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Surface plasmon resonance study of (positive, neutral, negative) vesicles rupture by AgNPs' attack for screening of cytotoxicity induced by nanoparticles

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Abstract–As the use of nanomaterials in industrial and commercial applications is growing, official reports concerning possible environmental and health effects of nanoparticles are also steadily increasing. Many toxicological studies on the adverse effects of silver nanoparticles (AgNPs) have used living organisms, which is a time consuming process. Therefore, we propose an alternative method to assess the *in-vivo* and *in-vitro* cytotoxicity of nanomaterials, involving a fast and simple screening procedure for vesicle rupture or fusion by the attack of AgNPs. With the assumption that particle interaction between AgNPs and vesicles is induced by electrostatic repulsion or attraction of surface charge, three vesicles with different charges (positive, neutral, and negative) were prepared and they were dispersed with AgNPs in different pH (3, 7, and 10) solutions to control the surface charge of AgNPs. Based on the results of vesicle rupture analyzed by SPR and TEM, screening of cell rupture through vesicles by AgNPs' attack is determined to be most suitable at pH 7.

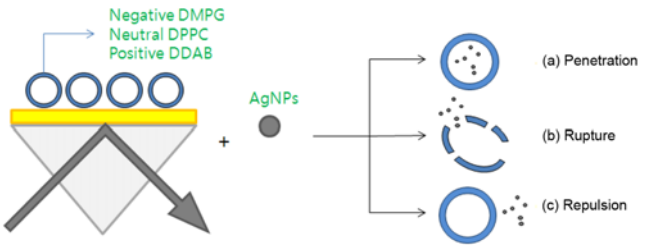
Key words: Silver Nanoparticles, Vesicles, Nanotoxicity, Cell Rupture, SPR

INTRODUCTION

With the rapid growth of nanotechnology, various nanomaterials are being produced and used as raw materials for nano-consumer products. Nanomaterials have unique physicochemical properties that are dependent on their base material and morphology. Therefore, many researchers have been focusing on developing novel synthesis and shape control methods of nanoparticles. However, environmental, health and safety risks of nanoparticles have recently emerged. For example, silver nanoparticles (AgNPs), which are known to be a strong antibiotic, were found to cause cell necrosis. It has come to light that nanoparticles might induce hazardous effects on bio-organisms by penetrating into the cell membrane [1-4]. Therefore, *in-vivo* and *in-vitro* cytotoxicity testing of as-made nanomaterials has become a necessary protocol to quantify and qualify the nanotoxicity of nanomaterials [4-8].

However, these *in-vivo* and *in-vitro* cytotoxicity test methodologies are time-consuming and are not suitable for rapid screening of various nanoparticles used in industrial applications. Moreover, the use of organisms or animals in cytotoxicity testing is faced with ethical difficulties. Therefore, a non-biological screening method, which should be fast, simple and better than *in-vivo* and *in-vitro* methodologies, as an alternative cytotoxicity test is required.

Recently, the Shiraki group suggested a simple test method for adsorption and disruption of lipid bilayers by nanoscale protein aggregation [9]. Supported lipid layers are known as well-defined models for the cell surface and for investigating molecular events in cell membranes [10]. The Shiraki group showed that the lipid bilayers could be used as a biomimetic cell membrane. Another study by Frank and coworkers investigated the development after exposure of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) vesicles to the amphipathic helix peptide (AHP) [11]. In addition, Chah and Zare presented evidence that POPC vesicles, which serve as a mimic for the cell membrane, are transformed into a lipid bilayer (by rupturing) using *in-situ* surface plasmon resonance (SPR) [12]. Therefore, previous studies have shown that vesicle rupturing or adsorption of AgNPs could be observed by mimetic lipid vesicles. Although the cytotoxicity mechanisms behind the activity of AgNPs on cells and bacteria are not yet fully elucidated, the three most common mechanisms have been proposed [1]: (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 the cell membrane by AgNPs attack. Among these mechanisms, the third mechanism was carried out as a proof-of-concept test through *in-situ* and *ex-situ* observations of vesicle rupturing or adsorption of AgNPs to verify the proposed methodology. As shown in Scheme 1, the adsorption of AgNPs on the vesicles induced by charge-matching between AgNPs and vesicles was induced for successive rupturing of vesicles. A weak electro-repulsive force between AgNPs and vesicles



Scheme 1. SPR experimental scheme to investigate the possible mechanisms (penetration, rupture, and repulsion) induced by the cytotoxicity of nanomaterials. Herein, the lipid bilayers of vesicles were used as biomimetic cell membranes for attacking of the bare AgNPs.

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can lead to the destruction of cell membranes.

In this work, we investigated the morphology change of cell membranes through direct attack of AgNPs using differently charged vesicles. Commercialized AgNPs without any additives are used here and called bare AgNPs. Vesicle rupturing was analyzed with spectroscopic and microscopic tools; *in-situ* SPR and *ex-situ* transmission electron microscopy (TEM).

EXPERIMENTAL

1. Preparation of Silver Nanoparticles without Any Additives

A nano-silver powder (Sigma-Aldrich, 99%, <100 nm) without suspension additives was prepared as a suspension of AgNPs in an aqueous phase via the tetrahydrofuran (THF) method [6], which is a procedure used to readily exchange with water and easily remove by evaporation. Ag powder was added to THF solution and stirred at approximately 300 rpm until THF had completely evaporated (1-2 days). After this, deionized water (DW) was added to replace THF. The resulting sample was then filtered through a polycarbonate membrane filter (50 nm isopure, Adventech).

2. Preparation of Vesicles

Vesicle was prepared via the chloroform dispersion method. Dimethyl dioctadecyl ammonium bromide (DDAB) was purchased from Sigma and dissolved in a chloroform solution to form lipids. The solution was added to a glass vial and the chloroform was slowly removed under a stream of nitrogen at a temperature above the transition temperature of 40-50 °C. The final traces of solvent were removed by pulling vacuum on the vial for 3-4 hours. Multilamellar vesicles were produced by resuspending the lipid in 4 mL of 25 mM phosphate buffered saline (PBS) at pH 7. The resulting solution contained positive DDAB vesicles [13]. Dipalmitoylphosphatidycholine (DPPC) solution, 50 mg/ml in chloroform, was purchased from Avanti Polar Lipids (Alabaster). The rest of the preparation processes for DPPC were identical to the preparation method of DDAB vesicles [14]. Sodium salt of 1,2-dimyristoyl-sn-glycero-3-[phospho-rac-glycerol] (DMPG) phospholipid was purchased from Avanti Polar Lipids. A lipid film was formed from the chloroform solution [15]. The remaining processes were identical to that of DDAB vesicles and the product was referred to as negative DMPG vesicles. The concentration of all vesicles and AgNPs for SPR analysis was 50 μM and 50 ppm, respectively. The flow rate of SPR analysis was set as 20 μL/min.

3. Characterizations

The morphology change of (positive, neutral, and negative) vesicles by the attack of AgNPs on the SPR gold chip was analyzed with SPR (SPR-Lab, K-mac) spectroscopy, namely, angle-resolved SPR analysis. In addition, to define the charge effect of AgNPs on the charge of vesicles, the pH of AgNPs solution was adjusted from 3 to 10. The final solution was sampled and TEM images were obtained for nine conditions (different pH and vesicles). The surface charge of AgNPs and vesicles was measured by electrophoretic light scattering (ELS-Z, Otsuka Electronics).

RESULTS AND DISCUSSION

The surface charge of AgNPs was easily changed by altering the pH conditions. Their surface charges are generally measured by ELS, and the point of zero charge (PZC) of AgNPs is found to be below pH 2 [16]. When the surface charge of AgNPs is close to zero mV, its colloidal stability is eventually decreased. In contrast, at a higher pH, AgNPs show a strong negative zeta potential, which signifies good stability. It is important to note that the charge of bare AgNPs without any additives is directly dependent on the pH and salts in solutions.

Table 1. Zeta potentials of vesicles in PBS and AgNPs in DW

Materials	Medium	Remarks	Zeta potential (mV)
Vesicles	PBS	DDAB (+)	+25.60
		DPPC (0)	−1.43
		DMPG (−)	−32.18
AgNPs	DW	pH 3	−2.13
		pH 7	−26.47
		pH 10	−29.48

Herein, bare AgNPs dispersed in DW were prepared by the THF/DW exchange method. A previous report confirmed that the rest of THF in AgNPs solution was not found [6]. Whereas, the raw silver powder obtained from the manufacturer had ca. 100 nm, bare AgNPs prepared via the THF method was approximately 10 nm. When the silver powder was dispersed, much of the particles settled. Thus, the supernatant was collected and used, followed by re-dispersion at pH 3, 7, and 10 solutions. The surface charge of AgNPs in DW media is summarized in Table 1, with its value at pH 3 to be −2.13 mV, which is close to zero. Zeta potentials at pH 7 and 10 were −26.47 and −29.48 mV, respectively, which are close values, indicating moderate stability by the American Society for Testing and Materials (ASTM) standard [17]. Because coagulation or flocculation between AgNPs easily occurs in long-term exposure to low pH, the exposure experiment for vesicle rupture by the attack of AgNPs was carried out promptly.

Vesicles prepared in this work were generally stable at pH 7. Three different charged vesicles (+, 0, −) were prepared in chloroform and were finally dispersed in PBS solution, a buffer solution commonly used in biological research. Since PBS is isotonic and non-toxic to cells, it was also used to test vesicle rupture as a bio-mimetic cell membrane [6,7,11,12]. Although the surface of vesicles could interact with various salts in PBS and could result in changes of the surface charge, three vesicles maintained the original sign (+, −) of the surface charge as shown in Table 1. The zeta potential of DDAB, positive vesicles, was +25.60 mV, and DPPC was −1.43 mV, which was an acceptable value for neutral vesicles. DMPG with −32.18 mV was prepared as negative vesicles.

Based on Coulomb's law, the electrostatic interaction between electrically charged particles is dependent on the product (q_1q_2) of charges (q_i) of two bodies. Its product values were large positive (i.e., repulsion) for DMPG-AgNPs for pH 7 and 10, while large negative (i.e., attraction) for DDAB-AgNPs for pH 7 and 10. Therefore, DDAB-AgNPs for pH 7 and 10 might have been unstable and thus showed cell rupture and fusion of vesicles.

This work is focused on the direct damage of the cell membrane by the attack of AgNPs. As shown in Scheme 1, AgNPs show electrostatic attraction or repulsion to vesicles with various charges, and then are adsorbed on the vesicle or penetrated into the lipid bilayer. Finally, this adsorption or penetration of AgNPs is enough to induce

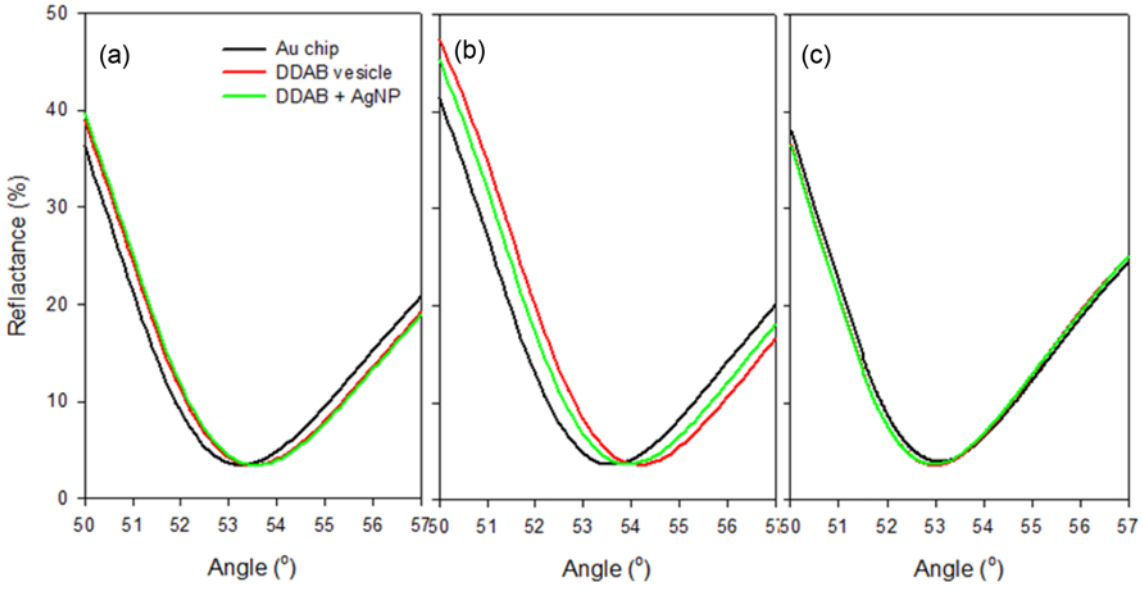

Fig. 1. Angle-resolve SPR data of DDAB (50 μM) and 50 ppm AgNP on SPR gold-chip at (a) pH 3, (b) 7, and (c) 10 solutions. Injection rate for all solutions was 20 μL/min.

direct damage to the lipid bilayer of vesicles. This feature is similar to the rupture or fusion of the cell membrane. When the repulsive force between AgNPs and vesicles is strong, the destruction of the cell membrane can be suppressed by keeping a distance based on the repulsion charge. When the electrostatic repulsive force between AgNPs and vesicles is weak, it can lead to the destruction of the cell membrane. Angle-resolved SPR analysis was carried out to define the possibility of direct damage of the cell membrane, i.e., vesicle rupture, by the charge attraction between AgNPs and vesicles.

As shown in Figs. 1 to 3, angle-resolved SPR data obtained as three vesicles were loaded on the gold film. Prior to injection of vesicles into the SPR cell, the reflectivity was scanned as a function of angle. The minimum reflectance of the bare gold chip was found at 52-53°. After sequential injection of vesicles and AgNPs onto the gold chip, the minimum reflectance was changed because of altered thickness and dielectric constant of the gold surface. When DDAB vesicles and AgNPs were injected into the SPR cell, angle-resolved SPR response changed in concert with pH of the solution (Fig. 1). SPR angle, showing the minimal intensity at the curves, shifted after the vesicles were loaded on the bare gold surfaces. Angle shifts after injection of vesicles and AgNPs are summarized in Table 2. The angle shift after injection of DDAB vesicles was large at pH 7 and small at pH 10. The reflectance change in SPR response was less changed, and the concentration of vesicles and AgNPs was not excessive to accumulate on the gold film. After injection of AgNPs in three pH conditions, SPR spectra and angle shift were changed with changing pHs, because AgNPs in three pH conditions did not have the same charge. Angle difference after injection of DDAB and AgNPs is larger at pH 7 (–0.30°), compared to that at pH 3 (+0.10°) and 10 (+0.04°). This result showed vesicles with positive charges represented a distinct change of SPR angles at pH 7. The same SPR test was performed for DPPC with a neutral charge (Fig. 2). In this case, a distinct change of SPR angle was observed at pH 7 (–0.42°). On the other hand, DMPG with a negative charge showed a smaller angle change at pH 7 (–0.10°) than pH 3 (+0.30°), as shown in Fig. 3.

Table 2. Angle change of angle-resolved SPR response after exposure of vesicles and AgNPs

Vesicles	pH	After exposure	$\Delta\theta$(°)	Angle difference
DDAB	3	Vesicle	+0.20	
		AgNP	+0.30	+0.10
	7	Vesicle	+0.60	
		AgNP	+0.30	−0.30
	10	Vesicle	−0.10	
		AgNP	−0.06	+0.04
DPPC	3	Vesicle	+0.48	
		AgNP	+0.46	−0.02
	7	Vesicle	+1.70	
		AgNP	+1.28	−0.42
	10	Vesicle	+0.20	
		AgNP	+0.12	−0.08
DMPG	3	Vesicle	+0.30	
		AgNP	+0.60	+0.30
	7	Vesicle	+0.90	
		AgNP	+0.80	−0.10
	10	Vesicle	+0.08	
		AgNP	+0.02	−0.06

SPR results between vesicles and AgNPs according to charge differences are related by the two points: i) dispersion stability of AgNPs at different pHs and ii) fusion and eventual rupture of vesicles at different pH conditions. To confirm these features, TEM analysis was carried out. As shown in Fig. 4, all vesicles in pH 3 were fused and ruptured, then formed the lipid bilayers. In addition, AgNPs were severely aggregated and grown up to tens of nanometers. The reflectance change of angle-resolved SPR data for pH 3 is the result of co-existing AgNPs aggregates with lipid bilayers, not spherical vesicles. Therefore, it is difficult to define the charge effect between AgNPs and vesicles at pH 3 by SPR and TEM analyses. All vesicles

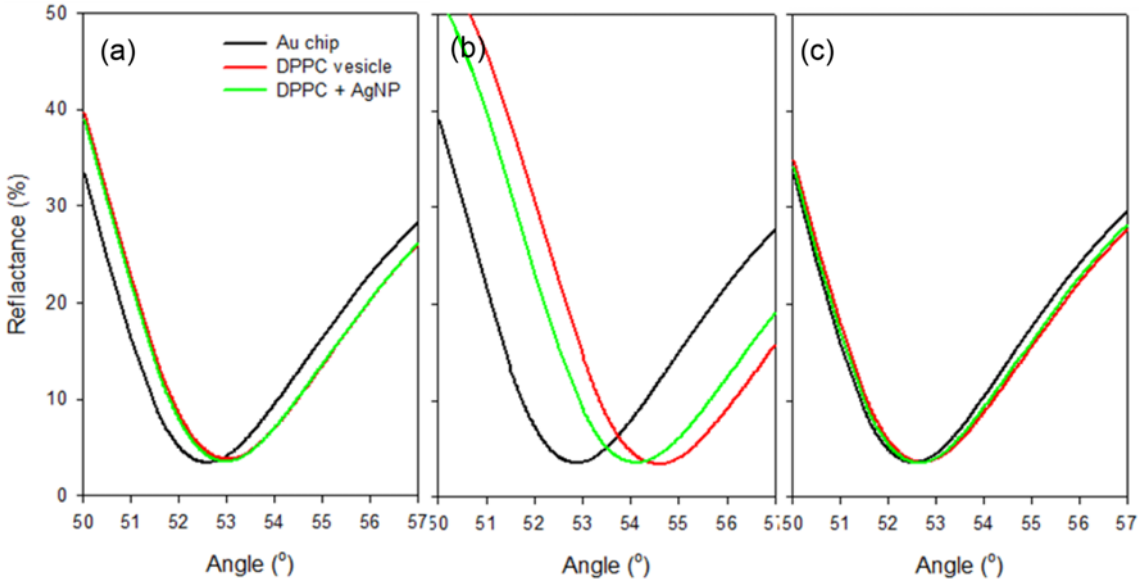

Fig. 2. Angle-resolve SPR data of DPPC (50 μM) and 50 ppm AgNP on SPR gold-chip at (a) pH 3, (b) 7, and (c) 10 solutions. Injection rate for all solutions was 20 μL/min.

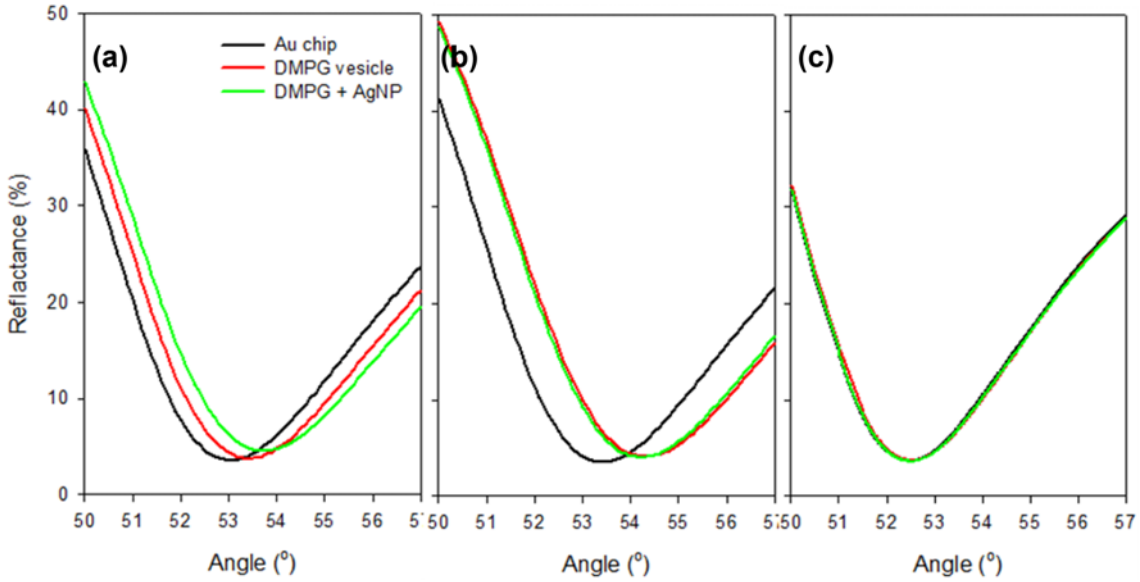

Fig. 3. Angle-resolve SPR data of DMPG (50 μM) and 50 ppm AgNP on SPR gold-chip at (a) pH 3, (b) 7, and (c) 10 solutions. Injection rate for all solutions was 20 μL/min.

at pH 10 maintained the spherical shape, but showed the smallest angle change in the SPR test. In the TEM image (Fig. 4i) for negative vesicles at pH 10, vesicles were the most stable and separated with AgNPs due to large electro-repulsion. The TEM image for neutral vesicles at pH 10 (Fig. 4f) appeared like blueberries, due to the adsorption of AgNPs on the outer surface of vesicles. AgNPs at pH 10 seemed to penetrate into the inner surface of positive vesicles (Fig. 4c). All vesicles at pH 10 maintained a partially spherical shape and AgNPs were partially adsorbed on the vesicle or penetrated into the lipid bilayers. Therefore, screening for the direct damage of the cell membrane by AgNPs at pH 10 is suitable for TEM analysis. At pH 7, three vesicles showed all other forms (sphere, rupture, and fusion). DMPG vesicles at pH 7 maintained a spherical shape and were separated with AgNPs, due to the large electro-repulsion similar to the case with DMPG at pH 10. Positive and neutral vesicles were partially fused and ruptured, and AgNPs were partially aggregated.

Consequently, a proof-of-concept test at pH 3 was not suitable because of the aggregation of AgNPs and spontaneous fusion/rupture of vesicles to form lipid bilayers. Even though the stability of vesicles at pH 10 is established and confirmed by TEM, SPR analysis is not suitable due to the very insensitive angle changes in the SPR response. Since AgNPs in the neutral condition before mixing with vesicles are stable in solution and vesicle condition is maintained as the initial pH, the screening test for vesicle rupture by AgNPs at pH 7 is reliable and easy. In addition, the charge effect between AgNPs

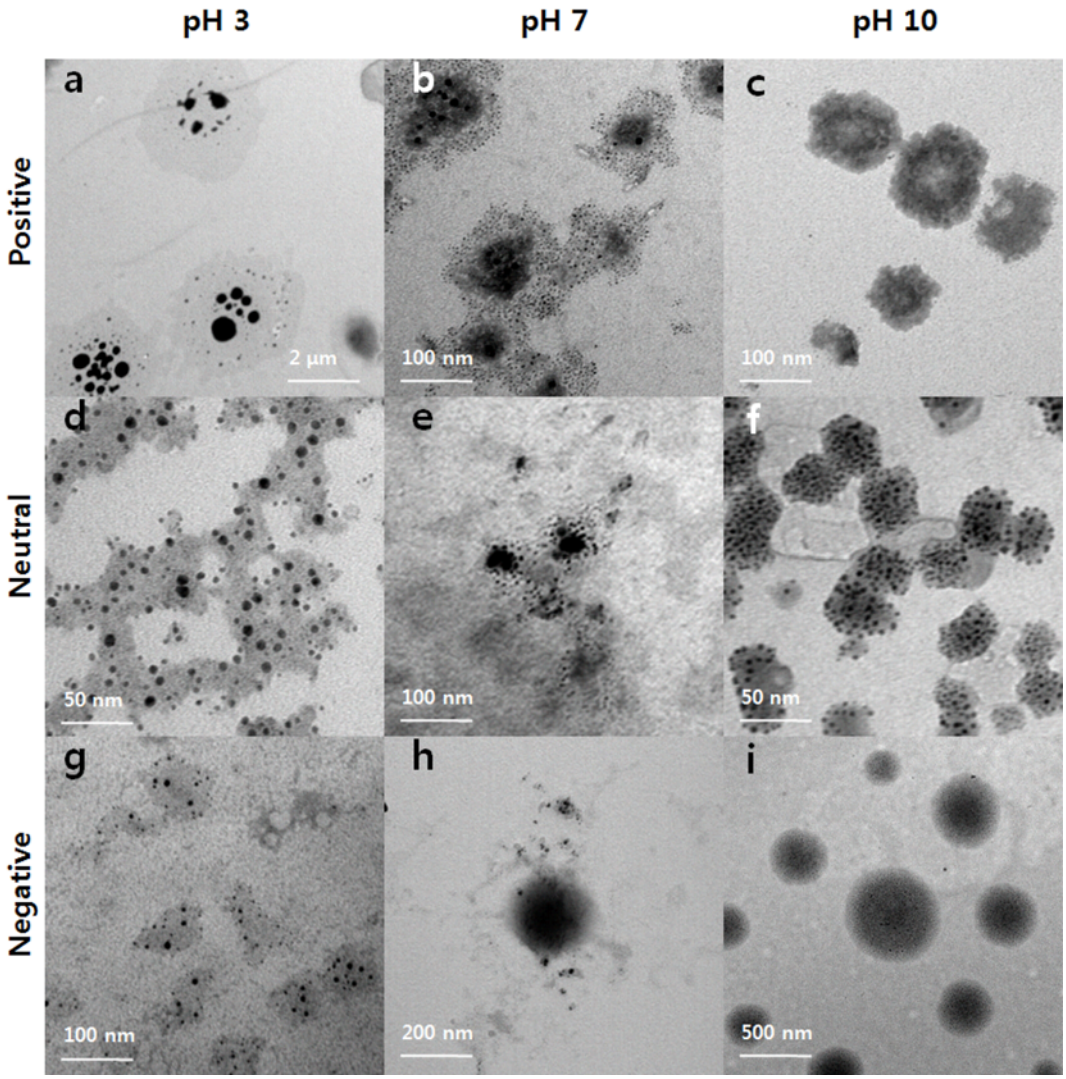

Fig. 4. TEM images of three different vesicles with AgNPs in various pH conditions. Morphology changes of (a)-(c) positive vesicles (DDAB), (d-f) neutral vesicles (DPPC), and (g-i) negative vesicles (DMPG) by the attack of bare-AgNPs in (a, d, g) pH 3, (b, e, h) pH 7, and (c, f, i) pH 10 solutions.

and vesicles in SPR was well matched with TEM results. Therefore, screening of cell rupturing of vesicles by AgNPs' attack using SPR and TEM at pH 7 is the most suitable alternative method to the *in-vivo* and *in-vitro* cytotoxicity test.

CONCLUSION

We have proposed a non-biological method to *in-vivo* and *in-vitro* cytotoxicity of nanomaterials, using vesicle rupture or fusion by the attack of AgNPs. The SPR and TEM data provided clues for vesicle rupture or fusion, which resembled cell rupture or fusion by the attack of AgNPs. The SPR signal change, i.e., angle change in angle-resolved SPR analysis was the largest at pH 7 and was less changed at pH 10. In TEM analysis, all vesicles at pH 3 and 10 were ruptured and maintained as spheres, respectively. Based on the SPR and TEM analyses, we concluded that screening of cell rupturing through vesicles by the attack of AgNPs is most suitable at pH 7. This proposed method is useful as an alternative method for the cytotoxicity test of nanoparticles instead of using biological organisms or animals. Although the SPR method is helpful to define the interaction between AgNPs and vesicles, we should investigate a more simple method (e.g., direct detection via dark field microscopy or fluorescence microscopy) that can provide direct information of cell rupturing by AgNPs.

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