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Spectroscopic and microscopic studies of vesicle rupture by AgNPs attack to screen the cytotoxicity of nanomaterials

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ABSTRACT

It is well-known that silver nanoparticles (AgNPs) with 1–10 nm of diameter attached to the surface of cell membrane were able to penetrate into the cell followed by rupturing of the cell. However, most studies for cytotoxicity used living organisms and were time-consuming. Therefore, in this study, we proposed a model method for fingerprinting of nanotoxicity of AgNPs in mimetic cell membranes using phosphor lipid. The vesicle rupturing mechanism by AgNPs attack was investigated using surface plasmon resonance (SPR) spectroscopy and atomic force microscopy (AFM).

1. Introduction

There has been an increased use of nanomaterials in industrial and commercial applications. The official reports concerning the possible environmental and health effects of nanoparticles are also steadily increasing. Especially, many epidemiological and toxicological studies regarding silver nanoparticles (AgNPs), which were included in the priority list of OECD (Organization for Economic Cooperation and Development), have reported on the adverse health effects [1–4]. According to these reports, exposure of AgNPs to bacteria cells or aquatic organisms cause adverse health effects including pulmonary inflammation and apoptosis by inducing oxidative stress and free radical generation [4–8]. However, these in vivo and in vitro cytotoxicity test are time-consuming and was not suitable to screen the various nanoparticles used in industrial applications rapidly.

Therefore, a fast and simple non-biological screening method as an alternative cytotoxicity test is required. A high-content screening assay has been tested by Kotov et al. and it was used as a universal tool for fingerprinting of nanotoxicity [9]. CdTe quantum dots and gold nanoparticles were exposed to NG108-15 (murine neuroblastoma cells) and HepG2 (human hepatocelluar carcinoma cells), and time- and dosage-dependent apoptosis were evaluated as fingerprinting of cytotoxicity of nanoparticles. This method can be done in 6 h which is reasonable fast but it is based on living cells. A study done by Nel group has reported the predictive toxicological paradigm for the safety assessment of nanomaterials by establishing and using mechanisms and pathways of injury at a cellular and molecular levels [10]. Recently, Shiraki group has suggested a simple test method for adsorption and disruption of lipid bilayers by nanoscale protein aggregation [11]. Even though the results of Shiraki group did not focus on the nanotoxicity, they showed that the lipid bilayer could be used as biomimetic cell-membrane.

In this study, we tried to test for a convenient and fast nanotoxicity assessment through the attack of nanoparticles on the phospholipid as biomimetic cell-membrane. Among various mechanisms of AgNPs' antibacterial interaction with cells [1,3,12], we evaluated the direct cell membrane damage done by the attack and penetration of AgNPs. Although lipid vesicle was not applied to assess nanotoxicity, Frank et al. have investigated on the exposure of POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) vesicles to the amphipathic helix peptide (AHP) [13]. In addition, Chah and Zare have presented evidence that the POPC vesicle, which serves as a mimic for cell membrane, is transformed into the lipid bilayer (rupturing) using in situ SPR (surface plasmon resonance) [14]. In the work, vesicle rupturing phenomena by the attack of AgNPs was observed using a mimetic lipid vesicle.

DPPC (dipalmitoylphosphatidycholine) vesicle, which is a phospholipid consisting of two palmitic acids and is capable of lowering surface tension to near zero levels, was selected as the biomimetic cell-membrane. Supported lipid layers are known as

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well-defined models for cell surface and for investigating molecular events in the membrane [15].

In this work, we aim to investigate the cell-membrane rupture via direct attack of AgNPs using the DPPC vesicle on the gold surface. The surface of AgNPs used in the study is not coated with any additives. Vesicle rupturing was analyzed with spectroscopic and microscopic tools using in situ SPR and ex situ AFM (atomic force microscopy).

2. Experimental

2.1. Preparation of PDDC vesicle

DPPC solution, 50 mg/ml in chloroform, was purchased from Avanti Polar Lipids (Alabaster). 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 42 °C. The final traces of solvent were removed by pulling vacuum on the vial for 3–4 h. Multilamellar vesicles were produced by resuspending the lipid in 25 mM PBS (phosphate buffered saline, Sigma) at pH 7. The multilamellar vesicles became the large unilamellar vesicles by sonicating the solution with 11 W for 5 min and extruding the lipid solution through a 100 nm pore size polycarbonate filter (Avanti Polar Lipids, Alabaster, PA). Both extrusion and sonication were performed at a temperature above the transition temperature. The size distribution of resulting vesicles was measured by electrophoretic light scattering measurements (ELS-Z, Otska).

2.2. Preparation of silver nanoparticles (bare AgNPs)

A nano-silver powder (Sigma–Aldrich, 99%, <100 nm) without suspension additives was prepared as a suspension of AgNPs in an aqueous phase via THF (tetrahydrofuran) method [6], which can be readily exchanged with water and is easily removed by evaporation. Ag powder was added to THF and the resulting solution was treated with sonication, followed by stirring at approximately 300 rpm until the THF completely evaporated (1–2 days). Following this, deionized (DI) water was added to replace THF. The resulting sample was then filtered through a polycarbonate membrane filter (50 nm isopore, Adventec). The size distribution was examined by HR-TEM (high resolution transmission electron microscopy, JEM-2010, JEOL) and an image analysis software (ImageJ).

2.3. SPR and AFM analysis using mixed SAM (self-assembled monolayer) on gold chip

When the lipid vesicle was exposed to a flat solid substrate, lipid bilayer easily formed due to fusion as well as rupture of the vesicle [15,16]. To overcome this drawback, gold surface for SPR analysis was treated with mixed SAM, namely hydrophobic and hydrophilic moieties with the same carbon length, which are prepared with 1-dodecanethiol (DDT, Sigma) and 11-mercaptoundecanoic acid (MUA, Sigma), respectively. Purified gold chip was treated in 10 mM DDT and MUA ethanolic solution for 4 h. Mixed SAM modified gold chip was rinsed with a copious amount of ethanol and DI water, and finally dried using nitrogen gas. The DPPC vesicle rupture was investigated by time-resolved SPR (SPR lab, K-mac) and AFM (XE-100, PSIA).

3. Results and discussion

3.1. Preparation of DPPC vesicle and mixed SAM

DPPC vesicles were easily attached on the hydrophilic surface from the vesicular solution of DPPC kept above the gel transition temperature. The size distribution of vesicles prepared showed 90% diameter of 100–200 nm and this was confirmed by TEM analysis. Although phopholipid vesicle is generally maintained as spherical form in the aqueous solution, lipid bilayer easily formed when it was loaded to the solid substrate. When vesicular solution is dropped on the substrate, their hydrophilic head group attached the vesicles on the substrate and some spontaneously adsorbed larger size vesicle rupture driven by the substrate-induced deformation [15]. This fusion and rupture of neighboring smaller vesicles may be another probable cause of the formation of the bilayer. Subsequently, propagation of such cascaded rupture events across several neighboring vesicles led to the formation of extended lipid bilayer on the solid substrate.

Therefore, the hydrophilicity of the gold surface was adjusted by treatment of mixed SAM, which could be providing a partial hydrophilicity and hydrophobicity on the gold substrate. This was done because localized hydrophilic gold surface may prevent the formation of vesicle fusion and eventual rupture of neighboring vesicles. The ratio of hydrophilic surface was adjusted with molar ratio of hydrophilic MUA to hydrophobic DDT, namely 5:5 and 1:9.

After DPPC vesicular solution was dropped on the mixed SAM treated gold surface, surface morphology was analyzed by AFM. As shown in Fig. 1, DPPC vesicles loaded on the gold surface showed not ruptured bilayer but partially fused vesicles. DPPC vesicles had a neutral charge and showed very a small zeta potential of −1.43 mV which was measured by electrophoretic light scattering (ELS-Z, Otsuka Electronics). Because the small and almost neutral charge of vesicle surface reduced the electrostatic repulsion between DPPC vesicles, the fusion of neighboring vesicles was induced and a large unilamellar structure was formed. More DPPC vesicles were loaded on the mixed SAM treated gold substrate than bare substrate. Its height on the mixed SAM and bare gold was 400 and 100–200 nm, respectively (cross-sectional lines in Fig. 1). It was noted that fusion of DPPC vesicles on the bare gold formed more easily compared to loading on the mixed SAM. While its width on the bare gold was approximately 3–5 μm, one on the mixed SAM was 2 μm. Therefore, control of hydrophilicity by treatment of mixed SAM on the gold surface was effective to prevent the propagation of rupture events across several neighboring vesicles on the solid substrate.

Fig. 2 shows angle-resolved SPR data obtained as lipid vesicles were loaded on three different gold surfaces. Prior to the 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 51.5° and that of the mixed SAM treated gold chip was less due to the change of both thickness and dielectric constant of the SAM loaded on the gold surface. When DPPC vesicles were injected into the SPR cell, angle-resolved SPR response was changed with the type of substrate. SPR angle, at which the intensity is minimal at the curves, shifts after the vesicles were loaded on the bare and mixed SAM treated gold surfaces. Shift angles for bare and 1:9 and 5:5 mixed SAM treated surface was 0.4°, 0.8°, and 1.2°, respectively. The enhancement effect of SPR signal was obtained through treatment of mixed SAM on the gold chip. It might be due to the large loading of DPPC vesicles without rupturing and a full fusion with neighboring vesicles, as shown in AFM analysis.

Time-resolved SPR data (Fig. 3) was obtained for bare and mixed SAM after the injection of DPPC vesicles as a prior step to the attack of AgNPs on the vesicle. The reflectivity was measured in real time as we changed conditions on the gold surface (a zone in Fig. 3). At 400 s, DPPC vesicles were injected into the cell (b zone in Fig. 3). The reflectance of the bare gold chip was found to have drastically increased from 26° to 33° (40° for 5:5 and 44° for mixed SAM). A slight changed in reflectivity was observed when the gold chip was washed with PBS buffer to remove any weakly