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Article

Toxicity of Silver Nanoparticles Increases during Storage Because of Slow Dissolution under Release of Silver Ions

S. Kittler,[†] C. Greulich,[‡] J. Diendorf,[†] M. Köller,*[,‡] and M. Epple*[,†]

[†]*Inorganic Chemistry and Center for Nanointegration Duisburg-Essen* (*CeNIDE*), *University of Duisburg-Essen, Universitaetsstrasse 5-7, 45117 Essen, Germany, and* [‡]*Bergmannsheil University Hospital/ Surgical Research, Ruhr-University Bochum, Bürkle-de-la-Camp-Platz 1, 44789 Bochum, Germany*

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The dissolution of citrate-stabilized and poly(vinylpyrrolidone)-stabilized silver nanoparticles in water was studied by dialysis for up to 125 days at 5, 25, and 37 °C. The particles slowly dissolve into ions on a time scale of several days. However, in all cases, a limiting value of the released silver was observed, i.e., the particles did not completely dissolve. In some cases, the nanoparticles released up to 90% of their weight. Formal kinetic data were computed. Rate and degree of dissolution depended on the functionalization as well as on the storage temperature. The release of silver led to a considerably increased toxicity of silver nanoparticles which had been stored in dispersion for several weeks toward human mesenchymal stem cells due to the increased concentration of silver ions. Consequently, "aged" (i.e., immersed) silver nanoparticles are much more toxic to cells than freshly prepared silver nanoparticles.

Introduction

The antimicrobial action of silver has led to its application in numerous devices, ranging from consumer commodities like mobile phones, refrigerators, and clothes to medical devices like catheters, implant surfaces, and plasters.[1−3] In addition, silver nanoparticles are increasingly popular for these purposes, partly because they offer a slower release of silver ions.[4−6] Other applications involve imaging and immune-detection, making use of the surface-enhanced Raman scattering (SERS) effect of silver nanoparticles.[7,8] There is a general agreement that dissolved silver ions are responsible for the biological action that is especially pronounced against microorganisms.[3,9−12] The lethal silver concentration of silver nanoparticles for human mesenchymal stem cells is about 3 times higher than that of silver ions (in terms of the absolute concentration of silver in a given solution).[13,14] Only very little is known about the rate of dissolution of silver nanoparticles.[15,16] As this rate directly determines the concentration of silver ions in the vicinity of a nanoparticle, it is highly important for any antimicrobial application of silver nanoparticles, and also for assessment of the toxicity of silver nanoparticles in humans. In addition, the final fate of silver nanoparticles that are released into the environment (e.g., from silver-containing clothes into sewage plants) depends on these data. It is likely that the rate of dissolution depends not only on the chemical species (i.e., "metallic silver in nanoparticulate form") but also on the particle size, the surface functionalization, and the particle crystallinity. In addition, the temperature and the nature of the immersion medium (e.g., the presence of salts or biomolecules) will be major factors.

We have therefore prepared and characterized silver nanoparticles with different surface functionalization and studied their dissolution in ultrapure water at three different temperatures. In addition, cell culture experiments with human mesenchymal stem cells (hMSCs) as reporter cells were carried out to elucidate the effect of aging on the toxicity of silver nanoparticles. Mesenchymal stem cells represent tissue cells and are neither transformed cell lines

*Corresponding author. E-mail: matthias.epple@uni-due.de.

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nor immortalized cells but represent primary cells which can be cultured over several passages.

Experimental Section

Synthesis of Silver Nanoparticles. PVP-coated silver nanoparticles were synthesized by reduction with glucose in the presence of PVP according to Wang et al.[17] Briefly, 2 g of glucose and 1 g of PVP were dissolved in 40 g of water and heated to 90 °C. Then, 0.5 g of $AgNO_3$ dissolved in 1 mL of water was quickly added. The dispersion was kept at 90 °C for 1 h and then cooled to room temperature. The particles were collected by ultracentrifugation (30 000 rpm; 30 min), redispersed in pure water and collected again by ultracentrifugation. Thereby, NO_3^-, excess glucose and its oxidation products, excess PVP, and excess Ag^+ were removed. The silver nanoparticles were then redispersed in water. The typical yield with respect to Ag was about 5%.

Citrate-stabilized silver nanoparticles were prepared by dissolving 9 mg of silver nitrate in 50 mL of water and bringing it to boiling. A solution of 1% sodium citrate (1 mL) was added under vigorous stirring. The solution was kept at a boil for 1 h, and then allowed to cool to room temperature. The silver nanoparticles were purified by ultracentrifugation (30 min at 30 000 rpm), followed by redispersion in water. The typical yield of citrate-stabilized silver nanoparticles was around 65% (with respect to silver). The final silver concentration in all dispersions was determined by atomic absorption spectroscopy (AAS).

Poly(vinylpyrrolidone) (PVP K30, Povidon 30; Fluka, molecular weight 40 000 g mol^{-1}), trisodium citrate dihydrate (Fluka, p.a.), silver nitrate (Fluka, p.a.), and D-(+)-glucose (Baker) were used. Ultrapure water was prepared with an ELGA Purelab ultra instrument.

Cell-Biological Studies. Human mesenchymal stem cells (hMSCs, third to seventh passage, Cambrex Bio Science, Walkersville Inc., MD) were cultured in cell culture medium RPMI1640 (GIBCO, Invitrogen GmbH, Karlsruhe, Germany) containing 10% fetal calf serum (FCS, GIBCO, Invitrogen GmbH) and L-glutamine (0.3 g L^{-1}, GIBCO, Invitrogen GmbH) using 24-well cell culture plates (Falcon, Becton Dickinson GmbH, Heidelberg, Germany). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Subconfluently growing hMSCs were incubated at 37 °C in the presence or absence of different concentrations of silver nanoparticles for 24 h under cell culture conditions. PVP-coated spherical silver nanoparticles (hydrodynamic diameter of 85 nm) were dispersed in sterile ultrapure water at 1 mg mL^{-1} as stock solution. The final concentrations of 50, 25, 20, 15, 5, 2.5, and 1 mg L^{-1} were achieved by the addition of 50 μL of silver nanoparticle dispersion to a 1 mL cell volume prior to cultivation. The solutions for addition to the cell cultures were prepared by serial dilutions of a 1 g L^{-1} stock solution with sterile ultrapure water. This stock solution was stored until dilution at 5 °C in a closed vessel. After 3 days, 1 month, and 6 months, respectively, particles were taken for the cell biological studies.

The cell viability and the morphology of incubated cells were analyzed using calcein-acetoxymethylester (calcein-AM, Calbiochem, Schwalbach, Germany) fluorescence staining. After culture for 24 h in RPMI1640 supplemented with 10% fetal calf serum (FCS; note that the particles did not agglomerate under these conditions[18]), the nanoparticle-treated cells were washed twice with RPMI1640 and then incubated with Calcein-AM (1 μM) for 30 min using cell culture conditions. Subsequently, the adherent cells were washed again with RPMI1640 and then analyzed by fluorescence microscopy (Olympus MVX10, Olympus, Hamburg, Germany). Fluorescence microphotographs were taken with an F-view camera (Olympus) and digitally processed using the Cell P software (Olympus). Quantification of viable adherent cells was performed by digital image processing using phase analysis (software Cell P).

The data are expressed as mean ± SD of three independent experiments. Statistical analysis was performed by the *t*-test. Group differences were analyzed by the Mann−Whitney *U* test. *P* values of less than 0.05 were considered to be statistically significant.

Analytical Methods. Scanning electron microscopy (SEM) was performed with a FEI Quanta 400 ESEM instrument in high vacuum without sputtering. The hydrodynamic diameter and the zeta potential were measured by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS. In all cases, the z-average value was used as average particle diameter. The polydispersity index (PDI) was below 0.3 in all cases. The concentration of silver was determined by atomic absorption spectroscopy (AAS; Thermo Electron Corporation, M-Series). The detection limit was 1 μg L^{-1}.

Dialysis experiments were carried out in dialysis tubes (Spectra/Por Biotech; cellulose ester; MWCO 100 000) filled with either 4 or 5 mL solution or dispersion and immersed within the 100fold volume of ultrapure water (400 or 500 mL). The dialysis was carried out under slow stirring with a magnetic stirrer at 5, 25, and 37 °C. For control experiments with the solution outside the dialysis tube, ultracentrifugation was performed with 66 100 g, followed by nanofiltration with a syringe filter (0.02 μm pore size, Anotop 25).

Results and Discussion

Dissolution Experiments of Silver Nanoparticles. Silver nanoparticles were functionalized by two different compounds. In both cases, they were spherical with a diameter of the metallic core of 50 ± 20 nm as shown by electron microscopy. Spherical citrate-stabilized nanoparticles carried a strongly negative charge (zeta potential −30 mV; hydrodynamic diameter 85 nm). PVP-stabilized nanoparticles carried a less negative charge (zeta potential −17 mV; hydrodynamic diameter 85 nm). High-resolution transmission electron microscopy (HRTEM) showed that both kinds of nanoparticles were crystalline, but highly twinned (see ref 18 for HRTEM data).

The silver nanoparticles were dispersed in ultrapure water after centrifugation, i.e., all counterions or side compounds from synthesis were removed. The dispersions were brought into a dialysis tube which was immersed in a 100-fold excess volume of pure water (Figure 1).

This method implies that the diffusion of the released silver ions through the dialysis membrane is faster than the release of the silver ions from the nanoparticles. In addition, an irreversible interaction of the silver ions with the dialysis membrane must be excluded. Therefore, we have carried out control experiments with dissolved silver nitrate within the dialysis tube and monitored the silver concentration outside the dialysis tube. As it is obvious from Figure 2, the diffusion of Ag^+ out of the dialysis tube was very fast and practically completed after a few hours.

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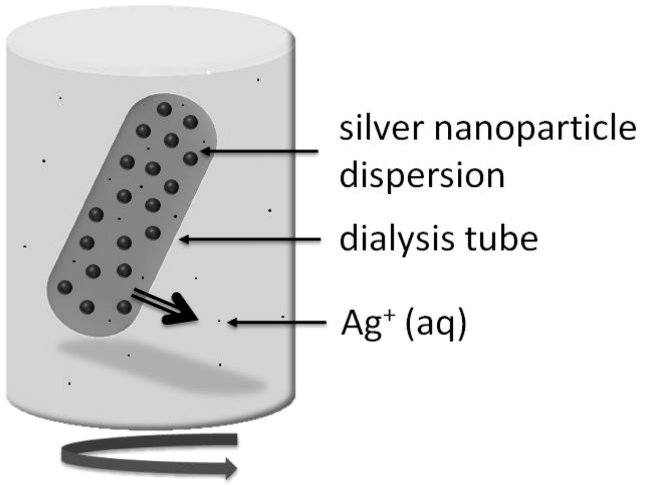

Figure 1. Setup used to monitor the dissolution of silver nanoparticles.

As each silver nanoparticle contains thousands of silver ions, it must be ensured that no nanoparticles are present in the analyzed solution outside the dialysis tube. Therefore, we have carried out a number of control experiments where this solution also underwent ultracentrifugation and nanofiltration to remove any silver nanoparticles that might have escaped the dialysis tube. In all cases, there was no difference between the silver concentrations; therefore, it can be safely assumed that the detected amounts of silver are due to only silver ions and not silver nanoparticles.

Figures 3 and 4 show the dissolution curves for both citrate- and PVP-stabilized silver nanoparticles at different temperature. Interestingly, the dissolution was only partial in all cases, i.e., in no case did the nanoparticles completely dissolve. This was confirmed by analyses of the dispersion inside the dialysis tube after the completion of the experiments where we still found nanoparticles by dynamic light scattering (see Figure 5). The degree of dissolution depended on the surface functionalization (citrate of PVP) and the temperature. An increase in temperature led to an increased degree of dissolution. The final degree of dissolution did not depend on the absolute concentration of silver nanoparticles, i.e., the final concentration of silver ions was not constant (see Table 1). This indicates that the observed final value does not correspond to a physicochemical "equilibrium" in which the concentration of ions in a solution is independent of the amount of solid being present (like in the case of a solubility product). Therefore, the observed degree of dissolution seems to be an intrinsic (but temperature-dependent) property of the nanoparticles. Importantly, the diffusion of silver ions through the dialysis membrane was always much faster than the dissolution process and can therefore be neglected in the kinetic consideration.

The dissolution of silver nanoparticles is obviously a complicated process which cannot be described by a simple model. Nevertheless, all release curves have a deceleratory shape that led to a final value at longer time.

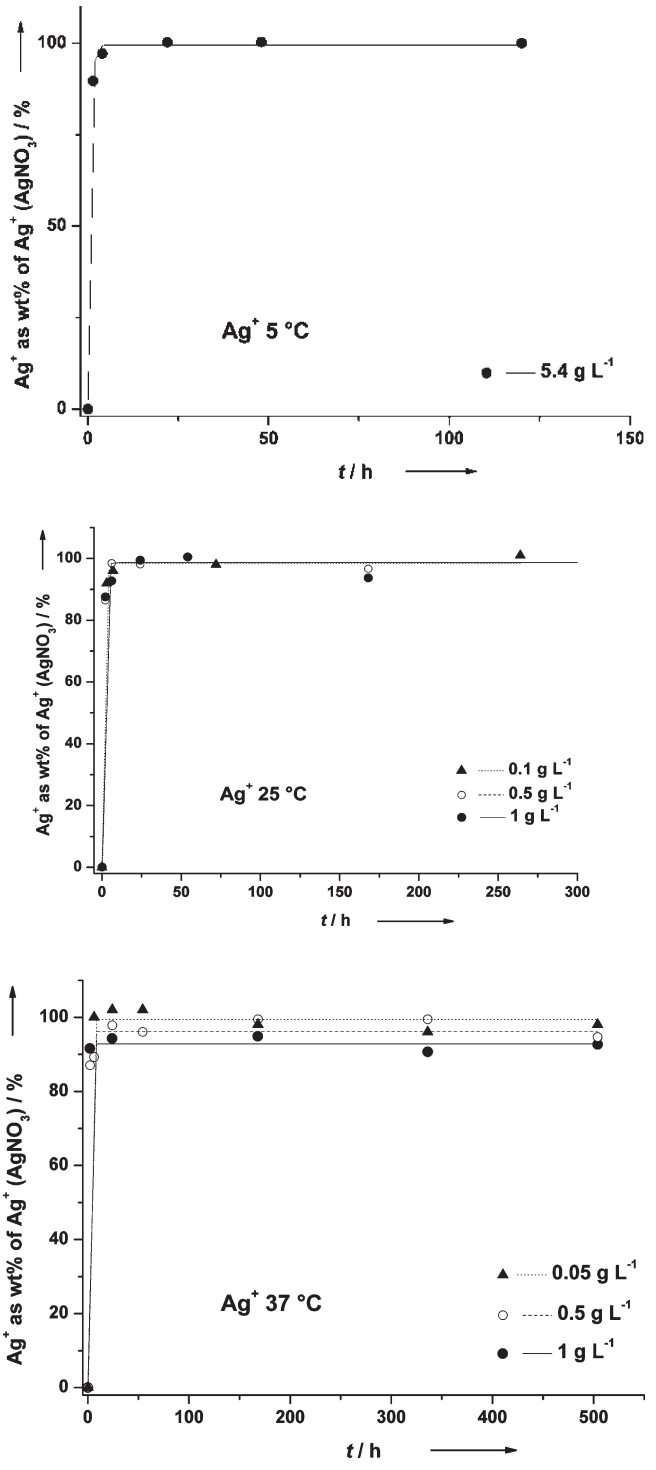

Figure 2. Data collected for the diffusion of Ag^+ (from $AgNO_3$) at 5, 25, and 37 °C. The data were fitted to a modified first-order reaction equation (see Table 1).

We have tentatively described these data by a modified first-order reaction kinetic rate equation of the form

$$y(t) = y(\text{final})(1 - \exp(-kt)) \quad (1)$$

with $y(t)$ the released amount of silver ions (in % of the original value), y(final) the final value of the released silver ions, i.e., extrapolated to $t \rightarrow \infty$ (in % of the original value), k a rate coefficient, and t the time in hours. The values for y(final) and k were extracted from the data by

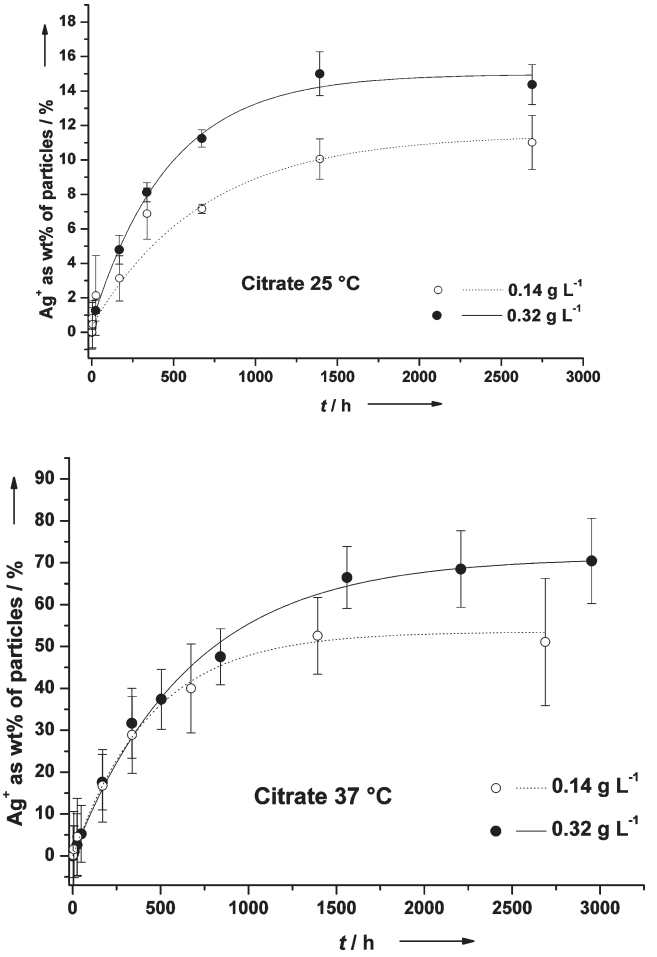

Figure 3. Data collected for the dissolution of citrate-stabilized silver nanoparticles at 25 and 37 °C. The data were fitted to a modified first-order reaction equation (see Table 1).

suitable least-squares fitting. Mathematically, this corresponds to the product formation of a first-order reaction, normalized to y(final), which may be smaller than one (as found for all investigated nanoparticles; y(final) is the degree of dissolution). This treatment has the advantage that the time τ needed to reach y(final)/2 (the half-life time) can be easily computed as

$$\tau = \ln(2)/k \qquad (2)$$

This allows a quick estimation of the time needed for the partial dissolution. Of course, a real half-life time with a turnover of 50% cannot be defined in all cases because y(final) is sometimes smaller than 0.5. Note that these kinetic data are just given for an easier interpretation of the data and do not carry a specific mechanistic meaning. A mechanistic interpretation must take into account inter alia exact particle size and shape distribution data and chemical models about the interactions between silver ions and functionalizing agents. This will be addressed in future studies. All results are shown in Table 1.

The rate of dissolution and the final degree of dissolution were higher for PVP-stabilized nanoparticles than for citrate-stabilized nanoparticles. The reason for this is not known, but it may be speculated that the citrate coating acts as chemical barrier because it is able to reduce

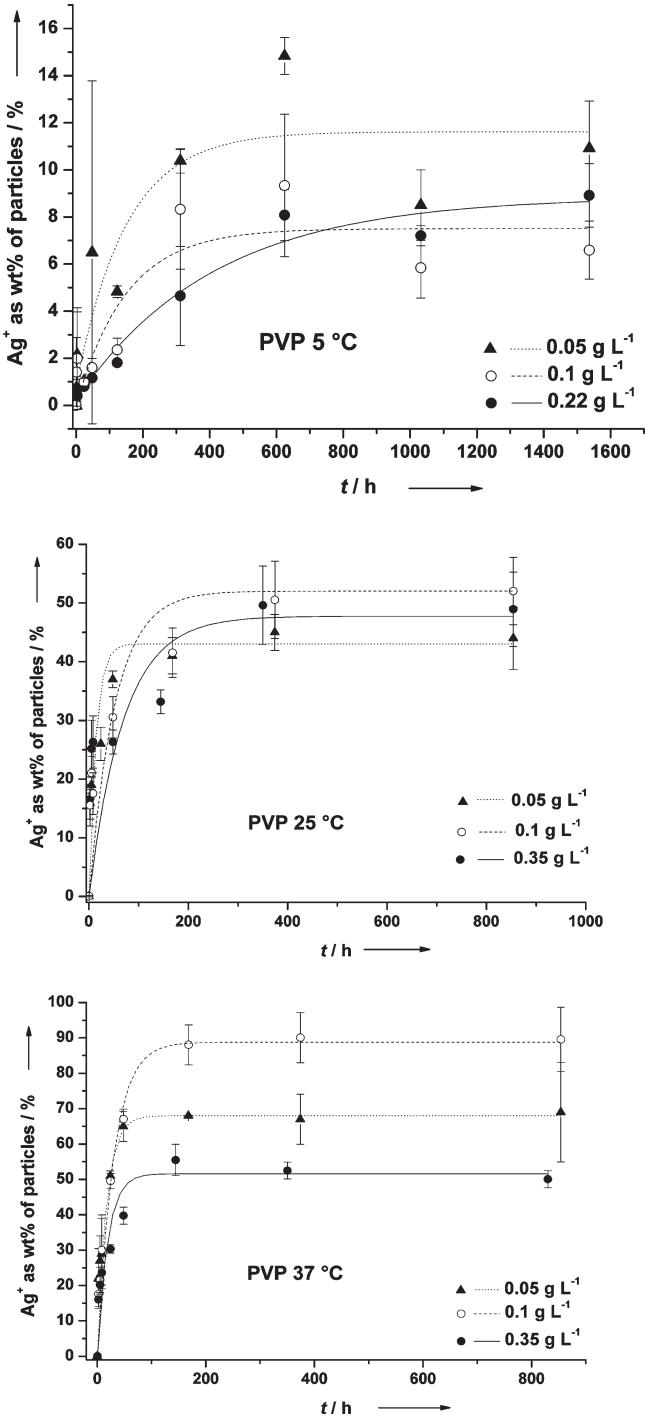

Figure 4. Data collected for the dissolution of PVP-stabilized silver nanoparticles at 5, 25, and 37 °C. The data were fitted to a modified first-order reaction equation (see Table 1).

the outgoing silver ions (as in the synthesis). The nature of the oxidizing species is also not known. Thermodynamically, the only species that would be able to oxidize metallic silver is dissolved oxygen.[15] The dissolution experiments were not carried out under inert gas atmosphere or in an airtight vessel, and therefore this is a reasonable possibility. However, it can be excluded that the rate of diffusion of oxygen from the environment or the amount of initially dissolved oxygen are responsible for the observed dissolution kinetics. In the first case, we would expect a complete dissolution of the nanoparticles

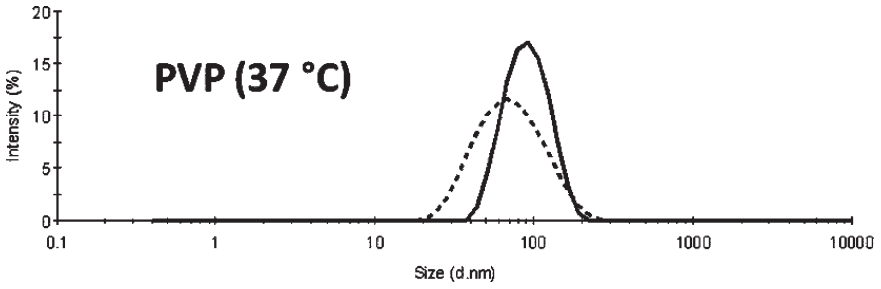

Figure 5. Dynamic light scattering data for PVP-stabilized silver nanoparticles immediately after the preparation (84 nm; PDI 0.295; solid line) and after 35 days at 37 °C in a dialysis tube (56 nm; PDI 0.270; dotted line). The scattering intensity as a function of particle diameter is shown.

Table 1. Formal Kinetic Data for the Dissolution of Silver Nanoparticles, Computed for Formal First-Order Kinetics to a Final Reaction Extent of *y*(final)[a]

sample	T (°C)	β(Ag) (g L^{-1})	β(Ag, final) (mg L^{-1})	y(final) (%)	k (h^{-1})	τ (h)
$AgNO_3$	5	5.4	54.1	100 ± 0.5		< 1.5
$AgNO_3$	25	1	9.7	98 ± 2	0.44	1.6
$AgNO_3$	25	0.5	4.9	99 ± 1	0.75	0.9
$AgNO_3$	25	0.1	0.97	98 ± 2	0.54	1.3
$AgNO_3$	37	1	9.8	99 ± 1		< 2
$AgNO_3$	37	0.5	4.9	96 ± 3	0.39	1.8
$AgNO_3$	37	0.05	0.48	93 ± 2		< 2
citrate-stabilized	25	0.32	0.48	15.0 ± 1	0.0021	336
citrate-stabilized	25	0.14	0.15	11.5 ± 1	0.0017	410
citrate-stabilized	37	0.32	1.8	56 ± 3	0.0023	307
citrate-stabilized	37	0.14	0.73	53 ± 3	0.0016	444
PVP-stabilized	5	0.22	0.19	8.9 ± 0.7	0.0017	411
PVP-stabilized	5	0.1	0.074	7.5 ± 1	0.00047	149
PVP-stabilized	5	0.05	0.057	11.6 ± 1	0.00047	149
PVP-stabilized	25	0.35	1.7	48 ± 3	0.0052	134
PVP-stabilized	25	0.1	0.51	52 ± 3	0.0086	81
PVP-stabilized	25	0.05	0.21	43 ± 3	0.017	42
PVP-stabilized	37	0.35	1.8	52 ± 4	0.025	27
PVP-stabilized	37	0.1	0.88	89 ± 4	0.026	27
PVP-stabilized	37	0.05	0.34	68 ± 4	0.061	11

[a] The relative errors in the rate constants and the half-life times are estimated to ±25%.

over time; in the second case, we would expect always the same amount of dissolved silver.

It must also be emphasized that the tabulated thermodynamic data for silver are valid for the bulk phase only. A given mass of silver nanoparticles will have a higher enthalpy of formation compared to the same amount of bulk silver because of its higher specific surface area and also to energy-rich defects in the surface of the nanoparticles. In addition, the high specific surface area will also enhance the rate of this oxidation reaction in comparison to a macroscopic piece of silver.

The diameter of dispersed nanoparticles decreased during storage. Figure 5 gives a typical image. As the weight and the diameter of a spherical particle are related by the third power, a release of 70% of the silver in ionic form would reduce the diameter by only 33%. Given the fact that the hydrodynamic radius also includes a water-swollen polymer layer, this reduction will be even smaller and may often remain undetected.

Cell-Biological Experiments. Human mesenchymal stem cells were used as reporter cells to analyze the release of silver ons from silver nanoparticles with respect to cell viability and morphology. We have shown previously that a given amount of silver ions is much more toxic than the same amount of silver in the form of nanoparticles.[13,14] Therefore, the cells were cultured in the presence of silver nanoparticles of different "age" and their viability was analyzed by fluorescence microscopy of living cells (green fluorescent calcein). Briefly, the hydrophobic nonfluorescent cell-permanent calcein-acetoxymethylester (calcein-AM) is converted by intracellular esterase activity into the hydrophilic fluorescent dye calcein which is well retained within living cells. Figure 6 shows representative fluorescence micrographs of these experiments. Evidently, aged silver nanoparticles, i.e., after 1 month and 6 months of immersion, caused a complete cell death (no signals of cell viability) compared to silver nanoparticles of 3 days age, which led to a reduction in viability to 70% (control: incubation without silver, i.e., 100% viability). Quantitative analyses of the cell-biological data indicated that the toxicity of the nanoparticle-containing solutions strongly increased with storage time (Figure 7). Nanoparticles that were stored in dispersion for 6 months had a lethal concentration that was about 20 times smaller than that of freshly prepared nanoparticles. This is corroborated by the dissolution experiments (Figures 2− 4), and can be clearly ascribed to silver ions which were released during storage. However, the released silver ions are probably bound by proteins and therefore are rendered less toxic,[18] and they are also probably precipitated in the culture medium as silver chloride or silver phosphate. Consequently, the toxicity of the nanoparticles is not as high as it could be expected from the release curves. Note that the storage before the cell biological experiments took place at 5 °C in aqueous

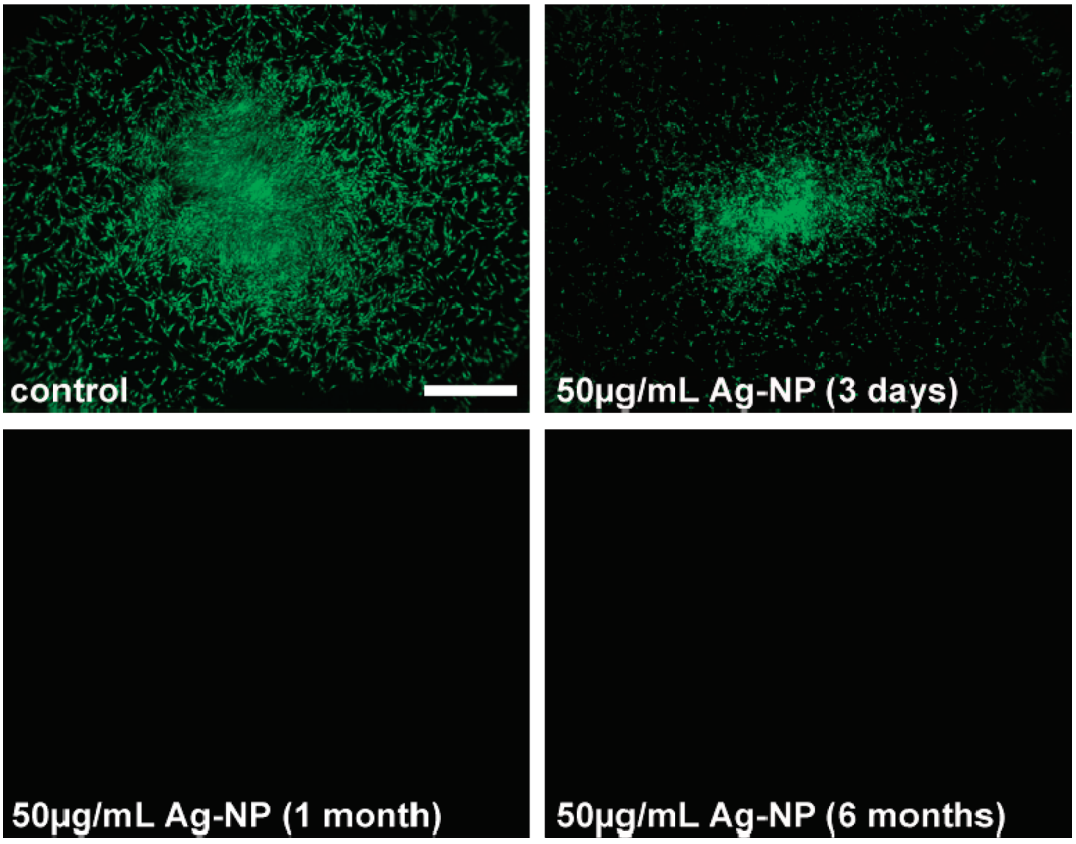

Figure 6. Influence of added silver nanoparticles (Ag-NP) on the viability of human mesenchymal stem cells (hMSCs). Individual wells of a 24-well cell culture plate are shown. The cells were treated with 50 μg mL^{-1} silver nanoparticles of different ages (i.e., immersed in water at 5 °C) for 24 h under cell culture conditions. In the control, no silver was added. Viable cells are indicated by green fluorescence (calcein-AM staining). Scale bar: 2 mm.

dispersion (in pure water), i.e., without contact with biomolecules or other ions, and therefore the dissolution experiments are directly comparable with the cell-biological studies. The instant when the partially dissolved silver nanoparticles in water were given to the cell culture was their first contact with biomolecules or other ions. Ag^+ has been reported to interact with a variety of biomolecules within a cell such as nucleic acids,[19] cell wall components, or sulfhydryl groups of metabolic enzymes.[20,21] Thereby, proliferation, membrane permeability, and different metabolic pathways of cells are interrupted. The mechanism of cell killing is obviously related to more than a single pathway; however, we never observed apoptosis under these conditions (data not shown).

We have carried out a control experiment to confirm that the presence of released silver ions is responsible for the increased toxicity of aged silver nanoparticles. For this, a mixture of freshly prepared PVP-stabilized silver nanoparticles and of silver ions (as silver acetate) was prepared in the silver weight ratio of 90:10. This corresponds to the dissolution at 5 °C, which was the storage temperature of the aged nanoparticles (Figure 4; top).

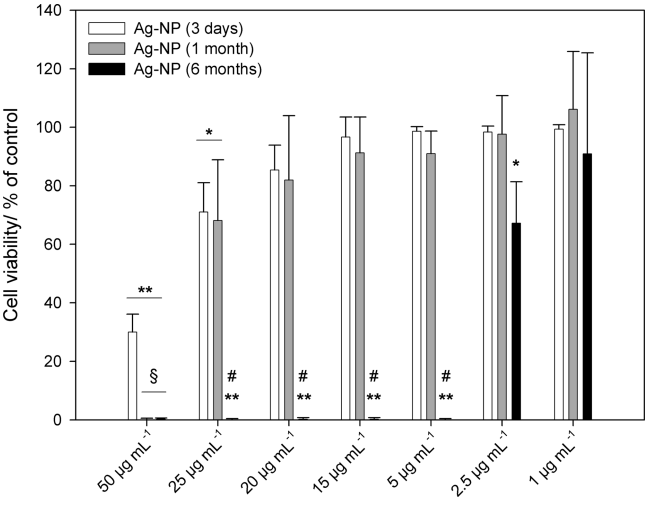

Figure 7. Effect of the age of silver nanoparticles (Ag-NP) after immersion for 3 days, 1 month, and 6 months at 5 °C on the viability of hMSCs. The cells were treated with different concentrations of silver nanoparticles for 24 h under cell culture conditions. Vital cells (green fluorescence) were quantified by digital image processing (phase analysis). The data are expressed as mean ± SD (N = 3 independent experiments) given as percent of control (cells cultured without silver). Significant differences in comparison to the control: * ($p < 0.05$), ** ($p < 0.001$); significant difference compared to 3 day old silver nanoparticles: § ($p < 0.05$); significant difference compared to 3 day and 1 month old silver nanoparticles: # ($p < 0.05$).

The cell viabilities were 98% for 10 and 15 μg mL^{-1}, 43% for 20 μg mL^{-1}, and 0% for 25 μg mL^{-1}. This is between the values for 1 month old and 6 month old silver

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nanoparticles (Figure 7) and confirms the higher toxicity of the aged silver nanoparticles that have partially dissolved. It must be stressed again that the dissolution behavior of nanoparticles in biological media is certainly different from that in pure water because the agents present in these solutions may lead to the deposition of sparingly soluble salts on the particle surface (AgCl, Ag_3PO_4). The fact that released silver ions may precipitate as sparingly soluble AgCl or Ag_3PO_4, may be reduced by sugars or other biomolecules (RPMI contains 2 g L^{-1} glucose), and may be coordinated by small molecules like amino acids (RPMI contains about 0.7 g L^{-1} amino acids) or by proteins (the medium contained 10% fetal calf serum) represents an additional complexity. These fates, however, would be suffered by both silver ions released by nanoparticles and ions added as such into the medium.

Conclusions

Silver nanoparticles are releasing silver ions after immersion in water. This is in line with earlier reports on the dissolution of dispersed nanoparticles by Kühnel et al. on WC/Co,[22] by Xu et al. on FePt,[23] and by Liu et al. on silver.[15] The rate and degree of the dissolution of silver nanoparticles depend on their surface functionalization, their concentration, and the temperature. In a given system under given conditions, a steady state was reached after several hours, i.e., the nanoparticles do not fully dissolve. This will change in a dynamic environment, e.g., during a perfusion experiment. Such changes in the nanoparticle dispersions may escape the attention of the experimentalist because the classical analytical methods (e.g., dynamic light scattering, electron microscopy, or ultracentrifugation) are insensitive to released ions and because the particle diameter undergoes only a minor change. A dynamic light scattering experiment of aged particles would typically be accepted as quality control that the particles did not change during storage, but this experiment would not reveal such dissolution phenomena.

The toxicity of nanoparticles in the body and in the environment is currently under intense discussion and investigation.[24,25] The biological action of freshly prepared and aged nanoparticles is strongly different due to the different amounts of released ions. Unfortunately, the dissolution in a biological medium is much more complicated to measure and describe because of the presence of various compounds in the medium, and the fate of the released silver ions is also unclear. Therefore, the dissolution in pure water gives first indications on the fate of immersed silver nanoparticles in biological environments. Nevertheless, nanoparticles are typically not stored in biological media but in water before they are tested for their biological action, and therefore the reported results represent typical laboratory investigations of nanoparticle toxicity studies. Some published discrepancies in reported toxicological levels may be explained by this fact. Of course, if nanoparticles are stored in the dry state, they will not dissolve, but this is not typical for surface-functionalized, dispersible nanoparticles because of redispersion problems. "Dry" silver nanoparticles that could be embedded in a matrix will also partially dissolve under release of silver ions when they come into contact with water, e.g., with washing water or with rain if applied outdoors.

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