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Research paper

Influence of gastrointestinal environment on free radical generation of silver nanoparticles and implications for their cytotoxicity

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ABSTRACT

With the increasing number of applications of silver nanoparticles (Ag NPs) in consumer products, including food contact applications, it is important to understand potential health effects of ingestion of Ag NPs. The biosafety analysis of Ag NPs in various mammalian cells has been widely studied; however, the influence of the gastrointestinal environment on the physicochemical properties and toxicity of Ag NPs has not been fully addressed. In the present study, we investigated the impact of simulated human gastrointestinal tract (GIT) fluids on the physicochemical properties of Ag NPs and their impact on the *in vitro* cytotoxicity of Ag NPs in intestinal epithelial (Caco-2) cells. Polyvinylpyrrolidone (PVP)-coated Ag NPs of different sizes (30, 50, 100 nm) were incubated in three GIT fluids that differed in composition and pH (1.6, 5.0, 6.5) and were designed to mimic human gastric fluid in a fasted state (FaSSGF), human intestinal fluid in a fasted state (FaSSIF), and human intestinal fluid in a fed state (FeSSIF). Time-dependent decreases in UV–Vis absorption and increases in dynamic light scattering (DLS) were observed during the three-hour incubation of Ag NPs in GIT fluids. The results of transmission electron microscopy (TEM) coupled with energy dispersive X-ray spectroscopy (EDS) suggested that the Ag NPs aggregated and precipitated as AgCl salts in the presence of FaSSGF and FeSSIF. In the presence of FaSSGF and hydrogen peroxide (H_2O_2), a particle size- and concentration-dependent generation of hydroxyl radicals (·OH) was detected by electron spin resonance (ESR) spectroscopy. When compared to the effects observed with the incubation of Ag NPs in H_2O, incubation of Ag NPs in FaSSGF showed decreased cellular uptake in Caco-2 cells. In contrast, the incubation of Ag NPs in FeSSIF demonstrated increased cellular uptake and cytotoxicity. The results of this study demonstrate that GIT fluids have a significant influence on the physicochemical properties and cytotoxicity of Ag NPs and they provide valuable information that may be used in the safety evaluation of Ag NPs for consumer products.

1. Introduction

Nanomaterials are widely used in the food industry for their attractive physicochemical properties and for improving the taste and texture of foods, increasing nutrient bioavailability, and prolonging shelf life (He and Hwang, 2016; Rashidi and Khosravi-Darani, 2011). Silver nanoparticles (Ag NPs) have garnered widespread interest because of their antimicrobial properties (Benn and Westerhoff, 2008; Luoma, 2008). A primary route of exposure for Ag NPs in mammals is *via* ingestion, either intentional or inadvertent, with subsequent absorption of these particles *via* cells within the gastrointestinal tract (GIT) (Bergin et al., 2016; Boudreau et al., 2016). Migration of Ag from food containers into different food simulants have been reported and both Ag NP and Ag ion were detected in different food simulants (Addo Ntim et al., 2015; Cushen et al., 2014; Echegoyen and Nerín, 2013). To understand the potential effects associated with oral exposure to Ag NPs, it is important to understand how the fundamental physical and chemical processes affect Ag NPs within the GIT in the presence and absence of digestive materials.

Absorption is what carries Ag NPs into the intestinal membrane and

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determines their toxicological effects *in vivo*. The physicochemical properties including size, surface charge, and aggregation can greatly affect the interaction of nanoparticles with cells lining the intestinal membrane (Misra et al., 2012). The *in vitro* toxicity of Ag NPs with different physicochemical properties has been evaluated in a wide range of studies (Chen et al., 2016; Chen et al., 2014; Foldbjerg et al., 2009; Jiang et al., 2015; Jiang et al., 2017; Zhang et al., 2015). However, these studies used commercially available nanoparticles suspended, primarily, in as-prepared or cell-specific mediums. Gastrointestinal fluids are complex mixtures, and the components of these mixtures vary by sites within the GIT (Mudie et al., 2010). The physicochemical properties of Ag NPs may be modified during their passage through the GIT system; therefore, it is essential to characterize the extent of modifications that occur to the physicochemical properties of ingested Ag NPs within the gastrointestinal fluid to better interpret the toxicity data from *in vitro* and *in vivo* studies.

The main digestive organs in humans include the stomach, where ingested food material is digested and mixed with gastric fluids, and intestine, in small intestine, chyme is further digested by digestive enzymes and absorbed by intestinal cells. In large intestine, only small portion of ingested materials will be further digested by the gut microbiota and absorbed in colon. Several recent studies have reported on the interactions of food matrix and ingested nanomaterials and proposed an integrated method to assess their biokinetics and cytotoxicity (DeLoid et al., 2017; Li et al., 2017; McClements et al., 2016; McClements et al., 2017). The pH of the GIT fluids has a strong influence on the dissolution and absorption of ionizable particles, and the pH is influenced by many variables, including the prandial condition (Mudie et al., 2010). The gastric fluid is composed of saliva, gastric secretions, dietary food and liquids. The stomach secretes hydrogen ions in the form of hydrochloric acid, and the concentration of hydrogen ions is reflected in the pH of the gastric fluid, which is approximately 1–2 under fasted conditions and contains small amount of bile salts, primarily as glycochenodeoxycholate and glycocholate (Vertzoni et al., 2008). Shortly after a meal, the pH of the gastric fluids tends to rise (pH ~3–7) but then decreases back to fasted state levels within 1–4 h (Kalantzi et al., 2006). The composition of the intestinal fluid is made up of the chyme from the stomach, as well as bile salts from the liver, bicarbonate from the pancreas to neutralize the gastric fluids, and mineral ions, in particular sodium and chloride (Mudie et al., 2010). Under fasted conditions, the intestinal fluid is at neutral pH (~6.5) and contains similar levels of bile salts as the stomach fluid. The composition of the intestinal fluid in the fed state can vary greatly from the composition in the fasted state, because the presence of food triggers many secretions into the intestine. The pH of the intestinal fluid tends to be lower in the fed state (pH ~5), but the solubilizing capacity of the intestinal fluids is enhanced due to the increased secretions of bile salts and pancreatic secretions. The compositions of the gastrointestinal fluid and food matrix can greatly influence the bioavailability and physicochemical properties of ingested engineered nanoparticles (DeLoid et al., 2017; McClements et al., 2017). To estimate the physicochemical and biological changes that occur to Ag NPs in the stomach and intestine of humans, we used simulated human gastric and intestinal fluids.

Several studies have examined the transformation of Ag NPs in gastric fluids and have shown that gastric fluids can modify the aggregation state, zeta potential and morphology of Ag NPs (Axson et al., 2015; Rogers et al., 2012; Walczak et al., 2012). The extent to which Ag NPs are modified within the various regions of the GIT and how these modifications might influence Ag NP bioavailability and cytotoxicity in intestinal cells is a topic for further investigation. In this paper, we report the findings of a study that examined the impact of simulated human gastric and intestinal fluids, including FaSSGF, FaSSIF and FeSSIF, on the physicochemical properties of Ag NPs when compared to distilled water, using UV–Vis, DLS, TEM, and ESR applications. In addition, the cytotoxicity and cellular uptake of Ag NPs were evaluated in Caco-2 cells following incubation of Ag NPs in simulated human gastric and intestinal fluids.

2. Materials and methods

2.1. Reagents and chemicals

Polyvinylpyrrolidone (PVP)-coated Bio-Pure Ag NPs (1 mg/mL in H_2O) of different sizes (30, 50, 100 nm) were purchased from nanoComposix (San Diego, USA). The three simulated-human GIT fluids (FaSSGF, FaSSIF, FeSSIF) were purchased from Biorelevent.com (London, United Kingdom). The composition information of the GIT fluids was provided in the Supporting Information (Tables S3–S5). Cell culture medium EMEM was purchased from ATCC (Manassas, VA, USA). Penicillin-Streptomycin (10,000 U/mL), Fetal bovine serum (FBS), Phosphate buffered saline (PBS), Trypsin-EDTA (0.5%), Alamar Blue™ and Pierce™ LDH Cytotoxicity Assay Kit were purchased from ThermoFisher Scientific (Waltham, MA, USA). 5-tertbutoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO) was purchased from Donjindo (Tokyo, Japan). Cell counting slides were purchased from BIO-RAD (California, USA). Mili-Q water (18 MΩ·cm) was used for preparation of all solutions.

2.2. Physicochemical characterization of Ag NPs

The size and morphology of Ag NPs of different sizes (30, 50, and 100 nm) were characterized using JEM-1400 TEM (JEOL, Tokyo, Japan). The hydrodynamic diameter and zeta potential of Ag NPs were measured using Zetasizer Nano ZS90 (Malvern, Worcestershire, UK).

2.3. Ag NP dissolution in GIT fluids

The Ag NP dissolution in GIT fluids was characterized by Cary 300 UV–Vis spectroscopy (Agilent, Santa Clara, CA, USA) and DLS. Before measurement, Ag NPs were dispersed in 1 mL of ddH_2O or one of the three simulated human GIT fluids (FaSSGF, FaSSIF, and FeSSIF) to a concentration of 150 μM. The 1 mL solution was transferred to a quartz cuvette and the absorption spectrum was measured by UV–Vis, and the hydrodynamic size was measured by DLS at predetermined time intervals. The UV–Vis absorption of Ag NPs in GIT fluids was normalized by subtracting the absorption of the GIT fluids.

2.4. Ag species analyzed by TEM coupled with elemental mapping

The Ag NP (1 mM) were incubated in ddH_2O or each of the simulated human GIT fluids for 3 h, and an aliquot of 6 μL of each suspension was deposited on individual carbon TEM grids. The TEM grids were rinsed with distilled water to remove loosely attached particles and dried at room temperature. A JEOL JEM 1400 TEM equipped with an X-Max 80 EDS spectrometer (Oxford, MA, USA) was used to collect images and conduct elemental mapping.

2.5. ESR measurement of free radical generation

ESR measurements were carried out using a Bruker EMX ESR spectrometer (Billerica, MA) at room temperature. Samples were prepared by mixing Ag NPs with H_2O_2 in the three different GIT mimic fluids, and a 50 μL aliquot of control or sample solution were injected into a glass capillary tube with an internal diameter of 1 mm and sealed. The capillary tubes were inserted in the ESR cavity and hydroxyl radical generation by a spin trap BMPO. The ESR spectra were recorded at selected times. Other settings for the ESR measurement were: 1 G field modulation, 100 G scan range, and 20 mW microwave power.