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A Dose Metrics Perspective on the Association of Gold Nanomaterials with Algal Cells

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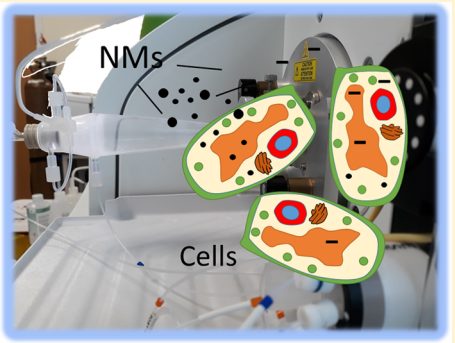
Supporting Information

ABSTRACT: A single-cell inductively coupled plasma mass spectrometry technique was used to explore the influence of particle properties on the association of nanomaterials (NMs) with algal cells. We investigated the effect of particle size, shape, and surface chemistry [citrate and natural organic matter (NOM) coating] on the association of gold (Au) NMs with algal cells using particle mass, particle number, surface area (SA), and volume-specific surface area (VSSA) as dose metrics. Particle number was found to be a better dose metric than particle mass, SA, and VSSA in view of the strong correlation obtained between the number of associated Au NMs with cells and the number of Au NMs in the exposure medium. When particle number was used as the dose metric, there was no selectivity of Au NM cellular association irrespective of particle size and shape, and the cellular association was proportional to the effective number of particles to which the cells were exposed. The surface chemistry of the Au NMs, however, decreased the level of cellular association of some NMs (60 nm spheres). Particle number is the main element used for the classification of NMs according to the recommended definition for NM by the European Commission. The key finding of our study supports the implementation of this definition for safety purposes.



INTRODUCTION

Nanotechnology is developing rapidly into a fast-growing global market. Nevertheless, still little is known about the factors driving nanomaterial (NM) cellular uptake and association, as needed, e.g., to facilitate developing and enforcing nanomaterial regulations.[1] It is critical to understand the amount of associated NMs with microorganisms such as algal cells. Algae are the main producers of aquatic ecosystems and the first trophic level in aquatic food webs.[2] Association of NMs with algae can lead to the reduction of the level of algal photosynthesis[3] and the trophic transfer of NMs in food chains and, thus, the accumulation of these materials in predators at the top of the food webs. Previous studies already documented that NMs are attached to algal cells[4] and, consequently, enter aquatic food chains.[5,6]

In the environment, NMs occur in different sizes, shapes, and compositions, and these properties may influence the association of NMs with algal cells.[7] Limitations in analytics not only imposed the use of traditional principles, e.g., mass as a dose metric, to express the association of NMs with microorganisms[8] but also have hindered the implementation of the European Commission (EC)-recommended definition of NM for regulatory purposes (2011/696/EU). Mass concentration is still largely used as a dose metric to express the cellular association and adverse effect of NMs, whereas scientific communities have been emphasizing that mass alone may not be a suitable dose metric for presenting the influence of particle properties on NM fate and adverse effects in biota.[9−11] Moreover, in the recommended definition for NMs by the EC, mass is not an element for the classification of NMs. For example, NMs of the same type and shape but with different sizes have a different number of particles per specific volume. If algal cells are exposed to the same type and shape of NMs with a heterogeneous size distribution, the number of particles associated with the cells may differ. As a result, the number of NMs entering the food chain would vary. Nevertheless, most of the studies have used particle mass to describe the adverse effect of NM size, shape, composition, and/or surface chemistry.[12−15] Particle number and surface

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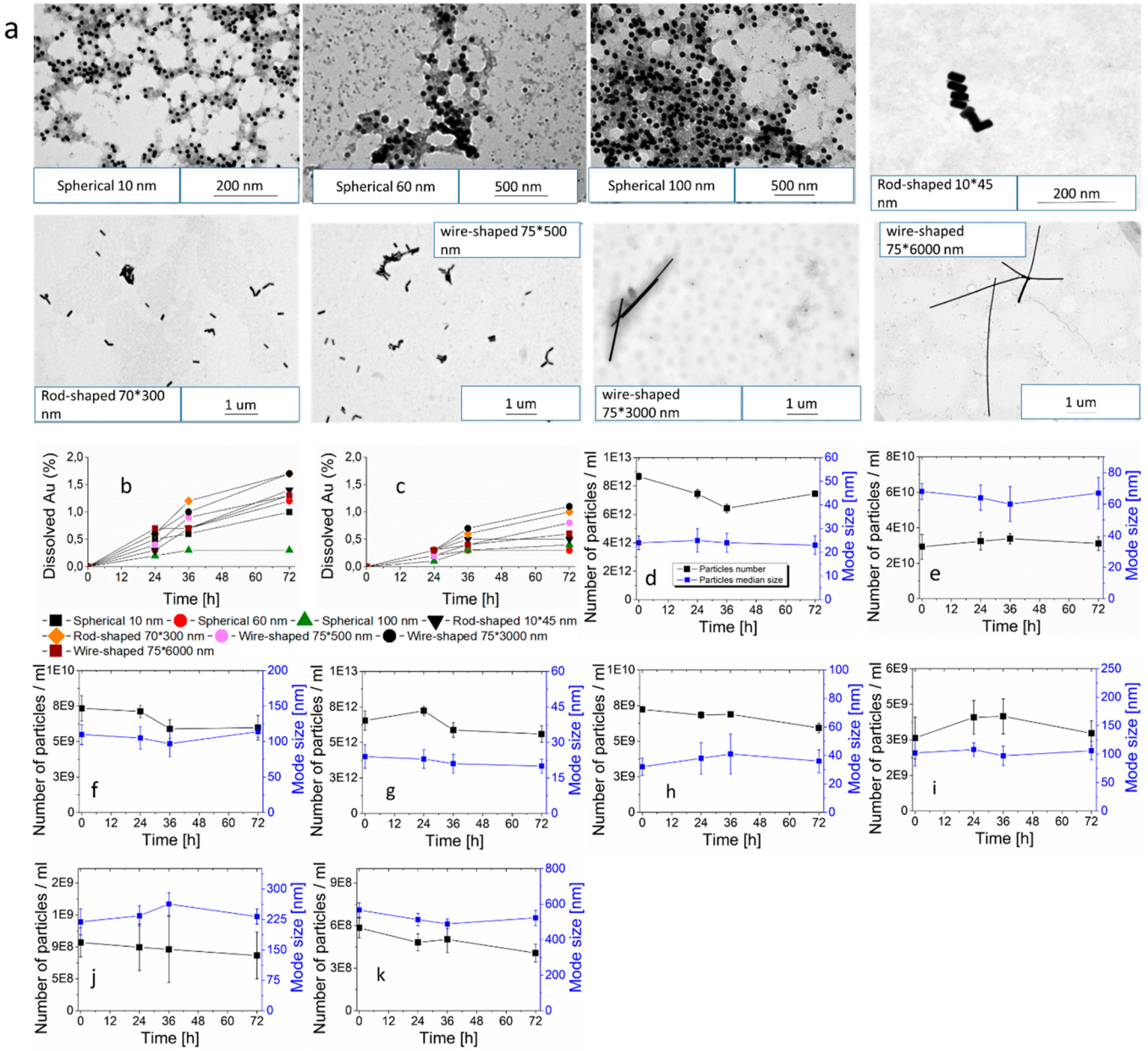

Figure 1. Characterization of the Au NMs. (a) TEM pictures of the citrate-coated Au NMs in MQ water. The percentage of dissolved Au mass to total Au mass was calculated by measuring the ionic Au mass in exposure media using spICP-MS. (b) Percentage of the dissolved Au released from the citrate-coated Au NM. (c) Percentage of the dissolved Au released from the NOM-coated Au NMs. (d–k) Particle numbers of Au NMs (black line) and mode sizes of the particle size distribution (blue line) over the duration of the exposure in exposure media without algal cells. The figures present spherical 10 nm Au NMs (d), spherical 60 nm Au NMs (e), spherical 100 nm Au NMs (f), rod-shaped 10 nm × 45 nm Au NMs (g), rod-shaped 70 nm × 300 nm Au NMs (h), wire-shaped 75 nm × 500 nm Au NMs (i), wire-shaped 75 nm × 3000 nm Au NMs (j), and wire-shaped 75 nm × 6000 nm Au NMs (k).

area (SA) have been proposed as alternative dose metrics and evaluated in few nanotoxicological studies,[14,15] although there is still disagreement among researchers.[9,11,14−17] No study is available that used a dose metric other than particle mass to demonstrate the amount of NMs associated with algae and to show how the properties of NMs influence their association with algal cells.

To date, investigation of the association of NMs with algal cells is mainly based on the traditional approaches such as using acid digestion methods followed by centrifugation or filtration methods and total mass quantification using, e.g., mass analysis by inductively coupled plasma atomic emission spectroscopy or inductively coupled plasma mass spectrometry (ICP-MS).[4,18,19] These approaches yield the average mass of the particles associated with cells. By averaging the NMs mass concentration over a population of cells, one loses information about particle number, size distribution, and SA.[8] This makes the adaptation of another dose metric rather than mass almost impossible.

The newly developed mode of the time-resolved inductively coupled plasma mass spectrometry (ICP-MS) technique, described as single-cell (sc)-ICP-MS, offers the possibility of quantitative analysis of elements within individual cells.[20−23] The sensitivity of the technique, at levels as low as attograms

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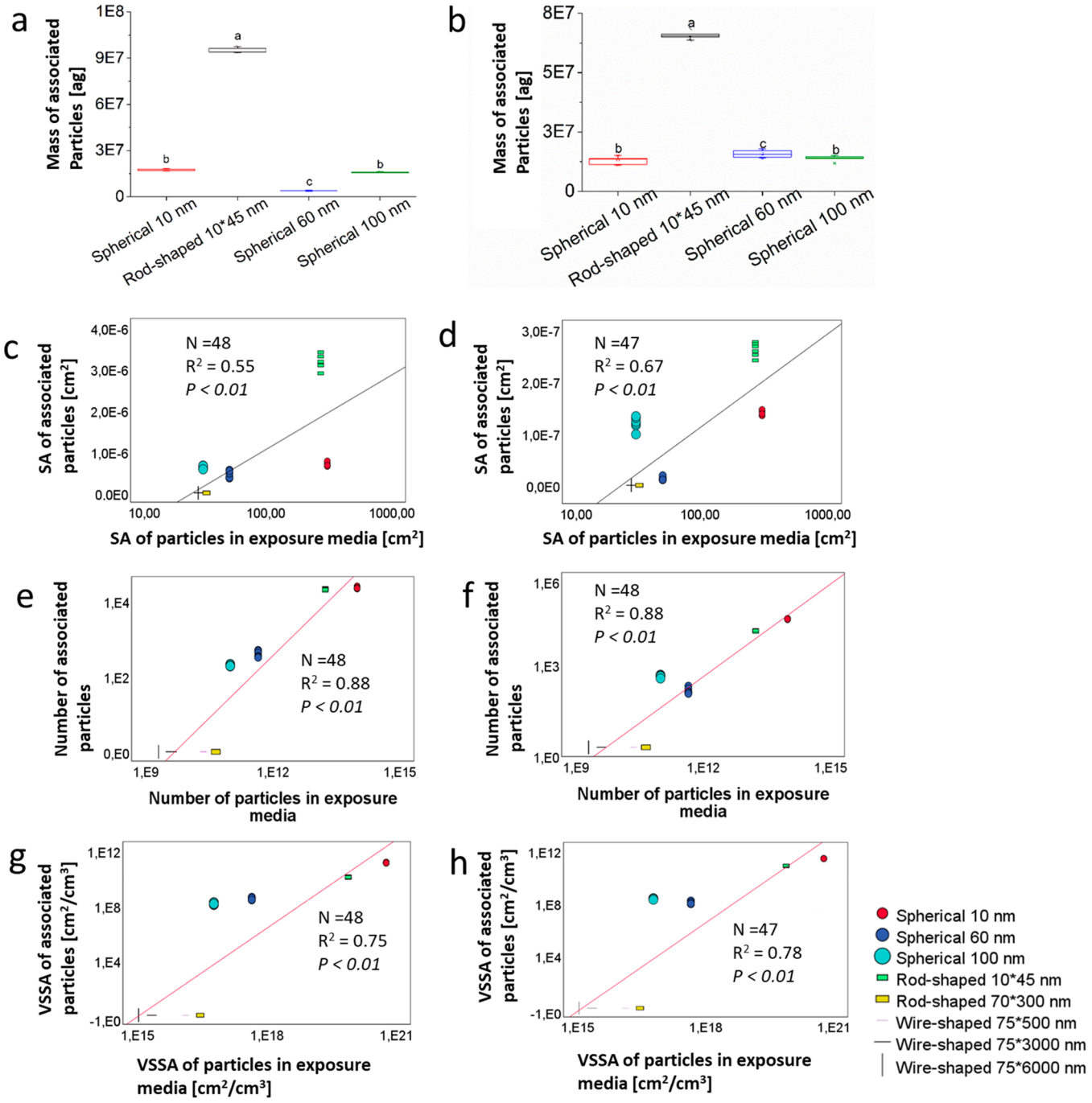

Figure 2. Total mass of the associated particles vs the total mass of the particles in exposure media for (a) citrate-coated and (b) NOM-coated particles. (c) Calculated surface areas of the associated particles (citrate-coated Au NMs) with the algal cells vs the calculated surface area of the citrate-coated particles in exposure media at the beginning of the exposure. (d) Calculated surface areas of the associated particles (NOM-coated Au NMs) with the algae vs the calculated surface area of the NOM-coated particles in exposure media at the beginning of the exposure. (e) Numbers of cellular associated citrate-coated Au NMs vs the number of citrate-coated Au NMs in exposure media. (f) Number of cellular associated NOM-coated Au NMs vs the number of NOM-coated Au NMs in exposure media. (g) Calculated VSSA of the cellular associated citrate-coated Au NMs vs the calculated VSSA of the citrate-coated Au NMs in exposure media. (h) Calculated VSSA of the cellular associated NOM-coated Au NMs vs the calculated VSSA of the NOM-coated Au NMs in exposure media.

(ag) per cell, allows quantification of the cellular association and uptake of NMs at trace levels[24] that cannot be determined using a bulk analysis. Recently, this technique was used for the quantification of internalized metals on a "by-cell" basis.[20,21,24] Despite the potential of this technique, very few studies used scICP-MS to quantify NMs at cellular levels.

In this Letter, we studied the cellular association of gold (Au) NMs with algal cells using scICP-MS by considering particle mass, number, SA, and volume-specific surface area (VSSA) as dose metrics. The potential of the dose metrics in elucidating the influence of particle properties, including particle size, shape, and surface chemistry, on the association of the NMs with algae was evaluated. Natural organic matter

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(NOM) is a complex mixture of different organic compounds, mostly resulting from the decay of organisms and plants, which is present in natural surface waters.[25,26] The addition of NOM to aquatic dispersions of particles leads to the formation of a NOM corona on the surface of NMs that provides particles with different surface chemistries and makes the study environmentally relevant. In this study, we have used NOM to understand how the variation in surface chemistry influences the association of NMs with algal cells.

MATERIALS AND METHODS

Chemicals and Materials. Spherical (10, 60, and 100 nm), rod-shaped (10 nm × 45 nm, 70 nm × 300 nm), and wire-shaped (75 nm × 500 nm, 75 nm × 3000 nm, and 75 nm × 6000 nm) Au NMs were purchased from Nanopartz (Nanopartz Inc.). The International Humic Substances Society (1R101N) supplied the Suwannee River NOM in this study. The incubation of the particles with NOM is reported in the Supporting Information (section S2). The NOM was added to form a NOM corona on the surface of the particles and to compare the cellular association of NOM-coated particles with the cellular association of citrate-coated particles.

Characterization of the Au NMs. The transmission electron microscope (TEM) images of Au NM dispersions in Milli-Q (MQ) water were obtained using a JEOL 1010 TEM operated at an accelerating voltage of 70 kV to determine the shape and size of the particles. The hydrodynamic size and ζ potential measurements were performed by means of a Zetasizer Nanodevice (Malvern Panalytical). Multiangle dynamic light scattering (MADLS) was performed using Zetasizer Ultra (Malvern, Panalytical) to measure the hydrodynamic size of the rod-shaped and wire-shaped 75 nm × 500 nm Au NMs. The size of the other wire-shaped particles was measured using the TEM. Single-particle ICP-MS (spICP-MS) was used to quantify size distribution and the dissolution profile of the particles in the algal culture media. The dissolution tests and the conditional setup for spICP-MS are described in section S3 of the Supporting Information. scICP-MS was applied to measure the particle number of wire-shaped particles. The dissolution of wire-shaped particles was carried out following the previous method.[4]

Algal Assay Setup. The unicellular alga *Pseudokirchinella subcapitata* was cultured (see section S4 of the Supporting Information) and used as the test cell. The algal cells were exposed to 1 mg/L citrate-coated or NOM-coated Au NMs with different particle sizes and shapes. This concentration of the Au NMs was arbitrarily selected to mimic the environmentally relevant concentration while allowing quantification of the NM association with the cell. The algae were exposed for 72 h, and the samples were subsequently kept at 4 °C to allow the algal cells to sediment for 48 h. The particles have a high thermal diffusion and do not sediment due to the gravitational force. This allows the cells to be separated from the unbound NMs upon sedimentation. After sedimentation for 48 h, the pellet of the algal cells was separated from the supernatant by gently discarding the supernatant. The remaining algal pellets were rinsed with 10 mL of PBS (0.1 mol, pH 7.4)[27] and centrifuged (4000 rpm) for 10 min at 4 °C to remove the unbound or loosely bound Au NMs to algal cells.[28] The latter step was repeated twice. Algae excrete various extracellular polymeric substances (EPS) into their immediate environment to mediate their adhesion to surfaces.[29] EPS is the first barrier to protect the inner microorganisms against external stressors.[30] It is likely that NMs are strongly attached to the surface of the EPS. To remove the strongly attached Au NMs from the surface of the EPS layer, the remaining algal pellets were treated with 5 mL of 0.02 M ethylenediaminetetraacetic acid (EDTA) for 20 min (see section S5 of the Supporting Information). The efficiency of the washing processes in the removal of the surface-attached particles is reported in section S5 of the Supporting Information.

Quantification of Au NMs on a Cell-by-Cell Basis. All scICP-MS measurements were performed on a PerkinElmer NexION 300D ICP-MS instrument operating in single-cell mode. The conditional setup is given in Table S3. The transport efficiency of the Asperon spray chamber was measured as being 41.14%. All data acquisition was accomplished with the Syngistix Single-Cell Application Module.

RESULTS AND DISCUSSION

Characterization of the Au NM. The Au NMs were characterized in terms of particle shape, size distribution, aggregation rate, dissolution rate, number concentration, and ζ potential. TEM images showed the shape and the projected size of the Au NMs (Figure 1a) in MQ water, which were in good agreement with the values reported by the supplier. The physicochemical properties of the Au NMs are summarized in Table S4.

The pristine particles (citrate-coated Au NMs) had a negative ζ potential in the exposure media (−16 to −20 mV), and the NOM corona significantly ($p < 0.05$) decreased the ζ potential to lower values (−27 to −31 mV). The dissolution profile of the citrate-coated (Figure 1b) and NOM-coated (Figure 1c) particles in exposure media without algae showed that the Au NMs did not undergo substantial dissolution and the released ions from the particles constitute <2% of the total Au mass. The number of particles in the exposure media without algae did not decrease significantly ($p < 0.05$) over 72 h, which shows that sedimentation did not occur (Figure 1d−k). The observation that the mode particle size did not change over time (Figure 1d−k) confirms that the particles do not agglomerate considerably in exposure media without cells. The characterization data showed that the algal cells were exposed to single-particle Au NM rather than dissolved or aggregated particles.

Association of Au NMs with Algal Cells. The association of Au NMs with algal cells as a function of particle properties, on the basis of different dose metrics, is reported in Figure 2. Six replicates were tested for each treatment. The figures show that the variation between the replicates of each treatment is low. This observation reflects the repeatability of the experiment and the robustness of the method. The statistical data are embedded in each figure. The algal cells did not take up the 70 nm × 300 nm rod-shaped, 75 nm × 500 nm wire-shaped, 75 nm × 3000 nm wire-shaped, or 75 nm × 6000 nm wire-shaped Au NMs. It is likely that these particles, which are large, could not penetrate the EPS layer and were removed during the washing processes.

Expression of the Association of Au NMs with Algal Cells Based on Mass. The mass of a single particle of each of the tested Au NMs is reported in Table S5. The average of the total mass of the associated Au NMs with the algal cells is plotted in panels a and b of Figure 2. The mass of a spherical 60 and 100 nm Au NM is several orders of magnitude higher

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than the mass of a spherical 10 nm Au NM. Averaging the total Au mass of the cellular associated particles results in the following trends: 10 nm × 45 nm rod-shaped > 10 nm spherical = 100 nm spherical > 60 nm spherical for the citrate-coated Au NMs and 10 nm × 45 nm rod-shaped > 60 nm spherical > 10 nm spherical = 100 nm spherical for NOM-coated Au NMs. Because the mass of a spherical 100 nm particle is higher than the mass of spherical 10 nm, spherical 60 nm, and rod-shaped 10 nm × 45 nm particles, considering the association based on particle number and SA would dramatically shift the observed trends.

Expression of the Association of Au NMs with Algal Cells Based on SA. The calculated SA of the Au NMs is reported in Table S5. Regression analysis showed a weak correlation (R^2 = 0.55) between the SA of the associated Au NMs with the algae and the SA of the Au NM in the exposure medium (Figure 2c,d). The SA of the spherical 10 nm Au NM attached to the cells was lower than the SA of the attached rod-shaped 10 nm × 45 nm Au NMs, while the SA of the spherical 10 nm Au NM in exposure media is higher than the SA of the rod-shaped 10 nm × 45 nm NMs. Similarly, the SA of the attached spherical 60 nm Au NMs to the cells was lower than the SA of the attached spherical 100 nm Au NMs, whereas the SA of the spherical 60 nm Au NMs in exposure media is higher. There is no clear trend for the cellular association of Au NMs when SA is considered as the dose metric, and the higher SA in the exposure media did not lead to a higher level of particle association with the cells. The regression (R^2 = 0.67) was stronger when the Au NMs were coated with NOM (Figure 2d). A larger surface area enhances the interaction of NMs with surrounding biological molecules and their toxicity to biota,[31] the capacity of transferring contaminants into organisms and the food chain, and release of the ion from the metallic NMs and subsequent toxicity of metallic ions.[32,33]

Expression of the Association of Au NMs with Algal Cells Based on Particle Number. The number concentrations of the Au NMs in exposure media at the start of the exposure tests are listed in Table S5. The number of Au NMs associated with the algal cells versus the number of particles in the exposure media is plotted in panels e and f of Figure 2. With an increase in the number of Au NMs in exposure media at the same total particle mass, the number of Au NMs associated with the cells increased. We observed a strong linear correlation (R^2 = 0.88) between the number of the cellular associated NMs and the number of NMs in the exposure medium for both citrate-coated and NOM-coated Au NMs. By considering the particle number as the dose metric, it is thus shown that particle shape and size did not influence the cellular association of the particles. The cellular association of Au NM with the algae is apparently not selective, and the number of the associated particles clearly reflects the exposure number concentrations. The surface chemistry, however, influenced the cellular association of Au NM by algae when the particle number is used as the dose metric. The number of associated spherical 60 nm particles was higher than the number of associated 100 nm Au NMs when the particles are coated with citrate. However, the number of associated spherical 60 nm Au NMs decreased to a level lower than the number of associated spherical 100 nm Au NMs when the particles were coated with NOM. Particle number is the main element used for the classification of NMs according to the recommended definition for NMs by the EC for safety purposes.[34,35] The higher the number of particles associated with algae, the higher the number of particles transferred in food chains. If a high number of NMs enter food chains, it is possible that number-based biomagnification takes place where the number of particles in the predator is significantly higher compared to the number of particles in the prey.[8]

Expression of the Association of Au NMs with Algal Cells Based on VSSA. By considering the same density for the Au NMs, the VSSA is the ratio between the external SA of the Au NMs and their volume. The calculated VSSA of the single-particle Au NMs is reported in Table S5. The VSSA of the particles in exposure media was calculated by considering a normal size distribution of the particles in the medium (a monodispersed particle size distribution) (see section S10 of the Supporting Information). Due to the dependency of VSSA on particle shape and size distribution, variation in these parameters would be reflected in the VSSA.[36,37] A strong regression was found (Figure 2g,h) between the VSSA of the associated Au NMs and the calculated VSSA of the particles in exposure media. With an increase in the VSSA of the particles in exposure media, the VSSA of the associated particles with the cells increased. The R^2 was equal to 0.75 for citrate-coated Au NMs and 0.78 for NOM-coated Au NMs when VSSA was considered as the dose metric. The R^2, however, was weaker when the VSSA was used as a dose metric compared to the case in which the particle number was considered the dose metric. VSSA plays an important role in the classification of NMs based on the recommended definition for NM by the EC.[34,35] Nevertheless, this study is the first that experimentally tested the applicability of VSSA to be a dose metric. VSSA is more applicable for understanding the adverse effects of agglomerates, aggregates, and porous NMs.[34,35] For example, the VSSA of porous materials may be large (as defined for >60 m^2/cm^3 NMs) even when the external size distribution of the materials is ≫100 nm, which forces them not to be classified as NMs according to the EU definition. Thus, such materials may lead to some nanospecific toxicity while they are disregarded as NMs.

In summary, we demonstrated how particle size, shape, and surface chemistry influence the association of Au NMs with algae when different dose metrics are applied. By evaluating the suitability of the dose metrics for NM cellular association based on empirical regressions, we can conclude that particle number followed by VSSA presents the most suitable dose metric. Our study for the first time documented that the cellular association of Au NMs in algae is not selective and the shape and size of particles do no influence the cellular association of particles. The surface chemistry of NMs plays an important role in their association with algae. This can be the case for other types of NMs. The application of VSSA as a does metric was documented for the first time in this study. In general, the findings derived from our study can initiate new research lines in nanotoxicology and environmental science studies to apply different dose metrics in investigating the adverse effects of NMs. The findings also pave the way to better understand the applicability of the EC-recommended definition for NMs and support the enforcement for nanosafety purposes. Our findings also offer a new approach to a better understanding of cellular association and uptake of NMs by other cells such as mammalian cells and/or bacteria, which is critical for nanomedicine and nanobiotechnology.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.estlett.9b00683.

Chemicals; preparation and characterization of natural organic matter; single-particle ICP-MS measurements; experimental design; algal extraction for quantification of Au NMs; quantification of Au NMs on cell-by-cell basis; data analysis; particle characterization; calculated particle mass of a single-particle Au NM and SA, number, and VSSA of the tested Au NMs; and particle SA, number, and VSSA calculation (PDF)

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Notes

The authors declare no competing financial interest.

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