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# Effect of Surface Coating on the Toxicity of Silver Nanomaterials on Human Skin Keratinocytes

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## Abstract

As nanotechnology field continues to develop, assessing nanoparticle toxicity is very important for advancing nanoparticles for daily life application. In this Letter, we report the effect of surface coating on cyto, geno and photo-toxicity of silver nanomaterials of different shapes on human skin HaCaT keratinocytes. We found that the citrate coated colloidal silver nanoparticles at 100 μg/mL level are not geno-, cyto- and phtotoxic. On the other hand, citrate coated powder form of the silver nanoparticles are toxic. We have demonstrated that coating of the silver nanoparticles with a biodegradable polymer prevents the toxicity of the powder. Toxicity mechanism has been discussed.

## Introduction

The nanoscience revolution that sprouted throughout 1990s is becoming a part of our daily life in the form of cosmetics, food packaging, drug delivery systems, therapeutics, biosensors, and others[1–8]. Due to antibacterial and odor-fighting properties of silver nanoparticles which are on the size scale similar to that of biological macromolecules, they are extensively used for a number of commercial products such as wound dressing, detergents or antimicrobial coatings already in the market[1–2,9–11]. As of 2007, the Project on Emerging Nanotechnologies at the Woodrow Wilson International Center for Scholars had compiled a list of more than 500 consumer products that claim to contain engineered nanomaterials, and of these products, about 20% contain silver nanoparticles[1–2]. Given the increasing use of silver nanomaterials in commercial products, their potential for release into the environment and effects on environmental health is of increasing concern[13–24]. For this purpose, it is inportant to understand whether nanomaterials retain their nominal nanoscale size, original structure, and reactivity in the environmental system. The lack of this scientific knowledge of environmental fate, coupled with the increased use of Ag-NMs in manufactured goods, has led to an increasing concern among the scientific community of the potential environmental impact Ag-NMs (nanomaterials) can be? Recently, there are reports of toxicity of spherical silver nanoparticles to bacteria and zebrafish[12–17], but there are no report how the surface coating can control the enovironmental fate and toxic effect of silver nanomaterials. More over, recent studies[18] have demonstrated that NMs undergo a multitude of transformations in the environment and environmental media, as a result, environmental fate and state of agglomeration/aggregation or dissolution depend on how the Ag-NMs are prepared, what type of surface coating they have and the conditions under which they are

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used. As a result, environmental fate is likely to be highly variable within a range of surface functionalizations that can make the same material biocompatible or biohazardous. This variability motivated us to do systematic investigation of the role of surface coating which can have important role in environmental fate of Ag-NMs with different shapes and ultimately, toxicity. In daily life applications, silver nanoparticles are mostly used in the powder form, and as a result, it is important to understand how the chemical properties and toxicity changes as we move from colloidal to powder form. Driven by the need, in this Letter, we have reported the fate and cyto and geno toxicity of silver nanomaterial in powder form and compared the results with their colloidal form. Since it is known in the literature that long-term exposure to silver nanomaterials based products by the skin may result in discoloration of the skin, called argyria[16–17], here we report the fate and toxicity of colloidal and powder silver nanomaterials in the presence of light. For this purpose, we performed two different experiments: 1) Silver nanomaterials in colloidal or powder form was first exposed to sunlight for 1–3 weeks before measurement of toxicity in HaCaT keratinocyte cells and 2) we monitored the photo-toxicity of silver nanomaterials, in colloidal or powder form in the presence of light. Since silver nanorod is known to be toxic due to the presence of CTAB surfactant as coating material[12–20], we have only tested toxicity of silver nanoparticle and nanoprism in colloidial and powder form.

## Experimental Methods

### Chemicals

Silver nitrate ($AgNO_3$), sodium borohydride ($NaBH_4$), ascorbic acid (AA), trisodium citrate (TSC), cetyl trimethylammonium bromide (CTAB), polystyrenesulfonate (PSS), polyvinylpyrrole (PVP), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO).

### Shape Dependent Silver Nanomaterial Synthesis and Characterization

Silver nanospheres of 30 nm diameter were prepared according to conventional citrate reduction method, as reported before[21,23]. Silver spheres show only one principal plasmon band around 430 nm. Spherical nanoparticles were also characterized by TEM images (Figure 1). Silver nanoprisms of 30 nm size were prepared using a two-step seed mediated procedure using PVP as reported recently[24]. Silver nanoprisms were characterized using TEM images (Figure 1) and absorption spectroscopy.

### Cell viability assay

To study the cytotoxicity of silver nanomaterials, human skin HaCaT keratinocytes, a transformed human epidermal cell line, was obtained from Dr. Norbert Fusenig of the Germany Cancer Research Center, Heidelberg, Germany. The HaCaT cells were grown in Dulbecco's Modified Eagle's Medicum (DMEM) with 10% FBS in 25 $cm^2$ culture flasks in a humidified incubator with 5% $CO_2$ at 37°C. After treating the cells with silver nanomaterials at different time intervals, cell viability was determined using the MTT assay as described before[15,22].

Comet Assay was used to study whether exposure to silver nanoparticles damages cellular DNA. The alkaline Comet assay procedure is described briefly as published before[15]. A total of 75 cells per sample were scored to calculate the amount DNA damage using a fluorescence microscope equipped with a DNA Damage Analysis Software (Loates Single Cell Gel/Comet assay software).

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### Phototoxic Effect

To understand the phototoxic effect of silver nanomaterials, two different experiments were performed: 1) silver nanomaterials in colloidal or powder form were exposed to sunlight for 1–3 weeks before cytotoxic test in HaCaT keratinocytes cell were carried out; 2) HaCaT keratinocytes mixed with silver nanomaterials in colloidal or powder form while irradiated by a 300 W Xe lamp for 60 min before cytotoxicity and genotoxicity tests were carried out using MTT and Comet assays. The light dose applied was 3.3 J/cm$^2$ of UVA and 6.3 J/cm$^2$ of visible light. After irradiation, the control and light irradiated cell plates were incubated for 30 min at 37°C before MTT and Comet assay.

## Results and Discussion

Our TEM data (Figure 2A and 2B) show that both the 30 nm silver nanoprisms and 30 nm silver nanoparticles can penetrate through HaCaT cells easily and accumulate in the nucleus. We have also seen that citrate and PVP coated gold nanomaterials can penetrate HaCaT cells easily. Our experimental results also show that silver nanomaterials are aggregated inside the cell.

The amount of silver nanoparticles inside the cell was determined using high-resolution ICP-OES and ICP-MS. As shown in Figure 2C, it takes about 7 hrs to reach saturation of the uptake for all the different silver nanomaterials (100 μg/ml) with similar dimensions. Our result clearly shows that (as shown in Figure 2C) for the silver nanomaterials of same size, the uptake is nearly the same. Uptake for dried NP was slightly lower and it may be due to the change of size of NP. We have also noted that uptake of silver level is almost the same when we exposed the cell to 10 μg/ml $AgNO_3$.

Figure 3 shows the viability of HaCaT cells after exposure to silver colloidal nanoparticles and nanoprisms at 100 μg/ml for 24 hrs. There is no difference in cell viability between treated or untreated cells with silver nanoparticles and nanoprisms in colloidal form. This indicates that spherical silver nanoparticles and silver nanoprisms in colloidal forms are not inherently toxic to human skin keratinocyte cells. To further confirm the lack of toxicity, we also tested silver nanoparticles of different sizes between 20 to 80 nm and extended the exposure to 48 hrs. All these tests did not result in observable toxicity (data not shown). On the other hand, silver nitrate, the starting material and a non-nano counterpart, is highly toxic even at 10 μg/ml level. As shown in Figure 4, 90% reduction of cell viability is observed after 24 hrs exposure. There are several possible ways $Ag^+$ can interact with cells aqueous solution[12–17], and it is well known in the literature. So our data show that 1) spherical silver nanoparticles and silver nanoprisms at 100 μg/ml level are not cyto-toxic even after 48 hours exposure, whereas silver nitrate is highly toxic at same dose level and 2) silver nanomaterials do not form micro gram (μg) levels of silver ions till 48 hours exposure on HaCaT keratinocytes cell line.

To understand whether silver nanomaterial induces genotoxicity, Comet assay was used to detect possible DNA damages caused by exposure to silver nanomaterials in colloidal form and compared their results with $Ag^+$. The alkaline Comet assay can detect both single and double stranded DNA breaks with high sensitivity. As shown in Figure 3B, the cells appear to be intact after exposure to colloidal silver nanoparticles or nanoprisms, while exposure to silver nitrate causes DNA fragmentation. This indicates that colloidal spherical silver nanoparticles and silver nanoprisms are not genotoxic, whereas silver nitrate is genotoxic even at 10 μg/ml. Since the early 20$^{th}$ century, doctors have known that silver nanomaterial or silver compounds can cause some areas of the skin and other body tissues to turn gray or blue-gray, called Argyria[25–26]. To understand the toxicity of colloidal silver nanomaterials in the presence of light, we performed two different experiments. Silver nanomaterial in

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colloidal form was exposed in sunlight for 1–3 weeks their toxicity in HaCaT keratinocyte cells was measured. As shown in Figure 3C, nanoparticles and nanoprisms in solution are not toxic even after 3 weeks of exposure to sunlight, whereas silver nitrate causes 98% of the cells to die after one week exposure to sunlight and with only one half level of the dose. Therefore, colloidal silver nanoparticles and nanoprisms are stable and do not generate microgram levels of silver ion. In the second experimental condition, HaCaT keratinocytes were incubated with silver nanomaterials in the colloidal form and concurrently irradiated by a 300 W Xe lamp for 60 min. Damage to the cellular DNA was evaluated using Comet assay. As shown in Figure 3D1, there is a clear DNA damage when exposed to and $AgNO_3$ and light. Figure 3D2 shows that silver nanoparticles and nanoprisms of different sizes do not cause any DNA damage after 60 min exposure to light, whereas $AgNO_3$ causes significant amount of DNA damage when irradiated with light, even at 10 μM concentration. To understand whether DNA damage happened due to the formation of the reactive oxygen species (ROS), ROS generation was determined using dichloroofluorescein diacetate (DCFH-DA based established fluorescence assay[30]. Hydrogen peroxide was used as a standard for ROS measurements and intracellular ROS concentrations due to silver exposure were determined with respect to $H_2O_2$. As shown in Figure 3E, our results showed that $Ag^+$ ions can generate intracellular ROS as also reported by others[25,27], whereas freshly prepared silver nanoparticles and nanoprisms do not generate ROS. Our ROS measurement results clearly shows that $Ag^+$produce reactive oxygen species (ROS), which may be an explanation as to why $Ag^+$ is toxic at even 10 μM concentration.

To produce powder form of silver nanomaterials from freshly prepared colloidal nanoparticles, silver nanoparticles were concentrated through several centrifugations and decanting of the supernatant. After that, the nanoparticles were dried under sunlight irradiation for several days or under mild heating (40°C). After the powder was produced, the original spherical silver nanoparticles were no longer soluble in water, buffer or cell media. To understand what type of structural changes happened in powder form, TEM images of the powder were taken. As shown in Figure 4A, spherical silver nanoparticles agglomerated in the powder form. Figure 4B is the TEM image of dried silver nanoparticles after exposure to sunlight for 2 weeks. It shows that nanoparticle agglomeration increases with exposure to sunlight. Since the powdered silver nanoparticles become very dark as viewed by TEM images in Figure 4A and 4B and are no longer soluble in aqueous solvents, we believe that the protective layer of citrate ions on the silver nanoparticles is lost during drying and/or sunlight irradiation process. As a result, the silver atoms in the nanoparticles are exposed to oxygen and other gases in the air directly and oxidation of silver occurred. It is reported that silver can be oxidized into silver oxide by air[27–28] and citrate ion can protect silver metal from oxidation[29].

On the contrary, there is no aggregation at all for the PVP-coated silver nanoprisms after drying and two further weeks under sunlight irradiation (Figure 4D). In fact, the powder form of nanoprism is highly soluble in water like their colloidal form. Since nanoprisms are coated by PVP and their powder form is soluble in water, we believe that PVP coated nanoprisms are highly stable in the powder form. To test our hypothesis, we also synthesized PVP coated colloidal silver nanoparticles and made powder form the same way as for the citrate coated silver nanoparticles. Surprisingly, PVP coated spherical nanoparticle powder is highly soluble in water. As shown in Figure 4C, PVP-coated spherical silver nanoparticles are highly stable even after two weeks of exposure to sunlight. Therefore, PVP coating is a must for using silver nanoparticles in powder form.

Since silver nanoparticles in powder form are not soluble in water or buffer or cell media, we have exposed the powder in cell media first followed by sonication and vortexing. After that, HaCaT keratinocyte cells were exposed to the powder form of silver nanoparticles for

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24 hrs. For the powder form of silver nanoprisms, it was dissolved in water and mixed with cells for 24 hrs. As shown in Figure 4E, silver nanoprisms are not toxic either in colloidal or powder form. Whereas, silver nanoparticles exhibit toxicity in the powder form, and not in colloidal form. We believe that this is mostly due to chemical change of spherical silver nanoparticle in the powder form to form silver oxides or ions. Since PVP coated silver nanoprisms do not exhibit toxicity, we also tested cell viability for PVP coated silver nanoparticles, keeping all other conditions the same. As shown in Figure 4E, PVP coated powder form of spherical silver nanoparticles are not toxic even after 3 weeks of exposure to sunlight. Therefore, the toxicity of silver nanoparticles in powder form is due to chemical changes in the powder form and it can be prevented by replacing citrate with a biocompatible and functionalization friendly stabilizing agent PVP. So our data clearly demonstrated that surface coating with biocompatible and functionalization friendly stabilizing agent PVP is very important parameter for daily life applications of silver nanomaterial.

## Conclusions

In conclusion, in this Letter, we have reported the effect of surface coating on shape dependent environmental fate, cellular uptake, and toxic effects of silver nanomaterials to human skin HaCaT keratinocyte cells. Our experimental observation through TEM images indicates that, silver nanomaterials are aggregated inside the cell. Toxicity experiments demonstrated that colloidal spherical silver nanoparticles and silver nanoprisms of 30 nm sizes are not cyto-, photo or genotoxic to human skin HaCaT keratinocyte cells after 48 hrs of incubation, whereas silver nitrate is toxic even at the concentration of 10 μg/ml and also photo toxicity increases in concentration dependent manner. The environmental fate data clearly show that silver undergoes transformation when citrate-coated spherical silver colloids are dried to form powder. Whereas, it remains similar for PVP coated silver nanoprisms as well as PVP coated silver nanoparticles. Our toxicity experiment clearly demonstrated that citrate coated silver nanopowder is toxic due to the chemical structure change during drying process, whereas PVP coated silver nanoprism or nanoparticle powder is not toxic even after 3 weeks of sunlight exposure. So, our result shows that replacing citrate with a biocompatible and functionalization friendly PVP is essential for daily life application. Much more studies are needed to evaluate the stability of these matrices in a variety of test systems to fully determine the potential for human exposure to the nanoscale components of commercially available products, as well as for future products. Future advances will require continued innovations by chemists in close collaboration with experts in environmental and biological fields.

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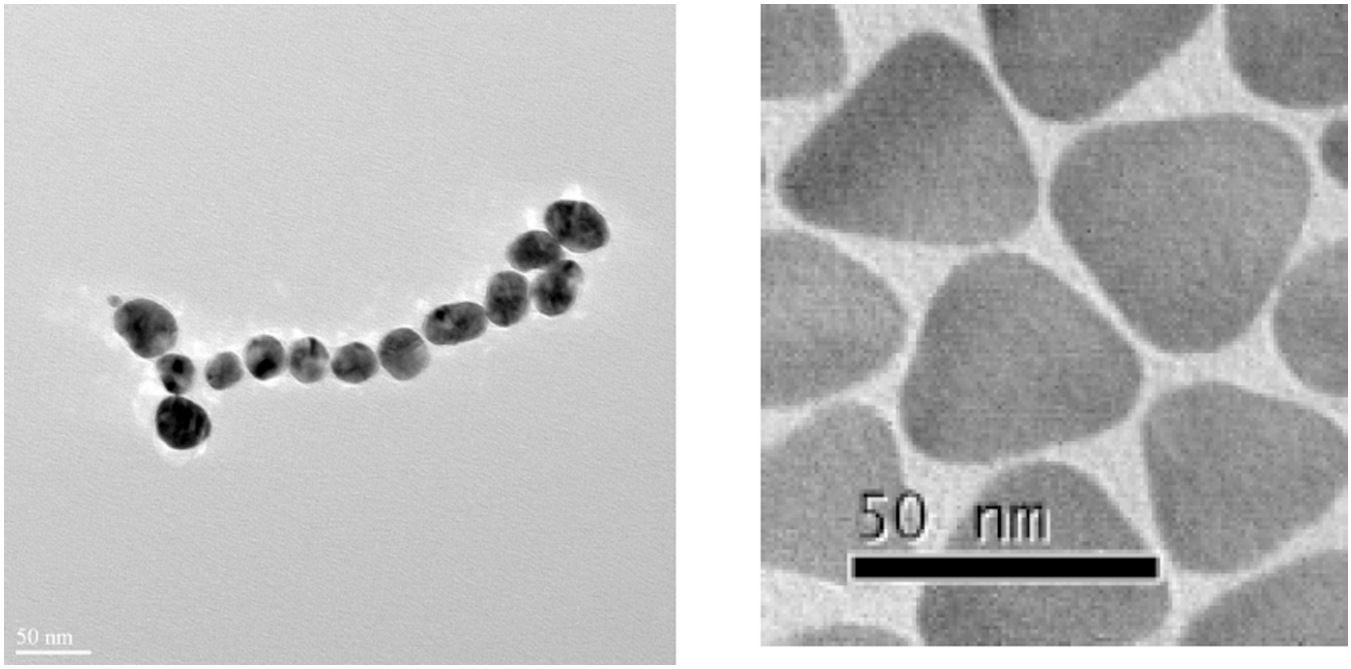


**Figure 1.**
TEM images of silver nanomaterial of ~ 30 nm size with different shapes, a) spherical nanoparticle, and b) nanoprism with edge length of 30 nm.

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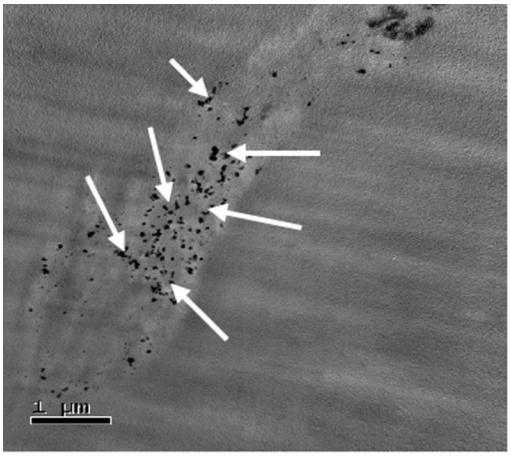


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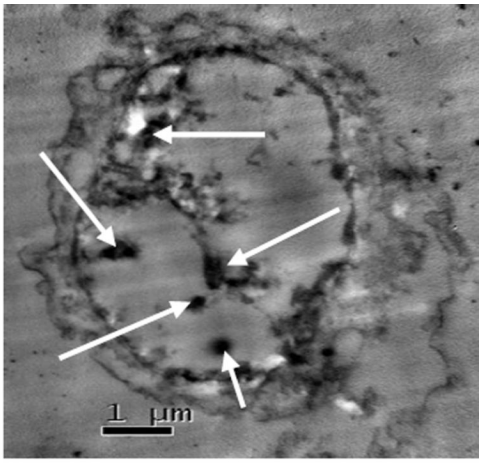


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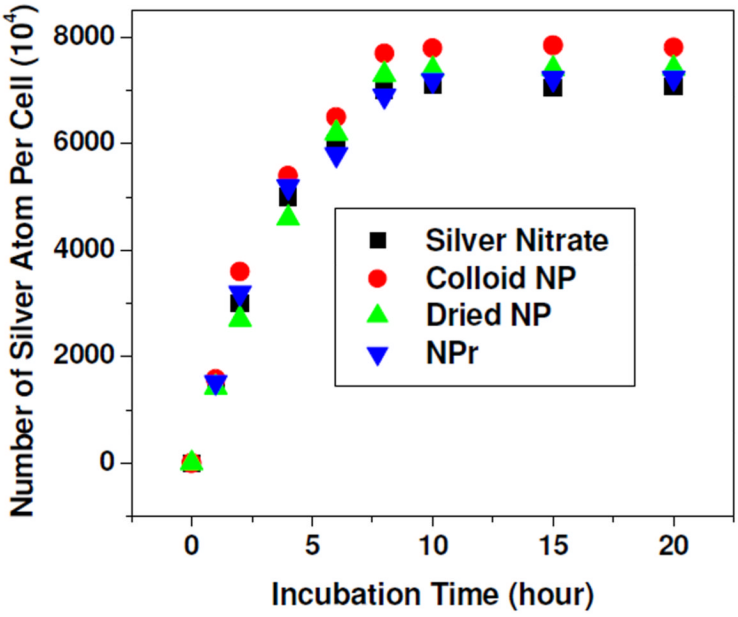


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**Figure 2.**

TEM images showing uptake of PVP coated silver nanomaterials inside the cell (arrows show the nanomaterials). A) Silver nanoprism (30 nm edge length) can be noted in the cycloplasm of the cell. B) Silver nanoparticle (30 nm size) within the granular bodies. C) Demonstrating cellular uptake at different times for various shapes silver nanomaterials (100 μg/ml) of same size and silver nitrate (10 μg/ml).

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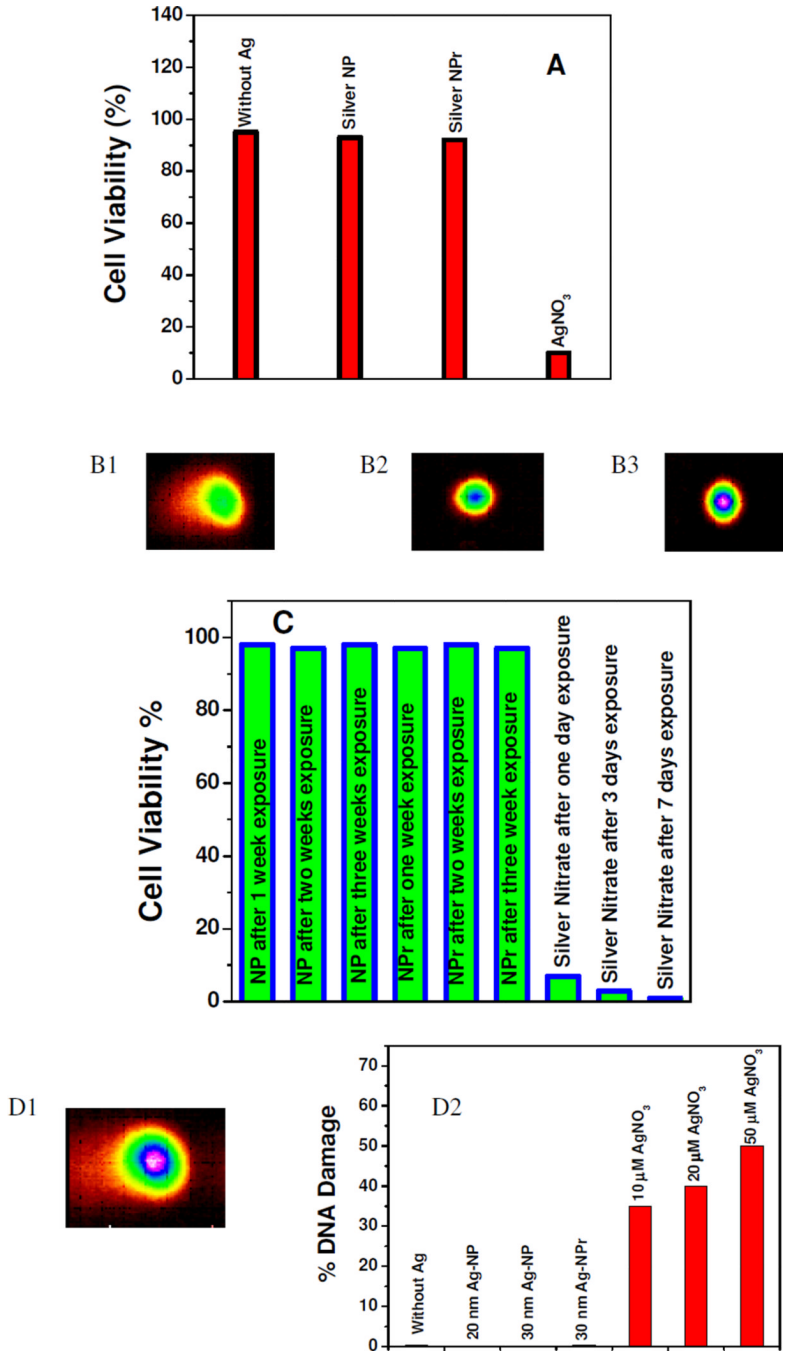

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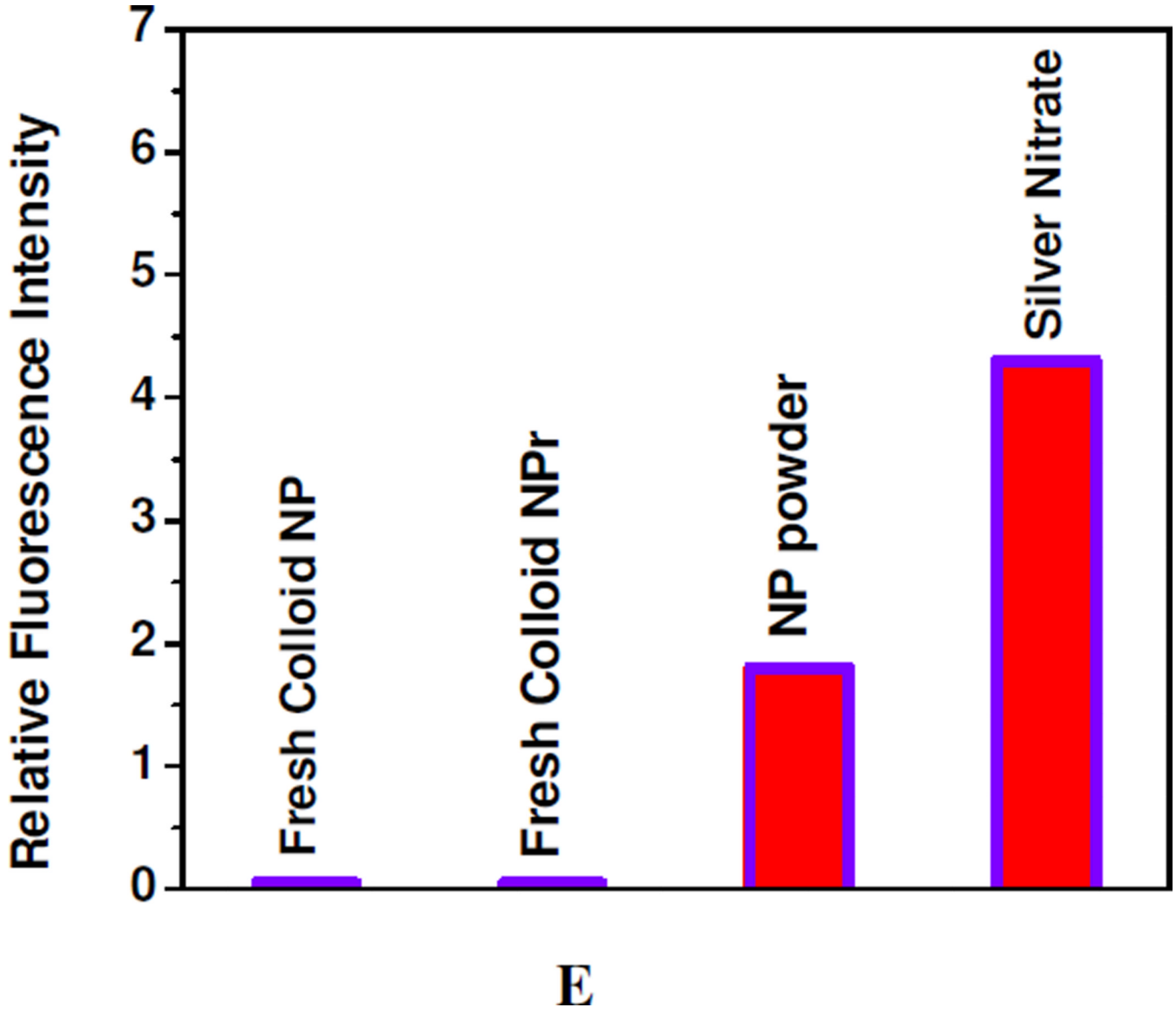


**Figure 3.**

A) Demonstrating HaCaT cell viability after exposure to 100 μg/ml silver nanoparticles (Ag-NP, 30 nm) and silver nanoprisms (Ag-NPr, 30 nm), or 10 μg/ml $AgNO_3$ for 24 hrs. B) DNA damage assessed by alkaline Comet assay. (B1) Silver nitrate (10 μg/ml). (B2) 30 nm silver nanoparticle (100 μg/ml); (B3) 30 nm silver nanoprism (100 μg/ml), C) Cell viability after exposure to sunlight-irradiated (1–3 weeks) colloidal silver nanoparticles (Ag-NP), silver nanoprisms (Ag-NPr) of 30 nm sizes, and $AgNO_3$ (10 μg/ml). HaCaT keratinocyte cell viability was determined at a dose of 100 μg/ml and 24 hours of incubation, D) DNA damage assessed by Comet assay for 1) silver nitrate (10 μM) and B) silver nanomaterials (100 μg/ml) and 2) silver nitrate of different concentrations after 60 min of light exposure. E) Relative fluorescence intensity (with respect to $H_2O_2$) demonstrates the cellular ROS formation capability by silver nitrate and silver nanomaterial.

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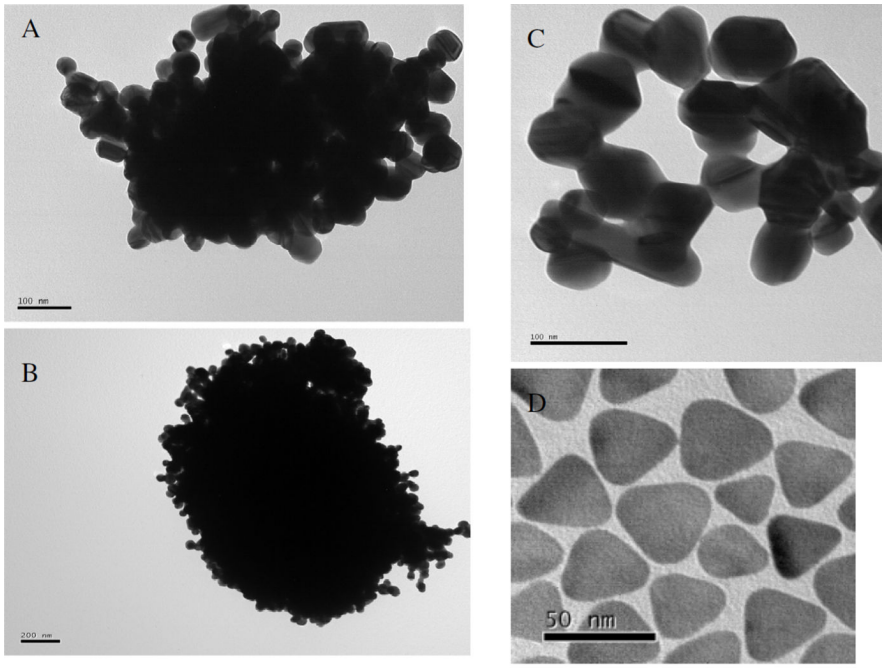


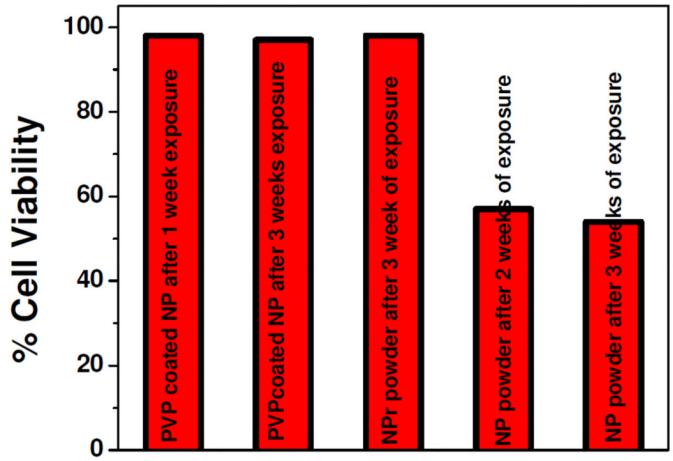


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**Figure 4.**

A: TEM images of spherical silver nanoparticles immediately after it was completely dried; B) after 2 weeks of exposure to sunlight; C) PVP coated dry silver nanoparticles after 2 weeks of exposure to sunlight and D) dried silver nanoprisms after 2 weeks of exposure to sunlight. E) HaCaT keratinocytes cell viability exposed to 100 μg/ml of powder form silver nanoparticles (Ag-NP) and silver nanoprisms (Ag-NPr) and 24 hrs incubation time. Nanomaterials were exposed to sunlight for 1–3 weeks.