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RESEARCH PAPER

Disentangling the effects of polymer coatings on silver nanoparticle agglomeration, dissolution, and toxicity to determine mechanisms of nanotoxicity

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Abstract Silver nanoparticles (AgNPs) are frequently coated with a variety of polymers, which may affect various interdependent mechanisms of toxicity or antimicrobial action, including agglomeration and dissolution rates. Here, we systematically measure how citrate, dextran, 5 and 20 kDa poly(ethylene glycol) (PEG), and poly(vinyl pyrrolidone) coatings affect AgNP agglomeration, dissolution, and toxicity. In addition, to disentangle the coatings' effects on agglomeration from their other effects, we produce multiple stable agglomerate sizes of several of the coated ~23 nm AgNPs ranging from singly-dispersed to mean agglomerate sizes of several hundred nanometers. These dispersions allow us to independently study the effects of agglomeration and polymer coating on dissolution rate and hemolytic toxicity. We find that both hemolytic toxicity and dissolution rate are highest for the 5 kDa PEG coating, and toxicity and dissolution rate decrease significantly with increasing agglomerate size independent of coating. This correlation between toxicity and dissolution rate suggests that both polymer coating and agglomeration may affect hemolytic toxicity largely through their effects on dissolution. Because both the AgNP dissolution rate and hemolysis decrease only moderately compared to the large increases in agglomerate size, AgNPs' hemolytic toxicity may be caused by their large surface area and consequently high dissolution rate, rather than from other size-specific effects. At the silver concentrations used in this work, silver dissolved from AgNPs is expected to be primarily in the form of AgCl NPs, which are therefore more likely than Ag^+ ions to be the primary drivers of hemolytic toxicity. In addition, all AgNPs we tested are much more toxic to horse red blood cells than sheep red blood cells, highlighting the complexity of toxic responses and the need to test toxicity in multiple biological systems.

Keywords Aggregation · Hemolysis · Nanosilver · Silver colloid · Dissolution

Introduction

Silver nanoparticles (AgNPs) are the most commonly used commercial NP, primarily for their antimicrobial properties (Liu et al. 2010; Sotiriou and Pratsinis 2010). However, systematic methods are still needed to test their toxicity and determine if they pose a threat to health and the environment. AgNPs are often coated with various types of polymers to influence their

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properties, particularly to improve their biocompatibility and stability against agglomeration, and these polymer coatings may affect toxicity as well (Cheng et al. 2011; Farkas et al. 2011; Qureshi et al. 2011). In addition, different polymer coatings have been shown to affect the dissolution rate of AgNPs in cell culture media (Zook et al. 2011a), which is important because dissolved silver is known to be toxic (Sopjani et al. 2009; Sotiriou and Pratsinis 2010; Yeo and Yoon 2009). However, the effects of polymer coatings on dissolution, agglomeration, protein adsorption/biocompatibility, and toxicity are complexly interdependent, with each effect influencing or dependent on each of the other effects (see Fig. 1). In order to understand mechanisms of toxicity, as well as to understand AgNPs' antimicrobial properties (Liu et al. 2010; Sotiriou and Pratsinis 2010) and AgNPs' applications as localized surface plasmon resonance and surface-enhanced Raman spectroscopy-based biosensors (Cao et al. 2002; Elghanian et al. 1997; Roca and Haes 2008), it is important to disentangle these effects. Therefore, here we produce different sizes of agglomerates of AgNPs with the same polymer coating to measure independently the effects of polymer coating and agglomeration on dissolution and hemolytic toxicity.

We recently described and validated methods to produce and characterize multiple agglomerate sizes of various types of nanoparticles (NPs) (Zook et al. 2011b). Well-dispersed NPs were produced by pipetting the NP solution into cell culture media containing bovine serum albumin (BSA) while vortexing rapidly. Rapid vortexing quickly disperses the NPs so that they do not agglomerate in regions of high local NP concentration before being coated with BSA. In order to produce different agglomerate sizes, the NPs were sequentially (1) injected into cell culture media without protein, (2) allowed to agglomerate to the desired size, and (3) coated with BSA to stabilize the agglomerates. These dispersions were generally stable for at least two days, and it was shown that larger agglomerates of citrate-coated AgNPs caused less hemolytic toxicity than smaller agglomerates or well-dispersed AgNPs (Zook et al. 2011b). By producing relatively stable agglomerates, we can avoid the complications of changing agglomeration states that confound the interpretation of many previous studies on dissolution and toxicity (Jiang et al. 2009; Murdock et al. 2008; Sharma 2009; Stebounova et al. 2011; Zhang et al. 2011).

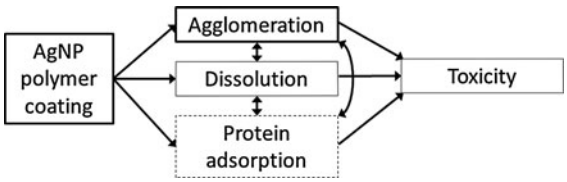

Fig. 1 Complex interactions (depicted by *arrows*) between the effects of polymer coating on agglomeration, dissolution rate, protein adsorption, and toxicity. In order to disentangle the effects of these parameters, in this work, we controllably vary parameters outlined in *thick black lines* and measure parameters outlined in *thin gray lines*, and the only protein in the solutions is bovine serum albumin (outlined with *dotted lines*)

The work presented here uses our previously developed methods (Zook et al. 2011b) for controlling agglomeration and measuring AgNP dissolution to explore the effects of five different polymer coatings on AgNP agglomeration, dissolution rate, and toxicity. Other methods developed to differentiate AgNP effects from silver ion effects include adding a thiol to complex the silver ions (Navarro et al. 2008), but thiols can also have other side effects, such as coating the AgNPs and modifying their toxicity, and mitigating oxidative damage because they are reducing agents. Some studies also use solutions with artificially low chloride ion concentrations to avoid complications of AgCl NP formation (Zhang et al. 2011). Here, by producing AgNPs with different polymer coatings and controlled agglomerate sizes in relevant biological media, we are able to study systematically the effects of both polymer coating and agglomeration on dissolution rate and hemolytic toxicity. The resulting correlation between dissolution rate and hemolytic toxicity suggests that hemolytic toxicity may be caused by silver dissolving from the AgNPs, which is primarily in the form of AgCl NPs.

Materials and methods

Citrate-stabilized AgNPs (~23 nm intensity-weighted mean diameter in water by dynamic light scattering (DLS)) were produced using a previously described method of reducing an aqueous solution of boiling silver nitrate in the presence of trisodium citrate dihydrate with sodium borohydride, all obtained from Sigma Aldrich (St. Louis, MO) (MacCuspie 2011). The polymers used to coat the AgNPs

were: two different molecular weights of poly(ethylene glycol)-thiols (5 kDa PEG and 20 kDa PEG) from Nanocs (New York, NY, #PEG3-0021 and #PEG3-0025, respectively), and approximately 10 kDa poly(-vinyl pyrrolidone) (PVP) and dextran from Sigma Aldrich (#PVP10 and #D9260, respectively). The polymer-coated AgNPs were prepared by ligand exchange from the citrate-coated AgNPs and concentrated to 1 mg/ml, and were the same solutions used previously in dissolution measurements (Zook et al. 2011a).

Dulbecco's modified eagle medium (DMEM) with 4.5 g/l glucose and sodium pyruvate but without phenol red or L-glutamine was obtained from Mediatech (Manassas, VA), which is buffered at pH 7.4. Bovine serum albumin (BSA) was from Sigma Aldrich (St. Louis, MO) (≥96 %, essentially fatty acid free). The antibiotics streptomycin and penicillin were from Invitrogen and were added at 100 μg/ml and 100 U/ml, respectively, to DMEM to reduce bacterial growth. Before use, DMEM + 4 % BSA by weight was filtered through a 0.2 μm polypropylene filter to remove large protein aggregates that would have interfered with the DLS measurements.

In order to produce different size distributions of stable NP agglomerates in cell culture media, we use a recently described method (Zook et al. 2011b). Briefly, the NPs are allowed to agglomerate in DMEM or 0.15 mol/l KNO_3 without protein for a specified period of time, and then 2 % (final concentration) BSA is added to coat the agglomerates and stop agglomeration. Specifically, to create the agglomerates, we (1) add 400 μl of DMEM or 0.15 mol/l KNO_3 to a 1.5 ml centrifuge tube, (2) add 100 μl of 50 μg/ml or 1 mg/ml AgNPs while vortexing and vortex for 1–2 s, (3) wait for the specified period of time, and (4) add 500 μl DMEM or 0.15 mol/l KNO_3 + 4 % BSA while vortexing. In order to produce the well-dispersed NPs ("0s"), we (1) add 400 μl of DMEM or 0.15 mol/l KNO_3 to a 1.5 ml centrifuge tube, (2) add 500 μl DMEM or 0.15 mol/l KNO_3 + 4 % BSA, and (3) add 100 μl of 50 μg/ml or 1 mg/ml AgNPs while vortexing at maximum speed.

The AgNP agglomerate dissolution experiments were performed using high speed ultracentrifugation followed by inductively coupled plasma-mass spectrometry (ICP-MS) measurements of the supernatant, as described previously (Zook et al. 2011a). In brief, the AgNP samples were immediately centrifuged at 20,800×*g* for 30 min at 2 °C to pellet the undissolved AgNPs. Then, the supernatant was taken and diluted with 0.1 mol/l HNO_3 for measurement by ICP-MS using a Thermo X7 ICP-MS instrument (Thermo Electron, Winsford, Cheshire, UK) operating in conventional (not collision cell technology) pulse counting mode. The expanded uncertainties of the ICP-MS measurements (calculated from the combined uncertainties of replicate measurements, calibration measurements, primary calibrant, instrument mass discrimination drift, instrument dead-time correction effects, instrument background correction, and weighing measurements (Guide 1993)) were approximately 2 % of the measured mean values.

After dispersing the NPs in cell culture media, the NPs were measured by dynamic light scattering (DLS) and UV–visible-near IR absorbance. In order to avoid effects of sedimentation, all solutions were vortexed immediately prior to measurement. DLS was performed using a Brookhaven Instruments (Holtsville, NY) ZetaPALS with a 660 nm laser and the detector at 90°. The combined mean size after 100 s was compensated for vibration and rotation of the agglomerates using the multiplicative factor described previously, which is close to one for small agglomerates but is greater than two for large agglomerates (Zook et al. 2011b). This compensated mean size is mass-weighted, which is smaller than the intensity-weighted mean size typically given. It should be noted DLS includes the hydration layer in its size estimate, though this hydration layer is most significant for small unagglomerated NPs. Bulk absorbance measurements were made using a Hewlett Packard 8453 spectrophotometer, measuring from 190 to 1100 nm and integrating for 0.5 s, with a stated wavelength accuracy of ±0.5 nm.

The hemolysis experiments were performed following the ASTM protocol "E 2524-08: Standard Test Method for Analysis of Hemolytic Properties of Nanoparticles" (Dobrovoiskaia et al. 2008), with the modifications described previously using DMEM + 2 wt % BSA instead of phosphate buffered saline to minimize changes in agglomeration during the experiment (Zook et al. 2011b). Most of the results presented in the figures below were carried out with heparinized horse blood diluted 5.5 fold with DMEM + 2 wt % BSA. Similar experiments were carried out mixing all of the coated AgNPs with heparinized sheep blood diluted 4.5 fold with

DMEM + 2 wt % BSA. The dispersed AgNPs were stored at room temperature at 100 μg/ml for 1 h prior to incubation with blood. The AgNPs were incubated with the blood for 3 h at 37 °C while constantly rotating the tubes to avoid sedimentation The tubes were centrifuged at $800\times g$ for 30 min to remove the red blood cells. Then, the supernatant was centrifuged at $20800\times g$ for 30 min to remove the AgNPs. Note that a higher centrifugal speed was needed than in previous experiments (Zook et al. 2011b) to remove all PEG-coated AgNPs because they absorb at 540 nm, which interfered with the absorbance-based measurement of hemoglobin. All samples were prepared in triplicate.

Statistical comparisons were performed with Matlab using one-way ANOVA and Tukey's honestly significantly different test with $P < 0.01$.

Results/discussion

Stable AgNP agglomerates with different sizes and coatings

We first chose two of the polymer-coated AgNPs (PVP and dextran) to study the effect of polymer coating on agglomeration and measure the stability of the agglomerates in the presence of BSA. We produced and characterized several mean agglomerate sizes of PVP- and Dextran- coated AgNPs using recently described methods, and agglomerates were vortexed immediately prior to the measurements to avoid sedimentation effects.(Zook et al. 2011b) The polymer-coated AgNPs were agglomerated for 5 or 30 s before adding BSA to stabilize the agglomerates, thereby producing mean agglomerate sizes (intensity-weighted but compensated for vibration and rotation of the agglomerates) ranging from 390 to 2800 nm, as shown in Fig. 2. Dextran-coated AgNPs agglomerated more rapidly than PVP-coated AgNPs and, therefore, had larger agglomerate sizes. In addition, relatively well-dispersed AgNPs were produced by pipetting AgNPs directly into DMEM + 2 wt % BSA while vortexing (time = 0 s), although the PVP and Dextran-coated AgNPs still agglomerated slightly due to their high concentrations. The two different PEG-coated AgNPs did not agglomerate even without BSA because the PEG sterically stabilizes the AgNPs. As shown in Fig. 2, most dispersions were relatively stable in size over two days, which is consistent with dispersions of citrate-coated AgNPs as agglomerates in our previous work (Zook et al. 2011b), although a few of the dispersions decreased in size, possibly due to deagglomeration or dissolution. However, the different sizes of agglomerates are still sufficiently separated to study the effect of agglomeration on dissolution and toxicity.

Effect of agglomeration on AgNP dissolution

Since agglomeration decreases the effective specific surface area of NPs, we hypothesized that it might thereby affect AgNP dissolution. No validated methods exist to measure the dissolution rate of moderate to high concentrations (>0.2 μg/ml) of agglomerated AgNPs in environmental or biological media (*e.g.*, DMEM) because dissolved silver and chloride ions form AgCl particles that cannot be distinguished from AgNPs with conventional measurement techniques (Zook et al. 2011a). Therefore, we tested the effect of agglomeration on dissolution in a solution without chloride (0.15 mol/l KNO_3 with 2 wt % BSA). Similar to the dispersions in cell culture media, the high KNO_3 concentration causes the AgNPs to agglomerate for a specified time, and then BSA is added to stabilize the agglomerates. In this solution, the agglomerates'

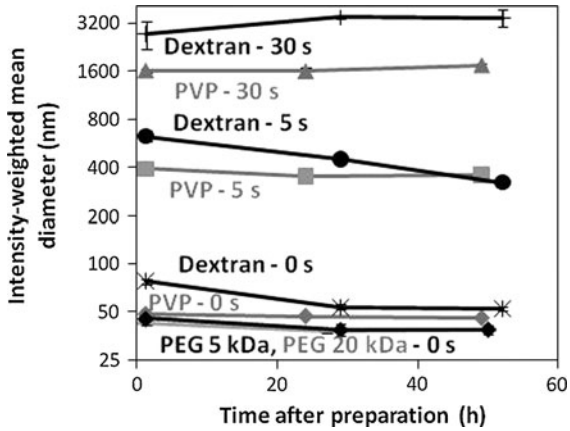

Fig. 2 Changes in the mean size of AgNP agglomerates dispersed at different mean agglomerate sizes over two days at a concentration of 100 μg/ml AgNPs in DMEM + 2 wt % BSA. The size is the intensity-weighted mean diameter measured by DLS, compensated for vibration and rotation of the agglomerates. Each point represents the mean of three separately prepared samples and the error bars indicate one standard deviation (*error bars* are smaller than the symbols if not visible)

size changes more than in cell culture media with BSA, decreasing in size by about 25 % over 27 h, possibly due to dissolution or deagglomeration. However, we were still able to produce three different well-separated sizes of agglomerates ranging from well-dispersed mostly single AgNPs to agglomerates with a mean size of 460 nm. As shown in Fig. 3, the dissolution rate is smaller for larger mean agglomerate sizes, as expected due to the decreased effective specific surface area of agglomerates compared to well-dispersed AgNPs. These results are for AgNP agglomerates in 0.15 KNO_3 because reliable dissolution measurements cannot be performed in DMEM, but the same qualitative trend is expected in DMEM since the specific surface area is probably driving the change in dissolution rate. This trend is compared to hemolysis below.

As an alternative qualitative measure of size and dissolution of agglomerated AgNPs, Fig. 4 shows selected absorbance spectra 15 min and 52 h after dispersion of dextran-coated AgNPs in cell culture media. Interaction between surface plasmons in nearby NPs within agglomerates causes a second, broad peak at longer wavelengths (Zook et al. 2011b; Zook et al. 2011c). The decrease in absorbance across all wavelengths suggests that dissolution is occurring, as observed previously for unagglomerated AgNPs in cell culture media (Zook et al. 2011a). However, because the decrease in absorbance is not completely uniform across all wavelengths, it is not a good quantitative estimate of dissolution (Zook et al. 2011a). In addition, the second localized surface plasmon resonance absorbance peak at longer wavelengths for AgNPs agglomerated 5 s and 30 s confirms qualitatively that AgNPs agglomerated for longer times have greater numbers of larger agglomerates, as expected (Zook et al. 2011b; Zook et al. 2011c). The small shoulder for the "0 s" AgNPs confirms the DLS measurements that a small amount of agglomeration occurs even for AgNPs dispersed in DMEM + 2 wt % BSA, probably because some AgNPs encounter each other before being completely coated with BSA (Zook et al. 2011b). Additional spectra for the PVP and PEG-coated AgNPs are shown in Figs. S1 and S2 in the supporting information.

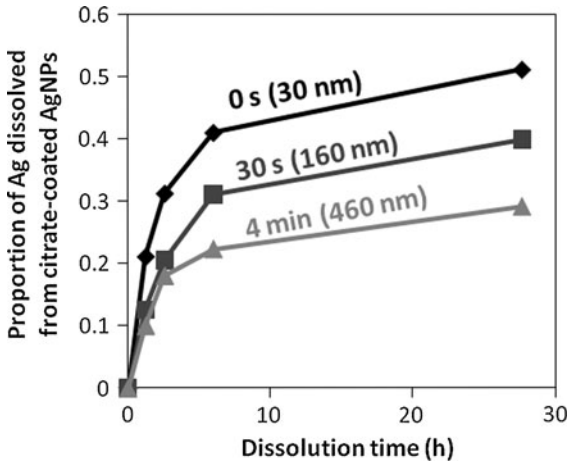

Fig. 3 Proportional dissolution of silver from 5 μg/ml citrate-stabilized AgNPs agglomerated in 0.15 M KNO_3 (instead of DMEM, to prevent formation of AgCl) for 0, 30 s, or 4 min before adding 2 wt % BSA to help stabilize the agglomerates. The "0 s" AgNPs had no detectable agglomeration at any time over 27 h. The intensity-weighted mean size (compensated for vibration and rotation) of the "30 s" AgNPs decreased from 160 to 110 nm over 27 h, possibly due to dissolution and deagglomeration. Similarly, the size of the "4 min" AgNPs decreased from 460 to 320 nm over 27 h. Dissolution was measured by ICP-MS after centrifugation to remove the undissolved AgNPs, and standard deviations of five ICP-MS measurements were smaller than the symbols

Effect of polymer coating and agglomeration on hemolytic toxicity

Finally, after characterizing and measuring dissolution from AgNPs with different polymer coatings and agglomeration states, we measured the effects of coating and agglomeration on AgNP hemolytic toxicity (see Fig. 5). These experiments were carried out in DMEM with 2 wt % BSA to produce stable AgNPs or AgNP agglomerates of different sizes in otherwise identical media. All AgNPs caused at least 75 % hemolysis at the highest concentration of 100 μg/ml, and caused no additional hemolysis compared to the DMEM at the lowest concentration of 10 μg/ml. Therefore, all statistical tests were performed for 50 μg/ml AgNPs, for which the sensitivity to changes in toxicity was highest. For 50 μg/ml AgNPs, the degree of hemolysis ranged from 10 to 40 %, depending on polymer coating and mean agglomerate size.

Hemolytic toxicity decreased significantly as mean agglomerate size increased for both PVP and dextran-coated AgNPs ($P < 0.01$), as shown in Fig. 5a. Three different mean agglomerate sizes of AgNPs coated with PVP and dextran were produced, with mean sizes ranging from 44 to 1100 nm for PVP and from 48 to

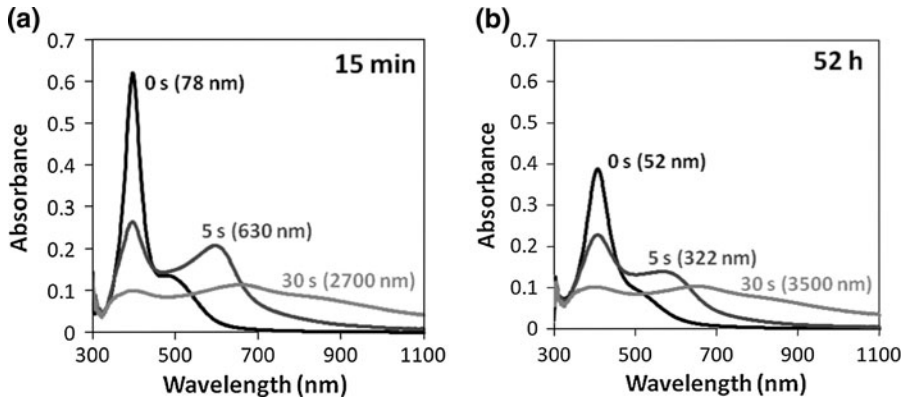

Fig. 4 Absorbance spectra of dextran-coated AgNPs agglomerated for 0, 5, or 30 s before adding BSA, and then measured (a) 15 min and (b) 52 h after adding BSA, with the intensity-weighted mean size compensated for vibration and rotation in parenthesis. These spectra are the mean of three samples, and correspond to the same samples as in Fig. 2

2100 nm for dextran-coated AgNPs. Figure 5b shows that hemolytic toxicity decreased with increasing agglomerate size for these AgNPs. These results are consistent with our previous measurements that showed that hemolytic toxicity decreased with increasing agglomeration of citrate-coated AgNPs (Zook et al. 2011b). However, the toxicity of 100 μg/ml 2100 nm dextran-AgNPs is still significantly greater than the toxicity of 50 μg/ml 48 nm dextran-AgNPs, so the toxicity decreases by less than a factor of two (in concentration) even though the mean size increases by a factor greater than 40. Therefore, the mechanism of toxicity is only weakly dependent on agglomerate size. Instead, the mechanism of toxicity may be primarily dependent on specific surface area, which only decreases moderately with agglomeration. The specific surface area of the agglomerates decreases only moderately due to the close proximity of NPs in the branched, fractal shape of agglomerates (see TEM examples in refs. (Zook et al. 2011b) and (Zook et al. 2011c)), which was shown by electron microscopy and atomic force microscopy in our previous work with agglomerates of similar NPs (Zook et al. 2011c) as well as in other work (Weitz and Oliveria 1984). Dissolution rate is an important factor that most likely depends on specific surface area, since oxidation happens only at the surface, and Fig. 3 confirms that dissolution rate decreases moderately with increasing agglomeration. Because $AgNO_3$ is much more hemolytically toxic than AgNPs (which has also been shown previously (Ruden et al. 2009)), silver dissolved from the AgNPs may be the primary driver of toxicity.

Although silver dissolved from AgNPs appears likely to be the primary driver of hemolytic toxicity, we calculated previously that, at these Ag concentrations, Ag dissolved from AgNPs (or Ag from $AgNO_3$) will primarily be in the form of AgCl NPs and the non-particulate aqueous Ag concentration will be small ($\sim$0.18 μg/ml) and almost independent of total Ag concentration (Zook et al. 2011a). These calculations predicting AgCl NP formation were supported by DLS and absorbance measurements of highly dissolved AgNPs that showed scattering from particles that were not AgNPs.(Zook et al. 2011a) Therefore, it is likely that the AgCl NPs rather than silver ions are the primary cause of the toxicity. The toxicity of AgCl particles may also depend on their size, so we measured the size of the AgCl NPs produced from $AgNO_3$. In our preparation of $AgNO_3$ in cell culture media, we pipetted $AgNO_3$ into DMEM + 2 wt % BSA while vortexing at a high rate for a total stock Ag concentration of 100 μg/ml. This dispersion method resulted in AgCl NPs (probably with BSA adsorbed) with an intensity-weighted mean size of 89 nm measured by DLS.

Some of the polymer coatings also had a significant effect on toxicity at 50 μg/ml AgNPs, as shown in Fig. 5. The 5 kDa PEG coating had the largest effect on toxicity, causing 40 % more hemolysis than citrate-coated AgNPs dispersed at 46 nm ($P < 0.01$), and the 20 kDa PEG coating also caused significantly more hemolysis than citrate-coated AgNPs dispersed at 46 nm ($P < 0.01$). Polymer coating may be hypothesized to affect toxicity in several ways, including its effects on agglomeration, biocompatibility, protein adsorption, and dissolution. All of these effects are intertwined with each of them affecting most of the others. However, by producing different sizes of controlled agglomerates and well-dispersed AgNPs, we were able to study the effect of agglomeration independently from the other effects. We recently used an absorbance-based technique to study the effect of

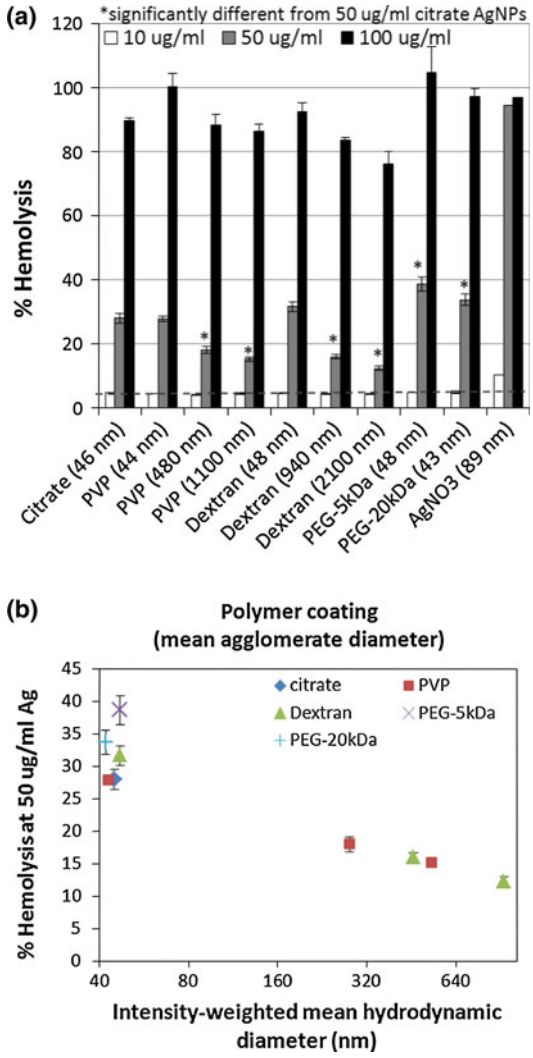

Fig. 5 Percent hemolysis caused by AgNPs with different coatings and in different agglomeration states during a three hour incubation with constant mixing. **a** The size in parentheses is the intensity-weighted mean DLS size compensated for vibration and rotation. The horizontal dotted gray line indicates the level of hemolysis (4 %) measured for the background cell culture media with 2 wt % BSA. All hemolysis experiments (except $AgNO_3$) were carried out in triplicate and error bars indicate one standard deviation. * Indicates the hemolysis for 50 μg/ml AgNPs was significantly different from the hemolysis caused by 46 nm citrate-stabilized AgNPs ($P < 0.01$, using one-way ANOVA and Tukey's honestly significantly difference test). All statistical tests were performed for 50 μg/ml AgNPs because their changes fall in the highest sensitivity region of the response curve. **b** % hemolysis versus mean agglomerate size for the 50 μg/ml AgNPs with different coatings in part (**a**). Error bars indicate one standard deviation and are smaller than the symbols if not visible. Hemolysis was measured with horse blood (see Fig. S3 in the supporting information for similar experiments showing little hemolysis with sheep blood)

these polymer coatings on dissolution of silver from the AgNPs.(Zook et al. 2011a) Those results demonstrated that the dissolution rate is significantly higher for 5 kDa PEG-coated AgNPs than for the other coatings, possibly because the coating is relatively thin and minimizes protein adsorption onto the AgNPs (see Table S1 in the supporting information). This correlation between dissolution rate and the effects of polymer coating and agglomeration on AgNP hemolytic toxicity suggests that the coatings' effect on hemolytic toxicity may be related to their effect on dissolution rate.

Interestingly, when the same citrate, dextran, PVP, and PEG-coated AgNPs were mixed with sheep blood instead of horse blood, they caused very little hemolysis (<5 % above background), even at a concentration of 100 μg/ml (see Fig. S3 in the supporting information). The reason for this difference is unclear, but it highlights how the toxicity response can change a large amount with only apparently small changes in the biology.

Conclusions

Polymer coatings on AgNPs may be hypothesized to affect toxicity through a variety of intertwined mechanisms, including the coatings' effects on biocompatibility, protein adsorption, agglomeration, and dissolution. In order to help disentangle these effects, we produced different agglomerate sizes of AgNPs with various polymer coatings and measured their toxicity. In addition, we measured independently the effects of agglomeration and polymer coating on dissolution. When changing agglomerate size or polymer coating, both toxicity and dissolution rate changed in the same direction. In addition, $AgNO_3$ caused significantly higher hemolytic toxicity than any of the AgNPs. These results suggest that the toxic effects of AgNPs on horse blood may be caused by the silver that dissolved from the AgNPs, which is primarily in the form of AgCl NPs based on our previous work (Zook et al. 2011a). In addition, the dissolution rate decreases only moderately with comparatively large increases in agglomerate size. This result is most likely explained by the branched fractal shape of the agglomerates, since large increases in agglomerate size only moderately decrease the specific surface area. Since hemolytic toxicity also decreased only moderately with large

increases in agglomerate size, hemolytic toxicity is probably dependent on mechanisms driven by surface area rather than size. These results exemplify how producing different controlled agglomerate sizes can help to distinguish between possible mechanisms of toxicity. In addition, the difficulty of generalizing results from nanotoxicity studies is highlighted by our results that the same concentration of AgNPs that caused near complete hemolysis in horse blood, caused very little hemolysis in sheep blood. Therefore, it is important to test toxicity in a variety of biological systems in addition to varying and/or measuring as many of the NPs' properties as possible. The methods presented in this work enable the systematic study of agglomeration and dissolution not only for nanotoxicity studies, but also for applications of AgNPs as antimicrobial agents and as localized surface plasmon resonance-based or surface-enhanced Raman spectroscopy-based biosensors.

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