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"Green" Synthesized and Coated Nanosilver Alters the Membrane Permeability of Barrier (Intestinal, Brain Endothelial) Cells and Stimulates Oxidative Stress Pathways in Neurons

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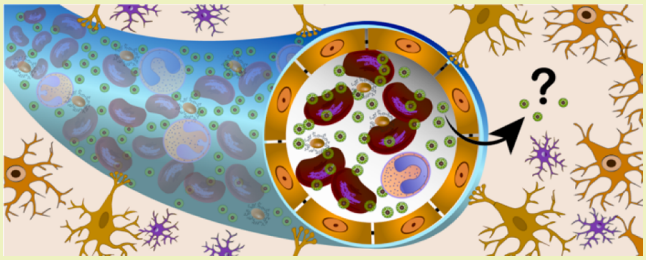
ABSTRACT: Nanosilver's (nanoAg) use in medical applications and consumer products is increasing. Because of this, its "green" synthesis and surface modification with beneficial coatings are desirable. Given nanoAg's potential exposure routes (e.g., dermal, intestinal, pulmonary), questions on its potential to move through these "port of entry" barriers and enter the body's circulatory system remain unanswered. In view of nanoAg's free radical activity and the brain's sensitivity to oxidative stress damage, the possibility that nanoAg particles can move from the systemic circulation, transport through the blood-brain barrier (BBB), and pose a neurotoxic threat is also a legitimate concern. Because of these issues, this study addresses an initial event of barrier transport, that is, if "green" synthesized nanoAg, coated with green tea polyphenols (GT) or glutathione (GSH), can alter the permeability of human intestinal epithelial (Caco-2) or rat brain endothelial (RBEC4) barrier cells. Additionally, it asks if such "green" synthesized nanoAg modifies its toxicity to oxidative stress-sensitive cultured neurons (N27). Physicochemical (PC) characterization of conventionally synthesized nanoAg and "green" synthesized nanoAg-GT or nanoAg-GSH indicated that all samples aggregated (>500−2500 nm) when suspended in cell culture exposure media. NanoAg-GSH showed the least electronegative zeta potential and largest aggregate size in both Caco-2 and RBEC4 exposure media, relative to conventional nanoAg. Transcellular resistance measures indicated that within 15 min of exposure to 6.5 ppm, both conventional and nanoAg-GSH altered the permeability of intestinal Caco-2 monolayers, and all nanoAg treatments altered the permeability of RBEC4 brain endothelial cells. To examine if a differential toxicity existed in the response of oxidative stress-sensitive neurons, a noncytotoxic (1.0 ppm) concentration of each nanoAg material was exposed (18 h) to rat dopaminergic neurons (N27), transfected with a NF$\kappa\beta$ reporter gene. Results indicated that all nanoAg samples significantly stimulated this oxidative stress pathway in the N27 neuron. Together, these data suggest that both conventional and "green" synthesized coated nanoAg alter the permeability of barrier cell membranes and activate oxidative stress pathways in target neurons, equivocally.



KEYWORDS: *"Green" chemistry, Biological barriers, Nanosilver, Surface coating, Surface modification, TER, Oxidative stress, Nanoparticle permeability*

INTRODUCTION

Because of its potent antibacterial, antifungal, antiviral and anti-inflammatory properties, nanosilver (nanoAg) is used in a wide array of medical products (e.g., antiseptics, fabric dressings for external wounds, burns and ulcers, embedded medical devices) and consumer products (e.g., domestic cleaning agents, water disinfectants, disinfectant sprays, odor-resistant textiles, baby bottle material, electronics, household appliances [washing machines], food packaging, cosmetics).[1−5] Potential routes of entry for nanoAg include oral, dermal, and inhalation. The "green" synthesis of nanoAg offers many advantages over conventional procedures, and surface modifying such particles with benign or beneficial biological coatings is desirable, given nanoAg's increasing use in biomedical applications and consumer products.

In view of nanoAg's potential exposure routes (e.g., dermal, intestinal, pulmonary), it is important to determine if nanoAg can alter the permeability of these "port of entry" barriers. More importantly, given its free radical activity[6,7] and the brain's extreme sensitivity to oxidative stress,[8,9] the possibility that nanoAg particles can move from the systemic circulation through the blood-brain barrier (BBB) and pose a neurotoxic threat to oxidative stress-sensitive neurons is a legitimate concern. This study asks if conventional or "green" synthesized coated nanoAg could alter the permeability of human lower

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intestinal epithelial (Caco-2) and rodent BBB endothelial (RBEC4) barrier cells. These nanoAg materials were also tested for their potential to activate oxidative stress pathways in vulnerable dopaminergic neurons (N27). In view of the relationship between the physicochemistry of nanomaterials and biological activation,[10−16] the relevance of their physical properties (e.g., aggregate size, zeta potential) on both permeability and neurotoxicity was evaluated under exposure conditions.

MATERIALS AND METHODS

Test Materials. Conventionally synthesized nanoAg particles (nanoAg) were made by mixing silver nitrate ($AgNO_3$) with sodium borohydride NaBH(4), a well-known reducing and capping agent.[17−19] NanoAg was also "green" synthesized and coated using the beneficial materials glutathione (GHS) or green tea (GT) as reducing and coating agents. NanoAg-GSH was made from $AgNO_3$ using a previously published assay.[20] To make nanoAg-GT, a modified procedure[21] was used that mixed boiled and filtered Chinese green tea (*Camellia sinensis*) with 0.1 N 99% $AgNO_3$ (Aldrich, Inc.). Acting as a reducing agent, the GT reduced the suspension and subsequently coated the nanosize Ag spheres to form nanoAg-GT. Samples were stored in distilled water until use.

Physical Characterization of Synthesized Materials. Transmission electron microscopy (TEM) of nanoAg-GT was done using a Phillips CM 20 TEM microscope. For this, aqueous suspensions of the material were loaded onto a carbon-coated copper grid and allowed to dry at room temperature before photography. The hydrodynamic size and surface charge (zeta potential) of conventional and "green" synthesized nanoAg samples were described under physiological exposure conditions (i.e., exposure media, time points, effective concentration, temperature) to parallel the biological response. Particle size distributions (PSD) were determined by dynamic light scattering (DLS) as a function of time and reported as volume percentage. The apparent zeta potential of each material was measured using the Smoluchowski equation to correlate particle mobility to zeta potential. Both the zeta potential and the PSD measured 10−20 ppm (μg/mL) suspensions of each material immediately after a 3 min sonication in cell culture media. Both the size and zeta potential were measured using a Zeta Sizer Nano ZS (Malvern, Inc., Southborough, MA).

Cell Models. The Caco-2 cell line was derived from a human colorectal adenocarcinoma and is commercially available (ATCC Manassas, VA). These cell lines form monolayers of polarized (apical, basal) epithelia held together by tight junctions. The Caco-2 cell line contains various drug transport systems and is an accepted model to study the mechanism of oral and intestinal drug permeability.[10,22−27] Caco-2 cells were grown and maintained in Dulbecco's modified Eagle medium (DMEM), with 10% fetal calf serum (FCS), 1% L-glutamine, and 5% penicillin/streptomycin (P/S). RBEC4 cells (a gift from M. Aschner, Vanderbilt University, TN) are rat brain vascular endothelial cells that have been immortalized (transfected) with an SV40 virus. Although RBEC4 cells express lower levels of the BBB-specific receptors, enzymes, intercellular adhesion molecules (e.g., ICAM-1), and transporter systems;[28−32] they have been proven adequately responsive to study its permeability and transport.[28,31,33,34] In these experiments, cells were grown on Type I rat tail collagen (Bectin Dickerson Bioscience, Bedford, MA) coated flasks in DMEM media supplemented with 10% FCS and 5% P/S. N27 cells (a gift from H.S. Hong, NIEHS, RTP, NC) are immortalized rat dopaminergic neurons dissociated from the mesencephaic cortex.[35,36] The neurons were grown and maintained in RPMI-1640 media supplemented with 10% FCS and 1% P/S. All cells were grown under standard incubation conditions. Stock suspensions were ultrasonicated using a Daiger Ultrasonic processor (Model GE 750 W) at a reduced amplitude of 20% for 1−2 min before preparing 10× exposure concentrations immediately before use.

Transcellular Resistance (TER). TER measures the electrical resistance of cell monolayers and is considered an index of membrane permeability.[23,37] The barrier cells (Caco-2, RBEC4) were tested as confluent monolayers grown in Millicell 96-well inserts fitted with 0.4 μm, carbonate filters, and coated with rat fibronectin. TER electrical recordings were taken using a robotic REMS instrument (World Precision Instrument, Sarasota, FL). Mini electrodes were inserted into each well, and the ratios of ionic concentrations found in the inside versus the outside chambers were calculated. Only cell monolayers showing a stable baseline resistance >200−220 Ω cm^2 were used in the permeability studies. For this, each well was exposed to noncytotoxic concentrations (6.5 ppm) of the individual nanoAg material, and electrode recordings were taken over a 15 min exposure time to minimize artifactual changes. Changes in stable baseline resistance values indicated permeability changes in the monolayer. $T = 0$ values were taken of each well before exposure to a noncytotoxic concentration of nanoAg (6.5 ppm). TER units were averaged (n = 6 well/treatment), normalized to their media control ($T = 0$) at each time point, and the data graphed (mean ± SD) using Excel software.

Reporter Genes. Reporter genes (RG), associated with NF$\kappa\beta$ oxidative stress pathways were transfected into N27 neurons using previously described techniques.[38,39] When activated, the RG-transfected N27 neurons responded by emitting a detectable chemiluminescent signal. Cells were exposed to noncytotoxic concentrations (six wells/concentration) of each nanoAg for 18 h and then washed, trypsinized, and assayed for their chemiluminescence using a luciferase based detection assay.

Viability. Levels of intracellular ATP, an index of cell viability, were measured with the lucerifase-based chemiluminescence assay CellTiter-Glo purchased from Promega.

Spectrophotography and Statistics. Data from both chemiluminescent assays above were collected on a Lmax III 96/384 plate reader using SoftMax Pro 5 software (Molecular Devices, Sunnyvale, CA.). Data were normalized to control values and analyzed using a one-way ANOVA followed by Dunnett's multiple comparison test. The data were graphed (mean ± SD, $p < 0.05$) using Excel software.

RESULTS

Physicochemical (PC) Characterization and Biological Measures. The conventional synthesis of nanoAg uses NaBH(4) to reduce $AgNO_3$ and form stable suspensions of negatively charged spherical silver nanoparticles <20 nm diameter.[40] The size of "green" synthesized nanoAg-GSH[20] indicated a 5−10 nm distribution when measured in water. In the present study, TEM indicated a variable size distribution of 5−20 nm for nanoAg-GT also measured in water (Figure 1 and insert). For the purpose of biological comparisons, PC measures were taken in cell culture exposure media. The zeta potential of each nanoAg sample, measured in Caco-2 media (10−20 ppm), indicated a negative zeta potential of −13 mV for conventional nanoAg. GSH and GT coated nanoAg showed less negative potentials of of −6 and −10 mV, respectively, suggesting that coating minimized the electronegativity of the "green" particles relative to conventional nanoAg. When measured by DLS in this media, nanoAg-GSH showed the highest agregate size (~2500 nm) relative to conventional nanoAg with the lowest aggregate size (~150 nm) (Figure 2 A,B). TER resistance changes recorded in the confluent Caco-2 monolayer, indicated that all nanoAgs (6.5 ppm) altered the permeability of the Caco-2 epithelia barrier within a 15 min exposure time although nanoAg-GT's effects were not significant (Figure 2 C).

PC measures were also taken in RBEC4 exposure media (10−20 ppm). "Green" synthesized nanoAg-GSH again showed the least electronegative zeta potential (−7 mV) and highest aggregate size (~2500 nm) relative to the −12.5 mV

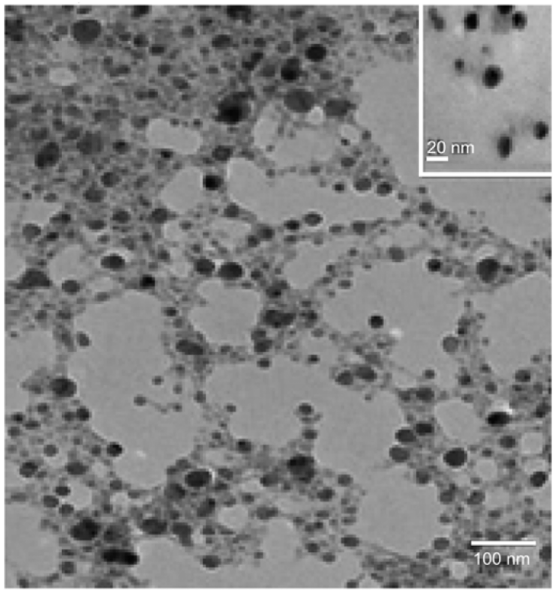

Figure 1. TEM image of silver nanoparticles coated with green tea (nanoAg-GT) revealed a population of particles with size ranges between 5 and 20 nm (insert). Photographs were taken using a Phillips CM 20 TEM microscope at an operating voltage of 200 kV. Magnification bars are shown on each micrograph.

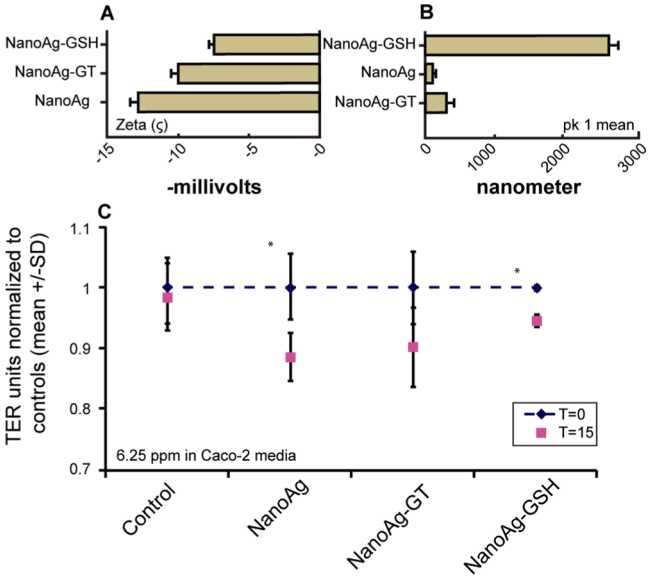

Figure 2. (A–C) PC measures of each material in Caco2-media. Zeta potentials (four averaged measures) indicated that GSH coated nanoAg showed the least negative zeta potential surface charge and highest aggregate size when measured by DLS. In contrast, conventionally synthesized nanoAg showed the most negative zeta potential and lowest aggregate size (A,B). TER data indicated that both conventional and "green" synthesized nanoAgs (6.5 ppm) significantly altered the membrane permeability of confluent Caco-2. TER units were averaged (n = 6 well/treatment), normalized to their media control (T = 0) at each time point, and graphed (mean ± SD) using Excel software. The cross hatch (#) indicates significance (p < 0.05).

zeta and ~150 nm size of conventional nanoAg (Figure 3 A,B). TER recordings indicated that each material significantly altered the membrane permeability within the 15 min exposure time. (Figure 3C).

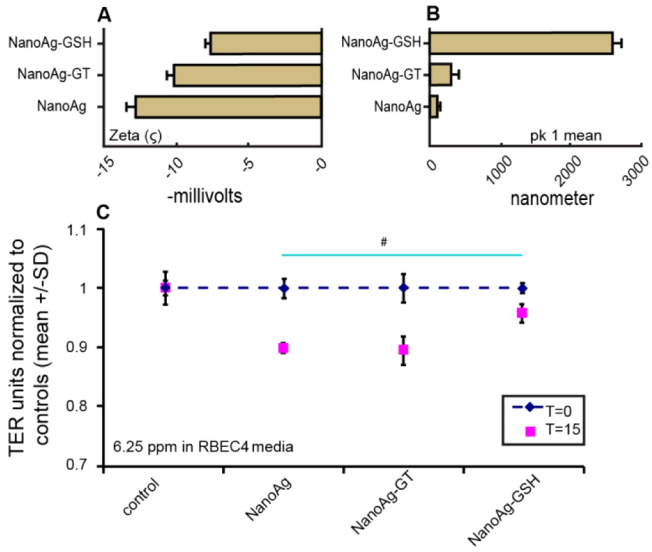

Figure 3. (A–C) PC measures of test particles RBEC4 media indicated that nanoAg-GSH again had the least negative zeta potential (A) and the highest aggregate size (B). Monolayer resistance, measured by TER indicated that all three materials signicantly altered the membrane permeability of the RBEC4 endothelial cells within 15 min (C). TER units were averaged (n = 6 well/treatment), normalized to their media control (T = 0) at each time point, and graphed (mean ± SD) using Excel software. The cross hatch (#) indicates significance (p < 0.05).

To determine if these test materials showed a differential toxicity to target neurons, N27 dopaminergic neurons, transfected with an NF$\kappa\beta$ RG, were exposed to noncytotoxic (data not shown) concentrations (1.0 ppm) of each nanoAg for 18 h and assayed for chemiluminescence emission (six wells/treatment). Results indicated that each nanoAg sample significantly stimulated NF$\kappa\beta$ mediated oxidative stress pathways, equivocally (Figure 4).

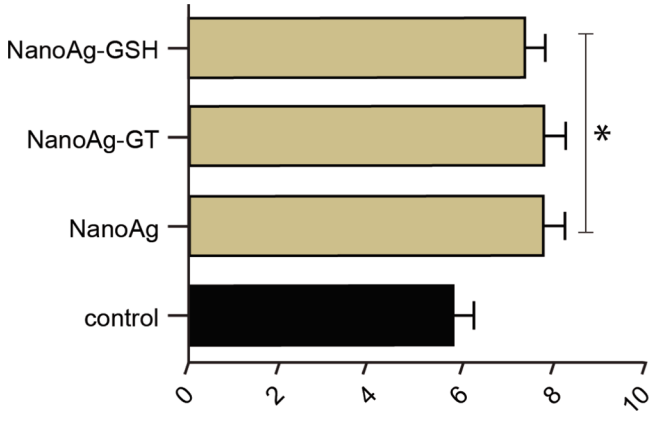

Figure 4. RBEC4 cells, transfected with a NF$\kappa\beta$ RG, were exposed to noncytotoxic (data not shown) concentrations (1.0 ppm) of each nanoAg for 18 h and spectrometrically measured for chemiluminescence as an indicator of RG activation. Each nanoAg was able to significantly stimulate the NF$\kappa\beta$ RG. Data (chemiluminescent units) are reported as standard deviation, and the asterisk is (*) used to indicate significance (p < 0.05).

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DISCUSSION

The "green" synthesis of nanomaterials has proven superior to conventional synthesis, assembly, and disposal methods in terms of time, costs, remediation, and overall environmental "friendliness".[41,42,42−51] Surface coating or "capping" is often done to limit the aggregation and oxidation of the nanomaterial and enhance its sustainability. However, it is important that these modified and relatively more sustainable materials are carefully evaluated for their efficacy and possible toxicity to the biological systems they may encounter during their production, use, and disposal.

The purpose of the present study was to determine if "green" synthesized and coated nanoAg differed from conventional nanoAg in their permeability of biological barriers or neurotoxicity. This is especially warranted in view of nanoAg's increasing presence in consumer and medicinal products. The biological materials (GSH, GT) used to coat the nanoAg are known for their antioxidant and anti-inflammatory properties and in the case of GT, antimicrobial properties. Our data show that both conventional and GSH coated nanoAg significantly altered the Caco-2 intestinal barrier cell's permeability, and all three altered the permeability of the more restrictive RBEC4 brain endothelial cell. It should be noted that TER recordings, although widely used as a permeability endpoint, only indicate that the cell membrane has been sufficiently altered in some way (e.g., junctional disruption, ion channels, transport systems, etc.) to allow measurable changes in the cellular membrane's ionic permeability.[23,37] TER does not indicate that the particles have physically moved through the cell, although this possibility is strengthened when the TER is followed by a lucifer yellow or sodium fluorescein tracer.[37,52] This technique was not possible in the current study because nanoAg is autorefractive,[53] resonating at the excitation wavelengths of the fluorescein isothiocyanate (FITC) tracer (∼490−520 nm) and would generate artifactual signals. However, confocal and TEM data from a companion study have demonstrated that PVP and citrate-coated nanoAg particles physically translocate (i.e., move through) RBEC4 cells within 30−60 min exposure,[54] and several animal studies have reported that injected or orally administered nanoAg particles transport from systemic circulation through the BBB and physically enter the brain.[55,56]

PC measures indicated that the GSH coating produced the least electronegative zeta potential and highest aggregate size relative to conventional nanoAg in both exposure media. Yet there was little difference in the permeability potential of the coated or the conventional nanoAg with its smaller size and higher electronegativity. Given these data, the particular PC endpoints (i.e., aggregate size and surface charge) taken under exposure conditions appear irrelevant to the permeability changes or neurotoxicity of the nanoAg materials. This stands in contrast to an extensive in vitro and in vivo literature base linking various PC characteristics (e.g., size, surface charge, shape, surface topography crystalline structure, etc.) with biological activation and toxicity.[10−16] One explanation for the failure of these PC endpoints to predict the biological activities of the coated nanoAgs could be their instability under physiological conditions. Although the coated particles remained stable in distilled water during their synthesis and storage,[20,21] the surface coatings could have dissociated from the nanoAg particle in the higher osmolarity of cell culture exposure media or could have been absorbed onto more highly charged components (e.g., globulins) found in the media. Another possibility might involve the coating's affinity for transport systems found in the plasma membranes of the Caco-2 epithelial[10,23−25,25−27] and RBEC4 endothelial barrier cells.[29−32] These systems are associated with the binding, uptake, and transcytosis of various exogenous nutrients and other materials. For example, both the BBB and RBEC4 endothelial cells have membranous transport systems that avidly take up GSH by Na+ dependent/independent mechanisms.[57] Although untested in the present study, this transport system could have facilitated nanoAg-GSH's permeability alteration of the RBEC4 cellular membrane.

Oxidative stress caused by the nanoAg exposure could also contribute to RBEC4's permeability. Various in vitro and in vivo studies indicate that oxidative stress alters BBB permeability[58−60] and more specifically that free radicals alter permeability in primary brain vascular[61] and RBEC4 endothelial cell.[62] More recently, it has been shown that PVP coated nanoAg stimulates multiple oxidative stress pathways in the RBEC4 barrier cell.[54] GSH is an important molecule that serves as an antioxidant and acts as a major determinant of the cell's redox microenvironment[63] and is depleted by oxidative stress in primary BBB endothelial cells.[59] It has also been reported that conventionally synthesized uncoated nanoAg produces inflammatory mediators in primary rat brain endothelial cells, which also increases their permeability.[64]

Green tea (*Camellia sinensis*) is known for its free radical scavenging, antimicrobial, and anticarcinogenic properties.[65−72] Tea flavonoids (catechins) have been shown to penetrate the brain barrier and protect against neuronal death in cellular and animal models of neurological diseases.[73,74] The mechanism underlying catechins' penetration of the BBB is unclear because there is no experimental evidence suggesting that the brain or RBEC4 endothelial cells house GT-sensitive receptors or transport systems. Yet, it has been experimentally demonstrated that the flavonoids and polyphenols of GT alter brain endothelial permeability[74,75] and transport.[75] One possible mechanism involves GT's ability to act directly on endothelial cell membranes[74,76] through hydrogen bonding to the surface of their membranous lipid bilayers.

In summary, these data indicate that both conventional and "green" coated nanoAg alter the membrane permeability of intestinal epithelial and BBB endothelial barrier cells and stimulate oxidative stress-mediated neurotoxicity. Although the physicochemical features of aggregate size and surface charge (measured under exposure conditions) appear irrelevant to these activities, explanations are proposed suggesting that the "green" coatings are unstable in physiological environments and/or that the coated particles could exploit transport systems inherent to the barrier cells themselves. Although such possibilities remain untested, these data underscore the need to refine the techniques and materials used in "green" synthesis and surface coating of nanoAg because modified particles represent promising delivery vehicles for therapeutic use.

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Notes

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