Environmental Toxicology and Pharmacology 47 (2016) 108–118

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Influence of silver and titanium dioxide nanoparticles on *in vitro* blood-brain barrier permeability

I-Chieh Chen, I-Lun Hsiao, Ho-Chen Lin, Chien-Hou Wu, Chun-Yu Chuang*, Yuh-Jeen Huang*

Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, 101, Section 2, Kuang-Fu Road, Hsinchu, 30013, Taiwan

ARTICLE INFO

Article history:
Received 29 April 2016
Received in revised form 31 August 2016
Accepted 14 September 2016
Available online 15 September 2016

Keywords:
Silver nanoparticles
Titanium dioxide nanoparticles
In vitro blood-brain barrier
Permeability
Silver ions

ABSTRACT

An *in vitro* blood-brain barrier (BBB) model being composed of co-culture with endothelial (bEnd.3) and astrocyte-like (ALT) cells was established to evaluate the toxicity and permeability of Ag nanoparticles (AgNPs; 8 nm) and TiO_2 nanoparticles (TiO_2NPs; 6 nm and 35 nm) in normal and inflammatory central nervous system. Lipopolysaccharide (LPS) was pre-treated to simulate the inflammatory responses. Both AgNPs and Ag ions can decrease transendothelial electrical resistance (TEER) value, and cause discontinuous tight junction proteins (claudin-5 and zonula occludens-1) of BBB. However, only the Ag ions induced inflammatory cytokines to release, and had less cell-to-cell permeability than AgNPs, which indicated that the toxicity of AgNPs was distinct from Ag ions. LPS itself disrupted BBB, while co-treatment with AgNPs and LPS dramatically enhanced the disruption and permeability coefficient. On the other hand, TiO_2NPs exposure increased BBB penetration by size, and disrupted tight junction proteins without size dependence, and many of TiO_2NPs accumulated in the endothelial cells were observed. This study provided the new insight of toxic potency of AgNPs and TiO_2NPs in BBB.

1. Introduction

Silver and titanium dioxide nanoparticles (AgNPs and TiO_2NPs) are the two most commonly engineered nanoparticles (NPs). AgNPs are regarded as great antibacterial agents, and are used extensively in textiles, masks, medicines, food packages or other spray products (Rai et al., 2009). TiO_2NPs have excellent photocatalytic activity and surface super-hydrophobic capacity so that can apply in paints, ceramic tiles, cosmetics, even food additives (Gupta and Tripathi, 2011). Along with the widely application in nanomaterials, there is numerous researchers have emphasized that these engineered NPs are involved in potential adverse impacts to the environment, health and safety (Maynard, 2014; Song et al., 2009).

In vivo studies have demonstrated that AgNPs and TiO_2NPs can translocate into central nervous system (CNS), and are detected in brain by main routes of introducing NPs into the body *e.g.*, oral (Ag (Loeschner et al., 2011; Park et al., 2010); TiO_2 (Ze et al., 2014b)), inhalation administration (Ag (Takenaka et al., 2001)), intranasally instillation (Ag (Genter et al., 2012)), subcutaneous (Ag (Tang et al., 2009)), intragastric (TiO_2 (Hu et al., 2010)) administration. Moreover, AgNPs and TiO_2NPs have been detected in various brain regions such as cerebral cortex, hippocampus, and cerebellum, and can stay in a long period of time (Skalska et al., 2015; Wang et al., 2008), which affects memory and learning behavior (Mohammadipour et al., 2014; Ze et al., 2014a). The accumulation level and half-life of NPs in CNS are size-dependent (Lee et al., 2013; Park et al., 2010; Sharma et al., 2013). Therefore, pre-evaluating the potential hazards of AgNPs and TiO_2NPs on CNS is necessary.

Pathways of NPs entering the brain are usually divided in two ways: via olfactory nerve or blood-brain barrier (BBB) penetration. BBB is an important structure between the CNS and circulatory system in the brain, which are composed of endothelial cells, basal lamina, astrocyte end feet and pericytes. Endothelial cells, the core anatomical element of BBB, have continuous intercellular tight junctions to reduce transport of substances by the paracellular pathway. Tight junction proteins are classified into two different protein groups: transmembrane proteins (*e.g.*, occludin,

Abbreviations: Ag^+, silver ions; BBB, blood-brain barrier; TiO_2NPs, titanium dioxide nanoparticles; AgNPs, silver nanoparticles; LPS, lipopolysaccharide; ROS, reactive oxygen species; TEER, transendothelial electrical resistance; CNS, central nervous system; ZO, zonula occludens; GLUT-1, glucose transporter; ABC, ATP-binding cassette; FI, fluorescence intensity; DCFH-DA 2′, 7′-dichlorofluorescin diacetate; BSA, bovine serum albumin; DLS, dynamic laser scattering; TEM, transmission electron microscopy; RT, room temperature.

* Corresponding authors.
E-mail addresses: ejchen00@gmail.com (I.-C. Chen), ilunshiou@gmail.com (I.-L. Hsiao), photorice2@gmail.com (H.-C. Lin), chwu@mx.nthu.edu.tw (C.-H. Wu), cychuang@mx.nthu.edu.tw (C.-Y. Chuang), yjhuang@mx.nthu.edu.tw (Y.-J. Huang).

http://dx.doi.org/10.1016/j.etap.2016.09.009
1382-6689/

claudin, junctional adhesion molecules) and peripheral proteins (*e.g.*, zonula occludens (ZO), cingulin). Both types of proteins seal the intercellular cleft, thus preventing free ion diffusion (Shin et al., 2006). Astrocyte end feet almost completely cover the surface of endothelium, and have many specific transport systems, such as ATP-binding cassette (ABC) transporters. The interaction between endothelial cells and astrocytes helps to modulate BBB function (Abbott et al., 2006). The main functions of the BBB are to serve as a guard to select solute, act as carrier to transport nutrients to the brain and remove metabolites from the brain. Even though *in vivo* studies have indicated that silver can be detected in the brain, arguments about whether silver really penetrates BBB and reaches neuron cells, or just accumulates in the endothelial cells is still unknown (Lin et al., 2015). Thus, establishing an *in vitro* BBB model to pre-screen the potential ability and mechanism of NPs to penetrate BBB is important.

One report has indicated that TiO_2NPs can pass through *in vitro* BBB (containing rat primary endothelial and glial cells), and cause BBB dysfunction (Brun et al., 2012). Similarly, AgNPs induce monolayer BBB inflammation by increasing oxidative stress and barrier permeability due to their small size, special modification and high exposure (Cramer, 2014; Trickler et al., 2010). However, these studies only used monolayer endothelial cells or co-culture of endothelial cells and astrocytes in insert and well bottom of Transwell® respectively, which is insufficient to mimic real BBB function and tightness. Moreover, these studies used substitute markers to evaluate the permeability of BBB, and neither quantify how much NPs penetrate BBB nor the amount of NP translocation from endothelial cells to astrocytes. In addition, it is still not clear whether the Ag^+ released from AgNPs or both (AgNPs and Ag^+) contributed the cytotoxicity in BBB. Eventually, neuro-inflammation occurs following almost all CNS pathologies, it is also crucial to understand if more NPs penetrate BBB under inflammatory state.

In this study, endothelial cells and astrocyte-like cells were cultured on different sides of a Transwell insert to mimic the BBB in the brain for evaluating potential hazard of AgNPs and TiO_2NPs on CNS under the pre-inflammatory condition resulting in lipopolysaccharide (LPS). This BBB model has been developed for drug delivery studies (Li et al., 2010), and is useful to observe the influence of elements on the neurovascular unit and the effect of nanotoxicity with cell-to-cell interaction (Naik and Cucullo, 2012; Wilhelm and Krizbai, 2014). After NPs exposure in endothelial cells, the integrity and permeability of BBB was evaluated by detecting the transendothelial electrical resistance (TEER) level, NP concentration in medium and cells, and tight junction protein expression ZO-1 and claudin-5. The cytotoxic effect of NPs as well as ions in BBB model was examined by cell viability, uptake potential, intracellular reactive oxygen species (ROS), and 23 kinds of cytokine secretion.

2. Materials and methods

2.1. NPs and silver ions

AgNPs were supplied by Gold NanoTech, Taipei, Taiwan, were produced by physical manufacturing, and contained no surface modifiers or stabilizers (Hsiao et al., 2015). A silver nitrate ($AgNO_3$) (Sigma-Aldrich, St. Louis) was used as a source of Ag^+ ions. Small- and large-sized TiO_2NPs (TiO_2NPs-S and TiO_2NPs-L) (ST-01, ST-21) (100% anatase, Ishihara Corporation, Japan) has been modified with alkaline solution to increase dispersity (Wu et al., 2016). Procedures were stated in Supporting information. Before characterizing NPs in water or cell medium, stock solutions of NPs were first ultrasonicated at 400W for 10 min (DC400H, DELTA new instrument Ltd., Taiwan) at 25 °C to achieve optimal dispersion. The NPs were characterized for shape/diameter by transmission electron microscopy (TEM, TECNAI 20, Philips, USA), crystal structure by X-ray diffraction analysis (XRD), concentration by inductively coupled plasma optical emission spectrometry (ICP-OES, Agilent 725, USA), and hydrodynamic diameter/zeta potential by Zetasizer Nano ZS (Malvern Instruments Inc., UK).

2.2. Cell culture

Murine brain astrocyte-like cells (ALT, BCRC-60581) were provided by Professor Chi-Shiun Chiang (Cancer Gene Therapy Laboratory, National Tsing Hua University, Hsin-Chu, Taiwan), sourced from *Mus musculus* mouse brain astrocytes with large-T antigen plasmid. Immortalized mouse cerebral endothelial cells (bEnd.3, BCRC-60515) were purchased from the Bioresource Collection and Research Center (Hsin-Chu, Taiwan). Both of the cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin mixture, and were cultivated in T75 flasks at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

2.3. Establishment of in vitro BBB model

The description of the experimental procedure for building up the *in vitro* BBB model according to (Li et al., 2010). First of all, the bottom side of the Transwell filters (0.4 μm pore sizes, PET, 6 or 12 well; Corning, USA) were coated with collagen type I (8 μg/cm^2 in 0.02 N acetic acid) for 1 h at room temperature (RT) to help cell attachment. Then, the filters were washed with 1 × phosphate-buffered saline (PBS), and ALT cells were seeded onto the back of the Transwell filters at a density of 2.44×10^4 cells/cm^2. After 3 h incubation, bEnd.3 cells were seeded onto the top side of the filters at a density of 10^5 cells/cm^2 and co-cultured with ALT cells for 4 days.

2.4. Exposure condition and dosages of NPs

After 4 days, both chambers were pre-treated w/or w/o LPS (2 μg/mL) from *Escherichia coli* 055:B5 (Sigma-Aldrich, St. Louis) in serum-free DMEM medium for 6 h. Then, the same volume of NPs or Ag^+ suspensions as pre-treatment were added to the top chamber of Transwell to achieve final concentration of LPS (1 μg/mL), TiO_2NPs (100 ppm), AgNPs (4 ppm) and Ag^+ (1.8 ppm) in DMEM/10%FBS medium for 24 h incubation.

2.5. Cell viability

AlamarBlue® (AbD Serotec, Kidlington, UK) as a cell viability indicator was used in monoculture testing. Briefly, after 24 h NP exposure, the alamarBlue® reagent was mixed 1:10 to DMEM/10%FBS followed by a 2 h incubation at 37 °C. Results of cell viability were determined by fluorescence at excitation wavelengths of 530 nm with emission at 590 nm. Cell viability was calculated using the expression (fluorescence intensity (FI) of test agent)/(FI of untreated control) × 100. Cell viability in BBB model was evaluated by flow cytometry (FACSCanto II, BD Biosciences, USA) with 10,000 cells collected, using a propidium iodide (PI) reagent. After NPs exposure for 24 h, the solution of both top and bottom chambers was aspirated to another plate for cytokines analysis, and then the cells were collected for PI staining (40 μg/mL). The dead cells were detected by flow cytometry. Cell viability was calculated using 100-(percentage of dead cell)%.

2.6. Cell uptake potential

After LPS and NPs treatment, the cells were collected and analyzed by flow cytometry. Potential cell uptake of NPs by flow