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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 exposure, 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 slow-release devices of metallic ions; continually stressing and modifying normal cellular functionality.

Keywords: Silver Nanoparticle; Ionic Dissolution; EGF Signaling; Stress Response; Lysosomal Fluid

1. Introduction
Nano-sized colloidal silver inherently possess a number of distinctive physicochemical properties that make it extremely advantageous for incorporation into consumer and medical applications. These properties comprise of 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.

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 rate 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 hours, 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 line was a kind gift from the Air Force Research Laboratory. HaCaT cells were maintained at 37 °C and 5% CO₂ 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 hours 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, 1x10⁶ HaCaTs were seeded in a 6-well plate and returned to the incubator for 24 hours. The cells were then washed and replenished with 2 mL of a AgNP solution, which consists of media supplemented with AgNPs to a final concentration of 5 μg/mL. After a 24 hour 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 2-well chambered slide at a density of 2x10⁵ per well and allowed 24 hours 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 hours. 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, 1x10⁶ HaCaTs were plated per well in a 6-well plate, adhered overnight, and then dosed with 2 mL per well of media supplemented with 5 μg/mL AgNPs. After 24 hours, the cells were fixed in 2% paraformaldehyde/2% glutaraldehyde (Electron Microscope Sciences) for 2 hours, 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 dichloroofluorescein diacetate (DCFH-DA). HaCaT cells were seeded at 2x10⁴ per well in a 96-well plate and incubated overnight. The following day, the cells were treated with 100 μM
DCFH-DA for 30 minutes, washed, then dosed as specified. Fresh solutions of either AgNPs, silver nitrate (AgNO₃), or hydrogen peroxide (H₂O₂) were prepared in media, with an exposure volume of 100 µL/well. After 24 hours 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 2x10⁴, returned to the incubator overnight, then treated with the denoted conditions for 24 hours prior to preparation for fluorescence evaluation. For AgNP, AgNO₃, or H₂O₂ 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) with the corresponding secondary antibodies and fluorescence levels monitored on a microplate reader.

2.7 Cell Lysate Preparation and Assessment of EGF Signaling Activation
HaCaTs were seeded in a 6-well plate at a density of 1x10⁶ per well and grown overnight. The cells were then washed and dosed with 2 mL/well of AgNPs (5 µg/mL), AgNO₃ (0.22 µg/mL), or H₂O₂ (20 µM) for 24 hours in serum free media. Controls were incubated in serum free media only. The HaCaTs were then stimulated with 10 ng/mL of EGF to activate signal transduction. After 2 hours, the cells were washed and lysed in a non-denaturing lysis buffer containing phosphatase and protease inhibitors (Cell Signaling Technology). The phosphorylation levels of Akt and Erk were determined through ELISA analysis using kits from Cell Signaling Technology, which targeted Ser473 and Thr202/Tyr204, respectively. Phosphorylation levels were normalized by the total amount of the same protein, also quantified through ELISA kits.

2.8 Prolonged Bioresponses Following AgNP Exposure
For the extended duration experiments, HaCaT cells were plated at a density of 5x10³ and 2x10⁵ cells per well, respectively, for 96- and 6-well plates and returned to the incubator for 24 hours. On the day of experimentation, a fresh 5 µg/mL AgNP dosing solution was made in HaCaT media. The cells were then incubated with the AgNPs for 24 hours, after which the cells were washed to remove excess particles and the cells were replenished with fresh growth media and returned to the incubator. For viability, ROS, actin, and ki67 the samples were left undisturbed for an additional 72 hours, followed by endpoint analysis as previously described. For Akt and Erk activation, the cells were incubated for 48 hours, after which they were washed and replenished with serum free media for an additional 24 hours. EGF stimulation and Akt and Erk evaluation were then carried out as previously stated.

2.9 Reproducing Sustained Stress Levels with H₂O₂
To identify the concentration of hydrogen peroxide (H₂O₂) that produced equivalent ROS production as the extended AgNP exposure, a dose dependent analysis was carried out. HaCaT cells were seeded on a 96-well plate at a density of 2x10⁴ cells per well and returned to the incubator. The following day, H₂O₂ was diluted in media at concentrations ranging from 0 to 100 µM. The cells were washed and replenished with the H₂O₂/media and incubated for 24 hours, after which ROS analysis was carried out as previously described. From this analysis the
concentration of H$_2$O$_2$ that produced an equal ROS level as seen with the extended exposure analyses (20 µM) was used for future experimentation. In addition to ROS production, actin expression, ki67 levels, and EGF signaling were evaluated following 24 hour exposure to 20 µM H$_2$O$_2$, as previously described.

2.10 Statistical Analysis
Data is expressed as the mean ± the standard error of the mean (SEM). A two way ANOVA with a Bonferroni post-test was run using Graph Pad Prism to determine statistical significance. Significance was indicated for a p-value less than 0.05, with an asterisk (*) indicating difference from untreated controls and a cross (†) indicating difference between different experimental conditions as denoted.

3. Results and Discussion

3.1 Characterization and Dosage Selection of AgNPs
As the fields of nanotechnology and nanotoxicology have developed, it has become abundantly clear that physicochemical properties of NPs strongly dictate the resultant biological responses [28,29]. Therefore, extensive characterization of the colloidal AgNPs was carried out prior to cellular introduction (Table 1). Firstly, TEM imaging confirmed spherical morphology (Figure 1A) and a primary particle size of 53 nm. The standard deviation of the primary size was only 3.8 nm, indicating excellent uniformity and quality AgNPs. The uniformity of the AgNPs was further verified through spectral signature identification (Figure 1B), which demonstrated a sharp plasmonic peak at approximately 430 nm, which is typical for colloidal, nano-sized silver.

![Figure 1](image)

**Figure 1: Characterization of the 50 nm silver nanoparticles.** (A) Representative TEM image of the AgNPs verified spherical morphology and was used to determine primary particle size. (B) The spectral signature of the 50 nm AgNPs was obtained through UV-VIS analysis.

Since EGF evaluation is carried out in serum free (SF) media, we characterized the AgNPs in this environment, in addition to water. This is due to the fact that NP properties are strongly dependent on their surrounding environment [30,31]. DLS analysis indicated that the particles did agglomerate in SF media, with an approximate hydrodynamic diameter of 150 nm. This increased degree of agglomeration is due to the strong ionic content of media that isn’t present in water. Furthermore, the small variance associated with agglomerate size indicated stable cluster formations. It is typically assumed that all the NPs will agglomerate in solution, but the best metric to qualitatively assess this is the polydispersion index (PDI), which indicates the uniformity or heterogeneity of the sample. A low PDI, generally less than 0.3, indicates good
uniformity with respect to agglomerate size and stability, whereas a high PDI denotes varying agglomerate sizes and a heterogeneous sample. As the PDI for these particles, both in water and in SF media, are low, it is safe to assume that all the AgNPs have agglomerated and that these agglomerates are stable and roughly uniform in size. This conclusion is supported by the low standard error associated with the DLS data.

The surface charge of AgNPs was found to be slightly negative for water and media, roughly -29 and -12 mV, respectively. Lastly, the extent of ionic dissolution was evaluated after 24 hours and found to be 1.4% and 4% in water and SF media at 37 °C. As SF media was not supplemented with serum proteins, the AgNPs had no protein corona shell around them. As the presence of a protein corona masks NP behavior and has been linked to reduced ion production, a lack of corona explains the increased degree of agglomeration and kinetic rate of dissolution [32].

Table 1: Silver Nanoparticle Characterization

<table>
<thead>
<tr>
<th>Primary Size (nm)</th>
<th>Agglomerate Size (nm)</th>
<th>Zeta Potential (mV)</th>
<th>Ionic Dissolution (%)</th>
<th>Deposition Efficiency (%)</th>
<th>LD_{50} (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>SF Media</td>
<td>Water</td>
<td>SF Media</td>
<td>SF Media</td>
</tr>
<tr>
<td>53.3 ± 3.8</td>
<td>70.0 ± 2.3</td>
<td>155.3 ± 7.2</td>
<td>-29.0 ± 1.8</td>
<td>-11.9 ± 2.7</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>PDI – 0.12</td>
<td>PDI – 0.25</td>
<td></td>
<td></td>
<td>88.1 ± 4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td>48.2</td>
</tr>
</tbody>
</table>

Previous research has shown that AgNPs can induce a strong cytotoxic response [6-9]. However, because the goal of this study was to explore the influence of AgNPs on EGF signal transduction, we needed a dosage within the sub-toxic region. Therefore, we performed a traditional dose-dependent cytotoxic assessment (Supplemental Figure 1) which generated an LD_{50} value of 48.2 μg/mL. A concentration of 5 μg/mL was then selected for this study based on the observed dose-dependent response and the fact that exposure to 5 μg/mL AgNPs was previously shown to disrupt EGF signaling [18]. While this selected dose is significantly above an average daily exposure level, it is still relevant due to the combined facts that exposure levels are continuously rising and that AgNPs have been shown to be retained and accumulated within skin cells [33].

3.2 Evaluation of the Nano-Cellular Interface

The nano-cellular interface, meaning interactions between engineered NPs and their surrounding cellular environment, has been linked to observed cellular outcomes and responses: identifying it as a key regulator for nano-bioeffects [34,35]. Therefore, beyond AgNP characterization, we sought to understand how the 50 nm AgNPs interacted with HaCaT cells. First, we evaluated the extent of AgNP deposition. After 24 hours, the cells either internalized or strongly bound 88% of the originally dosed AgNPs. This high deposition efficiency indicates that strong thermodynamic binding forces exist between HaCaTs and AgNPs [35]. Taking the evaluation of the nano-cellular interface one step further, this interaction was visualized with high resolution
microscopy (Figures 2A and 2B). While some mono-dispersed particles were visible, larger agglomerates predominated which indicate clustering during internalization.

**Figure 2: Evaluation of the AgNP-HaCaT interface.** Fluorescence microscopy visualized the nano-cellular interface for (A) untreated HaCaT cells and (B) following exposure to AgNPs. In these representative images, actin and nuclei are stained red and blue, respectively, with AgNPs appearing white. TEM images were used to demonstrate AgNP internalization following a 24 hour exposure. Representative images are shown of (C) control and (D) AgNP exposed HaCaT cells. Inset is a magnification of the area within the box.

Lastly, to verify that HaCaT cells were efficiently internalizing the AgNPs, uptake patterns were ascertained through TEM images (Figures 2C and 2D). These representative images clearly show AgNPs well within the cell membrane. Furthermore, the AgNPs are clustered together and can be seen within defined vacuole boundaries, indicating that they were internalized through endocytosis. As these groups of particles consistently appear in both the fluorescence and TEM images, these data support the observed deposition efficiency and entry mode of endocytosis.

### 3.3 Cause of EGF Signaling Disruption: Ions versus Particles

The next goal was to explore whether AgNP-dependent alterations to EGF signal transduction, as evaluated through Akt and Erk phosphorylation, were a result of particle-cellular interactions or the generation of silver ions. Figure 3 shows an approximate 25% reduction in Akt and Erk activity 24 hours after exposure to 5 μg/mL AgNPs, in agreement with our previous findings [18]. The ion only samples induced a nearly identical loss in signaling efficiency following stimulation with EGF, indicating that ionic dissolution is the predominant cause for changes to signaling behavior. This is further validated by the fact that the isolated AgNPs had a negligible impact on Akt and Erk phosphorylation levels.
Figure 3: AgNP dependent reduction in Akt and Erk signaling efficiency. (A) HaCaTs were exposed to either unseparated AgNPs/Ions, Ag ions, or isolated AgNPs for 24 hours followed by evaluation of EGF signaling. Stock AgNPs and ion samples decreased Akt and Erk phosphorylation equally, suggesting dissolution to be the driving force for EGF signaling interference. (B) To verify this conclusion, HaCaTs underwent a 24 hour exposure to silver nitrate (AgNO$_3$) at a concentration equal to the AgNP dissolution rate followed by evaluation of Akt and Erk activity. (Represents three independent trials, * denotes statistical significance from untreated control, † denotes significance from unseparated AgNPs/Ions, for both analyses $p < 0.05$)

To confirm that Ag ions disrupt EGF signaling and to remove the variable of the AgNPs themselves, we dosed HaCaT cells with a silver salt, silver nitrate (AgNO$_3$), at a concentration equivalent to the 24 hour dissolution rate. Comparing the EGF-dependent signaling responses between the AgNP/Ion and AgNO$_3$ conditions, we found that AgNO$_3$ controls produced nearly identical decreases in Akt and Erk phosphorylation levels (Figure 3B). These results support our previous conclusion that AgNP ionic dissolution is the primary mechanism behind Ag-NP induced interference of EGF signal transduction.

3.4 Sustained Effects by Internalized AgNPs
Nanomaterials are typically internalized through a combination of endocytosis mechanisms and are either marked for degradation, exocytosis, or retention [36,37]; as seen in Figure 2D. During this process, endosomes progress into lysosomes, introducing the internalized AgNPs to a low pH environment. Environmental factors have been shown to dramatically alter NP behavior,
including increased rates of ionic dissolution [31,30,38], thereby intensifying ion-dependent effects. The question then arises of whether this continual ion release could potentially result in modifications to cell functionality and basal stress levels over time. To explore this phenomena HaCaTs were dosed with 5 μg/mL AgNPs for 24 hours, after which the cells were washed, replenished with fresh media, and incubated for an additional 72 hours. Cellular responses were evaluated and compared after both the initial 24 hour exposure (Day 1) and the 72 hour recovery period (Day 4).

As AgNPs induce cellular death by generating reaction oxygen species (ROS) and activating other stress markers [6-9], we first evaluated key stress-induced endpoints. We assessed the expression of actin and ki67, as both are directly connected to the EGF-induced cellular response. During cellular stress, actin becomes inflamed and disorganized, impacting expression of EGF receptors [39]. In addition to its proliferative role, ki67 has a critical secondary function of repairing damaged DNA [40] and increases expression following exposure to AgNPs [19]. Therefore, modulation to basal expression of either actin or ki67 could disrupt the cellular balance and result in EGF-dependent signaling interference.

As expected, after a 24 hour AgNP exposure, the HaCaTs displayed a significant increase in all examined stress responses (Figure 4A). At Day 4, the HaCaTs not only showed signs of active stress, but the degree of cellular stress response was increased over Day 1 levels. These results indicated that the internalized or surface-bound AgNPs were able to sustain and enhance the NP-dependent bioeffects over time. The next goal was to determine if AgNP alterations to EGF signal transduction remained even after the removal of particles (Figure 4B). Most interestingly, EGF signaling displayed a two-phase response with the 25% reduction seen at Day 1 and an observed enhancement in Akt and Erk phosphorylation at Day 4. Taken together, the stress and EGF signaling responses demonstrate a HaCaT response significantly different from basal levels, indicating that the internalized particles are able to amplify and extend out modifications to cellular functionality even in the absence of excess AgNPs.
Figure 4: Sustained HaCaT response through internalized AgNPs. Cells were exposed to a 5 μg/mL dosage of AgNPs for 24 hours after which they were either evaluated (Day 1) or washed and allowed 3 days of recovery (Day 4). (A) Stress activation was compared for Day 1 and Day 4 time points, with an increased stress associated with the longer duration. (B) EGF signaling ability was determined through extent of Akt and Erk phosphorylation. A two phase response was identified, with an initial loss in signaling efficiency followed by an augmentation in Akt and Erk activity during cellular recovery. (represents three independent trials, * denotes statistical significance from untreated control, † denotes significance from Day 1 to Day 4, for both analyses p < 0.05)

3.5 Correlating the kinetic rate of AgNP dissolution to EGF disruption

In an effort to elucidate the mechanism behind this two-phase response, the kinetic rates of ionic dissolution were determined in water, media, and artificial lysosomal fluid. This analysis was carried out at 37 °C over a period of 3 days to understand how the AgNPs behaved over time following internalization. As shown in Figure 5, the dissolution rate varied as a function of time and composition. For water and media environments, the degree of ion generation was greatest in the beginning then stabilized, presumably due to the achievement of ionic equilibrium. In lysosomal fluid the AgNPs very quickly dissolved into a nearly pure ion form, as a result of the low pH of this fluid. The rapid dissolving of nanomaterials in lysosomal fluid was previously seen with zinc oxide, confirming this behavior [38]. However, even though the rates slowed with time, the total degree of ionic dissolution increased in lysosomal fluid, indicating that at extended periods the particles are still shedding ions.
Figure 5: Kinetic evaluation of AgNP ionic dissolution. AgNPs were resuspended in water, serum free media, or artificial lysosomal fluid at a concentration of 5 μg/mL and stored at 37°C. The percent dissolution was evaluated over 3 days. (B) Using the dissolution data, a kinetic rate production of ions, based off the initial concentration was determined at both initial and final phases.

While the deposition efficiency after 24 hours was shown to be 88%, there is no way of accurately predicting what fraction of the AgNPs are internalized versus being surface bound. Even though it is impossible to determine the true degree of ion generation occurring during the recovery period, we know that dissolution is occurring in both media and lysosomal environments, producing a cumulative rate higher than in media alone. Moreover, the cellular stress activation during this period is known (Figure 4A), providing a metric to evaluate the HaCaT response.

As high stress has been linked to modified EGF receptor expression and activity [39], we hypothesized that the second phase of AgNP-dependent signaling interference was brought on through the slow dissolution of internalized particles, resulting in a sustained stress response. To test this hypothesis and remove the variability of the AgNPs themselves, the stress response was recreated through the addition of hydrogen peroxide (H$_2$O$_2$) at a concentration which produced equivalent ROS (Figure 6A; Supplemental Figure 2). Following the addition of H$_2$O$_2$, kit67 levels were equal to the extended AgNP exposure, but while actin expression was increased it was not to the same level as seen with AgNPs. Following H$_2$O$_2$ exposure, there was a marked increase in EGF activation of both Akt and Erk, as seen in Figure 6B. While the phosphorylation levels were higher with H$_2$O$_2$ versus AgNPs, they did follow the same enhanced patterns of activation.
Figure 6: Connecting HaCaT cellular stress to modified EGF signaling. HaCaT cells were exposed to H$_2$O$_2$ at a concentration that induced an equivalent degree of ROS generation as AgNPs, followed by evaluation of (A) ROS, actin, and ki67 expression and (B) Akt and Erk phosphorylation. These results were directly compared to AgNPs (Day 4). (represents three independent trials, * denotes statistical significance versus untreated control, † denotes significance between Day 4 and H$_2$O$_2$ samples, for both analyses p < 0.05)

The discrepancy between these two sample sets is likely due to a combination of different transport mechanisms and the fact that H$_2$O$_2$ was added at a single time point instead of a slow dissolution. As previously discussed, NPs are typically internalized through a combination of endocytosis mechanisms. Small chemical compounds, such as AgNO$_3$ and H$_2$O$_2$, are transported into cells through different methods, such as direct diffusion and ion channels. Furthermore, the rate of cellular entry for these small molecules is orders of magnitude faster than AgNPs, which could easily account for any discrepancies noted between the stress and EGF levels in both the AgNO$_3$ and H$_2$O$_2$ trials. However, taken together, this data suggests that the second part of the biphasic EGF interference is brought on by a sustained stress response, likely from the dissolution of internalized particles.

3.6 Implications of These Findings
Owing to the facts that AgNPs are frequently utilized in consumer products and that these applications involve low exposure levels, identification of cellular responses in the sub-toxic range is of great interest. As AgNPs are retained in the skin [33], this means that even low
exposures can accumulate and introduce a potentially hazardous situation. Our findings demonstrated that over time, internalized 50 nm AgNPs were able to increase stress and EGF-dependent signaling responses. Chronic elevated stress levels have been linked to health implications such as cancer, Alzheimer’s disease, and modulations to the immune and inflammatory system [41]. Similarly, alterations to EGF-dependent effects can lead to cancer, metabolic diseases, and dysregulation of the inflammatory system [22,23]. Therefore even if NPs aren’t induce a cytotoxic response, over time they can lead to modified functionality and severe health problems.

This revelation poses novel concerns related to long term exposure and indirect effects brought on by the retention and slow dissolution of AgNPs. Through evaluation in lysosomal fluid, we identified an increased rate of ion production in physiological fluids. It was reported that the AgNPs incorporated into clothing display enhanced dissolution when in contact with sweat [42], demonstrating that this phenomena isn’t specific to lysosomal fluid. This report also confirmed that both AgNPs and silver ions are able to be directly transported from a product to the skin, resulting in a near continuous NP exposure. When combined with the knowledge that dissolution is the driving force for AgNP-derived stress effects, it highlights the crucial need to further explore the potential of sustained biological consequences of retained metallic nanomaterials.

4. Conclusions
In this study, we explored the ability of AgNPs to disrupt EGF signaling and defined the mechanism behind this interference. AgNPs induced a biphasic EGF signaling response, with an initial decrease followed by a latter augmentation of Akt and Erk phosphorylation. Owing to the fact that ionic dissolution correlates to AgNP stress activation, the elucidation of its role in signaling obstruction was a major focus of this work. Initial dissolution of AgNPs in media led to the early reduction in signaling efficiency. Through evaluation of dissolution kinetics, we found a high degree of silver ion formation in lysosomal fluid; supporting our hypothesis that internalized AgNPs act as a sustained source of silver ions and stress activation. This study verified that continual stress, even following the removal of excess AgNPs, brought about an increase of Akt and Erk phosphorylation following EGF stimulation.

In conclusion, we demonstrated that even in the absence of cellular death, 50 nm AgNPs with their high rates of ion generation and cellular internalization altered basic cell functionality, as assessed through stress activation and EGF signal transduction. We believe that the AgNPs act as a slow release mechanism of silver ions, which modify basal cellular responses over an extended period of time. While this study scratches the surface, the ability of AgNP dissolution to disrupt cellular behavior needs to be further explored. For example, do changes in primary particle size and surface coatings, which are known to influence dissolution rates [14-16], alter resultant biological consequences? Through exploring the implications of low level, sustained contact, more relevant conclusions can be drawn regarding the long term effects of AgNP exposure.

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