Interference of Silver, Gold, and Iron Oxide Nanoparticles on Epidermal Growth Factor Signal Transduction in Epithelial Cells

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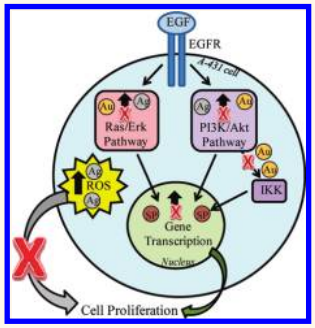
ABSTRACT Metallic nanomaterials, including silver, gold, and iron oxide, are being utilized in an increasing number of fields and specialties. The use of nanosilver as an antimicrobial agent is becoming ever-more common, whereas gold and iron oxide nanomaterials are frequently utilized in the medical field due to their recognized "biocompatibility". Numerous reports have examined the general toxicity of these nanomaterials; however, little data exists on how the introduction of these nanomaterials, at nontoxic levels, affects normal cellular processes. In the present study the impact of low levels of 10 nm silver (Ag-NP), gold (Au-NP), and iron oxide nanoparticles (SPION) on epidermal growth factor (EGF) signal transduction within the human epithelial cell line, A-431, was investigated. Following a biocompatibility assessment, the nanoparticle-induced interference at four specific targets within the EGF signaling process was evaluated: (1) nanoparticle-EGF association, (2) Akt and Erk phosphorylation, (3) Akt activity, and (4) EGF-dependent gene regulation. For all tested nanoparticles, following cellular exposure, a disruption in the EGF signaling response transpired; however, the metallic composition determined the mechanism of alteration. In addition to inducing high quantities of ROS, Ag-NPs attenuated levels of Akt and Erk phosphorylation. Au-NPs were found to decrease EGF-dependent Akt and Erk phosphorylation as well as inhibit Akt activity. Lastly, SPIONs produced a strong alteration in EGF activated gene transcription, with targeted genes influencing cell proliferation, migration, and receptor expression. These results demonstrate that even at low doses, introduction of Ag-NPs, Au-NPs, and SPIONs impaired the A-431 cell line's response to EGF.



KEYWORDS: nanoparticle · signal transduction · EGF · nanotoxicology · silver · gold · SPION

Nanotechnology has emerged as a multidisciplinary field with wide ranging applications extending into numerous military, industrial, medical, and scientific specialties. Three of the most prevalent nanomaterials to date are silver, gold, and iron oxide. Because of its antimicrobial properties nanosilver has become incorporated into various everyday products, including water disinfectants, bandages and medical devices, household appliances, cosmetics, and clothing.[1] Similarly, gold nanomaterials have become widespread in the medical field because of their relative biocompatibility[2] and unique optical properties with applications in cancer therapy and bioimaging.[3] Owing to their magnetic properties and nontoxicity, superparamagnetic iron oxide nanoparticles (SPIONs) are also attractive to biomedical areas with current applications including drug delivery, hyperthermia treatments, and MRI enhancement.[4,5] As nanomaterial prevalence increases, a corresponding rise in exposure levels is highly probable, of which the extent of biological interactions has yet to be fully determined. Extensive studies have undertaken the issue of nanoparticle toxicity; however, little research has been conducted into the disturbance of biological mechanisms by nanoparticles at doses that indicated no toxic response. These studies have, in general, demonstrated that nanoparticles produced an alteration in normal cellular behavior and responses to stimuli.[6−9] For example, Braydich-Stolle *et al.*[7] found that silver nanoparticles at 10 μg/mL specifically interacterd with Fyn kinase, thus inhibiting GDNF signaling in spermatogonial stem cells. Additionally, Speshock *et al.*[8] determined that both silver and gold nanoparticles reduced cathepsin activity in Vero cells at nontoxic doses, demonstrating the potential of nanomaterials to impact a host's immune system. Therefore, although a nanomaterial may not be lethal to a cell, it may be erroneous to assume that this material is not altering the cell's normal behavior. Consequently, widespread exploration into the impact of nanomaterials on cellular processes, such as signal transduction, is crucial

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TABLE 1. Characterization and Agglomeration Patterns of Nanoparticles

	primary nanoparticle size *via* TEM (nm) ± std. dev.	agglomerate size *via* DLS (nm) ± std. dev.			zeta potential (mV) ± std. dev.
sample		water	serum free media	media with serum	water
Ag-NP	9.15 ± 1.5	41.32 ± 2.8	110.87 ± 0.5	84.66 ± 0.5	−40.93 ± 1.9
Au-NP	9.97 ± 0.9	64.04 ± 2.8	328.10 ± 6.3	76.021 ± 3.3	−32.27 ± 1.0
SPION	10.17 ± 0.9	60.53 ± 0.5	79.34 ± 0.4	49.09 ± 0.4	−21.23 ± 0.9

to fully elucidate potential nanomaterial interactions in a physiological environment.

The ability of living cells to recognize and respond to chemical and physical stimuli in their surroundings is fundamental for cell survival. Signal transduction, typically driven by a growth factor or cytokine, is the main mechanism cells use to convert an external stimulus into a cellular outcome, such as proliferation, differentiation, or protein secretion. The mitogenic protein epidermal growth factor (EGF) and its receptor family is arguably the best studied and characterized ligand−receptor system to date.[10] Upon cellular introduction, EGF will bind to its specific receptor (EGFR) after which the receptor can dimerize and initiate intrinsic kinase activation, receptor phosphorylation, and signal transduction.[11−13] EGF has been shown to control the activation of numerous signaling pathways, including the Phosphoinositide 3-kinase (PI3K)/Akt pathway and the Ras/Extracellular signal-regulated kinase (Erk) cascade,[14] which results in an up-regulation of the gene transcription necessary for cell proliferation, survival, and migration.[15,16]

The aim of this study was to ascertain to what extent metallic nanoparticles of similar size and morphology, but of different composition, altered EGF-dependent signal transduction in the human epithelial cell line A-431. The A-431 cell line was selected for this study because of its well characterized overexpression of EGFRs[17] and its frequent use as a cell model for EGF signaling studies.[18] In essence, signal transduction serves a bridge that connects an external stimulus, EGF in this case, to the observed cellular outcomes. Therefore it is crucial to evaluate the degree of nanoparticle interference in signal transduction pathways, as a disruption could have a profound physiological impact. For instance, unregulated signaling from the EGFR is a leading cause for multiple classes of cancer including neck, bladder, and ovarian.[19] We selected four essential points of the EGF signaling response to investigate: (1) EGF-nanoparticle association, (2) Akt and Erk phosphorylation, (3) Akt activity, and (4) EGF-dependent gene regulation. Although EGF controls the activation of several signaling pathways we chose to evaluate the PI3K/Akt and Ras/Erk cascades due to their universal roles in the immune response, inflammatory response, and cellular proliferation. Akt activity was specifically assessed due to Akt's central role and importance in the majority of physiological processes. Our results establish that 10 nm, spherical silver, gold, and SPIONs were all able to disrupt the A-431 cell line's signaling response to EGF; however, each nanoparticle interfered at a different point in the signal transduction process.

RESULTS AND DISCUSSION

Selection, Characterization, and Biocompatibility of Nanoparticles. For this study, we investigated silver (Ag-NP), gold (Au-NP), and super paramagnetic iron oxide (SPION) nanoparticles as they are three of the most commonly used nanomaterials to date and thus potentially have the highest rates of exposure. Au-NPs and SPIONs were utilized owing to their known biocompatibility and substantial use and promise in the medical field. In fact, SPIONs are the only metal oxide nanoparticle clinically approved by the food and drug administration (FDA) and are frequently used for MRI screening and drug delivery.[20] Ag-NPs were selected to round out the experimental matrix with a well characterized, toxic, and commonly utilized nanomaterial.[21]

As the field of nanotechnology has developed, the need for in-depth characterization of nanomaterials has become increasingly apparent.[22,23] Proper characterization, which can include primary particle size, morphology, aggregation tendencies, and surface charge, is necessary to substantially contribute to the knowledge base of toxicology and nanomaterial interactions. As this study directly compared the biological effects of 10 nm, spherical Ag-NPs, Au-NPs, and SPIONs, characterization of these particles was carried out prior to cellular exposure. Transmission electron microscopy (TEM) analysis confirmed the primary size of approximately 10 nm and spherical shape for all nanoparticles utilized (Supporting Information, Figure 1, Table 1). Since it is known that nanoparticles will agglomerate to some extent when dispersed in media,[24] dynamic light scattering (DLS) was performed to determine size distribution and agglomerate size, in water, serum free media, and media with serum. As expected, the nanoparticles did undergo slight agglomeration once dispersed, with the largest sizes occurring in serum free media (Table 1). While there exists some disparity between agglomerate sizes, most notably Au-NPs in serum free media and SPIONs in media with serum, we believe that the results predominantly illustrate

a similarity in agglomeration patterns. Therefore, we conclude that as the effective sizes of the nanoparticles were similar prior to and following dispersion, any nanoparticle-dependent alterations observed can be attributed to composition and not size or morphology effects. Additionally, the zeta potential data demonstrated a negative surface charge for all three nanoparticles, again indicating composition as the key distinction between particles.

The biocompatibility of Ag-NPs, Au-NPs, and SPIONs in A-431 cells was assessed by evaluating the cytotoxicity and reactive oxygen species (ROS) production following nanoparticle exposure (Supporting Information, Figure 2).[25,26] The Au-NPs and SPIONs were found to be nontoxic with the viability remaining unaltered following exposure of up to 100 μg/mL and minimal change in ROS production. As anticipated, a considerable level of cytotoxicity was observed with the Ag-NPs coupled with a substantial increase in ROS levels.[21,27] This high toxicity induced by nanosilver is what makes it uniquely employable as an antimicrobial agent when incorporated into everyday objects and devices. The results of this biocompatibility evaluation allowed us to effectively design an experimental matrix for the signal transduction interference studies that captured our objective of low, subtoxic nanoparticle exposure concentrations

Nanoparticle Uptake and Surface Association. Both nanoparticle uptake and microscopy studies were performed to verify an interaction between the nanoparticle and the cellular system. The extent of nanoparticle uptake and cellular localization within A-431 cells was examined through TEM imaging of ultrathin sections of cells. Representative images illustrated that both Ag-NPs and Au-NPs were readily internalized following 24 h cellular exposure (Figure 1B,C). Additionally, these particles appeared to be internalized *via* an endocytosis mechanism, as Ag-NPs and Au-NPs were localized in intracellular vacuoles that, based on their size and shape, appear to be endosomes. Contrarily, as shown in Figure 1D, following SPION exposure, TEM images demonstrated no recognizable indications of particle internalization.

To further investigate nanoparticle-cellular association, studies were performed using ultrahigh resolution microscopy coupled with fluorescence staining. As shown in Figure 1F–H, for all three treatments, the nanoparticles clearly interacted with A-431 cells. Interestingly, Ag-NPs and Au-NPs appeared to be densely packed, indicative of clustering following internalization. However, SPIONs were dispersed around the cells, in almost a monolayer fashion. These results are in agreement with the observed lack of SPION uptake, and suggest that SPIONs bind tightly to the cell membrane but are not internalized. Taken together the TEM and microscopy data established that Ag-NPs, Au-NPs, and SPIONs all interacted with A-431 cells, thus

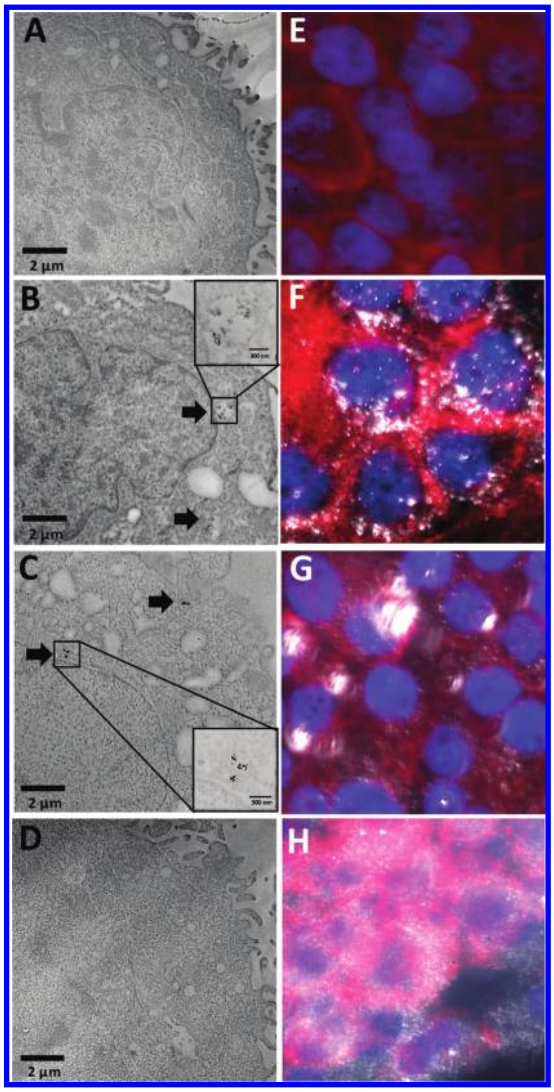

Figure 1. Internalization and cellular association and of 10 nm metallic nanoparticles. Internalization is demonstrated through representative TEM images of (A) control A-431 cells and following 24-h exposure to (B) Ag-NPs, (C) Au-NPs, and (D) SPIONs. Ultrahigh resolution microscopy depicts the interactions of A-431 cells and nanoparticles: (E) control, (F) Ag-NPs, (G) Au-NPs, and (H) SPIONs. In these images actin is stained red, nuclei are stained blue, and nanoparticles appear white.

providing an opportunity to interfere in EGF signal transduction.

Evaluation of EGF-Nanoparticle Binding. Prior to EGF introduction to a cell system, it had to first be determined if the presence of nanoparticles altered EGF levels in media *via* a nonspecific binding mechanism. If discovered that Ag-NPs, Au-NPs, or SPIONs were binding to EGF molecules, thus making them unavailable, it would greatly impede EGF dependent signal transduction by limiting EGF-EGFR pairing. To evaluate this potential interaction, 5 ng/mL EGF and 100 μg/mL of each nanoparticle were incubated together for 24 h, after which the nanoparticles were removed and the remaining EGF quantified. As shown in Figure 2, for all cases, the presence of nanoparticles did not diminish

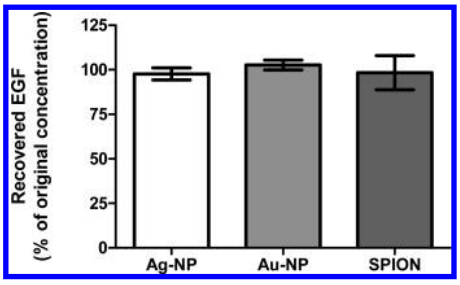

Figure 2. Effect of metallic nanoparticles on available EGF. Following co-incubation, the extent of nanoparticle-EGF binding was evaluated by determining the remaining EGF concentration (represents six independent trials).

EGF quantities, thereby, establishing that the EGF dose available to the A-431 cells was equivalent whether administered alone or with nanoparticles.

Nanoparticle Interference in EGF Dependent Signaling. Following EGFR activation the first stage of signal transduction is the induction of specific pathways including the PI3K/Akt and the Ras/Erk cascades. Generally speaking, a signaling pathway comprises a series of targeted cytosolic proteins that sequentially activate one another through specific phosphorylation in their active sites. In addition to the aforementioned reasons for investigating Akt and Erk, these proteins were desirable for this study because of their traditional use as a representative for their respective pathways and the availability of phospho-specific ELISAs. The experimental conditions chosen for this study (5 μg/mL Ag-NP and 5 and 25 μg/mL Au-NP and SPION) were based on the biocompatibility results and included only lower doses that did not significantly alter cell viability. A 25 μg/mL dose of Ag-NP was not performed due to the rapid induction of cytotoxicity. Our experimental design included 24 h of nanoparticle exposure prior to the introduction of EGF and the subsequent evaluation of Akt and Erk signaling. Regardless of composition, the nanoparticles reduced EGF-dependent phosphorylation levels of Akt (p-Akt) and Erk (p-Erk), though to varying degrees (Figure 3A,B). Au-NPs had the most significant impact, with an observed 15% inhibition of p-Akt combined with a 30−40% reduction of p-Erk. For both Akt and Erk, the SPIONs diminished protein phosphorylation in a dose-dependent fashion, with a scant effect observed at 5 μg/mL and an approximate 15% decrease associated with 25 μg/mL. Similarly, Ag-NPs inhibited both p-Akt and p-Erk by approximately 20%. To verify that the Ag-NP dependent alterations were brought about by the nanoparticles themselves and not the resulting ROS production, p-Akt and p-Erk levels were determined following exposure to hydrogen peroxide, a known proponent of ROS production. These results revealed that hydrogen peroxide induced no substantial reduction to either Akt or Erk phosphorylation states following EGF stimulation (Supporting Information, Figure 3),

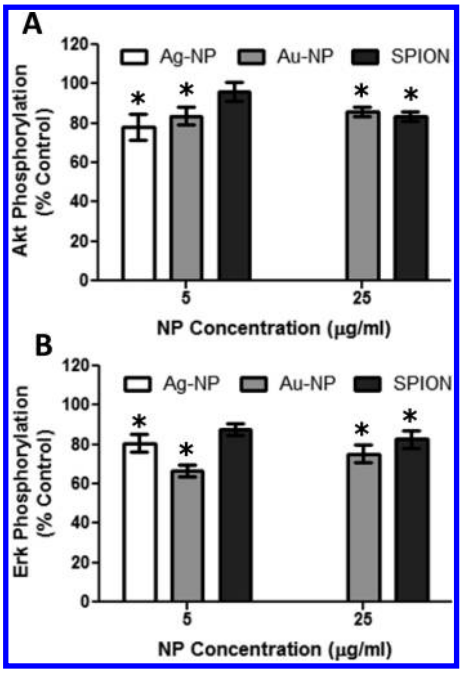

Figure 3. Interference in EGF signal transduction by 10 nm silver, gold, and SPION nanoparticles. Normalized phosphorylation levels of (A) Akt and (B) Erk following 24-h nanoparticle exposure and 2-h EGF stimulation (represents six independent trials, the asterisk (*) denotes $p < 0.05$).

indicating that the Ag-NPs, and not ROS, is responsible for the noted signaling interference.

Once dually phosphorylated, Akt, a serine/threonine protein kinase, seeks out several downstream, target proteins for phosphorylation, thus propagating the signaling response to EGF. Akt is paramount in the regulation of a diverse number of cellular processes, including metabolism, proliferation, cell survival, growth, and angiogenesis with over 100 reported effectors.[28] As its role in a plethora of biological outcomes and physiological processes has been elucidated, Akt has solidified its prominent position in signal transduction.[29] As such, nanoparticle induced alterations in Akt activity could be detrimental to a cellular system. Therefore, we next investigated whether 10 nm Ag-NPs, Au-NPs, and SPIONs were capable of interfering with the functionality of p-Akt. Akt activity was assessed following nanoparticle exposure by evaluating the phosphorylation level of a known downstream target, IKK-α (p-IKK-α), in response to EGF stimulation.[30] Surprisingly, Au-NPs decreased p-IKK-α levels by approximately 20%, while Ag-NPs and SPIONs produced no alteration (Figure 4). We believe that this revealed reduction in IKK-α signaling effectively demonstrates an obstruction in Akt activity by Au-NP, when compared with the Ag-NP and SPION results. Ag-NPs and SPIONs both induced an approximate 20% reduction in p-Akt levels, however, following exposure to these nanoparticles IKK-α signaling was equivalent to control cells, indicating full Akt functionality. As Au-NPs attenuated both p-Akt

and p-IKK-α by approximately 15−20%, we therefore conclude that Au-NPs both inhibited Akt phosphorylation and impeded its ability to act as a protein kinase. However, it is important to bear in mind the complexity of a signal transduction network and recognize that although Ag-NPs and SPIONs were not negatively regulating Akt activity, as measured by p-IKK-α levels, their reduction of p-Akt quantities is likely to influence other cellular mechanisms outside the scope of this study.

Impact of Metallic Nanoparticles on EGF-Dependent Gene Transcription. To evaluate the impact of 10 nm Ag-NPs, Au-NPs, and SPIONs on signal transduction downstream of Akt and Erk proteins, EGF dependent gene regulation was analyzed following nanoparticle exposure and subsequent EGF stimulation. Gene expression was calculated using the human EGF/PDGF signaling profiler PCR array which utilizes real-time PCR to monitor 84 genes controlled by the EGF and platelet-derived growth factor (PDGF) signal transduction networks. This array covers all dimensions of a signal transduction response, including genes that encode for growth factors and their receptors, signal transduction proteins, and targeted downstream molecules. Observed changes in gene expression are shown in Table 2 and demonstrated substantial alterations by SPIONs, but a minimal impact by Ag-NPs and Au-NPs. Previously, iron oxide nanoparticles were also shown to cause up-regulation of numerous genes in hTERT-BJ1 fibroblasts, indicating that this phenomenon is not unique to A-431 cells.[6] In most cases, the fold regulation changes induced by SPIONs were dose dependent indicating a direct correlation between SPION concentration and observed disruption of EGF signaling. Ag-NPs and Au-NPs produced slight alterations in EGF signaling gene expression, but in addition to being less numerous the resulting changes in transcription tended to be of a lower fold value.

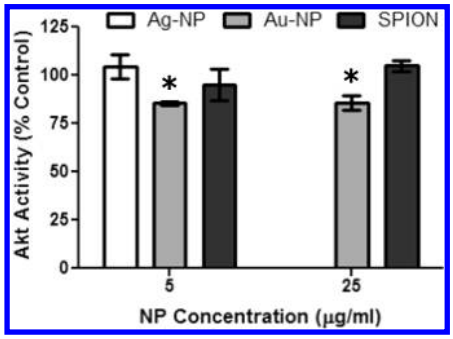

Figure 4. Nanoparticle induced attenuation of Akt performance. Akt activity, assessed by IKK-α phosphorylation, was evaluated following 24-h incubation with nanoparticles and 2-h EGF treatment (represents six independent trials, asterisk (∗) denotes $p < 0.05$).

The SPION induced shift in EGF signal transduction gene regulation can be classified into several function-based categories. First, our PCR results showed an amplification in genes encoding receptor production, including the EGFR and PDGF receptor (PDGFB), both of which are prominent in epithelial cells and are responsible for the regulation of proliferation, differentiation, and migration.[31] The upregulation of these genes implies that new EGF and PDGF receptors are being manufactured by the cell, most likely in an effort to enhance an inflammatory or immune response. Second, our results indicated that SPIONs modified genes which encode proteins involved in the epithelial cell's migratory ability, specifically fibronectin (FN1) and plasminogen activator (PLAT). In agreement with our results, it was previously reported that SPIONs can mediate microtube remodeling[32] which has the potential to influence cell motility. As fibronectin is crucial

TABLE 2. Metallic Nanoparticle Dependent Alterations in EGF Signaling Pathway Gene Regulation

		fold regulation (*vs* control)[a]				
		Ag-NP	Au-NP		SPION	
gene symbol	gene function	5 μg/mL	5 μg/mL	25 μg/mL	5 μg/mL	25 μg/mL
ATF1	ATF member that influences growth and survival	1.53†	2.02	1.83	—	—
BRAF	proto-oncogene invovled in Ras/Erk signaling	3.3	—	—	2.58	3.63
DUSP1	phosphatase that regulates Erk	—	—	—	3.97	4.98
DUSP6	phosphatase that regulates Erk	—	—	—	3.29	—
EGFR	EGF receptor	—	2.43	—	4.3	5.32
EPS8	part of the EGFR pathway with unknown role	—	—	—	−2.03	—
FN1	involved with cell adhesion and migration	—	—	—	3.24	3.11†
MAPK9	stress induced MAPK that induces apoptosis	−1.16†	—	—	−1.33†	−2.03
NFATC3	transcription complex downstream of Jnk2		—	−1.45†	−1.74	−2.72
PDGFB	PDGF receptor	−1.26†	—	—	2.49	4.37
PLAT	regulates cell migration and wound healing	—	—	—	−1.96†	−3.2
TP53	induces apoptosis	−1.45†	—	—	−1.91†	−2.5

[a] Reported genes have at least one condition with ≥2.0 fold regulation over control and statistical significance. Represents three independent trials. Unmarked samples denotes $p < 0.05$, "†" denotes $0.05 < p < 0.1$, and "—" indicates that no statistically significant change occurred.

in cell adhesion, growth, migration, and wound healing, the upregulation of FN1 indicates SPIONs are capable of enhancing these biological effects. Interestingly, the gene array data displayed evidence that one response to SPIONs was an attempt to enhance cellular proliferation through multiple mechanisms. Several key members of the MAPK−Jnk pathway (MAPK9, also known as Jnk2, NFATC3, and TP53), which is known to be induced by stress and often concludes in cellular apoptosis,[33] were down-regulated, suggesting a tendency toward enhanced cell survival. Furthermore B-Raf, an upstream kinase of Erk, was upregulated with a possible consequence of enhanced proliferation and cell cycle progression.[34] However, we speculate that this increase in Raf was antagonized by a concurrent augmentation in DUSP1 and DUSP6, both of which are protein phosphatases responsible for the negative regulation of the Ras/Erk cascade.[35]

Possible Implications of Signaling Interference by Silver and Gold Nanoparticles. The toxicity of nanosilver has been well established,[36] and is generally believed to be due to an increase in ROS production and the resultant DNA damage.[37,38] Our data supported that claim by reporting a drastic increase in ROS production following treatment with 5 μg/mL Ag-NP (Supporting Information, Figure 2). Therefore, as a possible secondary mechanism for cytotoxicity, we also investigated whether silver nanoparticles were able to interfere with growth factor signaling. Even though a subtoxic dose of Ag-NP diminished Akt and Erk phosphorylation, Akt activity was unaffected and gene transcription was minimally impacted. Therefore, while it is improbable that signaling interruption was a leading cause of silver nanoparticle toxicity, this phenomenon may work in conjunction with ROS production to increase cellular apoptosis. In addition, recent research has raised the question of whether nanosilver-dependent cytotoxicity is induced by the nanoparticles themselves, the dissolution of these particles into ions, or a combination of both.[39,40] While it was undetermined in this study if the Ag-NPs or the Ag ions are responsible for the interruption in EGF signaling, elucidating the role of ions in the disruption of biological events is worthy of further investigation.

Contrary to silver, we believe that the signaling interference provoked by gold potentially has significant cellular ramifications. Gold has been used for centuries as a medicinal agent and is particularly recognized for its treatment of rheumatoid arthritis.[41] Although the exact mechanism is unclear, it is believed that gold's therapeutic benefits arise from its ability to scavenge free radicals, thus reducing intracellular ROS.[42] Gold's ability to act as an antioxidant appears to be retained on the nanoscale,[43] with our results demonstrating a marginal reduction in ROS following Au-NP exposure (Supporting Information, Figure 2). More notably, we established that gold nanoparticles

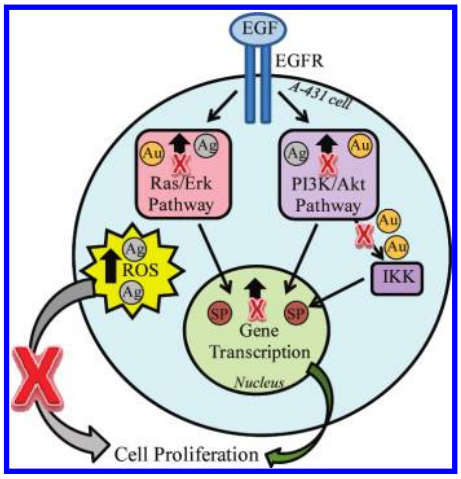

Figure 5. Sites of cellular disruption by metallic nanoparticles. This model depicts the different cellular events in which silver (Ag), gold (Au), and SPION (SP) nanoparticles were found to interfere.

were capable of obstructing Akt and Erk phosphorylation (Figure 3) and impeding Akt's function as a protein kinase (Figure 4) following EGF stimulation. Akt and Erk are both dominant players in the inflammatory response,[44,45] so blocking their activation could potentially negatively regulate inflammation and arthritis. We speculate that gold's capacity to reduce the efficacy of signal transduction, and thus impede the inflammatory response, is imperative to its therapeutic benefits. Therefore, through careful design and implementation, Au-NPs, and their subsequent attenuation of Akt and Erk signaling, could potentially be used in the development of therapeutics targeting cancer and inflammation.

SUMMARY AND CONCLUSIONS

The prevalence of Ag-NPs, Au-NPs, and SPIONs in everyday objects, military applications, and medical procedures is rapidly increasing. While the general toxicity or biocompatibility of these nanoparticles has been extensively studied, it is still unknown what biological repercussions may occur following chronic, low level exposure. In this study, it was discovered that 10 nm Ag-NPs, Au-NPs, and SPIONs all impeded EGF-dependent signal transduction, but each through a different mechanism as summarized in Figure 5. In addition to producing high ROS levels, Ag-NPs reduced Akt and Erk signaling. Au-NPs significantly diminished p-Akt and p-Erk levels as well as inhibiting Akt activity. Lastly, SPIONs caused considerable alterations to EGF-dependent gene transcription. Succinctly stated, pretreatment with these metallic nanoparticles reduced the effectiveness, performance, and consistency of the cellular response to EGF. However, one major challenge that is often associated with using an *in vitro* model is the difficulty in extrapolating results to an *in vivo* system. Nevertheless, we believe for this paradigm an *in vitro* model can serve as a general screening

method used to establish which signaling networks are prone to influence by selected nanomaterials, prior to *in vivo* experimentation. In conclusion, while low concentrations of nanoparticles appear to be biocompatible, based on cytotoxicity, we showed that alterations to cellular functions do occur on both a protein and genomic level. These findings raise the question of whether long-term exposure may create functional defects, and call for further investigation into the impact of nanomaterials on biological processes.

MATERIALS AND METHODS

Nanoparticle Characterization. The Ag-NPs and Au-NPs were received from nanoComposix (San Diego, CA) and the SPIONs were purchased from Nanocs (New York, NY) all dispersed in solution. Nanoparticle solutions used for dosing were made up fresh prior to each experiment to avoid extensive agglomeration. Primary nanoparticle size and morphology were verified using transmission electron microscopy (TEM) and viewed using a Hitachi H-7600. Dynamic light scattering (DLS) was used to determine the nanoparticle dispersion in cell culture media using the method previously established by Murdock *et al.*[23] Furthermore, a zeta potential analysis was performed on each nanoparticle to ascertain the surface charge and stability. DLS and zeta potential analyses were performed on a Malvern Zetasizer.

Cell Culture. The human epithelial A-431 cell line was purchased from ATCC (Manassas, VA) and utilized in this study, due to its well characterized overexpression of the EGF receptor.[17] The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (ATCC). Recombinant human epidermal growth factor (EGF) was used for cell stimulation and was purchased from Peprotech (Rocky Hill, NJ). During experimentation, A-431 cells were seeded in either 6- or 96-well tissue culture plates (Fisher Scientific, Pittsburgh, PA) and maintained at 37 °C and 5% CO_2. For the EGF stimulation assays, the cells were placed in serum free media for 24 h to ensure any response observed was due to the EGF and not another serum component.

Nanoparticle Uptake and Cellular Association. For TEM imaging of nanoparticle uptake, cells were seeded in a six-well plate at a density of 1×10^6 per well and allowed to adhere and grow for 24 h. The cells were then dosed with nanoparticle at a concentration of 15 μg/mL and incubated for 24 h to allow for internalization. Thereafter, the cells were fixed in 2% paraformaldehyde/2% glutaraldehyde for 2 h, postfixed with a 1% osmium tetroxide solution for 1 h, and dehydrated using increasing ethanol concentrations. Cell pellets were cured overnight in 100% LR White resin (Electron Microscopy Sciences, Hatfield, PA) at 60 °C. The samples were thin-sectioned on a Leica ultramicrotome and TEM imaged.

For cellular association studies, 2×10^5 A-431 cells were plated on a chambered slide and allowed to proliferate for 24 h. The cells were then exposed to 15 μg/mL of each nanoparticle and incubated for an additional 24 h. The cells were then fixed with a 4% paraformaldehyde solution and stained with Alexa 555-phallodin and DAPI (Invitrogen, Carlsbad, CA), to visualize the actin and the nucleus, respectively. The slides were sealed and imaged using the CytoViva 150 ultraresolution attachment on an Olympus BX41 microscope (Aetos Technololgies, Inc., Auburn, AL)

EGF Binding Assay. To assess nanoparticle-EGF nonspecific binding, 100 μg/mL of each type of nanoparticle was dispersed in serum free media containing 5 ng/mL EGF and incubated for 24 h at 37 °C. The samples were then centrifuged at 15000 rpm for 15 min to remove the nanoparticles from the media. The EGF concentration in the supernatant was then evaluated using the human EGF ELISA kit from Peprotech. The ELISA was performed according to the manufacturer's protocol, developed with ABTS substrate (Sigma Aldrich, St. Louis, MO), and the absorbance was read at 405 nm using a SpectraMAX Gemini Plus microplate reader.

Preparation of Cell Lysates and ELISAs. For cell lysate collection, A-431 cells were seeded in a 6-well plate at a density of 1×10^6 per well and grown for 24 h. The cells were then washed and incubated in serum free media containing the stated nanoparticles or concentration of hydrogen peroxide for 24 h. Cell samples not exposed to nanoparticles served as a control. The cells were then stimulated for 2 h with 5 ng/mL EGF. This EGF concentration was selected because it was found to induce Akt and Erk signaling to a half maximal value (data not shown), which is optimal for observing any alterations in the signaling response. After washing in cold PBS, the cells were lysed in nondenaturing lysis buffer (Cell Signaling Technology, Beverly, MA) containing phosphatase and protease inhibitors. The phosphorylation levels of Akt, Erk, and IKK-α were ascertained by performing an ELISA analysis on the cell lysates using kits from Cell Signaling Technology. These ELISAs determine the phosphorylation states of Ser473, Thr202/Tyr204, and Ser176/180 for Akt, Erk, and IKK-α, respectively. In all cases, protein phosphorylation was normalized by the total amount of the same protein, quantified in parallel according to the manufacturer's protocols.

Gene Expression Analysis. Real-time PCR was used to determine the interference of nanoparticles on EGF-dependent gene expression. Cells were seeded at a density of 1×10^6 cells per well in a 6-well plate and cultured in growth media overnight. The cells were then washed and dosed with nanoparticles dispersed in serum free media for a duration of 24 h. Control samples were incubated with serum free media only. All cultures were then stimulated by treating with 10 ng/mL human EGF. After 24 h the RNA was isolated from each culture using the RNeasy Mini Kit from Qiagen (Valencia, CA) and quantified using the NanoDrop spectrophotometer (ThermoScientific, Waltham, MA). Each RNA sample was processed and converted to cDNA using the RT2 First Strand kit from SABiosciences (Frederick, MD), and the human EGF/PDGF signaling PCR Array (SA Biosciences) was run in accordance with the manufacturer's protocol. The data collected from the PCR arrays was analyzed with the aid of the SABioscience software.

Statistical Analysis. Data is expressed as the mean $\pm$ the standard error of the mean (SEM). A two-sample *t* test (MS Excel, Microsoft Corporation, Redmond, WA) was used to determine statistical significance with an asterisk denoting a p-value ≤ 0.05.

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Supporting Information Available: TEM images of nanoparticles, biocompatibility procedures and results, and the impact of hydrogen peroxide on Akt and Erk signaling. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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