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Incubation of 20 nm silver nanoparticles with human serum albumin (HSA) stabilizes the nanoparticles against aggregation and dissolution and produces particles that can be lyophilized for longterm storage and redispersed in water consistently. Biological studies of this stabilized material showed reduced toxicity and cellular uptake compared to other 20 nm particles.

Nanoparticles are important active components of many commercial products and nanosilver, in particular, has been used in a wide array of products primarily because of its antimicrobial properties.[1] The intended uses of such products result in direct exposure to either embedded or released nanosilver that may pose a health risk.[2] The unique optical and plasmonic properties of silver nanoparticles (AgNPs) also make them a powerful tool for building biological and chemical sensors.[3] As a result, there is a need for reproducible toxicity studies to properly assess the risk different nanosilver-enabled products may pose to human and environmental health. The challenges in producing reproducible data have been highlighted by others who point out the need for detailed particle characterization prior to, during and after biological testing as well as a detailed description of sample preparation and experimental execution.[2] As both cells and particles are evolving over the course of an experiment, it is important to describe and monitor these changes in as much detail as possible in order to draw rational conclusions from the measured biological endpoints.

The synthesis, applications and biological activity of AgNPs have been reviewed.[1,2] There is little agreement; however, on the whether or not human or environmental toxicity should be a concern. While some papers report that silver nanoparticles pose no health risk,[4] others have shown them to bioaccumulate in aquatic organisms at environmentally relevant concentrations[5] or to persist for nearly a year in the brains of exposed mice.[6] Even with respect to the intended antimicrobial use of silver nanoparticles, there are conflicting reports with respect to the mode of action and whether or not the nanoparticles themselves impart activity or only the dissolved silver ions resulting from exposure of the particle surface to dissolved oxygen in aqueous environments.[7–9] Reasons for the breadth in observations include changes to experimental protocols and differences in the exact form of nanosilver used. Several parameters impact the biological activity of silver nanoparticles including size, charge and surface coating. Depending on how the silver nanoparticles are coated, they may be unstable in aqueous or biological environments and can dissolve or agglomerate, changing the properties of the species that interact with the cells or biomolecules. One challenge in comparing experiments across laboratories is a lack of reference materials for nanosilver to help baseline experiments. With both the biological systems and particles being highly variable, it is important to try and bring standardization to at least one aspect of these measurements in order to effectively improve the reproducibility of results. The use of reference materials would ensure a common starting point for monitoring changes that arise during exposure experiments where particles are evolving. There are currently only two reference materials available, a solution of silver citrate 35 nm nanoparticles from BAM or a 70 nm

Environmental significance

The ubiquitous use of silver nanoparticles as antimicrobial agents in commercial products has proceeded at a pace rapidly outstripping the regulatory science needed to ensure its safe use. Complications in the assessment of silver nanoparticles include its tendency to rapidly dissolve or aggregate in aqueous systems. A limited scope of available reference materials results in poor agreement between toxicological assessments. The development of protein stabilized silver particles that have prolonged stability in water and can be lyophilized for long-term storage as a dried material offer a new opportunity to fill a gap in the regulatory framework for nanomaterials.

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particle coated with polyvinylpyrrolidone (PVP) distributed by NIST.[10] We have chosen to examine the use of human serum albumin (HSA) as a coating agent as this protein has been reported to stabilize silver nanoparticles against aggregation.[11,12] Here we report the potential of using HSA to stabilize 20 nm AgNPs for use as a reference material and compare it to PVP and citrate stabilized particles in some preliminary biological assays.

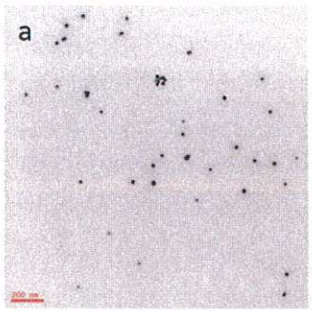
We started by determining how much HSA is required to stabilize 20 nm citrate stabilized silver nanoparticles. Solutions of 20 nm citrate stabilized nanoparticles were treated with excess protein for three hours. The resulting solutions were then divided into two aliquots, one stored in the dark overnight, the other lyophilized overnight and resuspended in water the following day. Both UV-Vis and dynamic light scattering (DLS) measurements were used to determine changes in the apparent particle size before and after lyophilizing (Table 1 and Fig. S1†). As the ratio of HSA to silver was increased to 50×, changes in DLS values and UV-Vis spectra became smaller and at 50× no change was observed in the UV-Vis spectra of the resuspended particles compared to the material before drying (ESI†). The amount of HSA needed is much greater than for the 70 nm PVP-coated particles which use a 10× by weight formulation of polymer. This, however, is not surprising as the surface area of the same weight of smaller particles is an order of magnitude greater and thus a greater amount of stabilizing agent is predicted to be needed. Other factors such as particle curvature may also impact the interaction between the stabilizing agent and particle and a more detailed study of these interactions is needed. Several groups have probed some of the details of such protein–particle interactions, however, there are still many unanswered questions as to the interfacial chemistry that arises at the nanoscale and drive the dynamics of particle–protein corona formation.[2,13,14] We also found that the 50× by weight ratio of HSA to silver was sufficient to stabilize 5, and 40 nm citrate-stabilized nanoparticles, resulting in the potential to create a series of stabilized particles that could be used as experimental reference points.

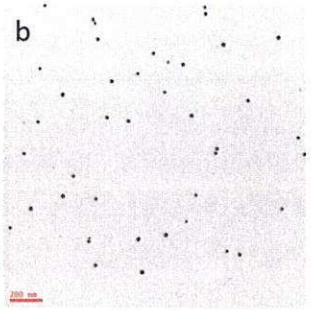
Table 1 *z*-Average hydrodynamic diameters of silver citrate stabilized 20 nm nanoparticles as determined by DLS after protein treatment for 3 hours made both before lyophilizing and after resuspending the dried material in water. 5, 10, 20 and 50× refers to the ratio of protein to silver. 50× means 50 mg of protein for each mg of silver. Diameters and PDI reported are average of five measurements with standard errors reported in parentheses

	Before lyophilizing particles	
	z-Average hydrodynamic diameter	Average PDI
5× HSA 20 nm AgNP	34.9 (0.3)	0.16 (0.01)
10× HSA 20 nm AgNP	34.9 (0.3)	0.17 (0.01)
20× HSA 20 nm AgNP	34.9 (0.3)	0.16 (0.01)
50× HSA 20 nm AgNP	35.4 (0.3)	0.27 (0.01)
50× BSA 20 nm AgNP	31.0 (0.2)	0.25 (0.01)
50× HSA 5 nm AgNP	25.9 (0.4)	0.50 (0.04)
50× HSA 40 nm AgNP	53.0 (0.4)	0.11 (0.01)
	After resuspending dried particles in water	
5× HSA 20 nm AgNP	89 (1)	0.20 (0.01)
10× HSA 20 nm AgNP	59.5 (0.3)	0.20 (0.01)
20× HSA 20 nm AgNP	44.0 (0.4)	0.19 (0.01)
50× HSA 20 nm AgNP	38.0 (0.3)	0.25 (0.01)
50× BSA 20 nm AgNP	37.0 (0.2)	0.25 (0.01)
50× HSA 5 nm AgNP	28.1 (0.6)	0.43 (0.04)
50× HSA 40 nm AgNP	57.0 (0.4)	0.10 (0.01)

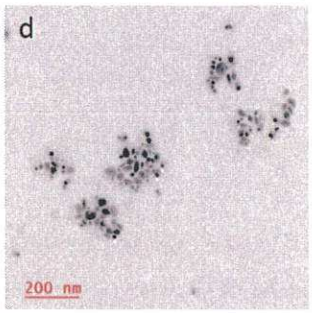
The stability of the HSA particles was monitored by TEM, to ensure that particle average size, and size distribution were stable over time both as a powder and suspended in water (Fig. 1). A time course was executed over several months, during which time a new aliquot of dried particles was resuspended each month. These were compared to a sample of dried 20 nm PVP-coated particles that were also resuspended each month. We found that both particles resuspended to their original size each time over 6 months, however, upon leaving the particles in water, agglomerates were observed in the PVP-coated particles after one month that were absent in the HSA-coated samples (Fig. 1). HSA coated particles were stable for three months without significant changes in their size distribution by TEM. PVP-coated particles showed a significant change after one month in aqueous suspension that also resulted in a change in colour of the suspension. The sizes reported in Fig. 1c exclude values for agglomerated particles that were present in the PVP samples (see ESI†). Changes were also observed by UV-Vis. After four months, the HSA-coated particles were still not agglomerated; however, a large number of smaller particles were observed suggesting that the particles were beginning to dissolve.

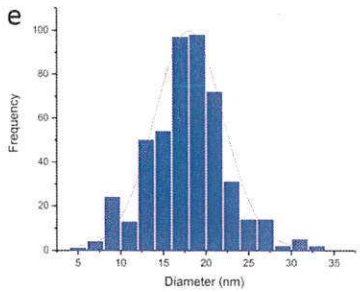
Next the cytotoxicity and uptake of these 20 nm particles were tested in four cell lines. Previous studies have shown that silver nanoparticles accumulate in both the liver and brain of mice, but that clearance from the brain is particularly low, resulting in long-term accumulation.[6] Cell lines were selected to match conditions of previous studies. We used human hepatocytes (HepG2), human neurons (SH-SY5Y), human glial cells (U87-MG), and mouse neurons (Neuro-2A) for comparison. While we had characterized the particles prior to the experiments, we wanted to also monitor the stability of the particles under experimental conditions. It should be noted that for all biological experiments, the samples were less than two months old, and no obvious changes were seen in either spectral properties, or measured toxicities over this time for repeated experiments performed over several weeks. Previous studies have reported on the stability of PVP and citrate coated particles in various media[15] but these experiments lacked serum and such studies should be included to track the evolution of particles over the course of a biological assay. We found that there was a significant change over the first few hours in the observed UV-Vis spectrum of the particles in media with 10% fetal bovine serum (FBS). Over the course of 60 hours, PVP particles exhibited a 10 nm shift in absorption, a broadening of the absorption





c	20 nm PVP-coated particles	20 nm HSA-stabilized particles
Initial measurement	16.6 ± 0.6	18.0 ± 0.4
After 1 month in water	17.5 ± 0.8	17.5 ± 0.6
After 2 months in water	18.3 ± 0.9	18.5 ± 0.4
After 3 months in water	18.1 ± 0.5	17.6 ± 0.8
After 4 months in water	18.2 ± 0.8	20 ± 1





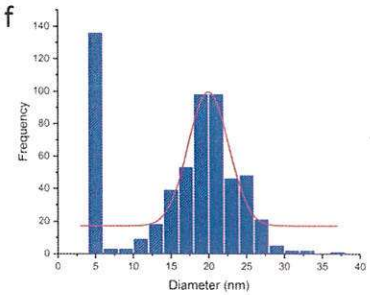
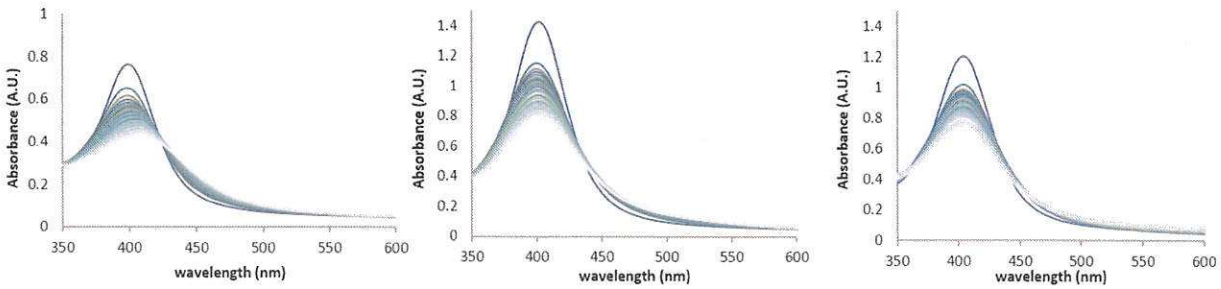


Fig. 1 TEM data was collected for 20 nm PVP stabilized particles (a) and HSA stabilized particles (b) upon suspending the particles in water. Suspended particles in water were measured every month for 4 months to determine the stability of the particles in water (c). Values are reported in nm ± standard error calculated from a minim of 400 particles per sample (excluding agglomerated particles). At one month larger agglomerates were observed for the PVP-stabilized particles (d), these were excluded form table (c). Profiles of the HSA-stabilized particle size distribution from initial suspension in water (e) and at 4 months (f) shows a significant increase in smaller particles present at month 4, though larger agglomerates were still not present.

spectrum and a decrease in absorption intensity (Fig. 2). Interestingly, while the same trend with respect to a decrease in absorption intensity was observed for the citrate and HSA-stabilized particles, no change was observed in the wavelength of the absorption maximum. This suggests that the PVP-particles undergo a different evolution in media than do the other two particles. Changes in media with 10% FBS were also observed by DLS. Here it was found that over three days a two to three-fold increase in hydrodynamic diameter was observed by DLS for all three particles in media with 10% FBS (ESI†).

Knowing that the particles are changing in media over the time course of the experiment allows for better analysis of observed trends in toxicity. First, the IC_{50} value of silver nitrate was determined against all four cell lines using the MTT assay.[16] This is important in order to include or exclude silver ions as a factor in the observed toxicity of the particles. The toxicity of silver nitrate against all four 4 cell lines was similar between 3.2 and 4.2 μM. The IC_{50} values of each of the three 20 nm particles was then measured against each of the four cell lines (Table 2). In order to ensure the reproducibility of the assay all experiments were performed with six replicates and repeated a minimum of three times. This is an important point as there is a high degree of scatter in the points at sub-lethal concentrations of the nanoparticles (ESI†). Samples were initially prepared in PBS; however, the citrate stabilized particles



Q4 Fig. 2 Change in UV-Vis spectrum of a) 20 nm PVP-coated particles in DMEM with 10% FBS over 60 hours, b) 20 nm citrate-coated particles in DMEM with 10% FBS over 60 hours and c) 20 nm HSA-coated particles in DMEM with 10% FBS over 60 hours. Each curve represents a two hour time point with t = 0 having the most intense peak at 398 nm.

Table 2 IC_{50} values are listed as μg mL^{-1} for silver nanoparticles and in micromoles per L for $AgNO_3$. Each experiment was performed 3 times with 6 replicates in each experiment. IC_{50} values are the average of 18 unique measurements with standard error reported in parathenses

Toxicity of silver particles suspended in PBS				
	SH-SY5Y	HepG2	U87-MG	Neuro-2A
20 nm citrate-AgNP	8.5 (0.5)	7.5 (0.8)	>10	>10
20 nm PVP-AgNP	0.8 (0.1)	0.6 (0.1)	>10	>10
20 nm HSA-AgNP	1.1 (0.1)	1.0 (0.1)	>10	>10
Toxicity of silver particles suspended in water				
20 nm citrate- AgNP	0.8 (0.1)	0.8 (0.1)	>10	>10
20 nm PVP-AgNP	0.8 (0.1)	0.7 (0.1)	>10	>10
20 nm HSA-AgNP	1.1 (0.1)	1.0 (0.1)	>10	>10
$AgNO_3$	3.7 (0.2)	3.2 (0.2)	4.2 (0.2)	3.6 (0.2)

rapidly agglomerated and settled out under these conditions. The toxicity of these samples was very low compared to the stable PVP and HSA coated particles in PBS. When experiments were repeated using dilutions of the particles in water, no precipitation of the particles was observed. Generally, we found that PVP-coated particles were very minimally more toxic than HSA-stabilized particles in both the SH-SY5Y and HepG2 cells. We also found that while the citrate particles precipitated in PBS and thus exhibited very low toxicities, when prepared in water and then added to media, the particles were stable and toxicity values were the same as PVP particles within experimental uncertainty (ESI†), and marginally more toxic than the HSA-protected particles. Some results for the toxicity of silver nanoparticles against SH-SY5Y cells have been previously reported.[16] The results in this paper exhibit a higher toxicity for our particles, as well as for silver nitrate. Several differences arise; however, from the source of the particles, the uniformity of the particles size distribution, and the composition of the culture medium for the cells. As a result, a direct comparison of the results is not possible as these factors need to be controlled to achieve reproducible outcomes. The toxicity of the particles was also measured against a human glioblastoma cell line and mouse neuronal cell line. In both cases, survival was greater than 80% at the highest doses of 10 μg mL^{-1} for all particles. Assuming that silver ion formation in media is the same for each experiment, we can rule out the role of extra-cellular silver ions as a primary cause of the measured toxicity, consistent with recent *in vivo* studies,[17] since all four cell lines have similar toxicities to silver ions, while the toxicities of the particles differ by orders of magnitude. All cell lines were cultured in the same media, and thus we can assume that the dynamics that would lead to the production of silver ions over the course of the experiment should be the same for all four cell lines.

Table 3 Silver uptake into cells reported in ng/10^6 cells. Each measurement was repeated on three different samples. Reported uptake values are the average with standard error in parentheses

Uptake of silver particles suspended in water				
	SH-SY5Y	HepG2	Neuro-2A	U87-MG
20 nm citrate-AgNP	15 (3)	70 (5)	5 (2)	60 (4)
20 nm PVP-AgNP	18 (3)	48 (5)	8 (2)	50 (4)
20 nm HSA-AgNP	3 (2)	20 (5)	6 (2)	10 (2)

The uptake of silver into the cells was then measured against all four cell lines (Table 3). Total silver content was considerably higher in the HepG2 cells. The HSA coated particles in the three human cell lines showed much lower uptakes than either the PVP or citrate stabilized particles (ESI†). There appears to be little correlation between silver uptake and toxicity. These results show that different cells can manage different nanoparticle loads before toxic effects are observed, and that the intracellular machinery responsible for protecting cells against nanosilver toxicity is cell type dependent. This also shows that uptake of particles stabilized with HSA is reduced in human cells but not the mouse cell line. This suggests a species specific role for the coating protein.

Conclusions

Human serum albumin can be used to stabilize silver nanoparticles against dissolution and aggregation. The protein corona established by HSA around the particles interferes with particle uptake into cells. These particles; however, remain toxic to human liver cells and neurons at concentrations only marginally higher than those of PVP- or citrate-stabilized particles. The mode of action of these particles requires further study. Using HSA as a stabilizing agent for the production of new nanosilver reference materials is a practical solution to overcome issues with particle stability and is effective across a range of particles sizes at the nanoscale. Species specific effects of protein coating suggest interesting possibilities for species tailored materials.

Conflicts of interest

There are no conflicts to declare.

References

1 P. Hartemann, P. Hoet, A. Proykova, T. Fernandes, A. Baun, W. De Jong, J. Filser, A. Hensten, C. Kneuer, J.-Y. Maillard, H. Norppa, M. Scheringer and S. Wijnhoven, *Mater. Today*, 2015, **18**, 122–123.
2 B. Reidy, A. Haase, A. Luch, K. Dawson and I. Lynch, *Materials*, 2013, **6**, 2295.
3 L. Ge, Q. Li, M. Wang, J. Ouyang, X. Li and M. M. Xing, *Int. J. Nanomed.*, 2014, **9**, 2399–2407.
4 B. Nowack, H. F. Krug and M. Height, *Environ. Sci. Technol.*, 2011, **45**, 1177–1183.
5 A. Orbea, N. Gonzalez-Soto, J. M. Lacave, I. Barrio and M. P. Cajaraville, *Comparative biochemistry and physiology*, Toxicology & pharmacology: CBP, 2017.
6 A. Antsiferova, B. Yu, V. Demin, P. Kashkarov, M. Kovalchuk and E. Petritskaya, *IOP Conf. Ser.: Mater. Sci. Eng.*, 2015, **98**, 012003.
7 N. Lubick, *Environ. Sci. Technol.*, 2008, **42**, 8617.

8 R. Foldbjerg, P. Olesen, M. Hougaard, D. A. Dang, H. J. Hoffmann and H. Autrup, *Toxicol. Lett.*, 2009, **190**, 156–162.
9 C. Beer, R. Foldbjerg, Y. Hayashi, D. S. Sutherland and H. Autrup, *Toxicol. Lett.*, 2012, **208**, 286–292.
10 R. I. MacCuspie, A. J. Allen, M. N. Martin and V. A. Hackley, *J. Nanopart. Res.*, 2013, **15**, 1760.
11 E. I. Alarcon, C. J. Bueno-Alejo, C. W. Noel, K. G. Stamplecoskie, N. L. Pacioni, H. Poblete and J. C. Scaiano, *J. Nanopart. Res.*, 2013, **15**, 1374.
12 D. McShan, P. C. Ray and H. Yu, *J. Food Drug Anal.*, 2014, **22**, 116–127.
13 J. H. Shannahan, X. Lai, P. C. Ke, R. Podila, J. M. Brown and F. A. Witzmann, *PLoS One*, 2013, **8**, e74001.
14 N. Durán, C. P. Silveira, M. Durán and D. S. T. Martinez, *J. Nanobiotechnol.*, 2015, **13**, 55.
15 M. Tejamaya, I. Romer, R. C. Merrifield and J. R. Lead, *Environ. Sci. Technol.*, 2012, **46**, 7011–7017.
16 T. Coccini, L. Manzo, V. Bellotti and U. De Simone, *Sci. World J.*, 2014, **2014**, 259765.
17 C. Recordati, M. De Maglie, S. Bianchessi, S. Argentiere, C. Cella, S. Mattiello, F. Cubadda, F. Aureli, M. D'Amato, A. Raggi, C. Lenardi, P. Milani and E. Scanziani, *Part. Fibre Toxicol.*, 2016, **13**, 12.