Challenges of Biological Complexity in the Study of Nanotoxicology

Andrew B. Northwick[a] and Erin E. Carlson[a,b,c,d]*

[a]*Department of Chemistry, University of Minnesota, 207 Pleasant Street SE, Minneapolis, Minnesota 55455, United States*

[b]*Department of Medicinal Chemistry, University of Minnesota, 208 Harvard Street SE, Minneapolis, Minnesota 55454, United States*

[c]*Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, 321 Church St SE, Minneapolis, Minnesota 55454, United States*

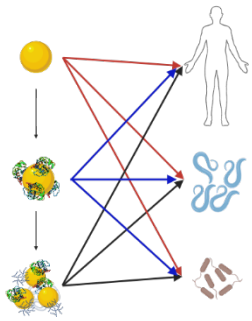
[d]*Department of Pharmacology, University of Minnesota, 321 Church St SE, Minneapolis, Minnesota 55454, United States*

**Corresponding Author*

Email: carlsone@umn.edu

The scale of nanoparticle use in consumer goods has grown exponentially over several decades owing to the unique properties of materials in this size range. At the same time, well-defined end of life cycle disposal strategies have not been developed for most materials, meaning that we are approaching the potential for a new ecological disaster with the release of millions of metric tons into the waste stream. The field of nanotoxicology has also expanded rapidly to investigate these potential hazards and has identified multiple mechanisms of toxicity to all tropes of life. While this research has been insightful, there are stipulations on how applicable many of these results are to real world applications. One of the major challenges in this research is that

nanoparticles are immediately transformed when introduced into an environment. For example, biomolecules, such as proteins, rapidly coat nanoparticles with a shell, called a corona, that can modulate the toxicity of the core materials or aid the internalization into cells. This additional layer of complexity and the non-covalent nature of the corona has made it difficult to identify consistent trends in the study of nanotoxicity using traditional methods. In this perspective, we will highlight the limitations with current techniques, discuss advances that have been made to aid in these studies, and outline remaining challenges.



Author Biographies:

Andrew Northwick – *Andrew Northwick is completing his PhD in the lab of Dr. Erin Carlson at the University of Minnesota. In his graduate work, he is developing analytical techniques from the field of chemical biology to obtain more information on the temporal and spatial dimensions of the biomolecular corona on nanoparticles.*

Erin Carlson – *Erin E. Carlson is a Smith Professor in the University of Minnesota Department of Chemistry. She is also graduate faculty in the Department of Medicinal Chemistry, Department of Biochemistry, Molecular Biology, and Biophysics, the Molecular Pharmacology and Therapeutics graduate program, and the Bioinformatics and Computational Biology graduate program. The Carlson Lab focuses on characterizing and inhibiting bacterial resistance mechanisms.*

Introduction:

One of the biggest technological advancements of the 21st century has been the development and application of nanoparticles. A nanoparticle is any material that has one dimension on the nano (10^{-9} m) scale and can be made from a variety of materials such as heavy metal oxides, precious metals, carbon and/or polymers. Their small size and large relative surface area means that nanoparticles have drastically different physical and chemical properties relative to the material's bulk counterparts. An example of this is the plasmonic effect with light that causes them to resonate with rather than absorb light waves, the phenomenon that causes gold nanoparticles to appear red rather than yellow.[1] In addition, the discreet layered sheets in some nanoparticles make them more capable of generating charge than their bulk counterparts. Nanoparticles can be synthesized in a variety of shapes to further alter interactions with light and electrons and surface modifications can be used to optimize bonding interactions. These properties have led to extensive research into their use as sensors, in energy storage, bioimaging, and drug delivery for a few examples. Because of their diverse applications, nanoparticle production is increasing at a rate that is difficult to estimate but best predictors suggest that ~13 million metric tons were produced in 2012, with an expected increase in annual production.[2] Allied Market Research places the economic impact of nanoparticles to be 16.3 billion USD in 2021 and projects it to grow to 62.8 billion USD by 2031.[3] *Keller et. al.* breaks down the manufacture, application, and end of life of ten types of nanoparticles found that ~60% of these materials end up in landfills, with the remainder entering the environment (Figure 1). [4] They also broke down where the major production of the particles occurs and highlight how this is on the verge of being a global scale problem.

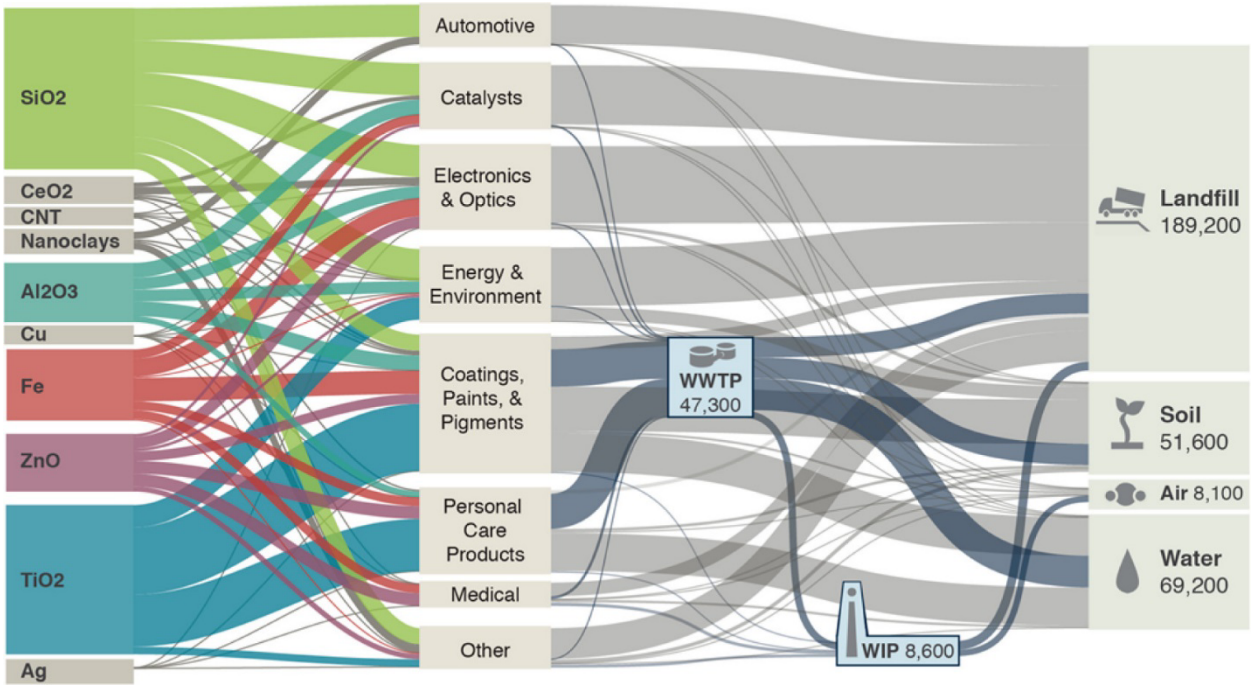


Figure 1: A breakdown of the production and waste stream of ten common nanoparticles. Figure taken from Keller A. et. al. without adaptation.[4]

However, the same properties that have led to the exponential increase in the use of nanomaterials also raise substantial concerns about their interactions and consequences in complex biological environments. Numerous studies over the last decade have investigated what happens when a variety of nanoparticles are introduced to these environments and have found that nanoparticles leech more heavy metal ions, facilitate the production of reactive oxygen species (ROS) causing DNA damage, cause membrane disruption and/or lysis, or are internalized into cells resulting in a variety of toxic effects relative to their bulk counterparts often as a result of their larger surface area-to-size ratio.[5-7] Their size and shapes can also cause them to be more readily internalized into cells, directly causing damage to internal structures and biomolecules.

The broad scope of production, use and disposal/exposure already presents a complex web of study for predicting the potential toxicity of nanoparticles (Figure 2), but there is another

factor when nanoparticles are exposed to a biological environment – biomolecules will generate a coating that encompasses the nanoparticle, forming what is called a corona.

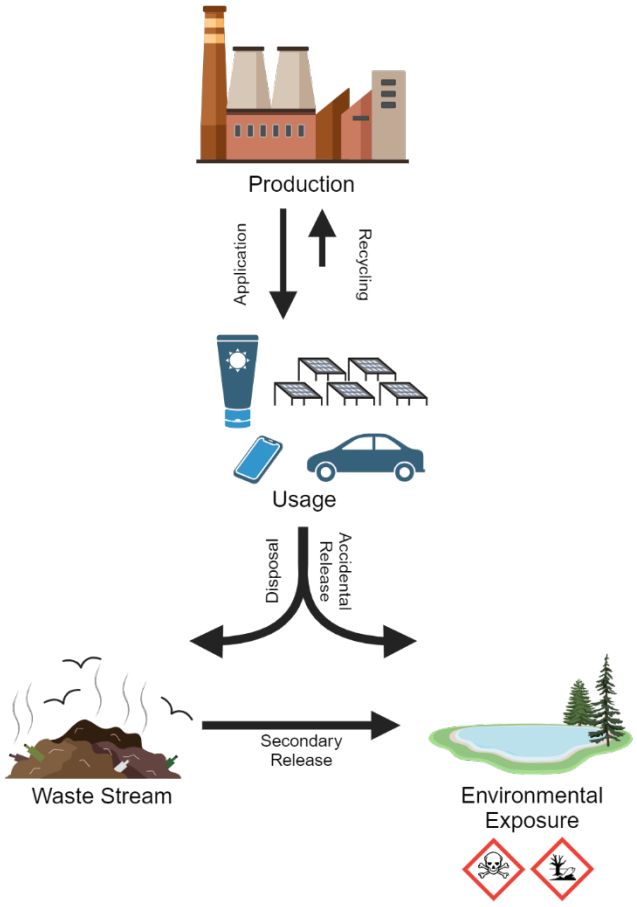


Figure 2: The life cycle of an engineered nanoparticle has multiple stages, but most end up in waste streams or released into environments where the materials were not designed for a present a new hazard to the organisms in that environment.

The adsorption of a corona onto nanoparticles is known to influence their toxicity to biologic systems in a variety of ways. For example, corona formation can cause particle aggregation or sequestration in specific parts of the environment increasing the effective dose. Corona constituents have also been shown to facilitate leeching of heavy metals out of the nanoparticle or make it easier for them to be internalized by cells to access organelles or DNA.[8-11] Because of the added influence of a corona on nanotoxicity, work has been performed to determine the driving

interactions between the nanoparticle and the corona. Currently, investigations have been largely limited to the composition of molecules in the corona. One of the most relevant discoveries is that the concentration of a biomolecule in the medium does not correlate to the amount of association onto the surface of the nanoparticle.[12-13] The primary example of this is the corona adsorption onto particles that were introduced into vascular systems. While albumin comprises ~60% of the protein in blood serum, it was only found in a low concentration on the particles. This is likely due to the well-documented phenomenon called the Vroman effect, wherein higher mobility biomolecules in a solution interact first with a surface but are eventually replaced with species that have a higher affinity for the surface. The Vroman effect in the context of corona formation on a nanoparticle leads to two distinct coronas, the hard and soft corona. The hard corona is composed of high affinity proteins that displace more weakly bound, highly mobile proteins in the initially formed soft corona. However, unlike the hard corona, the soft corona is likely primarily comprised of protein-protein interactions with minimal interactions directly with the nanoparticle, but this is not well defined. The differing levels of surface affinity has largely limited study of nanoparticle corona formation to the characterization of hard corona composition. However, even discovering that there are difference in affinity implies that there are important interactions that drive adsorption and favor interactions in the corona despite it being comprised of noncovalent interactions.

Given the difficulty in studying the interface between nanoparticles, the environment, and biological systems, trends that drive toxicity and accumulation are not well understood. This stems from limitations in available techniques to probe these complex interactions. With the increased application of nanoparticles in commercial goods rising, we must work to uncover the short and long-term toxic effects of various nanoparticles. For this to succeed, a better understanding of the

forces that contribute to the toxic properties of nanoparticles is needed so that wholistic design standards can be implemented. In this perspective, we aim to summarize the current state of nanotoxicology research, highlighting advancements that showcase the possibilities for particle redesign to decrease their toxicity, and outline what advancements and additional research are needed for deeper understanding and to assist in the development of better predictive models.

The Current State of Research

Over the last two decades, the field of nanotoxicology has primarily been focused on investigating cause-and-effect relationships. A nanoparticle and model organism are studied in combination, a variable of interest within the nanoparticle is typically changed, and outcomes are measured against one another. The potential combinations for these experiments are theoretically limitless. The breadth of nanoparticles ranges from metal oxides to carbon to polymers to Cd or Zn quantum dots, as some examples. The organisms can range from bacteria to caronamids and fish to mammals and birds and results are dependent upon how nanoparticles are introduced to the samples (e.g., lysed cells, whole organisms). Even when rigorous experiments are designed to investigate the toxicity of a specific nanoparticle or a specific organism's response to a variety of particles, this still leads to an over-simplified model that is only applicable to those exact experimental parameters. This picture is also muddied by the fact that the behavior of many organisms is dependent on environmental conditions.

Some examples of this can be seen from the investigation of *Li et. al.*, who investigated the toxicity of zinc oxide nanoparticles to *E. coli* in five different growth mediums ranging from highly nutrient deficient (ultrapure water) to nutrient rich (Luria-Bertani broth).[14] Across their study, they found that toxicity of nanoparticles was dependent upon the dissolution of zinc to zinc oxide. In PBS and LB, the particles were less toxic relative to when the bacteria were exposed in ultrapure

water because they could form alternative zinc species that were less toxic. A similar effect can be observed even within the same growth medium. Römer *et. al.* investigated the toxicity of silver nanoparticles to *D. magna* in various concentrations of the same growth medium but altered the dilution factor to account for changes in toxicity from particle aggregation in concentrated media but not in diluted media.[15] They observed significant aggregation in the undiluted and two-fold diluted media samples with no aggregation in the ten-fold dilution. This increase in aggregation caused a ~two-fold decrease in the EC_{50} of the nanoparticles to *D. magna*. The negative correlation between aggregation and toxicity can be attributed to the lower available fraction of the nanoparticles in solution for ingestion and silver dissolution. The differences in growth media are even more pronounced in a nutrient-rich environment, because of the capability of the proteins and other biomolecules to form a corona on the nanoparticles. When a corona adsorbs onto the surface of a nanoparticle, the relative complex size increases and the surface that is presented now looks like a ball of proteins. The NP-corona complex has been shown to allow nanoparticles to have different translocation properties than the bare particles. The interplay between these three factors has made it difficult to design experiments that can untangle the driving factors of nanotoxicity. Furthermore, the same nanoparticle can have different toxicity mechanisms depending on the model organism. Lithium cobalt oxide (LCO) nanoparticles and its derivatives, for example, are lethal to *S. oneidensis* through a combination of heavy metal oxide leeching and ROS production.[16] In *D. magna* though, LCO causes a drop in iron concentration and aggregates in the digestive tract affecting nutrient adsorption.[17-18]

Broadening the Scope of Research

In the face of this complex triad of factors, there has been progress made in reducing the toxic effects of nanoparticles in the environment. One technique used is to cap the surface of the nanoparticle with a thin layer of a secondary material to act as a barrier between the environment and the toxic material. One of the most studied examples of this has been the reduction in the toxicity of cadmium-selenide based quantum dots (QDs) following the addition of a zinc shell or sulfate-containing small molecules.[19-20] QDs are known for their photobrightness and color tuneablilty. These properties have popularized their use in LCD displays and solar panels for around two decades. Unsurprisingly though, the particles are highly toxic if they are removed from the display as cadmium leeches from the particles causing heavy metal poisoning.[21-23] However, this leeching can be mitigated by the addition of a zinc or silica coating onto the surface of the cadmium quantum dot.[24] The addition of this coating helps limit the photocorrosion during repeated reduction-oxidation cycles and limits cadmium leeching from the system while retaining function because the zinc shell is also naturally fluorescent. Shivaji *et. al.* have discovered a second interesting option using tea leaf extract in the synthesis of QDs.[25] They found that incorporation of the plant material not only limited the degradation of the particles by stabilizing the sulfur ions in the QD, thus limiting the leeching of cadmium, but also that the photoactive chemicals had a synergistic effect with QDs, increasing their photocatalytic activity.

Another material garnering interest from the nano field is red phosphorus because of its potential use as an energy storage material, as well as its common use as a semi-pyrophoric material in matches and explosives. One downside of this material is that phosphorus readily converts to phosphoric acid in water or phosphine gas in oxygen/water vapor, both of which are undesired products. Kinsley *et. al.* found that introducing a thin carbon coating onto the surface of red phosphorous nanoparticles reduces the release of phosphine gas two-fold.[26] The thickness of

this coating is time-dependent and can be tuned to the desired depth required for storage and function.

Another possibility to lower nanotoxicity is to alter the composition of the nanoparticle. LCO is the main component of rechargeable batteries for small electronics and electric vehicles because of its excellent storage capacity and energy generation as the lithium shuttles through the cobalt oxide nanosheets. One downside of this material is that cobalt is a rare precious metal and often found with nickel contamination, making it highly expensive. LCO has also been shown to be toxic to many tropes of life because it readily produces ROS in aqueous environments, leeches toxic cobalt ions, and disrupts metabolism in smaller organisms.[27] However, there have been modifications made to the composition of LCO incorporating nickel and manganese to form a complex metal oxide sheet called lithium nickel magnesium cobalt oxide (NMC), taking advantage of the natural contamination of nickel in the process of cobalt mining. NMC has also been shown to be an efficient battery material but retains the toxic properties of LCO. Gunsolus *et. al.* sought to determine if changing the composition of NMC, lowering the amount of nickel and cobalt and increasing the composition of manganese in the metal oxide sheets, could alter the toxicity.[28] They found that material comprised of ~70% manganese leeched less of the toxic metal ions into solution, generated less ROS, and minimally impacted the growth of their model bacterium. These examples show that less toxic nanoparticles can be generated, but this approach is limited as it requires that there is a way to modify the particles while also retaining their desired functionality. With the coating approach, the modified materials were either comparable to the original function in the case of the quantum dot fluorescence or unimpactful in the case of the carbon-coating of phosphorous, while for the nanoparticle reformulation, LCO was amenable to the addition of nickel and manganese while retaining its function.

Understanding and reducing toxicity cannot solely rely on modification of the nanoparticles, especially since there are already examples in the literature wherein the same type of particles will yield different biological outcomes, even when the organisms are grown in the same medium.[19] A key reason for this disconnect is that nanoparticle toxicity is typically characterized by the measurement of cell or organism death (e.g., IC_{50} or LC_{50}) under a specific set of well-defined conditions. While this metric is useful in determining toxicity and can potentially identify trends, it is limited in scope because the experiments hold many variables, such as growth time, exposure time, growth medium, and organism, constant or are limited to a few examples as nanomaterial quantities are often limited.

To enable more high-throughput investigation of nanoparticle toxicity, *Qiu et. Al* devised a plate-based method called the growth-based viability assay. This method facilitates the investigation of many samples simultaneously, but also avoids nanoparticle interference in optical assays.[29] They found that by using a range of nanoparticle dilutions and varying the starting dosage of bacteria, they can track the rate of growth by optical density absorbance measurements and use differences in the growth curves to quantify the impact of nanoparticles on the rate of growth (Figure 3). While the initial trial conditions were limited, the reduced culture size, the multiple bacteria and nanoparticle concentrations, and adaptability for different media and organisms enables the rapid generation of toxicity trends for various nanoparticles in multiple media types.

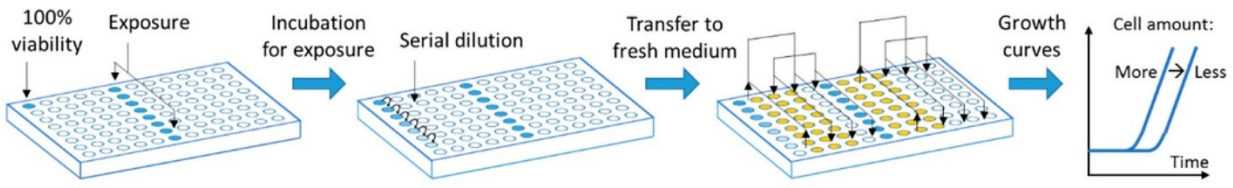


Figure 3: A schematic walkthrough of an alternative method developed to trial viability conditions for nanoparticles as a higher throughput method. Figure taken from Qui. T., et. al. without adaptation.[26]

The ability of organisms to adapt to nanoparticles upon exposure must also be taken into consideration. We have investigated the effect of chronic exposures of sub-lethal doses of NMC to *S. oneidensis*. Mitchell *et. al.* discovered that after prolonged exposure to NMC nanoparticles, *S. oneidensis* are able grow at NMC levels up to twenty-fold higher than the wild-type organism and that this tolerance remains after multiple generations of growth in the absence of nanoparticles.[30] Initial investigations into the resistant strain found that the nanoparticle-exposed bacteria were significantly filamented, often a response to ROS, and that they production more riboflavin early in the exposure period. Sharan *et. al.* continued investigating the role of ROS in *S. oneidensis* resistance.[31] In their studies, they determined that the addition of NMC not only caused increased production of ROS in the extracellular matrix, but also inside the cells. This increased amount of ROS was correlated with an increase in DNA damage and likely promotes the observed filamentation. They also found that the bacteria had higher mutation rates than the wild-type, as evidenced by increased resistance generation frequencies to several common antibiotics. These chronic studies indicate that the bacteria not only survive NMC exposure, but that they have likely undergone mutations that enable sustained NMC resistance.

Studies to date have identified common trends, such as how positively-charged particles have higher membrane association, likely due to interactions with the negatively-charge constituents, and are generally more toxic to cells than neutrally or negatively-charged nanoparticles of similar composition.[32-33] However, assessment of how corona formation contributes to the observed outcomes has been challenging, in large part due to the fact that the corona associates with the nanoparticle non-covalently. It is generally essential to isolate particles

before the nanoparticle-biomolecule complex can be characterized and more weakly associated biomolecules are lost in this process. Thus, it has only been possible to identify the composition of the hard corona but information about how it develops and changes over time, potential secondary protein-protein interactions between the hard and soft coronas, and specific information about critical nanoparticle-biomolecule interactions remains elusive. This is further complicated by a recent study from *Hoang et. al.* comparing the traditional isolation on the nanoparticle-corona complex with the "softer" magnetic pulldown isolation . [34] They found that the two techniques yielded different compositions of the hard and soft corona, as well as some proteins were isolated in the hard corona for one technique but in the soft corona for the other technique. In addition, they resuspended the isolated complex in fresh serum solution and reisolated the particle batches using both techniques to determine if preincubation changes the corona composition differences. They interestingly found that some proteins change from a being primarily in the hard corona to being in the soft corona and from the soft corona into the hard corona.

Some techniques have been developed to isolate more weakly associated corona components such as fractional field-flow chromatography and the aforementioned magnetic-core nanoparticles, but they only enable assessment of corona composition.[34-35] While some molecular trends can be gleaned by the comparison of particle type and preferred protein, methods to identify specific interactions that drive the stability of the hard corona and to determine the orientation of the biomolecules in the corona must be developed. Recent work has begun to address these major challenges through the investigation of single proteins on the surface of nanoparticles. Shrivastava *et. al.* investigated how cytochrome C, RNase A, and lysozyme bind to the surface of silica particles by chemically modifying the lysine residues with acetic anhydride and measuring the acetylation changes and location with MALDI-TOF/TOF analysis.[36] Tollefson *et. al.* confirmed

these results with cytochrome C on gold nanoparticles, implementing a similar chemical protein footprinting technique but reducing the reaction time from 40 min to 1 min to minimize corona perturbation during the labeling period.[37] Both studies identified common regions of cytochrome C that contact the nanoparticle surface (K22, K25, K27), as well as a differing secondary sites specific to their model nanoparticle. In addition, both studies found that other regions of cytochrome C underwent increased labeling, implying that the conformation of the protein changes in the presence of nanoparticles. This postulation was confirmed by Tollefson *et. al.* using circular dichroism measurements to observe protein structural changes. Tollefson's study was also performed in concert with a computational group to model and predict the most stable confirmations of the adsorption of cytochrome C to the particle surface. Across their investigations, they measured the approach distance of cytochrome C of different faces of the protein and calculated the predicted distance and binding energy for each protein face to the surface of the nanoparticle. They found that there was preference for two different approaches, which agreed with the experimental data. These results imply that while the corona is primarily driven by non-covalent forces, it is more complex than just random association and that there are likely preferred binding regions on proteins for a given nanoparticle.

Another strategy to investigate preferred biomolecule-nanoparticle interactions is through computational modeling of binding kinetics. Vilanova *et. al.* have investigated the binding kinetics of three different proteins both computationally and experimentally and have shown that there are competitive effects in the adsorption process that change the binding rates of proteins when they are co-incubated compared to individually incubated.[38] The authors also describe what they call a "memory effect" on exchange of proteins in the corona when particles are sequentially exposed to proteins. Their model accurately predicted the integration of a protein with lower binding affinity

into a pre-formed corona, displacing proteins with a higher binding affinity. However, it predicted a much lower exchange than experimental data found when a more strongly binding protein integrated into a corona comprised of weakly binding protein. They hypothesize that addition of the nanoparticle changes the protein-protein interactions in a way that their model does not account for. The authors test this by adapting their model to a three-body system, which can account for interactions between the nanoparticle and each protein, as well as the protein-protein interactions, yielding a more accurate model. This illustrate the complexity of understanding nanoparticle-corona dynamics even when only two proteins are interacting on the surface, much less when there are hundreds of proteins and other biomolecules, as is the case in real world exposures. These examples also highlight that processes such as assay development, sample preparation, clean-up and processing can complicate or even bias measurements and modeling.

How do we move forward

As briefly highlighted, over the past two decades of research, the field of nanotoxicology has determined trends in nanoparticle toxicity. More detailed design principles and an understanding of how environmental factors affect these trends remain elusive. Most investigations have evaluated the toxicity of a specific material under a small and defined set of conditions. While this strategy has provided critical new information and is the most practical way to investigate nanoparticle-organism interactions, this approach has left multiple questions unanswered, such as:

- What are the forces that drive the adsorption of the corona, and can this process be controlled or directed?

- How does the adsorption of a corona affect the mechanisms of toxicity and long-term stability of the particles?
- Does the nanoparticle-corona complex have different interactions with the environment than the particle or biomolecules independently?
- Does preincubation with a biological coating limit the toxicity of the materials or change the mechanisms of toxicity?
- How do these factors change in non-aqueous mediums, such as soil?
- What is an acceptable balance between toxicity and function when designing particles for use?
- What happens to materials we have released for potential remediation nutrient development?

To address these questions, we must move past "simplified" model experiments with single proteins or defined serum systems to long-term exposures in increasingly complex environments (Figure 4). We must also find ways to more readily investigate the dynamic interactions of the nanoparticle-corona complex as it develops into the hard corona and maintains this steady state once formed. The study of dynamics is exceptionally difficult given that the experimental

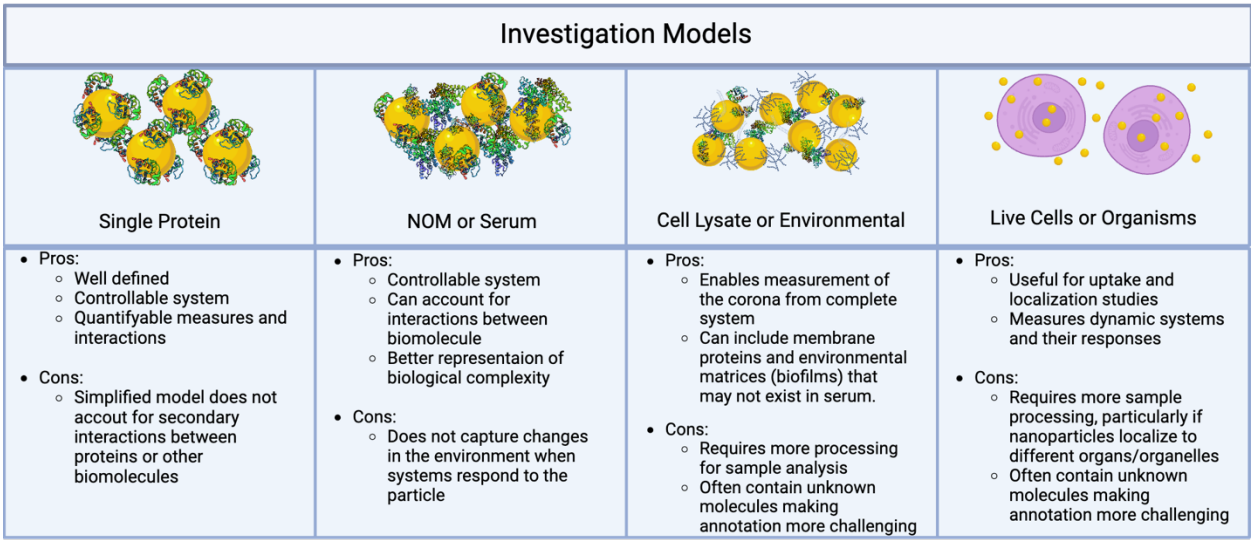


Figure 4: There are various levels of complexity to consider when designing an experiment to probe nanotoxicity. The ideal experiment is one that can collect high-resolution data from a simplistic single nanoparticle-biomolecule study from the highly complex system of a living organism. Currently, we are gated by the isolation techniques biasing toward high-affinity corona interactions and loss of all spatial resolution upon collecting native species.

techniques must be rapid enough to prevent changes at the timepoint of interest and must avoid disrupting the native nanoparticle-corona complex. In addition, such methods should enable investigation of a wide variety of nanoparticles and environments, as well as provide sensitive and accurate measures in complex environments. Some of these challenges will likely have to be tackled with techniques not yet developed, but strategies from other fields can also continue to be implemented in nanotoxicology studies. For example, the protein footprinting method, originally used to investigate protein-protein interactions, was employed by Shrivastava *et. al.* and Tollefson *et. al.* to identify lysine residues important in nanoparticle-protein interactions. This methodology could be further expanded to investigate the roles of other protein residues using established labeling reactions, such as amidation of aspartate and glutamate, or implementation of the many other chemoselective bioconjugation reactions that have been developed in the field of chemical biology.[39-43]

Stepping back further, we must also consider how nanoparticles transform not just upon introduction to a biological environment but also through their chemical and physical use. Many materials are designed for energy storage or chemical reactions that involve repeated redox reactions or mechanical processing that can cause uncontrolled modifications and damage. It has already been demonstrated that ion leeching is one of the driving forces behind nanotoxicity, so particles that are already partially degraded or are predisposed to leech metals could have differing toxicity than expected. Deformities could also create physical changes that affect biomolecular corona formation. It is known that increasing the size of a nanosphere alters the packing density of the corona on the surface and that different nanoparticle shapes preferably localize biomolecules on different areas.[44-45] These are not simple challenges to surmount given the complex and varied conditions that could arise, especially if the end goal is to predict or direct the outcomes of nanoparticles in the environment. With the breadth of techniques and guidelines that have been developed to investigate these questions for the toxicity of small molecules (e.g., DTT, carcinogens), there is a strong baseline to assist in the growth of the field of nanotoxicology. The adaptation of these techniques and development of novel assays to quantify nanotoxicity will be essential to answer the questions that we have outlined above, in addition to many as-of-yet unknown challenges we must overcome to minimize harm to ecological and environmental health.

Author Contribution

E.E.C. and A.B.N. conceived the idea for this manuscript. A.B.N. provided the first draft and edits. E.E.C. provided secondary edits. Both authors were involved with the final editing and approval of the submitted manuscript.

Conflict of interest

There are no conflicts to declare.

Acknowledgements

This manuscript is based on work supported by the National Science Foundation under Grant No. CHE-2001611, the NSF Center for Sustainable Nanotechnology (CSN). A. Northwick was also supported the National Institute of Health Chemical Biology Training Grant (T32GM132029). The table of contents figure, figure 2, and figure 4 were made using BioRender.

Abbreviations

LCO – Lithium Cobalt Oxide

NMC – Nickel Manganese Cobalt Oxide

ROS – Reactive Oxygen Species

QD – Quantum Dots

References

1. Ghosh, S. K.; Pal, T., Interparticle Coupling Effect on the Surface Plasmon Resonance of Gold Nanoparticles: From Theory to Applications. *Chemical Reviews* **2007,** *107* (11), 4797-4862.
2. Piccinno, F.; Gottschalk, F.; Seeger, S.; Nowack, B., Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. *Journal of Nanoparticle Research* **2012,** *14* (9), 1109.
3. Nanomaterials Market Size, Share, Competitive Landscape and Trend Analysis Report by Material Type, by End Use Industry : Global Opportunity Analysis and Industry Forecast, 2021-2031. *Allied Market Research* **2022**.
4. Keller, A. A.; Lazareva, A., Predicted Releases of Engineered Nanomaterials: From Global to Regional to Local. *Environmental Science & Technology Letters* **2014,** *1* (1), 65-70.
5. Wang, D.; Lin, Z.; Wang, T.; Yao, Z.; Qin, M.; Zheng, S.; Lu, W., Where does the toxicity of metal oxide nanoparticles come from: The nanoparticles, the ions, or a combination of both? *Journal of Hazardous Materials* **2016,** *308*, 328-334.
6. Ganguly, P.; Breen, A.; Pillai, S. C., Toxicity of Nanomaterials: Exposure, Pathways, Assessment, and Recent Advances. *ACS Biomaterials Science & Engineering* **2018,** *4* (7), 2237-2275.
7. Roy, J.; Roy, K., Evaluating metal oxide nanoparticle (MeOx NP) toxicity with different types of nano descriptors mainly focusing on simple periodic table-based descriptors: a mini-review. *Environmental Science: Nano* **2023,** *10* (11), 2989-3011.
8. Bertoli, F.; Garry, D.; Monopoli, M. P.; Salvati, A.; Dawson, K. A., The Intracellular Destiny of the Protein Corona: A Study on its Cellular Internalization and Evolution. *ACS Nano* **2016,** *10* (11), 10471-10479.
9. Francia, V.; Yang, K.; Deville, S.; Reker-Smit, C.; Nelissen, I.; Salvati, A., Corona Composition Can Affect the Mechanisms Cells Use to Internalize Nanoparticles. *ACS Nano* **2019,** *13* (10), 11107-11121.
10. Lesniak, A.; Fenaroli, F.; Monopoli, M. P.; Åberg, C.; Dawson, K. A.; Salvati, A., Effects of the Presence or Absence of a Protein Corona on Silica Nanoparticle Uptake and Impact on Cells. *ACS Nano* **2012,** *6* (7), 5845-5857.
11. Wan, S.; Kelly, P. M.; Mahon, E.; Stöckmann, H.; Rudd, P. M.; Caruso, F.; Dawson, K. A.; Yan, Y.; Monopoli, M. P., The "Sweet" Side of the Protein Corona: Effects of Glycosylation on Nanoparticle–Cell Interactions. *ACS Nano* **2015,** *9* (2), 2157-2166.
12. Fernández-Iglesias, N.; Bettmer, J., Complementary mass spectrometric techniques for the quantification of the protein corona: a case study on gold nanoparticles and human serum proteins. *Nanoscale* **2015,** *7* (34), 14324-14331.
13. Schäffler, M.; Semmler-Behnke, M.; Sarioglu, H.; Takenaka, S.; Wenk, A.; Schleh, C.; Hauck, S. M.; Johnston, B. D.; Kreyling, W. G., Serum protein identification and quantification of the corona of 5, 15 and 80 nm gold nanoparticles. *Nanotechnology* **2013,** *24* (26), 265103.

14. Li, M.; Zhu, L.; Lin, D., Toxicity of ZnO Nanoparticles to Escherichia coli: Mechanism and the Influence of Medium Components. *Environmental Science & Technology* **2011,** *45* (5), 1977-1983.
15. Römer, I.; Gavin, A. J.; White, T. A.; Merrifield, R. C.; Chipman, J. K.; Viant, M. R.; Lead, J. R., The critical importance of defined media conditions in Daphnia magna nanotoxicity studies. *Toxicology Letters* **2013,** *223* (1), 103-108.
16. Hang, M. N.; Gunsolus, I. L.; Wayland, H.; Melby, E. S.; Mensch, A. C.; Hurley, K. R.; Pedersen, J. A.; Haynes, C. L.; Hamers, R. J., Impact of Nanoscale Lithium Nickel Manganese Cobalt Oxide (NMC) on the Bacterium Shewanella oneidensis MR-1. *Chemistry of Materials* **2016,** *28* (4), 1092-1100.
17. Bozich, J.; Hang, M.; Hamers, R.; Klaper, R., Core chemistry influences the toxicity of multicomponent metal oxide nanomaterials, lithium nickel manganese cobalt oxide, and lithium cobalt oxide to Daphnia magna. *Environmental Toxicology and Chemistry* **2017,** *36* (9), 2493-2502.
18. Niemuth, N. J.; Curtis, B. J.; Laudadio, E. D.; Sostare, E.; Bennett, E. A.; Neureuther, N. J.; Mohaimani, A. A.; Schmoldt, A.; Ostovich, E. D.; Viant, M. R.; Hamers, R. J.; Klaper, R. D., Energy Starvation in Daphnia magna from Exposure to a Lithium Cobalt Oxide Nanomaterial. *Chemical Research in Toxicology* **2021,** *34* (11), 2287-2297.
19. Hu, L.; Zhong, H.; He, Z., The cytotoxicities in prokaryote and eukaryote varied for CdSe and CdSe/ZnS quantum dots and differed from cadmium ions. *Ecotoxicology and Environmental Safety* **2019,** *181*, 336-344.
20. Nagy, A.; Zane, A.; Cole, S. L.; Severance, M.; Dutta, P. K.; Waldman, W. J., Contrast of the Biological Activity of Negatively and Positively Charged Microwave Synthesized CdSe/ZnS Quantum Dots. *Chemical Research in Toxicology* **2011,** *24* (12), 2176-2188.
21. Pace, H. E.; Lesher, E. K.; Ranville, J. F., Influence of stability on the acute toxicity of CdSe/ZnS nanocrystals to Daphnia magna. *Environmental Toxicology and Chemistry* **2010,** *29* (6), 1338-1344.
22. Wroblewska-Wolna, A. M.; Harvie, A. J.; Rowe, S. F.; Critchley, K.; Butt, J. N.; Jeuken, L. J. C., Quantum dot interactions with and toxicity to Shewanella oneidensis MR-1. *Nanotechnology* **2020,** *31* (13), 134005.
23. Mo, D.; Hu, L.; Zeng, G.; Chen, G.; Wan, J.; Yu, Z.; Huang, Z.; He, K.; Zhang, C.; Cheng, M., Cadmium-containing quantum dots: properties, applications, and toxicity. *Applied Microbiology and Biotechnology* **2017,** *101* (7), 2713-2733.
24. Sun, H.; Zhang, F.; Wei, H.; Yang, B., The effects of composition and surface chemistry on the toxicity of quantum dots. *Journal of Materials Chemistry B* **2013,** *1* (47), 6485-6494.
25. Shivaji, K.; Sridharan, K.; Kirubakaran, D. D.; Velusamy, J.; Emadian, S. S.; Krishnamurthy, S.; Devadoss, A.; Nagarajan, S.; Das, S.; Pitchaimuthu, S., Biofunctionalized CdS Quantum Dots: A Case Study on Nanomaterial Toxicity in the Photocatalytic Wastewater Treatment Process. *ACS Omega* **2023,** *8* (22), 19413-19424.

26. Kinsley, P. C.; Zeng, A.; Hedlund Orbeck, J. K.; Debow, S.; Zander, Z. B.; Heaney, P. J.; Hamers, R. J., Reactivity passivation of red phosphorus with thin plasma-deposited carbon coating. *Applied Surface Science* **2022,** *587*, 152791.
27. Cheng, X.; Tian, X.; Wu, A.; Li, J.; Tian, J.; Chong, Y.; Chai, Z.; Zhao, Y.; Chen, C.; Ge, C., Protein Corona Influences Cellular Uptake of Gold Nanoparticles by Phagocytic and Nonphagocytic Cells in a Size-Dependent Manner. *ACS Applied Materials & Interfaces* **2015,** *7* (37), 20568-20575.
28. Gunsolus, I. L.; Hang, M. N.; Hudson-Smith, N. V.; Buchman, J. T.; Bennett, J. W.; Conroy, D.; Mason, S. E.; Hamers, R. J.; Haynes, C. L., Influence of nickel manganese cobalt oxide nanoparticle composition on toxicity toward Shewanella oneidensis MR-1: redesigning for reduced biological impact. *Environmental Science: Nano* **2017,** *4* (3), 636-646.
29. Qiu, T. A.; Nguyen, T. H. T.; Hudson-Smith, N. V.; Clement, P. L.; Forester, D.-C.; Frew, H.; Hang, M. N.; Murphy, C. J.; Hamers, R. J.; Feng, Z. V.; Haynes, C. L., Growth-Based Bacterial Viability Assay for Interference-Free and High-Throughput Toxicity Screening of Nanomaterials. *Analytical Chemistry* **2017,** *89* (3), 2057-2064.
30. Mitchell, Stephanie L.; Hudson-Smith, N. V.; Cahill, M. S.; Reynolds, B. N.; Frand, S. D.; Green, C. M.; Wang, C.; Hang, M. N.; Hernandez, R. T.; Hamers, R. J.; Feng, Z. V.; Haynes, C. L.; Carlson, E. E., Chronic exposure to complex metal oxide nanoparticles elicits rapid resistance in Shewanella oneidensis MR-1. *Chemical Science* **2019,** *10* (42), 9768-9781.
31. Sharan, D.; Wolfson, D.; Green, C. M.; Lemke, P.; Gavin, A. G.; Hamers, R. J.; Feng, Z. V.; Carlson, E. E., Chronic exposure to complex metal oxide nanomaterials induces production of reactive oxygen species in bacteria. *Environmental Science: Nano* **2023,** *10* (8), 1978-1992.
32. Jiang, Y.; Huo, S.; Mizuhara, T.; Das, R.; Lee, Y.-W.; Hou, S.; Moyano, D. F.; Duncan, B.; Liang, X.-J.; Rotello, V. M., The Interplay of Size and Surface Functionality on the Cellular Uptake of Sub-10 nm Gold Nanoparticles. *ACS Nano* **2015,** *9* (10), 9986-9993.
33. Jeon, S.; Clavadetscher, J.; Lee, D. K.; Chankeshwara, S. V.; Bradley, M.; Cho, W. S., Surface Charge-Dependent Cellular Uptake of Polystyrene Nanoparticles. *Nanomaterials (Basel, Switzerland)* **2018,** *8* (12).
34. Hoang, K. N. L.; Wheeler, K. E.; Murphy, C. J., Isolation Methods Influence the Protein Corona Composition on Gold-Coated Iron Oxide Nanoparticles. *Analytical Chemistry* **2022,** *94* (11), 4737-4746.
35. Weber, C.; Simon, J.; Mailänder, V.; Morsbach, S.; Landfester, K., Preservation of the soft protein corona in distinct flow allows identification of weakly bound proteins. *Acta Biomaterialia* **2018,** *76*, 217-224.
36. Shrivastava, S.; Nuffer, J. H.; Siegel, R. W.; Dordick, J. S., Position-Specific Chemical Modification and Quantitative Proteomics Disclose Protein Orientation Adsorbed on Silica Nanoparticles. *Nano Letters* **2012,** *12* (3), 1583-1587.
37. Tollefson, E. J.; Allen, C. R.; Chong, G.; Zhang, X.; Rozanov, N. D.; Bautista, A.; Cerda, J. J.; Pedersen, J. A.; Murphy, C. J.; Carlson, E. E.; Hernandez, R., Preferential Binding of

Cytochrome c to Anionic Ligand-Coated Gold Nanoparticles: A Complementary Computational and Experimental Approach. *ACS Nano* **2019,** *13* (6), 6856-6866.
38. Vilanova, O.; Mittag, J. J.; Kelly, P. M.; Milani, S.; Dawson, K. A.; Rädler, J. O.; Franzese, G., Understanding the Kinetics of Protein–Nanoparticle Corona Formation. *ACS Nano* **2016,** *10* (12), 10842-10850.
39. Zhang, H.; Liu, H.; Blankenship, R. E.; Gross, M. L., Isotope-Encoded Carboxyl Group Footprinting for Mass Spectrometry-Based Protein Conformational Studies. *Journal of the American Society for Mass Spectrometry* **2016,** *27* (1), 178-181.
40. Ge, J.; Du, S.; Yao, S. Q., Bifunctional Lipid-Derived Affinity-Based Probes (AfBPs) for Analysis of Lipid–Protein Interactome. *Accounts of Chemical Research* **2022,** *55* (24), 3663-3674.
41. Zafra, F.; Piniella, D., Proximity labeling methods for proteomic analysis of membrane proteins. *Journal of Proteomics* **2022,** *264*, 104620.
42. Kozoriz, K.; Shkel, O.; Hong, K. T.; Kim, D. H.; Kim, Y. K.; Lee, J.-S., Multifunctional Photo-Cross-Linking Probes: From Target Protein Searching to Imaging Applications. *Accounts of Chemical Research* **2023,** *56* (1), 25-36.
43. Varnaitė, R.; MacNeill, S. A., Meet the neighbors: Mapping local protein interactomes by proximity-dependent labeling with BioID. *PROTEOMICS* **2016,** *16* (19), 2503-2518.
44. García-Álvarez, R.; Hadjidemetriou, M.; Sánchez-Iglesias, A.; Liz-Marzán, L. M.; Kostarelos, K., In vivo formation of protein corona on gold nanoparticles. The effect of their size and shape. *Nanoscale* **2018,** *10* (3), 1256-1264.
45. Piella, J.; Bastús, N. G.; Puntes, V., Size-Dependent Protein–Nanoparticle Interactions in Citrate-Stabilized Gold Nanoparticles: The Emergence of the Protein Corona. *Bioconjugate Chemistry* **2017,** *28* (1), 88-97.