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# Dispersion stability of citrate- and PVP-AgNPs in biological media for cytotoxicity test

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**Abstract**−Silver nanoparticles (AgNPs) with antimicrobial property have been used for years in various applications, such as antibacterial coating and air/water purification to eliminate microorganisms. In recent years, a number of studies on the potential risk of nanomaterials in humans and the environment have appeared, and thus a large number of studies regarding the toxic effects of AgNPs on cells and micro-organisms have been reported. In *in-vivo* and *in-vitro* cytotoxicity tests, various biological media are used, and thus the dispersion stability of AgNPs in media is important to a successful toxicity test. Therefore, we investigated the dispersion stability of AgNPs in various biological media; PBS (phosphate buffered saline), FBS (fetal bovine serum), and de-ionized water (pH 2, 7, and 9) with and without photo-irradiation. Herein, citrate and PVP (polyvinylpyrrolidone) stabilized AgNPs were used as target materials. In short- and long-term stability tests, we found that PVP-AgNPs stabilized by steric hindrance have good dispersion stability in biological media, compared to citrate-AgNPs stabilized by electrostatic repulsion.

Key words: Silver Nanoparticles, Cytotoxicity, Dispersion Stability, Citrate, PVP

## INTRODUCTION

Nanomaterials show unique and considerable physicochemical (PChem) and biological properties, compared to their bulk counterpart. Metal nanoparticles are useful in areas such as catalysis, biological labeling, optoelectronics and plasmonic sensors [1]. In particular, colloidal silver is of great interest due to its good conductivity, chemical stability and antibacterial activity [2]. Generally, silver nanoparticles (AgNPs) are prepared by a chemical reduction method and are stabilized in water or organic solvents [3]. AgNPs with antimicrobial property have been used for years in antibacterial applications and air/water purification to eliminate microorganisms [4].

However, a number of studies on the potential risk of nanomaterials in humans and the environment have recently appeared. For example, it has been reported that engineered nanomaterials can cause various effects including oxidative stress, catalysis of cell necrosis, and protein aggregation in systems ranging from human cells to bacteria [5]. Therefore, over the last decade, a large number of studies have been conducted regarding the toxic effects of AgNPs on cells and micro-organisms via *in-vivo* and *in-vitro* testing [6].

A cytotoxicity test for new chemicals is mainly carried out by high-dose testing involving extrapolation from high to low doses. This traditional test is expensive, time-consuming, and has questionable relevance in predicting risks for humans. Therefore, an alternative vision proposed by the U.S. National Research Council called for moving away from traditional high-dose animal studies to an approach based on the perturbation of cellular responses using well-designed *in-vivo* and *in-vitro* assays [7]. Various biological objects from cells to animals, such as lung cells, *Escherichia coli*, *Daphnia magna*, zebrafish, and mice, are used in the nanotoxicity test [8]. In the toxicokinetic study involving intravenous injection as a route of exposure in mice, AgNPs must be dispersed in blood isotonic solution [9]. In addition, in the cell vitality test, AgNPs should be stabilized in culturing media, such as PBS (phosphate buffered saline), FBS (fetal bovine serum) with 10% MEM (minimal essential medium), and de-ionized water (DW) [10]. PBS is a buffer solution commonly used in biological research due to non-toxicity to cells and matching osmolarity and ion concentration with the human body (isotonic). However, culturing media consist of several salts (NaCl, KCl, and $Na_2HPO_4$), and thus can easily cause flocculation between neighboring AgNPs [11]. FBS is the portion of the remaining plasma after coagulation of blood, and thus is the most widely used serum-supplement for *in-vitro* cell culture. Proteins in FBS compete for

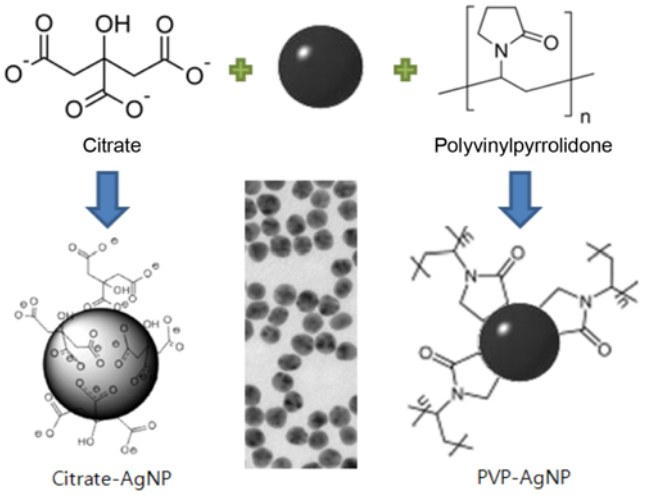


**Fig. 1. Citrate- and PVP-AgNPs stabilized in the aqueous phase by electrostatic repulsion and steric hindrance, respectively.**

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the AgNPs surface, leading to the formation of a so-called protein corona, which largely defines the biological identity of the particle [12]. Therefore, it is important to maintain the pristine stability of AgNPs in biological media and external conditions during the nanotoxicity test.

In this work, we investigated the dispersion stability of AgNPs in various biological media: PBS, FBS, DW (pH 2, 7, and 9) with and without photo-irradiation. AgNPs prepared by chemical reduction were obtained and stabilized with citrate and PVP (polyvinylpyrrolidone). As shown in Fig. 1, citrate- and PVP-AgNPs were stabilized with different mechanisms: electrostatic repulsion and steric hindrance, respectively. To define the stability of AgNPs in media, hydrodynamic diameter (HDD) with exposure time and zeta potential were measured. In addition, the stability by photo-irradiation was tested at 455 and 630 nm LED-light.

## EXPERIMENTAL

Citrate-AgNPs with a diameter of *ca.* 40-50 nm were purchased at ABC Nanotech (Korea), a company which produces standard AgNPs following specifications in the OECD's (Organization for Economic Co-operation and Development) WPMN (Working Party on Manufactured Nanomaterials) [13]. PVP-AgNPs with *ca.* 70 nm were prepared by the well-known polyol method [14]. Both AgNPs were initially dispersed and stabilized in the water phase. PBS, FBS, and DW were selected as biological media. HDD change of 10 ppm AgNPs in PBS, FBS, DW (pH 2, 7, and 9) was analyzed by electrophoretic light scattering (ELS-Z, Otsuka, Japan). Surface charge was also measured by ELS. The photo-irradiation test of AgNPs was carried out with 700 Lux of 455 and 630 nm LED lamp (Maestor Korea, Korea) for 24 hr. The morphological change of AgNPs was analyzed by using transmission electron microscopy (TEM, JEM-1010, JEOL, Japan) and UV-vis spectrophotometer (UV-1800, Shimadzu, Japan).

## RESULTS AND DISCUSSION

In the synthesis of nanoparticles, capping agents such as surfactants and polymers are used to prevent their aggregation through electrostatic repulsion, steric repulsion or both. AgNPs can be prepared using various stabilizers, but OECD WPMN recommends citrate and PVP coated AgNPs for the cytotoxicity test [13]. If the stabilizer has its own toxicity, it is difficult to define the intrinsic toxicity of AgNPs itself. Therefore, stabilizers with less toxicity such as citrate and PVP were recommended by OECD WPMN. Citrate with three carboxyl groups ($COO^-$) immobilized on the surface of AgNPs revealed a strong negative charge of the AgNPs. Thus, citrate-AgNPs were stabilized by electrostatic repulsion to reduce the aggregation between neighboring citrate-AgNPs. Whereas, PVP with the pyrrolidone group (cyclic organic) immobilized on the surface of AgNPs induced steric hindrance and maintained an appropriate separating distance between neighboring PVP-AgNPs. In addition, carbon and oxygen double bonds in pyrrolidone induced a negative charge, which caused PVP-AgNPs to show a weak negative surface charge [15]. As a result, PVP-AgNPs were stabilized by combination of steric hindrance and electrostatic repulsion.

HDD of citrate- and PVP-AgNPs in DW are approximately 50 and 70 nm, and the surface charges of two AgNPs were −45 and −35 mV, respectively. In UV-vis spectra, both AgNPs showed single surface plasmon absorption band with a maximum of 420 nm, indicating the presence of spherical AgNPs.

AgNPs were dispersed in the most widely used biological media (PBS, FBS, and DW) and then the dispersion stability was analyzed with ELS (HDD and zeta potential). The results obtained in this study may be useful as fundamental data for *in-vivo* and *in-vitro* cytotoxicity tests (i.e., aged PChem) regarding citrate- and PVP-AgNPs in target biological media, not pristine PChem of AgNPs. Short-term stability of AgNPs was assessed to determine the aggregation of AgNPs within one hour at intervals of 5 min, and then the

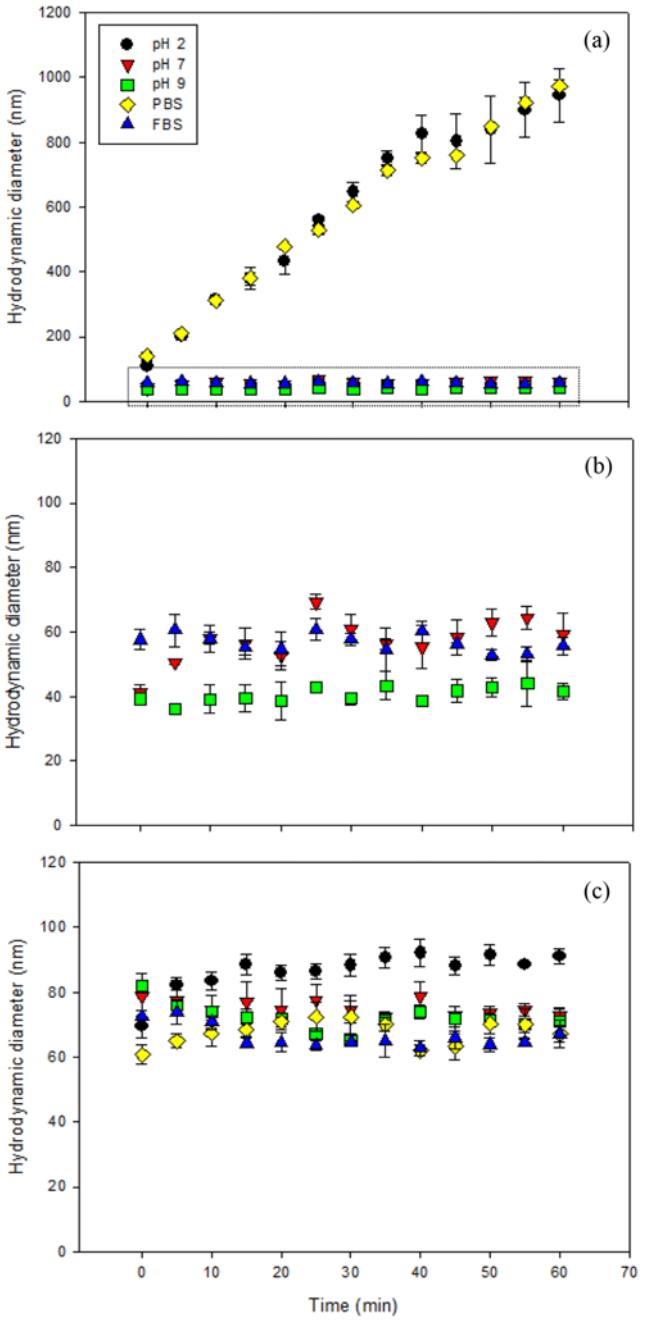


**Fig. 2. Hydrodynamic diameters of ((a) and (b)) citrate-AgNPs and (c) PVP-AgNPs under various media.**

**Table 1. Aggregation rate constant ($m^3$/min) of citrate- and PVP-AgNPs in short-term stability test**

| Media | Citrate-AgNPs | PVP-AgNPs |
|---|---|---|
| pH 2 | $2.13\times10^{-33}$ | $1.77\times10^{-35}$ |
| pH 7 | $3.80\times10^{-35}$ | $-5.63\times10^{-36}$ |
| pH 9 | $9.51\times10^{-36}$ | $-9.48\times10^{-36}$ |
| PBS | $2.10\times10^{-33}$ | $-1.59\times10^{-36}$ |
| FBS | $3.49\times10^{-36}$ | $-1.21\times10^{-35}$ |

change of HDD was measured using ELS data (Fig. 2) and aggregation rate (Table 1) was calculated based on the previous reported method [6]. Long-term stability was analyzed by the change of HDD and zeta potential (Table 2) after AgNPs were exposed in target media and light (455 and 630 nm) for 24 hr.

The short-term stability test (Fig. 2) revealed that HDD of citrate-AgNPs was dramatically increased in only two media: DW with pH 2 and PBS solution. This feature could be quantified by the calculation of aggregation rate constant (Table 1). Citrate-AgNPs in pH 2 and PBS showed large aggregation rate ($2.1\times10^{-33}$ $m^3$/min), compared to others. PVP-AgNPs in all media showed a slight change of HDD with time. When we measured the isoelectric point (IEP) of AgNPs by titration [16], IEP of citrate- and PVP-AgNPs was 2.7 and 3.2 of pH, respectively. IEP is the pH at which a particular molecule carries no net electrical charge, and thus electrostatic repulsion between neighboring AgNPs does not get diminished at this pH. Therefore, pH 2 to 3 is important to define whether aggregate of AgNPs occurs or not. As shown in Fig. 2(a), HDD of citrate-AgNPs in pH 2 steadily increased and reached above 1 μm in 60 min. In addition, the particle size of PVP-AgNPs was also slightly increased from 65 to 85 nm. This difference of two AgNPs is due to the additional stability mechanism (i.e., steric hindrance) of PVP-AgNPs. No change of HDD in pH 7 was observed for both AgNPs. In pH 9 DW, AgNPs were negatively charged and caused the greatest electrostatic repulsion, thus providing excellent particle dispersion as shown in Fig. 2(b) (citrate-AgNPs) and 2C (PVP-AgNPs).

In PBS, citrate-AgNPs showed a similar feature as pH 2, due to the diminishing electrostatic repulsion by matching between carboxyl groups of citrate-AgNPs and positive charged salts in PBS. However, HDD of PVP-AgNPs was not changed because its stability was maintained mainly by steric hindrance. As described before, FBS contained proteins and MEM can form protein coronas on the surface of AgNPs. As a result, particle size could be slightly increased: 60 nm for citrate-AgNPs. Their initial HDD was maintained during the short-term stability test. Therefore, the formation of protein corona (steric hindrance) in FBS and additional $OH^-$ (electrostatic repulsion) in pH 9 affect positively the particle dispersion stability in media.

The results of the long-term stability test are summarized in Table 2. As shown in the short-term test, citrate-AgNPs exposed in pH 2 and PBS media showed steadily increasing particle size, and finally it settled at the bottom of the beaker due to the formation of bulk particles after 24 hr. Therefore, HDD and zeta potential of citrate-AgNPs cannot be measured in two media. HDD and zeta potential of citrate-AgNPs in pH 7 were almost the same as those of pH 9. The particle size in FBS was increased by 20 nm compared to pH 9, and the zeta potential was decreased to −15 mV, which is not a good stability value of surface charge. Due to the effect of protein corona in FBS, citrate-AgNPs maintained its slightly increased particle size (66 nm). This feature was also observed for PVP-AgNPs (75 nm). For PVP-AgNPs, HDD in pH 7, 9 and PBS media was almost the same. Although the zeta potential of PVP-AgNPs in pH 2 and PBS media approached a zero value, HDD was less changed due to the effect of steric hindrance of PVP on the surface of AgNPs.

The light exposure of citrate-AgNPs leads to photo-oxidation of adsorbed citrate, destroying the strong citrate double-layer kinetic stabilization, which may subsequently change the size and shape of AgNPs [17]. For the photo-irradiation test of AgNPs solution, the HDD and zeta potential were measured during 24 hr irradiation with 455 and 630 nm LED-light. As shown in Fig. 3, UV-vis spectra of PVP-AgNPs were not changed, but that of citrate-AgNPs were greatly decreased for irradiation of 455 nm. While PVP-AgNPs maintained their initial size regardless of photo irradiation, citrate-AgNPs were markedly increased and reached 3.6 μm. Citrate undergoes photochemical oxidation on AgNPs through the following reaction [18]: citrate+$1/2O_2$→acetone-1,3-dicarboxylate+$CO_2$+$OH^-$. That is, Ag plasmon-induced photo-oxidation of adsorbed citrate injects electrons into the silver nanocrystal, and then these electrons accumulate on the Ag particle, leading to particle growth [19]. The charge-transfer photo-oxidation is irreversible due to fast decarboxylation of oxidized citrate, which thus destroys the strong citrate double-layer kinetic stabilization of the initial colloid [17]. Therefore, the long-term stability of citrate-AgNPs was weak in pH 2 and PBS media and under 455 nm irradiation. The same results were observed in the short-term stability test. Both short- and long-term stability of PVP-AgNPs was great in all conditions.

**Table 2. Change of hydrodynamic diameter and surface charge of citrate- and PVP-AgNPs under various conditions after 24 hr**

| Medium | Light | Citrate-AgNP | | PVP-AgNP | |
|---|---|---|---|---|---|
| | | HDD (nm) | Zeta potential (mV) | HDD (nm) | Zeta potential (mV) |
| pH 2 | Dark | N.D.* | N.D. | 66.5±1.5 | −6.3±0.1 |
| pH 7 | | 42.4±1.2 | −40.5±5.9 | 71.9±0.6 | −35.2±1.3 |
| pH 9 | | 45.8±2.5 | −48.9±0.8 | 72.7±0.8 | −37.8±1.3 |
| PBS | | N.D. | N.D. | 70.7±1.9 | −4.3±2.4 |
| FBS | | 66.1±0.1 | −15.0±2.1 | 75.7±1.6 | −14.2±5.3 |
| pH 7 | 455 nm | 3670.1±347.2 | −22.4±1.8 | 71.9±2.0 | −22.0±2.0 |
| | 630 nm | 53.4±6.7 | −22.9±0.8 | 71.5±0.9 | −20.6±2.1 |

*N.D.: not detectable due to aggressive aggregation and sedimentation of particles

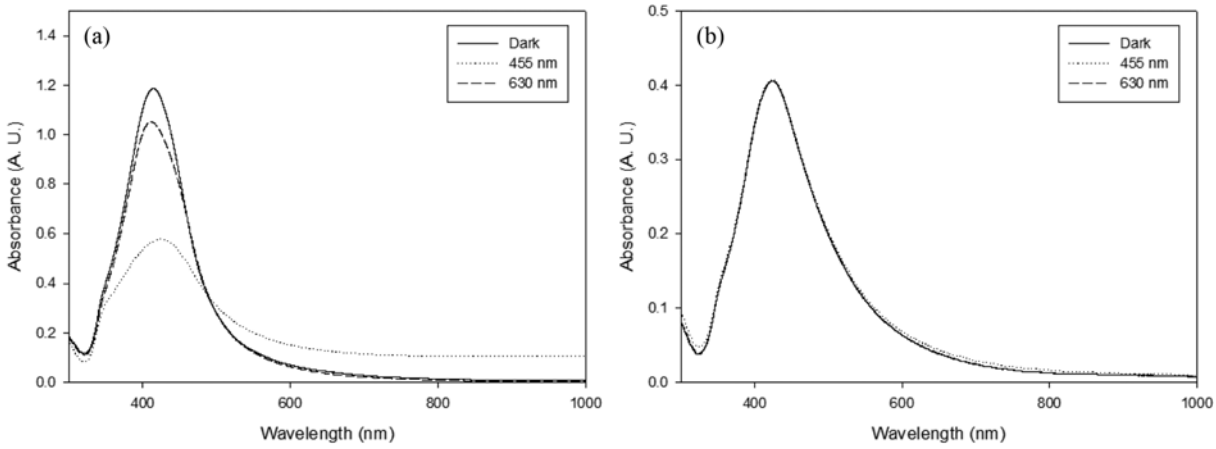


**Fig. 3. UV-vis spectra of (a) citrate- and (b) PVP-AgNPs in pH 7 in darkness and light of 455 and 630 nm for 24 hr.**

## CONCLUSION

We investigated the dispersion stability of AgNPs in various biological media (PBS, FBS, and DW) by analyzing the change of HDD, zeta potential, and UV-vis spectra. The stability mechanism of citrate-AgNPs was different with PVP-AgNPs; namely, electrostatic repulsion vs. steric hindrance. Therefore, short- and long-term stabilities of both AgNPs were different from each other. Citrate-AgNPs were easily aggregated in the vicinity of IEP because the electrostatic repulsion of AgNPs was minimized. In addition, salts in PBS solution were readily combined with the carboxyl groups of citrate-AgNPs and then the aggregated silver particles were obtained. In FBS solution, the formation of protein corona between AgNPs and proteins positively affected the dispersion stability in media. Photo-irradiation was also an important factor for dispersion stability in media, and 455 nm of irradiation for citrate-AgNPs showed only particle aggregation. Therefore, PVP-AgNPs stabilized by steric hindrance has good dispersion stability in biological media, compared to citrate-AgNPs stabilized by electrostatic repulsion. The results obtained here may be used as fundamental data in the nanotoxicity test. However, there is one point to be considered. Nanoparticles with good dispersion stability will be adaptable in *in-vivo* and *in-vitro* tests, but their residence time in environmental media after environmental exposure will be longer. Therefore, these particles may have other side effects in humans and bio-organisms.

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