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# Slow release of ions from internalized silver nanoparticles modifies the epidermal growth factor signaling response

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ABSTRACT

Due to their distinctive physiochemical properties, including a robust antibacterial activity and plasmonic capability, hundreds of consumer and medical products contain colloidal silver nanoparticles (AgNPs). However, even at sub-toxic dosages, AgNPs are able to disrupt cell functionality, through a yet unknown mechanism. Moreover, internalized AgNPs have the potential to prolong this disruption, even after the removal of excess particles. In the present study, we evaluated the impact, mechanism of action, and continual effects of 50 nm AgNP exposure on epidermal growth factor (EGF) signal transduction within a human keratinocyte (HaCaT) cell line. After AgNP expose, EGF signaling was initially obstructed due to the dissolution of particles into silver ions. However, at longer durations, the internalized AgNPs increased EGF signaling activity. This latter behavior correlated to sustained HaCaT stress, believed to be maintained through the continual dissolution of internalized AgNPs. This study raises concerns that even after exposure ceases, the retained nanomaterials are capable of acting as a slow-release mechanism for metallic ions; continually stressing and modifying normal cellular functionality.



## 1. Introduction

Nano-sized colloidal silver inherently possesses a number of distinctive physicochemical properties that make it extremely advantageous for incorporation into consumer and medical applications. These properties comprise a robust antimicrobial ability, high electric and thermal conductivity, catalytic behavior, and a unique plasmonic spectral signature [1,2]. There are currently hundreds of consumer products available across a variety of sectors that incorporate AgNPs, including clothing, personal hygiene, cleaning items, purification systems, photovoltaics, sensors, and antibacterial coatings [2,3]. The medical field, in particular, incurs a high degree of AgNP utilization through applications such as water purifiers, bandages, antibacterial coating of surgical equipment, and bio-imaging techniques [4,5].

However, while significant progress has been achieved through the employment of AgNPs for applications, nano-silver has also been shown to be extremely detrimental to biological environments, with known responses including significant loss of viability, induction of cellular stress, and activation of the immune system [6–9]. Recent advances have identified ionic dissolution as a key contributor to these AgNP dependent consequences [10,11]. However, some uncertainty remains with regards to the exact mechanism behind ion-induced effects [12]. Previous dissolution studies have indicated that the kinetic rate of silver ion generation is dependent on a number of variables, including particle size, extent of agglomeration, morphology, surface chemistry, and temperature [13–15]. Primary size, in particular, is a predominant factor, with smaller particles inducing both a higher degree of cytotoxicity and rate of ionic dissolution in comparison to their larger counterparts [16].

Recently, a new research focus has emerged that explores the ability of AgNPs and other nanomaterials to disrupt normal cellular functionality in the absence of a cytotoxic response. Previous studies have identified that at sub-toxic levels, AgNPs interfered in both epidermal growth factor (EGF) and nerve growth factor signal transduction [17,18]. Furthermore, it was shown that following chronic exposure to AgNPs in the pg/mL range, cells displayed an augmented stress profile and modified EGF signaling efficiency [19]. While it is known that AgNPs will disrupt the cellular response to EGF, the mechanism behind this perturbation has yet to be explored. Nevertheless, these results suggest that at dosages more representative of actual exposure scenarios, in the sub-toxic regime, modification to normal cellular behavior and functionality poses a real concern.

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Signal transduction is the primary mechanism through which cells recognize and respond to an external stimulus. EGF and its corresponding receptor family is arguably the best studied and characterized ligand–receptor system to date; controlling responses ranging from proliferation and survival to wound healing and migration [20]. This is accomplished following activation of the EGF signal transduction network through receptor dimerization, induction of intrinsic kinase activity, and phosphorylation of downstream signaling targets [21]. Two of the primary signaling pathways activated by EGF are the Phosphoinositide 3-kinase (PI3K)/Akt and the Ras/Extracellular signal-related kinase (Erk) cascades. Phosphorylation and subsequent activation of Akt and Erk are key regulators of observed EGF-dependent responses and serve as representative proteins to gauge signaling performance. Akt is a critical player in a multitude of processes, including immune functionality, metabolism, cell survival, and growth [22]. Similarly, Erk activation has been shown to control proliferation, regulation of mitosis, and cell differentiation; making mutation of this pathway a primary cause of cancer [23]. As such, modulation of basal Akt and Erk introduces the potential for far-reaching health implications.

The two primary goals of this study were: (1) to explore the root cause behind AgNP-dependent EGF signaling interference and (2) to identify if internalized AgNPs are able to introduce prolonged cellular perturbations, assessed through signaling efficiency. Of particular interest was how the kinetic rate of ionic dissolution, as a function of both environment and time, influenced activation of stress and signaling pathways. For this study, a human keratinocyte, HaCaT, cell model was selected due to the considerations that skin is a primary exposure route for most nano-silver applications and HaCaTs have become a model cell line for NP behavior [24–26]. Our analyses revealed that AgNPs induced a two-phase disruption of EGF signal transduction, which were directly related to the rates of stress activation and ionic dissolution. These results suggest that internalized AgNPs have the ability to act as slow release devices for silver ions, which over time can lead to augmented cellular stress and modified biological functionality.

## 2. Materials and methods

### 2.1. Silver nanoparticle characterization and ionic dissolution

The 50 nm, citrate coated colloid AgNPs were purchased from NanoComposix in concentrated solution form. To avoid excessive agglomeration the dosing solutions were made up fresh prior to each experiment. Primary size analysis and morphology were verified using transmission electron microscopy (TEM) on a Hitachi H-7600. The spectral signature of the AgNPs was obtained through UV–vis analysis on a Varian Cary 5000. Dynamic light scattering (DLS) and zeta potential analyses were performed to evaluate particle agglomeration tendencies and surface charge, respectively, on a Malvern Zetasizer.

The extent of AgNP dissolution was determined by separating generated ions from the particles through a tangential flow filtration (TFF) process (Kros Flo Research System, Spectrum Labs) and silver content quantified through inductively coupled plasma mass spectrometry (ICP-MS; Perkin Elmer NexION 300D) [11]. Stock particles (AgNPs/ions), were incubated in serum free media at 37 °C for 24 h, after which the ions and particles were separated using TFF. Dosing solutions were made of either stock AgNPs/ions, the reconstituted particles (AgNP), or Ag ion solutions (Ions). Artificial lysosomal fluid was comprised of a complex salt solution and was adjusted to an acidic pH of 4.70 as previously reported [27]. The extent of ionic dissolution in lysosomal fluid was carried out in an identical manner.

### 2.2. Keratinocyte cell culture

The human keratinocyte, HaCaT, cell maintained at 37 °C and 5% $CO_2$ in RPMI 1640 culture medium supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin–streptomycin (ATCC). For EGF signaling experiments, HaCaTs were serum starved for 16 h prior to EGF stimulation (Peprotech) to ensure observed responses were not due to another serum component.

### 2.3. Evaluation of the AgNP–HaCaT interface

For deposition efficiency, $1 \times 10^6$ HaCaTs were seeded in a six-well plate and returned to the incubator for 24 h. The cells were then washed and replenished with 2 mL of AgNP solution, which consists of media supplemented with AgNPs to a final concentration of 5 μg/mL. After a 24 h exposure the media was removed and the quantity of silver within the media was determined using ICP-MS.

For visualization of the nano-cellular interface, HaCaTs were seeded on a two-well chambered slide at a density of $2 \times 10^5$ per well and allowed 24 h to adhere and grow. The cells were dosed with a freshly prepared 5 μg/mL AgNP/media solution (1.5 mL per well) and incubated for an additional 24 h. The HaCaTs were then fixed with 4% paraformaldehyde and stained with Alexa Fluor 555-phalloidin for actin and DAPI (Invitrogen) for nuclear imaging. The slides were then sealed and viewed using a CytoViva ultraresolution attachment on an Olympus BX41 microscope (Aetos Technologies).

### 2.4. Extent of AgNP internalization by HaCaT cells

For internalization studies, $1 \times 10^6$ HaCaTs were plated per well in a six-well plate, adhered overnight, and then dosed with 2 mL per well of media supplemented with 5 μg/mL AgNPs. After 24 h, the cells were fixed in 2% paraformaldehyde/2% glutaraldehyde (Electron Microscope Sciences) for 2 h, stained with a 1% osmium tetroxide, and dehydrated using increasing ethanol concentrations. Cell pellets were encased in LR White Resin and cured overnight at 60 °C in a vacuum chamber, thin-sectioned on a Leica ultramicrotome, and visualized via TEM.

### 2.5. Induction of ROS production

The generated reactive oxygen species (ROS) was monitored via the fluorescent probe dichlorofluorescein diacetate (DCFH-DA). HaCaT cells were seeded at $2 \times 10^4$ per well in a 96-well plate and incubated overnight. The following day, the cells were treated with 100 μM DCFH-DA for 30 min, washed, then dosed as specified. Fresh solutions of either AgNPs, silver nitrate ($AgNO_3$), or hydrogen peroxide ($H_2O_2$) were prepared in media, with an exposure volume of 100 μL/well. After 24 h incubation the fluorescence was measured using a SpectraMAX Gemini Plus fluorescent microplate reader (Molecular Devices) with an excitation of 485 nm and an emission of 538 nm. Untreated wells served as the negative control.

### 2.6. Fluorescence evaluation of actin and Ki67 quantities

In a 96-well plate, HaCaT cells were seeded at a density of $2 \times 10^4$, returned to the incubator overnight, then treated with the denoted conditions for 24 h prior to preparation for fluorescence evaluation. For AgNP, $AgNO_3$, or $H_2O_2$ conditions, fresh dosing solutions were made in media the day of experimentation. After exposure, cells were washed, fixed with 4% paraformaldehyde, permealized with 1% Triton X-100, and blocked with 1% bovine serum albumin (Thermo Scientific). The fixed cells were then probed with primary antibodies specific to actin and ki67 (Thermo Scientific)