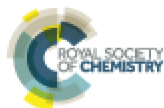

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Journal:	*ChemComm*
Manuscript ID	CC-FEA-08-2018-006473.R1
Article Type:	Feature Article

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FEATURE ARTICLE

Linking nanomaterial properties to biological outcomes: analytical chemistry challenges in nanotoxicology for the next decade

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Tian A. Qiu,[a†] Peter L. Clement[a] and Christy L. Haynes*[a]

The field of nanotoxicology has evolved rapidly in the past two decades. Starting from simple nanomaterials and established toxicity assays, researchers' foci have shifted towards understanding the mechanisms underlying nanotoxicity. Furthermore, an important goal has been linking nanomaterial properties to biological responses to build predictive models for safer nanomaterial design. Here, we provide our perspectives, as analytical chemists, on the analytical challenges in nanotoxicology as the field is entering its third decade. We have identified these challenges to include understanding causal relationships in mechanistic studies of nanotoxicity, overcoming nanomaterial interferences for accurate nanotoxicity assays, connecting nanoparticle interactions to cellular responses at the single-cell level, and making chemical measurements at the nano-bio interface in real-time and *in situ*.

1.1 Introduction

The development of nanotechnology has enabled advances in far-ranging fields. Though the full extent to which nanomaterials (NMs) will revolutionize society remains to be seen, a conservative assessment of their impact to date would find that NMs have made significant and lasting contributions to a number of fields, especially medicine, computing, and energy production and storage.[1-4]

The explosion of nano-enabled products is made more impressive by the fact that the field is relatively new—arising in the second half of the twentieth century. At recent count, there are thousands of commercial products that contain NMs[5], not to mention industrial processes that use ENMs.[6] To date, the Nanodatabase in Europe lists 3038 products containing NMs and The Project on Emerging Nanotechnologies has documented 1827 NM-containing consumer products.[7, 8] The rapid advance in nanotechnology over the past half-century is due in no small part to the National Nanotechnology Initiative (NNI) funded by the government of the United States of America, championed by Mihail Roco and brought to fruition in 2000. In 2004, Roco outlined four generations of NMs: 1) passive materials that don't differ too significantly from bulk materials but enhance bulk properties; 2) active nanomaterials which comprise materials that are the active participants in processes; 3) 3-D nanosystems and systems of nanosystems; and 4) heterogenous nanosystems in which different individual components play roles in the performance of the whole.[1] Over the past decades, as predicted, the complexity of NMs and related nanosystems has remarkably increased and will not likely stop expanding in the near future (**Figure 1**).

From the early days of the NNI, it was clear that NMs would interact with living systems in engineered (e.g. medicine, agriculture, etc.) and incidental capacities.[1, 9] In light of this, wide-spread calls were made for understanding the environmental health and safety of NMs, and specifically for understanding mechanisms of nanotoxicity at the molecular level.[9, 10] The study of the environmental health and safety of NMs has made great strides in recent years and provided key insights into chemical interactions between NMs and organisms of varying complexity.

Pre-dating the NNI, a large community was interested in the human health implications of ultrafine particles and aerosols.[10, 11] This "zeroth generation" of nanotoxicologists was largely concerned with either natural NMs (e.g. asbestos) or unintentionally synthesized NMs (e.g. carbonaceous materials

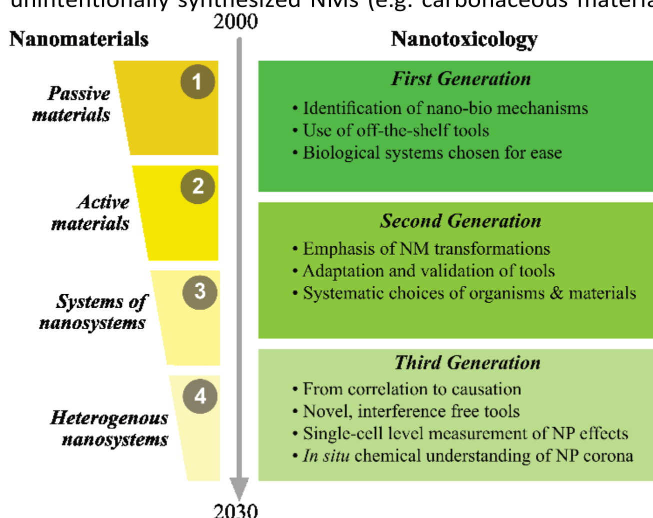

Figure 1 Evolution of nanotoxicology and nanomaterial complexity.

[a] Department of Chemistry, University of Minnesota, 207 Pleasant St SE, Minneapolis, MN 55455, United States
* Email: chaynes@umn.edu
† Current address: Beckman Institute, University of Illinois, Urbana-Champaign, 405 N Mathews Ave, Urbana, IL 61801, United States

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from combustion) and used language that may be unfamiliar to the current field. However, their questions were the same: By what chemical mechanisms do nanoscale materials impact organisms? And are these interactions fundamentally different than those of small molecules?

The first generation of nanotoxicologists heeded the call from the NNI and looked to answer how engineered NMs may impact cell lines and organisms. These groups studied the interactions between a wide range of NMs and organisms. In the process, those in the first generation of nanotoxicology identified some of the primary mechanisms of nanotoxicity and built foundational knowledge that current scientists rely upon when testing hypotheses, such as generation of reactive oxygen species (ROS). However, in this wide-open field, the NMs studied were often chosen for ease of access, either synthetically or commercially, rather than to systematically test hypotheses. Similarly, testing was performed on a number of cell lines and whole organisms, but the choices of biological systems were often arbitrary. These non-systematic choices have been problematic for the field-at-large. Most limiting, groups, including our own, often used off-the-shelf tools developed for small molecule toxicology. It has now been well-documented that established tools, such as the MTT viability assay or fluorescence assays can suffer from false-positive or –negative results due to properties of nanomaterials.[12, 13]

We assert that the field of nanotoxicology is currently in its second generation. Choices of NMs and organisms used for exposures are intentional and systematic, enabling analysis of trends across organisms or between types of NMs.[14-16] During the second generation of nanotoxicology, it has also been realized that there was huge discrepancy in nanotoxicity data acquired on similar materials, creating confusion and hindering accurate risk assessment of nanomaterials to both human health and environment.[17, 18] Unlike small molecules, which are the objects of study in traditional toxicology, the unique properties of NPs could affect accurate measurement of nanotoxicity. For instance, the status of nanomaterials under different testing conditions, such as aggregation state and surface-adsorbed molecules (“corona”), could vary drastically from one testing medium to another and result in changes in observed toxicity.[19] In addition, nanotoxicity assays are subject to the optical, catalytic, and other properties that are specific for each kind of NM and lead to under- or over-estimation of toxicity.[18] Managing these discrepancies has been a challenge to analytical chemists and toxicologists. Off-the-shelf tools started to be validated, and new tools were developed.[18, 20] Methodologies for characterization of NM transformations in complex biological and environmental matrices and the nano-bio interface when NMs encounter biological system have been developed and we believe that they will continue to grow in the future.[21]

The strong foundation built in the zeroth, first, and second generations has provided a launching point for chemists to better understand molecular mechanisms of nanotoxicity. A critical effort that was started by the second generation of nanotoxicologists was to build predictive models that link nanoparticle physiochemical properties to the biological outcome of NM exposure in a quantitative manner. As we are entering the third decade of nanotoxicology, NMs are rapidly being designed and produced, and with increasing complexity, as predicted.[1]. We contend that p roactive establishment of safe NP design rules has never been as critical as it is right now. Ideally, a powerful predictive model can provide information on potential toxicity and preliminary risk assessment of a theoretically designed NM, when the relevant parameters of this designed NM are input into the model. This grand challenge has motivated the field to explore various approaches to link the physiochemical properties of NMs to biological outcomes and build the desired predictive model.

To build predictive models for the biological outcome of NP exposure, two general approaches can be taken: the first is to create NP libraries and test different exposure scenarios, and the second is to explore underlying mechanisms that potentially play roles across numerous scenarios and can be attributed to certain NM characteristics. The former approach relies on large-scale data generation and analysis usually through construction of NM or cell libraries, high-throughput assays, and statistical modeling. The mechanistic approach depends on bioanalytical techniques to characterize biological processes as thoroughly as possible. Such approaches can be either hypothesis- or discovery-driven. The two approaches contribute to predictive models in different ways. The data-demanding approach will provide a large amount of data points to better reveal the

Dr. Tian (Autumn) Qiu completed her PhD in Chemistry at University of Minnesota in 2018 and BS in Chemistry at Peking University in 2012. Her PhD work focuses on understanding bacterial responses to nanomaterials at the molecular level using bioanalytical tools in the laboratory of Prof. Christy Haynes as part of the Center for Sustainable Nanotechnology. She is currently a Beckman Postdoctoral Fellow at University of Illinois, Urbana-Champaign, working with Prof. Jonathan Sweedler and other collaborators on the chemical basis of microbiome-gut-brain axis.

Mr. Peter L. Clement received his BA in chemistry at Williams College (2014). He is currently pursuing a PhD in the laboratory of Professor Christy Haynes in the department of chemistry at the University of Minnesota as part of the Center for Sustainable Nanotechnology. His research focus is the development of methods that provide better insight into nano-bio interactions.

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Prof. Christy L. Haynes is the Elmore H. Northey Professor of Chemistry at the University of Minnesota where her research group explores both biomedical and environmental questions at the interfaces of analytical, biological, and materials chemistry. She is also the Associate Director of the National Science Foundation-funded Center for Sustainable Nanotechnology, a Center for Chemical Innovation.

trends, while mechanistic information helps to reduce omitted variable bias and improve predictive power. We assert that together, the two approaches give the foundation of predictive models.

In this feature article, we have identified four analytical challenges that should be addressed in the third generation of nanotoxicology to better elucidate mechanisms of nanotoxicity and build predictive models that link NM/nanoparticle (NP) properties to biological outcomes. Here, the terms NMs and NPs are used interchangeably. The four challenges are: 1) shifting the paradigm of the mechanistic approach from correlation to causation, 2) overcoming NM interferences in *in vitro* toxicity assays, 3) connecting NP interaction to biological responses at the single-cell level, and 4) measuring the nano-bio interface in real-time and *in situ* with chemical information. The sections that follow detail the motivations for each of these foci and discuss current work and methods that show promise in addressing these challenges.

1.2 From correlation to causation: shifting the paradigm of the mechanistic approach

Fully understanding mechanisms of NP toxicity means knowing the causal relationship among NP exposure, affected biochemical processes, and final biological outcomes (**Figure 2**). NPs for exposure can vary in their physiochemical properties, such as composition, size, charge, and surface modification/adsorption. Biochemical processes, the events on the cellular and molecular levels involving species such as protein and RNA, can be affected differently depending on the NP traits. Final biological outcomes, depending on the scope of the study, can be cytotoxicity for *in vitro* tests or whole-organism outcomes such as survival and reproduction for *in vivo* studies. The causal relationships among these three variables are key in understanding how NPs affect biological organisms and furthermore, impact human health and environment.

A common practice to reveal causal relationships is to systematically tune the properties of pristine NPs and measure NP-affected biochemical processes and whole-organism endpoints. Such approaches have effectively demonstrated that the toxicity of NPs can be affected by their properties, such as composition,[22] size[23, 24] and surface coating.[25] In some early work from our group, we synthesized and characterized either mesoporous silica nanospheres or graphene oxide varying in size, pore character, composition and/or surface modification, and found connections among NP characteristics, their hemolytic activities, and cytotoxicity.[26, 27] Recently, using well-controlled AuNPs of various surface coatings, we also related the extent of NP-cell association and gene expression responses to NP surface properties.[28, 29] With such studies, however, there are several things that may result in revealing only a correlative, rather than causal, relationship.

When varying one NP property, it is extremely difficult not to affect other traits as many physiochemical properties are interconnected, as has been extensively documented.[30] For example, altering NP size likely impacts the coverage of surface modifications and the propensity for surface adsorption. These unintended and interlinked changes could act as confounding variables. In these cases, omitted factors may govern outcomes and lead to inaccurate conclusions. Efforts to fill the gap include construction of NP libraries to systematically represent various combinations of NP properties,[14, 31, 32] thorough characterization of NPs,[30, 33] and development of accurate descriptors that account for interconnected NP characteristics. Burello et al. extensively reviewed both theoretically and experimentally driven descriptors to portray NP properties that can be applied to establish NP quantitative structure-activity relationship (nano-QSAR) models.[34] Using descriptors of NP

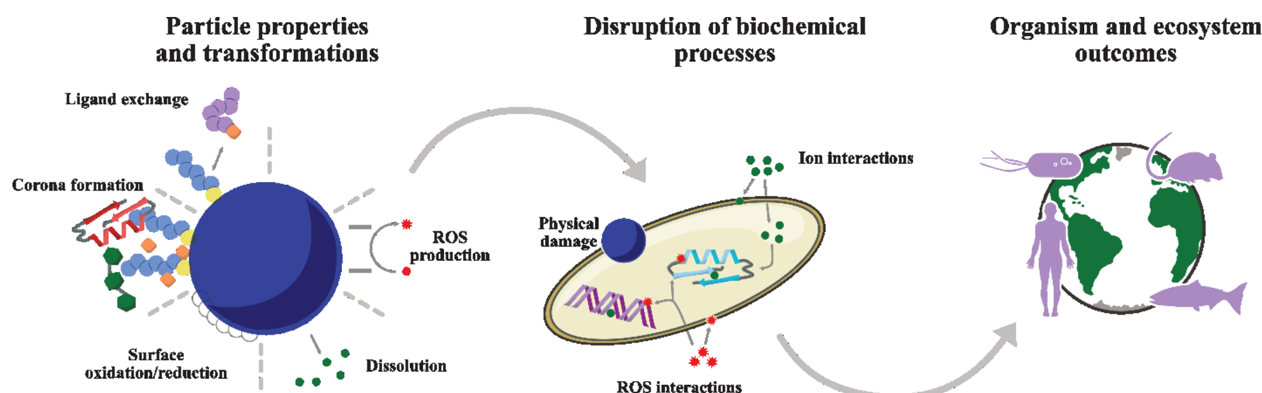

Figure 2 Causal relationships among NP properties, affected biochemical processes and final biological outcomes.

characteristics, Singh et al. built nano-QSAR models that outperform previous studies with a robust, reliable ensemble learning approach.[35] Lynch et al. further suggest mapping interlinked physicochemical properties into three principle components as overarching descriptors.[36] By performing meta-analysis on pertinent knowledge extracted from existing studies using random forest regression models, Oh et al. sorted out key attributes of cadmium-containing quantum dots to cellular toxicity.[37] Advances in NP synthesis, characterization, and statistical modeling will help nano-QSAR deconvolute interacting and interfering variables—allowing understanding of major routes of interaction.

When attributing observed biological effects to NP properties, one gap is the often-omitted role of NP surface transformation in nanotoxicity studies. The importance of NP surface coating in determining nanotoxicity has been well established, including consideration of surface charge, layer thickness, ligand type, etc.[25, 38] However, the pristine NP surface can undergo changes when entering new matrices, such as changes in surface ligands, structure, and composition as well as NP aggregation states.[39, 40] Much research has shown that the altered NP surface can affect the apparent toxicity compared to pristine NPs.[41, 42] To approach this knowledge gap, efforts have been made to include NP surface transformation in the causal relationship between NP properties and biological effects. As an excellent example, Walkey et al. established a multivariate model that used the "fingerprints" of proteins adsorbed to various NP surfaces to predict NP-cell associations.[43] The importance of NP surface characteristics and an overview of available analytical measurements for NP surface characterization is presented in Section 1.5 of this perspective.

Another limitation of the common practice, in our opinion, lies is tunnel vision. Often in practice, due to the limited resources and time, several individual biochemical tests are done to probe toxicity mechanisms based on specific hypotheses. Detection of intracellular reactive oxygen species and metabolites, assays for various enzyme activities and membrane integrity, and measurement of changes in certain biomarkers such as DNA lesions and gene expression are all among popular assays for probing biochemical processes. However, each of these individual functional tests provides only a small window onto a large system. Results from these narrow measures are observed, and we may conclude correlations among several observations. However we could certainly omit other important variables, and with limited observations cannot deduce causal relationships. Failure to elucidate causal relationships among biochemical processes and final biological outcomes may mislead efforts in future mechanistic studies and diminish the power of predictive models for nanotoxicity.

One obvious way to gain a whole picture is application of -omics, part of system toxicology approaches.[44] By definition, -omics refer to measurements on the whole collection of events in biological organisms. An -omics approach can be applied at different molecular levels including DNA (genomics, DNA adductomics), epigenetics (DNA methylation and histone modification), RNA (transcriptomics), proteins (proteomics, redox and affinity proteomics), metabolites (metabolomics) and other molecules (lipidomics, glycomics). Various recent reviews have covered the application of -omics in analytical nanotoxicology and suggested that, combined with bioinformatic approaches like pathway analysis, -omics can help to identify perturbed pathways, discover new biomarkers, and form new hypotheses.[45-48] Schnackenberg et al. and Matysiak et al. outlined the application of metabolomic and proteomic approaches in nanotoxicology with an emphasis on human health risk assessment.[49, 50] In ecotoxicology, Revel et al. recently presented -omics as powerful tools in aquatic nanotoxicology.[51] Integration of -omics at different molecular levels further adds the power of these systems biology approaches. Pillai et al. revealed dynamic toxicity response of algae to silver NP exposure by integrating transcriptomics, proteomics, and phenotype data.[52] A recent review by Shin et al. discussed the integration of metabolomics and transcriptomics ("metabotranscriptomics") in nanotoxicology.[53] In the future, we expect increasing application of diverse -omics in nanotoxicology on both human health and ecological systems, especially integrated -omics across different levels, to depict more complete pictures of molecular mechanisms of NP toxicity.

However, due to limitation of resources and complexity in performing -omics experiments, biochemical assays will most likely remain methods of first choice when testing nanotoxicity. Thus, we assert that it is important to incorporate these individual assays in a framework that emphasizes the causal relationships among biological events. Adverse outcome pathways (AOPs), a recently developed conceptual framework that links existing toxicological knowledge on biological events between a molecular initiating event (MIE) to an adverse outcome (AO) that is relevant to risk assessment, depict a series of causally related key events (KE) that span multiple biological levels (**Figure 3**).[54] The causal relationships between key events are defined as key event relationships (KERs). The AOP framework provides possibilities to integrate existing knowledge on different biological levels, predict toxicity pathways of new chemicals, and guide discovery of biomarkers. This tool shows promise in reducing animal testing as it facilitates the extrapolation of chemical effects across species.[55] Establishment of AOPs is fed by studies on toxicity mechanisms, including both individual toxicity tests and system toxicology approaches. Many efforts have been made to build AOPs for chemicals, and multiple databases have been developed, such as AOP wiki, AOP knowledge base, and Effectopedia. Using existing, but mostly fragmented, knowledge on nanotoxicity mechanisms, researchers have recently started developing AOPs for nanotoxicity. Vietti et al. reviewed toxicity literature of lung fibrosis induced by carbon nanotubes to draft an AOP on the fibrotic potential of CNTs.[56] Knowledge gaps in toxicity mechanisms were identified while developing the AOP for CNTs. Instead of reviewing literature, Labib et al. developed an AOP for lung fibrosis upon MWCNT exposure based on *in vivo* transcriptomic data, showing the potential of -omics data being used to reveal causal relationships among events.[57] Two challenges in building AOPs for nanotoxicity were pointed out by Gerloff et al.[58] The first one is to identify characteristics of

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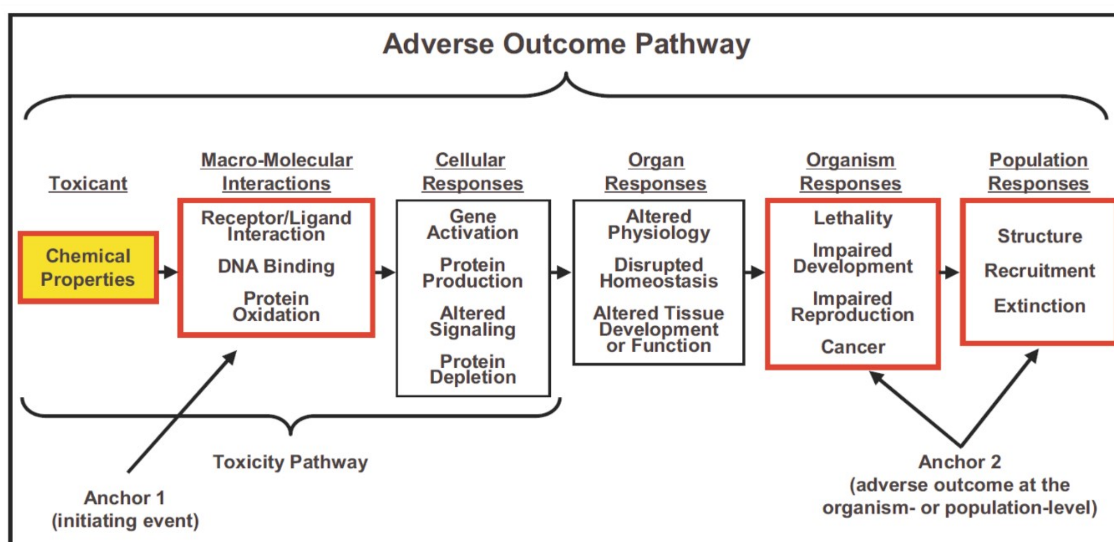

Figure 3 Illustration of adverse outcome pathway (AOP). (Reprinted with permission from **Ref 54**. Copyright 2010 Wiley InterScience.)

NPs that can initiate a molecular event. A great example is relating the oxidative potential of NPs to ROS generation as an MIE. As oxidative stress is proven to be an important mechanism for nanotoxicity, NP characteristics that lead to potential oxidative stress, such as bandgap and hydration energy, have been identified as predictors for nanotoxicity both experimentally and theoretically.[31, 59-62] However, other traits that could potentially be related to MIEs are still vague and lacking sufficient evidence, leaving plenty of room for future research. The second challenge is that the nature of MIEs for nanotoxicity can be different from chemicals. While the molecular interactions at the nano-bio interface inform potential MIEs that initiate an AOP, the nano-bio interaction might be physical or mechanical, thus not necessarily a traditional "molecular event". The authors suggested that instead of using MIEs, the first key event following MIE, called initial KE, might be used instead as the first event in a nano-specific AOP. In their recent work, Gerloff et al. reviewed the liver toxicity induced by both chemicals and metal oxide NPs and suggested that establishment of an AOP should not be limited to nano-specific toxicology literature but also expand to established knowledge on chemical AOPs, as they may share downstream key events despite differences in MIEs and early KEs.[58] With this in mind, it is important, when designing experiments, to consider incorporating individual analytical tests in the framework of potential AOPs to help researchers form reasonable hypotheses and integrate future new knowledge into the existing database of nanotoxicity.

As mentioned at the very beginning of this section, a common practice for mechanistic study is to expose organisms of interest to NPs of various properties and measure induced biological responses. An under-utilized approach by analytical chemists and toxicologists is the manipulation of biological processes and components to infer causal relationships between exposure and biological outcomes. Our work with collaborators demonstrated the power of this approach, revealing that lipopolysaccharides (LPS) on Gram-negative bacterial membranes are the primary binding site for AuNPs wrapped with positively charged polymers.[63] Chemically depleting a fraction of LPS on the bacterial cell outer membrane led to a significant decrease in NP-cell association compared to unmodified cells. Combined with experiments on solid-supported LPS-containing lipid bilayers interacting with NPs, this work represents strong evidence that positively charged NPs interact with bacterial cells through LPS. Another example is the use of a library of *E. coli* knockout mutants for toxicity screening; mutants that are more sensitive than the wild type can inform important pathways involved in response to nanomaterials.[64, 65] Similar to the concerns about controlling NP characteristics via synthesis, manipulating one biological variable might be accompanied by undesirable, yet inevitable, changes in other variables. Therefore, thorough characterization of manipulated processes and potentially affected processes is necessary. Nevertheless, using tools from biochemistry, chemical biology, and molecular biology, biological processes and components can be controlled or blocked, providing evidence of toxicity mechanisms from another angle different from simply measuring responses upon exposure. Thus, we emphasize the importance that analytical chemists and toxicologists consider implementation of these available tools outside analytical chemistry through broader collaboration across chemistry and biology to gain complementary information to complete the picture of toxicity mechanisms.

1.3 Overcoming NP interference in *in vitro* toxicity assays

There are many ways that NPs can interfere with an *in vitro* biological assay (**Figure 4**). The intrinsic optical properties of NPs can interfere with assays that rely on measuring changes in absorbance or fluorescence of molecular indicators for cellular activities. Due to their high surface reactivity, NPs can adsorb,[66]

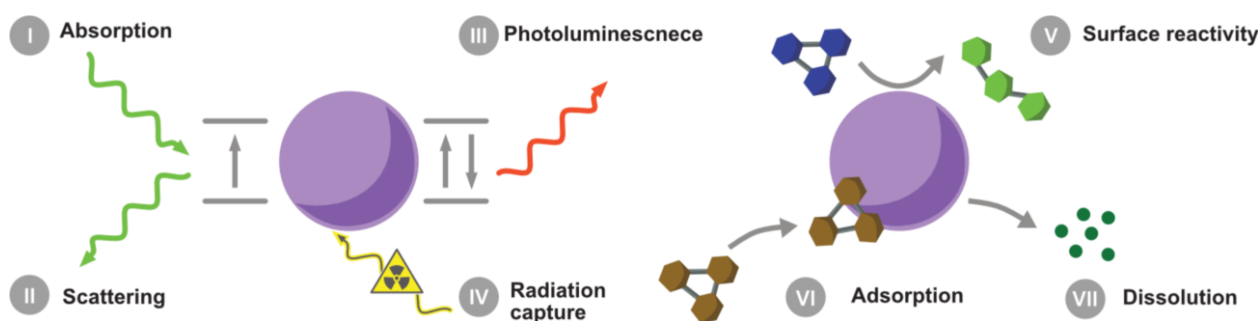

Figure 4 Potential sources of interferences of NMs with measurements. Optical interferences by I) emission, II) scattering, and III) photoluminescence are known to interfere with many colorimetric, fluorometric, and scattering-based assays. IV) Capture of radiation has implications for radiolabeling experiments. And V) surface reaction and VI) adsorption of assay components or VII) interaction of dissolved species with assay components complicates many traditional assays.

react with assay reagents,[67] or release chemical species[68] in testing media that alter the behavior of dyes and enzymes in assays. In some cases, catalytic NPs can cause side reactions such as ROS production. All these interferences contribute to discrepancies observed in nanotoxicity studies and has been extensively reported.[18, 69-71] Two analytical challenges in the field of nanotoxicology have been, and will continue to be, the development of toxicity assays that are free of NP interference and improvement of well-established toxicity protocols where interference cannot be avoided nor neglected.

Optical interference, including absorbance, scattering, and luminescence, have the potential to be corrected via proper control and background subtraction. Many well-established toxicity protocols have been re-validated on NPs, and optical interference from NPs in *in vitro* assays have been checked and taken into account in data analysis.[72-77] However, background subtraction does not always work, especially when reagents (converted or not) react with NPs or side products from catalytic reaction with NPs. More well-considered and complicated controls need to be done in this case. For instance, Semisch et al. found that copper ion released from CuO NPs caused the reduction of tetrazolium salt, the dye for the WST cytotoxicity assay.[68] In this case, the WST assay is not appropriate to test the toxicity of CuO NPs, unless NP concentration for exposure is low enough that the interference from released copper ion can be neglected. Interference from catalytic properties can potentially be mitigated by controlling experimental conditions, such as avoidance of UV illumination for TiO_2 NPs in the presence of assay reagents.[78]

Studies have pointed out that NP interference cannot be predicted as they are highly specific to different assays and type and concentration of NP exposure.[13, 79] Thus, it is critical to include proper controls when designing each set of experiments. Rosslein et al. have provided a good example by using cause-and-effect analysis to design a well-controlled MTS assay layout on 96-well plates, taking NP interference into account.[80] Drasler et al. discussed different controls that should be added for *in vitro* nanotoxicity studies, including measurement on (1) NPs alone dispersed in testing medium for background correction, (2) untreated cells with NPs being added at the end of the actual exposure to test if NPs interfere with converted reagents (such as formazan in the MTT assay), and (3) centrifuged supernatants from NM-exposed cells to remove NP agglomerates.[17] In addition to the above controls, it is also beneficial to test if the reagents themselves will react with NPs without the presence of cells.[67] Another factor that contributes to inaccurate conclusions in nanotoxicity is insufficient purification of colloid suspensions. Studies have shown that impurities from insufficient purification of synthetic products can dominate the observed apparent toxicity in standard assays, leading to over-estimation of nanoparticle toxicity.[81-83] We thus suggest that the supernatant from NP suspensions following proper ultracentrifugation should always be included as a control in nanotoxicity assays.

Besides implementation of adequate controls, researchers have been seeking analytical methods that are free of or less sensitive to NP interference. For mammalian cell lines, visualization of cell morphology under microscopy is considered a technique that complements cytotoxicity assays, but it is not sufficient to quantitatively measure toxicity endpoints. Colony counting assay for microbial cells is widely accepted as a standard microbial toxicity assay that is not affected by NP interference.[17] Microbial cells are treated with NPs and spread onto solid medium; after overnight incubation, colonies grown from individual viable cells are counted as colony forming units (CFUs). As the biological endpoint is the number of visible colonies, it is unlikely that NPs will play a role in colony forming and counting beyond any bacterial toxicity that they display. However, both cell visualization and colony counting assays suffer from very low throughput and high labor demand. Our group recently developed a simple but effective assay for high-throughput bacterial nanotoxicity screening.[20] After an exposure, the bacterial suspension is largely diluted into fresh medium to allow re-growth. Optical interference from NPs is negligible due to the large dilution, and the re-growth profiles are quantitatively related to the relative number of viable cells, thus viability. This assay is performed on 96-well plates, allowing fast and robust bacterial toxicity screening. Respirometry, where oxygen consumption by a microbial population or other organisms is measured to reflect growth and viability, is another NP interference-free test, and it has been used to to study the effects of NP on bacterial population growth.[28, 29]

Impedance-based measurements are promising in terms of overcoming NP interference with fast speed and high throughput.[84-86] Impedance spectroscopy measures the

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resistance at electrode surfaces. Cells can attach to electrodes, and any change in the cell-electrode interface will result in changes in electrode capacity and resistance. Thus, impedance spectroscopy can be used to monitor changes in cell morphology, cell density, viability, and extent of cell adhesion. Otero-Gonzalez et al. developed a real-time cell analyzer (RTCA) based on impedance spectroscopy and measured the response of human bronchial epithelial cells upon exposure of 11 different inorganic nanomaterials.[87] Besides being interference-free, impedance spectroscopy is label-free, non-invasive, high-throughput and real-time. However, we must give the caveat that the biological meaning of impedance measurement is not as clear as other well-established cytotoxicity assays, making results hard to interpret and to compare with other biological endpoints. In addition, the versatility of impedance assays is limited as adhesion of cells onto the electrode surface is required to perform this assay.

Researchers have also been expanded the use of biomarkers instead of apical biological endpoints. In toxicology, a biomarker is a molecule or molecular change that can be measured that is indicative of the level of exposure, the extent of biological response, and toxic effect or susceptibility.[88] Measurements on biomarkers are intrinsically at the molecular level and provide information on toxicity mechanisms.

Genotoxicity assays that measure toxic effects on DNA have been used to evaluate the risk of nanoparticles, especially nanomedicines, and whether current genotoxicity assays are suitable for nanotoxicology has also been discussed.[18, 78, 89] The Comet assay is a genotoxicity assay that measures DNA strand breakage using single-cell electrophoresis. After treatment of NPs, cells are embedded in agarose, lysed *in situ*, and released DNA is imaged after electrophoresis. Concerns about NPs directly reacting with either naked DNA released from cells or lesion-specific endonucleases as reagents have been examined, and it is generally believed that the Comet assay is adequate for NP genotoxicity, though caution still needs to be taken due to possible interference.[90-95] The Ames test, chromosome aberration, and micronucleus assays were used to study AuNP genotoxicity, and the authors concluded that the latter two assays are more suitable for NP genotoxicity studies as chances are low for reagents to directly interact with DNA.[96] Further tests need to be done to validate genotoxicity assays, focusing on understanding interference and expanding genotoxicity assays from mammalian cells to microbes.

Measurements at the mechanistic level beyond genotoxicity, such as gene expression, metabolite secretion, immunotoxicity, and changes in epigenetics have been explored and discussed recently as alternative ways to evaluate the toxicity of NPs.[97] These measurements, while more complicated, are always free of NP interference because NPs are usually excluded during sample preparation and not present at the point of measurement. One challenge for these assays is to validate proposed biomarkers and relate molecular-level measurement to organism- or cell-level biological endpoints such as population survival. Elucidation of all biological processes related to one specific biomarker is also desired for biomarker validation.

While interference is still a possibility, as in many of the aforementioned measurement approaches, toxicity evaluation of NPs must avoid relying on one single assay. Multiple assays on the same, similar, or related endpoint should be performed to confirm a certain effect. An example mentioned earlier is investigating AuNP genotoxicity using four different assays.[96] While a fluorescence assay for ROS might suffer from NP interference, examining gene expression changes on ROS-related genes can be used to complement the fluorescence assays.[98] Even for cytotoxicity assays, it is good practice to use multiple assays to reach a more solid conclusion. In addition, standardized operating procedures across labs is also desired to minimize the discrepancies in studies reported from different labs. Toxicity response is, to an extent that is not fully realized by the nanotoxicology community, surprisingly dependent on a variety of factors other than NP dosage, including cell density, cell status, assay background signal, reagent concentration, etc.[30] Furthermore, NPs can be quantified in different ways (e.g. mass/particle/surface area concentration), and this dosimetry complicates understanding NP toxicity.[30] Piret et al. reported an inter-laboratory study on a series of *in vitro* cytotoxicity and pro-inflammation assays to test NP toxicity and examined potential sources of variation in these protocols.[99] Standardized procedures among labs, plus as many details as possible being reported in publications, will help to find the source of discrepancy among different labs.

1.4. Connecting NP interaction to biological responses at the single-cell level

In the first and second generations of studying nano-bio interactions, one dominant focus has been on the response of cell populations to NM exposures. Such ensemble approaches have been used widely in cell viability and functional assays. Easy-to-perform, ensemble measurements have established strong foundational knowledge of the types of interactions between NMs and cells and the biological response by populations of cells to NMs.

Many researchers have realized the importance of studies on single cell levels, due to the heterogeneity of cell populations and nanoparticle distributions. Xia et al. outline that cell populations can differ cell-to-cell depending on genetic variations, different cell cycle phase, biochemical state (namely protein concentrations/bursts), and microchemical environments.[100] Nanomaterials, different than traditional molecular toxicants, can vary from one particle to another within a population in terms of size and structure; thus the load of NPs to individual cells within a population can vary. Assessing NP effect at the population level of biological systems risks averaging out the real effect of NP exposure, and results in difficulty distinguishing a bimodal response, where some cells respond weakly and others respond strongly, from a unimodal response in which all cells exhibit similar responses. The averaging of biological responses may hamper the development of molecular level insights into mechanisms of interactions and likely contributes to the high level of disparate data in

nanotoxicology studies. Thus, we believe it is critical to push past the limitation of population measurements towards single-cell level analysis.

Two general categories of analytical methods are available to study NPs interacting with single cells: imaging-based and cytometry-based techniques. Imaging-based, i.e. microscopic, methods are frequently used to study NP uptake, co-localization, and trafficking in cells, as they can resolve fine details of NPs interacting with sub-cellular components and monitor kinetics of NP-single cell interactions over time. While lacking structural and temporal information, flow cytometry and mass cytometry provide much higher throughput, better quantification, and the possibility to separate cells into sub-populations for subsequent studies. To correlate cellular responses to effective NP loads at a single-cell level, measurements on both NP uptake/localization and biological responses needs to be achieved simultaneously on the same individual cells. This represents one big challenge for analytical nanotoxicologists, and current single-cell techniques need to be expanded and integrated to achieve this goal. In this section, we highlight recent studies that correlate cellular NP load to response on a single-cell level and discuss related recent analytical advances toward this goal.

Case study: high-content imaging to correlate cellular NP load to single-cell response. Microscopy has long been a powerful tool to study NP-cell interactions. Depending on the purpose of the study (co-localization, trafficking or quantification), the nature of biological samples (fixed or living cells), and the size of NPs (small or large), a whole collection of microscopy methods ranging from electron to optical microscopy can be potentially useful. Amongst all, fluorescence microscopy is the most widely used based on its versatility and simplicity. Combined with a wide range of fluorescent dyes available to label cellular components and indicate cellular activity, fluorescence microscopy can co-localize NPs in fixed cells or track single particles dynamically in living cells at single-cell resolution.[101-105]

Recent work from Manshian et al. made the first attempt to correlate the concentration of QDs at the single-cell level using high-content imaging with confocal microscopy.[106] High-content analysis (HCA) is a multi-color imaging-based technique frequently used for drug discovery.[107] Using different fluorescent tags, multiple cellular phenotypes can be monitored with simultaneous readout. Jan et al. used an HCA approach to stain neural cells with four fluorescent dyes: Hoeschst for nuclei, TMRM for mitochondrial membrane potential, Fluor-4 for free calcium concentration, and propidium iodide for membrane integrity, to evaluate if a single cell is healthy, impaired, or dying upon QD exposure.[108] Other work using zebrafish embryo,[109] fibroblast cell lines,[110] and various other cell lines[111] has demonstrated that HCA is a powerful tool to study cellular response at a single-cell level. Pushing it further, Manshian et al. simultaneously quantified the relative QD load of individual cells based on QD intensity and area in cells, and binned data on single cell biological responses into categories based on NP load (**Figure 5**). As a result, they found conflicting mechanisms such as apoptosis and autophagy in cell

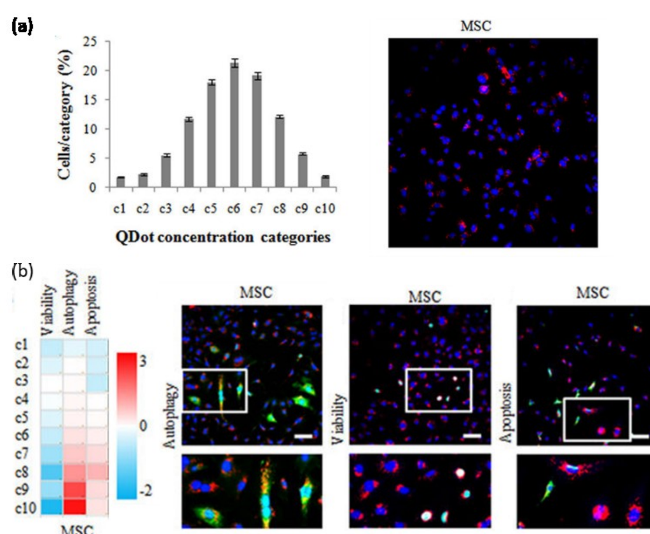

Figure 5 (a) Histograms indicating the number of mouse mesenchymal stem cells (MSC) cells per QD-load category and a representative image of MSCs exposed to 10 nM QDs. The population was divided into 10 categories spanning the range of determined QD load for individual cells. (b) Heatmap showing cell response in viability, autophagy and apoptosis clustered for each category of NP load and representative HSC images for each kind of response. (Adapted from **Ref 106** under the Creative Commons license.)

subpopulations with medium to high QD load at apparent sub-cytotoxic concentrations. Together with other work, this study revealed that cell populations do not respond to NPs unimodally, and the distribution of single-cell response is correlated to NP load per cell.

The work highlighted above by Manshian et al. used microscopy to obtain data to correlate NP load to cell response at the single-cell level. Such microscopic studies can benefit from (1) precise quantification and dynamic tracking of NPs and (2) visualization of cellular processes with high resolution; both rely on the development of state-of-the-art microscopic techniques and the integration of multiple imaging techniques to obtain data about NP load and cell response simultaneously. Recently, Vanhecke et al. reviewed advances in quantifying NPs at single-cell levels,[112] and Ivask et al. presented a comprehensive review on methodologies for NP-cell interactions.[21] Generally, conventional optical microscopy is semi-quantitative for NP quantification, due to the diffraction limit, fluorophore quenching, and optical interference from samples. Confocal fluorescence microscopy has been extensively used to visualize NPs with limited capability for NP quantification. Label-free optical methods have also been applied to image non-fluorescent NPs, such as hyperspectral imaging for QDs and AuNPs[113, 114] and Raman spectroscopy for carbon nanotubes and TiO_2 NPs[115, 116] Lasne et al. presented a home-built imaging system to track individual 5-nm gold NPs in living cells by detecting the laser-induced scattering around a nanoabsorber (LISNA).[117]

Beyond the optical diffraction limit, electron microscopy achieves resolution below the size of NPs. Traditional biological transmission electron microscopy (bio-TEM) requires sample preparation including fixation and slicing into ultrathin sections, making quantification difficult unless 3D imaging can be re-constructed from 2D stacks, and even then, the small sampling

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volume presents challenges to quantitation. Advances in liquid cell TEM have enabled whole-cell imaging in aqueous medium, though its resolution still needs improvement.[118] In addition, conventional bio-TEM is not able to clearly differentiate between high mass contrast natural cellular components and exogenous NPs.[119] Dark-field TEM, applied for nanotoxicology by our lab, helps to solve this problem by looking for the unique diffraction patterns from crystalline nanoparticles but not stained biological components.[120] Besides electron microscopy, super-resolution imaging, a collection of optical imaging techniques that overcome the diffraction limit, has also been employed to acquire fine details on NP localization and trafficking in cells. A direct comparison was made between confocal fluorescence microscopy, stochastic optical reconstruction microscopy (STORM), and electron microscopy to co-localize NPs with organelles in HeLa cells, showing that previously unknown details at nano-scale resolution can be revealed by super-resolution microscopy.[121] Using structural illumination microscopy (SIM), we revealed co-colocalization of QDs on the cell membrane of Gram-positive and Gram-negative bacterial cells.[122] Compared to electron microscopy, super-resolution microscopy provides possibilities to image live cells and multiple cellular components along with NPs simultaneously, though requirements for particular fluorophore characteristics (e.g. photoswitchable dye for STORM) still limit universal application.

Mass spectrometry imaging provides chemical information that is not available through optical imaging. Laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) vaporizes the surface of a sample and coupled with mass spectrometry, allows for bioimaging of metals.[123] As an example of its application in nanotoxicology, Elci et al. applied this technique to characterize the biodistribution of AuNPs in mice organs.[124] However, LA-ICP-MS cannot differentiate between (1) NPs and biomolecules if they contain the same element (e.g. carbon nanotubes), and (2) elements in different forms (e.g. Ag NPs vs. Ag ions). Laser desorption/ionization mass spectrometry imaging (LDI-MSI) applies laser irradiation to ionize molecules on a sample surface and analyzes the generated ions with a mass analyzer. With the assistance of a solvent ("matrix"), matrix-assisted LDI-MSI (MALDI-MSI) allows detection of a wide range of biomolecules.[125] Chen et al. mapped a carbon cluster fingerprint with MALDI-MSI to reveal the distribution of carbon nanomaterials in mice organs and tissues.[126] By tuning laser fluency, LDI-MS can be used to differentiate cell surface-bound and internalized AuNPs.[127] Higher resolution MS imaging can be achieved by secondary ion mass spectrometry (SIMS), a surface analysis technique achieved by sputtering the surface of sample using highly focused primary ion beams and analyzing the ejected secondary ions. SIMS has been used to examine metabolites and nanoparticle distribution in single cells.[128-131] Overall, mass spectrometry imaging provides good quantification of NPs and simultaneous mapping of biological matrices through elemental and mass analysis.

The power of combining tools in microscopy remains largely untapped for NP-single cell interaction studies. Le Trequesser et al. assessed uptake of TiO_2 NPs into individual primary human foreskin keratinocytes cells by using multimodal correlative microscopy: fluorescence microscopy for sub-cellular compartments, scanning electron microscopy (SEM) for surface analysis, and ion beam analysis (IBA) for in-depth elemental analysis and *in situ* quantification of TiO_2 NPs.[132] Similarly, quantification of ZnO NP uptake, distribution, and dissolution was done by combination of ion beam milling, X-ray fluorescence microscopy, and SEM.[133] A more complicated system, mass spectrometry imaging and super-resolution microscopy were integrated in a home-built microreactor known as the "system for analysis at the liquid vacuum interface (SALVI)" to perform ToF-SIMS and SIM on the same sample.[129] SIM was used to characterize cells and guide subsequent ToF-SIMS analysis. ToF-SIMS identified lipid fragments and ion transportation upon NP exposure and showed direct evidence of ZnO NP uptake as well. Multi-modal correlative microscopies provide details that complement each other from the same sample, showing numerous possibilities for studies on correlating the status of NP in cells to biological responses at the single-cell level. A great example is from Liu et al., who used combination of bright field microscopy, fluorescence microscopy, and AFM for estimation of NP load, cytoskeleton and ROS production, and single-cell mechanics, respectively.[134] The level of NP uptake was related to changes in cell stiffness, ROS production, and damage to the actin network. Multimodal correlative microscopy, combined with other assays, provides information at multiple dimensions and thus helps to understand causal relationships between NP exposure and biological consequences. The power of multimodal correlative microscopy and to potential to understand the cascades of biological changes at the single cell level is yet to be fully exploited in nanotoxicology studies.

Microscopy can also be combined with other single-cell measurement techniques such as electrochemical analysis, whose application in nanotoxicity has been reviewed.[84] Our group pioneered using carbon fiber microamperometry (CFMA) to monitor exocytosis from single mast cells and other cell types upon AuNP, AgNP and TiO_2 NP exposure.[135-139] Exocytosis is a highly conserved cell function across cell types and plays important roles in chemical communication among cells. By using microelectrodes, the Haynes group was able to monitor the process of single-cell exocytosis and correlate changes in single-cell exocytosis to bulk measurements of NP uptake and cytotoxicity. Using a microelectromechanical system (MEMS)-based sensing array that can trap cells, Shah et al. presented a platform for electrochemical impedance spectroscopy of single cells and small cell populations upon metal oxide NP exposure.[86] As slow speed might be one drawback of single-cell electrochemical measurements, MEMS systems with cell capturing capability and sensing arrays present a future direction for the integration of single-cell microscopic and electrochemical measurements with fast speed and relatively high throughput.[140, 141]

Case study: flow cytometry, and single-cell RNA-Seq for cells with varied QD loads. There are two circumstances beyond

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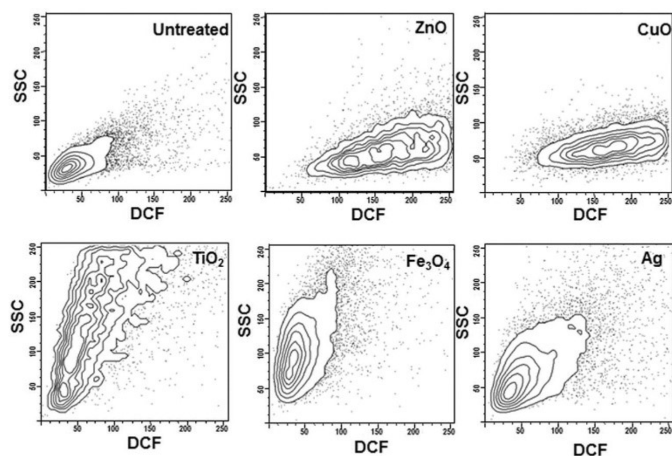

Figure 6 Contour plot patterns of SSC and DCF intensity showed correlation between NP association and ROS production in single cells. Reprinted with permission from **Ref 144**. Copyright © 2012 American Chemical Society.

reach of imaging techniques despite their versatility: when analysis of millions of cells is desired or when single cells need to be separated first for subsequent analysis. Flow-based single-cell techniques, including flow cytometry, mass cytometry and cell sorting, have provided solutions for both research needs. Cells and particles are suspended in a fluid and passed through one or several lasers in a fluid stream as separated single cells. Using different detectors, multiparametric detection of each individual cell's optical characteristics such as light scattering and fluorescence can be achieved simultaneously. Coupled with ICP-MS, cytometry can perform elemental analysis on single cells, called mass cytometry. Originally, mass cytometry specifically referred to the detection of metal ion labelled antibodies in single cells,[142] but now researchers have adapted this technique for NP quantification in cells.[143] Cell sorting techniques further allow the separation of single cells based on their characteristics such as the amount of associated NPs and further analysis like single-cell -omics.

Here, we highlight two studies to demonstrate the power of flow-based single-cell techniques. The first study is from Toduka et al. where flow cytometry was used to correlate NP association and cellular response at the single cell level.[144] Compared to microscopy, flow cytometry provides orders of magnitude higher throughput. Side scattering (SSC) can be implemented to evaluate NP association with mammalian cells[145, 146] and bacteria[28, 147] and combined with fluorescence staining to determine cellular states. Pan et al. used SSC to gate a NP-loaded cell subpopulation and evaluate the states of these gated cells as healthy, apoptotic, or necrotic based on fluorescence staining on the same cell population.[148] Pushing it further, Toduka et al. plotted SSC versus fluorescence induced by intracellular ROS after their simultaneous detection with flow cytometry upon metal oxide and Ag NP exposure. Results showed clear correlation between NP association and ROS production in single cells (**Figure 6**). With analysis of millions of cells, flow cytometry provides richer information than bulk experiments. Recent development of imaging flow cytometry further enables analysis of cell morphology and particle tracking.[149] Such combination of microscopic and flow cytometry analysis makes it a powerful tool for future high-throughput and high-content single-cell analysis for nanotoxicology, as it has been applied.[150]

For more accurate quantification of NP association, as stated earlier, Yang et al. adapted mass cytometry for high-throughput quantification of AuNPs in single cells.[143] Compared to flow cytometry, mass cytometry showed higher sensitivity and lower limits of detection for measuring NP load. Combined with metal-chelated antibodies, cell types and NP load per cell were determined simultaneously for each individual cell. While achieving precise quantification and high sensitivity, mass cytometry removes information on the chemical status of elements, e.g. dissolved ions vs. NPs. In the future, we predict that integration of flow cytometry and mass cytometry on one platform will yield precise and comprehensive information on NP-single cell interactions.

Flow-based cell sorting techniques provide capability to separate and collect sub-populations of cells for subsequent analysis. In fluorescence-activated cell sorting (FACS), individual cells in the fluid stream are given different electrostatic charges based on detected fluorescence and deflected to containers at different sides by passing between two charged plates.[151] Other optical characteristics can be used for cell sorting as well. As an example, our group used both fluorescence and side light scattering to sort live cells with AuNP association, and dark-field microscopy plus hyperspectral imaging was subsequently used to confirm that the sorted cells were associated with AuNPs.[63] Another recent study by Mitchell et al. combined cell sorting techniques and single-cell -omics.[152] Gene expression and transcriptomics have been extensively used in nanotoxicology on both prokaryotic and eukaryotic cell lines to reveal NP toxicity mechanisms, while studies on single-cell levels are scarce. In this study, a fluorescence-activated cell sorter determined the level of QD load to each single cell based on QD fluorescence intensity and harvested the portion of the cell population associated with high or low levels of QDs. Individual cells were then analyzed by single-cell RNA-Seq, an -omics technique allowing global gene expression profiling in one single cell. Results showed that cells carrying lower QD loads responded with multiple strategies that are different for each QD type, while high loads of QD induced more uniformly, mostly down-regulated processes shared across QD types. This study clearly linked cellular NP association to gene expression profiles which would have been averaged and likely not observed in bulk experiments. As single-cell -omics are emerging in all -omics areas, including transcriptomics, proteomics, metabolomics, genomics and epigenomics,[153, 154] it is promising to combine them with flow cytometry and advanced cell sorting platforms[141] and reveal otherwise unavailable details on the molecular level.

1.5 Real-time and *in situ* measurements with chemical information at the nano-bio interface

The interface between NPs and biological systems is dynamic. The NP surface, the solid-liquid interface when NPs interact

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with surrounding medium, and the contact zone where the solid-liquid interface encounters a biological system are closely interconnected and constantly undergoing changes.[19] Upon entering a new biological or environmental matrix, pristine NPs instantly gain new "identities" via interacting with surrounding molecules, including chemical reactions and dissolution, biological transformations, and physical adsorption of surrounding molecules.[155, 156] NPs with the new "identity" then interact with biological systems like cell membranes. Such interactions can induce cellular responses, change the behavior of cell membranes in contact with NPs, and further alter NP characteristics in turn. The dynamic nature of nano-bio interactions thus requires powerful physical and analytical tools for real-time and *in situ* measurements to resolve the kinetics of nano-bio interactions without disturbance. Depending on the pristine surface of NPs, surrounding environment, and biological systems, nano-bio interactions can be loose or tight, as non-specific as electrostatic interactions or as specific to certain binding sites as antigen-antibody binding.[155] Understanding the chemical nature of nano-bio interactions will greatly contribute to the establishment of nano-QSAR and other predictive models that are mentioned in previous sections of this perspective. With all this in mind, an ideal technique to monitor the nano-bio interface should be both real-time and *in situ*, and capable of providing chemical information. Here, we discuss techniques used for capturing the kinetics at the nano-bio interface on the two aforementioned interfaces: interactions between NPs and surrounding molecules in the medium and interaction of NPs with model membrane and biological systems.

Measurement on NPs interacting with surrounding molecules. The new "identity" of NPs when entering a new matrix is decided by their pristine surface features, chemical reactions, physical adsorption, and biological transformation. Chemical transformation of NPs in aqueous medium includes dissolution, oxidation, ligand exchange, surface coating degradation, photocatalytic reactions, etc.[156] Although not necessarily a "nano-bio" interaction, chemical reactions are an integral part of NP transformations. Mechanisms of chemical reactions on NP surface, especially dissolution, photochemistry, and catalysis have been extensively studied.[156-158]

Adsorption of ions, small molecules, and biomacromolecules have all been observed and are governed by forces including hydrodynamic, van der Waals, electrostatic and static interactions, plus hydrophobic effects.[19] Physical adsorption of molecules has been widely studied in the context of human health[155] and the environment,[159] and is believed to be the main source of NPs' identity in biological and environmental matrices. As a prominent example, the protein "corona", referring to layers of tightly or loosely bound proteins on NP surfaces, has undergone extensive investigation in the past decade.[160] A common practice to study the protein corona is the separation of NPs from aqueous matrices via centrifugation and stripping off the NP-bound proteins for further analysis such as gel electrophoresis and mass spectrometry. Such separation-based techniques have been used to study protein corona evolution at the time scales of minutes to hours.[161-163] To study protein adsorption kinetics in a much shorter time scale, correlation spectroscopy has been employed. Vilanova et al. used fluorescence correlation spectroscopy (FCS), a sensitive fluorescence technique to measure concentration and size of particles by monitoring fluorescence fluctuation in solution to estimate the fraction of protein binding to the NP surface and adsorption kinetics.[164] Scattering correlation spectroscopy and surface plasmon resonance were also used for *in situ* measurement of protein interaction with gold NPs[165, 166]. Spectroscopic techniques to extract kinetic parameters of protein adsorption on NPs have been thoroughly reviewed elsewhere.[167]

While techniques based on absorbance, fluorescence, scattering (e.g. dynamic light scattering) and surface plasmon resonance can be used to determine kinetic parameters, chemical information is not available from these studies. Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy is a surface analysis technique capable of real-time and *in situ* characterization of surface dynamics with chemical information. ATR-FTIR utilizes the evanescent field of the total internal reflection (TIR) of infrared radiation at the solid-liquid interface, and the surface sensitivity stems from the short penetration depth (at the magnitude of 1 micrometer) of the evanescent field. NPs are typically immobilized on the ATR crystal, and adsorption of molecules onto NP surfaces is monitored over time; specific functional groups in adsorbed molecules can be thus identified by examining detected IR absorption bands. Mudunkotuwa et al. has given a detailed review on the application of ATR-FTIR spectroscopy to investigate nanoparticle surface adsorption[168], and numerous studies have shown the application on probing NP surface adsorption of proteins[169-171] and small molecules such as surfactants[172] and phosphate.[173] A vibrational spectroscopy, Raman spectroscopy also provides chemical information at the nano-bio interface. By using plasmonic NPs as substrates, surface-enhanced Raman spectroscopy (SERS) amplifies Raman signals near metallic substrates, thus it is a great platform for probing nano-bio interactions. Bonifacio et al. systematically studied the SERS spectra of Ag and AuNPs in blood plasma and serum to fingerprint biological fluid composition.[174] In another study, SERS spectra of serum albumin protein and cysteine adsorption onto AgNPs were acquired and compared with Raman spectra of free protein and cysteine, showing a strong decrease in S-S bond signal in the adsorbed protein.[175] Micro Raman spectroscopy is also used to study NP interactions with small molecules such as ATP.[176] Overall, we maintain that the capability of both ATR-FTIR and Raman/SERS is yet to be fully explored for dynamically probing NP surface adsorption of surrounding molecules, especially in complex matrices that contain multiple species.

Solution nuclear magnetic resonance (NMR) has been a tool to examine protein and ligand adsorption onto NP surfaces. Binding and exchange of ligand on NP surface from small molecules to polymers and proteins can be characterized and quantified using ^{1}H NMR, while NPs usually need to be centrifuged or dried followed by re-suspension for ^{1}H NMR analysis. [177, 178] Wang et al. achieved *in situ* measurement on

proteins adsorbing to 15 nm AuNP surface using solution ^{1}H NMR and two-dimensional TROSY NMR with ^{15}N-labeled proteins.[179] No centrifugation was needed prior to NMR measurement, and the measurement time was reduced to as little as 10 minutes. Combined with data from a binding competition assay and results from other work, the authors concluded a three-step model for protein adsorption onto AuNP surfaces: reversible association, followed by rearrangement/reorientation, and finally irreversible binding via cysteine at the Au surface. Using relaxation-based solution NMR, Ceccon et al. revealed the global motions and exchange kinetics on the microsecond scale of a model protein, ubiquitin, binding to negatively charged lipid nanoparticles (liposomes).[180] Another NMR technique, solid-state NMR has also been used to characterize the interaction between ligands and NP surfaces.[181, 182] Together with other spectroscopic methods, NMR represents a powerful technique to study the kinetics of molecule adsorption onto NP surfaces with chemical information.

Measurement on nano-bio interaction with model membranes and cells. When NPs reach a biological organism, the first interface they will likely to encounter is a cell envelope.[19] Thus, the interaction between NPs and membranes has been a focus for researchers interested in nano-bio interactions. The characteristics of the cell membrane can vary drastically among different organisms. For example, Gram-negative bacterial cell membrane features a large coverage of lipopolysaccharides (LPS), while the Gram-positive bacterial cell membrane is covered with a thick peptidoglycan layer and teichoic acids. Membranes of most eukaryotic cells contain proteins, glycolipids and components other than phospholipids. Nevertheless, phospholipids compose the main structure of a cell membrane. Model membrane systems are manually constructed lipid bilayers to mimic real cell membranes. By tailoring the composition (e.g. ratio of different phospholipids and other components) and architecture (e.g. vesicle, planar supported bilayer, etc.), model membrane systems have been an effective approach to study cell membrane functions, the role of different membrane components, and membrane interactions.[183] Naturally, this approach has been widely applied to understand the interaction between NPs and membranes. A recent review by Rascol et al. has discussed in detail the application of model membrane systems for investigating nano-bio interactions.[184]

Depending on the hypothesis of interest, three architectures are among the most commonly used model membrane systems: lipid bilayer vesicles, planar lipid bilayers, and supported lipid bilayers. Lipid vesicles, i.e. liposomes, are structurally the closest to real cells and range from tens of nanometers to hundreds of micrometers. Microscopy and dye leakage assays, wherein vesicle-encapsulated dye molecules are released upon membrane disruption, can be used to assess lipid bilayer permeation upon NP exposure and NP association and colocalization with vesicles.[185-188] Planar lipid bilayers can be tethered between two bridges to separate two chambers filled with electrolytic solution for capacitance measurements. Changes in planar lipid bilayers upon NP interaction can be probed via measuring changes in their capacitance.[189] While these measurements on vesicles and planar bilayers provide visualization and dynamics of NP-membrane interaction, they are not quantitative in determining the extent of NP binding to model membranes and also lack chemical information.

In contrast to free-floating lipid vesicles and tethered planar lipid bilayers, supported lipid bilayers (SLB) are formed on planar solid surfaces. The solid surface helps to stabilize the SLB, providing high flexibility to tune composition, architecture, and fluidity of model membranes. Quartz crystal microbalance with dissipation monitoring (QCM-D) has been extensively used to quantify the extent of NP binding and membrane disruption as well.[184, 190, 191] When mass is added to or removed from a piezoelectric crystalline quartz, changes in the quartz's mechanical resonance frequency is proportional to the mass change. Using QCM-D, the deposition kinetics of graphene oxides on SLBs were determined.[190] Combined with atomic force microscopy (AFM) and structured illumination microscopy (SIM), Melby et al. explored the interaction of 4-nm AuNPs on model membranes that contained segregated domains.[192] Besides QCM-D, AFM and other microscopies, as well as electrochemical techniques are also used for real-time and *in situ* measurement of membrane change. Lu et al. showed fabrication and application of microfluidic lipid bilayer arrays for high-throughput electrochemical measurement on NP-membrane interactions.[193]

As mentioned at the beginning of this section, the capability to provide chemical information about nano-bio interactions can be enlightening. While QCM-D and microscopic techniques complement each other as quantitative and qualitative measurements, sum-frequency generation (SFG) spectroscopy is a surface-/interface-sensitive technique that performs real-time and *in situ* measurement on molecular symmetry. In two recent studies, the dynamics of inner and outer leaflets of lipid bilayers upon interacting with AuNPs[194] or chitosan NPs[195] were determined by collecting a time series of SFG spectra. Combined with ATR-FTIR, Hu et al. concluded that SLBs undergo a flip-flop movement upon AuNP interaction.[194] A comprehensive study performed by Troiano et al. using SFG along with a series of other techniques determined the dynamics of positively or negatively charged AuNPs interacting with SLBs with different lipid compositions.[196]

Currently, most model membrane systems are quite simple, consisting of a few phospholipids and occasionally incorporating other membrane components. Increasing the complexity of model membranes by adding more components such as lipopolysaccharide (LPS)[63] and membrane proteins and exploring different composition ratios will bring the system closer to real cell membranes. Extracted cell membranes might also be used and compared with model membrane systems of controlled composition. In addition, spectroscopic methods that provide chemical information, such as SFG, ATR-FTIR, SERS and NMR, used in combination with microscopy will further facilitate *in situ* measurement of the NP-membrane interface. As evidence from model membrane experiments has been used to complement both experimental data on cell-NP interaction[197] and computational simulation,[186, 198] studies on

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NP-membrane interaction will continue to serve as a bridge that connects nano-bio interaction systems at different levels of complexity.

Table 1 Examples of various techniques used to probe interactions of NMs with biomolecules, and classification of if measurements are done in real-time, *in situ*, provide chemical information, and are selective for interactions at the nano-bio interface.

Measurement technique	Biological subject studied	Real-time	*in situ*	Chemical information	Interface sensitive	Ref.
Mass spectrometry	Proteins, lipids, NOM, and others	No	No	Yes	No	161-163
Fluorescence correlation spectroscopy	Proteins	Yes	Yes	No	No	164
Scattering correlation spectroscopy and surface plasmon resonance	Proteins	Yes	Yes	No	Yes	165-167
Attenuated total reflectance – infrared absorption	Proteins, small molecules, ions	Yes	Yes	Yes	Yes	168-173
Raman/surface-enhanced Raman spectroscopy	Blood proteins	Yes	Yes	Yes	Yes	174, 175
Nuclear magnetic resonance	Protein with liposomes	Yes	Yes	Yes	No	178-182
Dye leakage assays	Lipid vesicles	Yes	Yes	No	Yes	185-188
Capacitance	Supported phospholipid bilayers	Yes	Yes	No	No	189
Quartz crystal microbalance with dissipation monitoring	Supported phospholipid bilayers	Yes	Yes	No	Yes	190
Structured illumination microscopy	Supported phospholipid bilayers	No	Yes	Yes	No	192
Atomic force microscopy	Supported phospholipid bilayers	No	Yes	Yes	Yes	192
Sum frequency generation/second harmonic generation	Supported phospholipid bilayers	Yes	Yes	Yes	Yes	194-196

On a more complex scale that is beyond the scope of this perspective, *in vivo* biodistribution and metabolism of NPs in animal bodies represents another important interface that has been extensively studied, mostly in the field of drug delivery, but also in nanotoxicology.[199, 200] Reviews have pointed out the close relationship among NP properties, *in vivo* biodistribution, and nanotoxicity.[201, 202] For example, Khlebtsov et al. presented an intensive review on biodistribution and toxicity, both *in vitro* and *in vivo*, of engineered gold NPs, revealing several general rules for how NP properties affect biodistribution and toxicity.[202] Many analytical techniques are available to study NP biodistribution, including fluorescence microscopy,[203] inductively coupled plasma mass spectrometry (ICP-MS),[204] radioactive analysis (RA),[205, 206] positron emission tomography (PET),[207] magnetic resonance imaging (MRI),[208] etc. In the context of nanotoxicology, the challenge remains to precisely track the circulation, distribution, and metabolism of NPs in animal bodies and relate the biodistribution data to toxicity and risk assessments.

1.6 Outlook

Analytical chemistry contributes much to nanotoxicology, ranging from characterization of NP properties to measurement of biological response upon NP exposure. As we are entering the third decade of nanotoxicology, building predictive models between NP properties and biological outcomes for safe NP design is more critical than ever, as confusion and discrepancies prevent accurate assessment of NP toxicity due to the unique characteristics of nanomaterials. Herein, we identified four analytical challenges in nanotoxicology for the next decade, including shifting studies of nanotoxicity mechanisms from correlative to causative, overcoming NP interferences for accurate *in vitro* nanotoxicity assays, connecting NP interaction to cellular responses at the single-cell level, and developing a kinetic understanding of various nano-bio interfaces with chemical information. With these challenges in mind, analytical chemists, including our own research group, will continue to develop tools to solve puzzles in nanotoxicology and build predictive models together with wide collaboration with researchers in biology, materials science, and computational chemistry.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

This work was supported by National Science Foundation under the Center for Sustainable Nanotechnology, CHE-1503408. The CSN is part of the Centers for Chemical Innovation Program.

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