nanoparticles.

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