A functional assay-based strategy for nanomaterial risk forecasting

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38 **Graphical Abstract**

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Abstract

The study of nanomaterial impacts on environment, health and safety (nanoEHS) has been largely predicated on the assumption that exposure and hazard can be predicted from physical-chemical properties of nanomaterials. This approach is rooted in the view that nanoöbjects essentially resemble chemicals with additional particle-based attributes that must be included among their intrinsic physical-chemical descriptors. With the exception of the trivial case of nanomaterials made from toxic or highly reactive materials, this approach has yielded few actionable guidelines for predicting nanomaterial risk. This article addresses inherent problems in structuring a nanoEHS research strategy based on the goal of predicting outcomes directly from nanomaterial properties, and proposes a framework for organizing data and designing integrated experiments based on functional assays (FAs). FAs are intermediary, semi-empirical measures of processes or functions within a specified system that bridge the gap between nanomaterial properties and potential outcomes in complex systems. The three components of a functional assay are standardized protocols for parameter determination and reporting, a theoretical context for parameter application and reference systems. We propose the identification and adoption of reference systems where FAs may be applied to provide parameter estimates for environmental fate and effects models, as well as benchmarks for comparing the results of FAs and experiments conducted in more complex and varied systems. Surface affinity and dissolution rate are identified as two critical FAs for characterizing nanomaterial behavior in a variety of important systems. The use of these FAs to predict bioaccumulation and toxicity for initial and aged nanomaterials is illustrated for the case of silver nanoparticles and *C. elegans*.

62 **Keywords:** dissolution rate, functional assay; nanoEHS, nanomaterial risk assessment; reference
63 systems; risk framework; surface affinity

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Introduction

The incumbent framework for assessing nanomaterial risk has been one based on an underlying assumption that exposure and hazard can be predicted from physical-chemical properties of engineered nanomaterials (ENMs). This framework is rooted in the view that nanoobjects essentially resemble chemicals with additional particle-based attributes that must be included among their intrinsic physical-chemical descriptors. The behavior of nanomaterials within this chemical framework is thought to be amenable to description using structure-activity relationships and similar approaches that seek to map intrinsic nanomaterial properties to hazard and exposure. However, with the exception of the trivial case of predicting that nanomaterials made from toxic or highly reactive materials should be toxic, this approach has yielded few actionable guidelines for predicting nanomaterial risk.

Progress is being made in understanding the general factors affecting ENM fate and effects. Important data on the toxicity of a few (primarily) pristine nanomaterials are now available (ICN 2014) across a biological continuum. Factors influencing the environmental fate of nanomaterials are being identified (Alvarez, Colvin et al. 2009, Bone, Colman et al. 2012, Klaine, Koelmans et al. 2012, Lowry, Gregory et al. 2012, Nowack, Ranville et al. 2012, Unrine, Colman et al. 2012, Westerhoff and Nowack 2012, Barton, Therezien et al. 2014), and theoretical models are being developed to predict nanomaterial behavior and impact from these measured behaviors (Scheringer 2008, Praetorius, Scheringer et al. 2012, Dale, Lowry et al. 2013, Hendren, Badireddy et al. 2013, Therezien, Thill et al. 2014, Dale, Casman et al. 2015, Quik, de Klein et al. 2015). The growing and necessary focus on the many complex interactions and transformations that impact nanomaterial behavior is being aided by the development of computational methods for data integration (NRC 2012, NRC 2013, NNI 2014).

Nonetheless, the complexity and breadth of potentially relevant materials (e.g. nanohybrids used to harvest photons and shuttle electrons in photovoltaics (Saleh, Aich et al. 2015)) and the wide variety of elemental compositions, functions, variations in surface chemistry, and morphology, coupled with a vast number of system-dependent transformations, biouptake processes, and resulting by-products, makes it challenging to know what nanomaterial properties and environmental variables enable us to describe, predict and therefore control nanomaterial behavior, be it beneficial or harmful.

For all the novelty of nanomaterials, the challenges they present from a risk forecasting perspective are similar to those that have been confronted in addressing previous environmental issues ranging from predicting the environmental impacts of synthetic organic chemicals to managing wastewater discharges. In these cases, decision-making was aided by measurements of material behavior in prescribed conditions representing useful simplifications of the environment(s) into which these materials would be introduced. Predictions of oxygen depletion caused by the degradation of complex mixtures of municipal wastes were not obtained from a first principles analysis of the chemical composition of waste water and the composition of microbial populations present in the waste and in surface water (even if these factors were studied later). Rather, functional measurements of oxygen consumption by the bacteria actually present in the waste entering surface waters were obtained and the concept of biochemical oxygen demand (BOD) was introduced. Similarly, environmental fate and bioaccumulation of synthetic organic chemicals (SOCs) was correlated to measurements of the partitioning behavior of compounds between water and a representative organic phase. Trends in chemical partitioning behavior between water and a specific reference organic phase such as octanol (K_{ow}) could be

predicted subsequently from first principles (Schwarzenbach, Gschwend et al. 2005). These examples of what we refer to as functional assays illustrate the utility of making measurements of key variables in complex systems (e.g. oxygen demand) without the need for complete mechanistic understanding of the processes that yield that behavior.

As in the cases of BOD and SOC_s, the complexity of a system's effects on nanoparticle properties tends to obscure predictive links between intrinsic properties of nanomaterials and their impacts. An expedient alternative to directly linking ENM properties to impacts is to focus on standardized reproducible measurements of relevant nanomaterial behaviors in relevant systems that can inform near-term decision-making. We refer to procedures that produce such measurements as **functional assays**. To forecast nanomaterial behavior we will need parameterized predictive models, and functional assay measurements provide such parameters as well as the range of values for those parameters. Ideally, functional assays should be pertinent not only for first generation nanomaterials (that roughly conform to the chemical model) but also for more complex nanoobjects ranging from hybrid materials to active nano-scale devices. In this communication we outline the general features of functional assays for nanoobjects, focusing on the need to designate reference systems in performing these assays and identifying two key assays that can be easily applied to parameterize models for exposure and, potentially, biouptake.

Definition of a functional assay

We define functional assays as procedures for quantifying parameters that describe a specific process (or function) occurring within a given (often complex) system. Functional assays provide quantitative information on the rates at which processes occur (e.g., rates of aggregation,

134 bioaccumulation or precipitation) or indications of performance or final states (e.g., distribution
135 between air/water, living/dead (LD₅₀), or Young's modulus). Functional assays (FAs) inherently
136 incorporate linkages between nanomaterials properties, nanomaterial function, system
137 characteristics, and the exposure or hazard endpoints of interest; they describe the convergent
138 properties of nanoobjects and the systems in which they are present (Figure 1b). The parameters
139 measured in these assays may be semi-empirical, expressing the aggregate effects of numerous
140 predicate processes that may in turn be deconstructed to ultimately yield a description of the
141 overall process based on first principles, including knowledge of all component processes as well
142 as the nanomaterial and system properties. FAs therefore bridge individual empirical
143 measurements and long-term generalizable theory in support of near-term nanoEHS guidance
144 and sustainable product development. These assays allow for parameterization of models for
145 transport, transformation, reactivity and effects of nanomaterials.

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147 This approach adds a crucial halfway point along the path that is typically discussed with regard
148 to predictive risk assessments for nanomaterials, which often focus on pursuit of quantitative
149 structure-activity relationships (QSARs) and attempts at mapping entire adverse outcome
150 pathways (AOPs). Figures 1a and b illustrate (for a fate- and transport-focused example) that the
151 insertion of functional assay measurements as an intermediate, currently measurable predictor of
152 key behaviors that will determine fate and effects of nanomaterials provides an achievable
153 interim goal while also setting the stage for future mechanistic investigation. FAs are particularly
154 needed to predict rates of processes; these measurements group together complex behaviors and
155 imply upstream mechanistic processes that determine their values. FAs do not entirely replace
156 the need for measurements of the nanoparticle properties, system properties and social and

engineered properties; indeed, these measurements and estimates (in particular production volumes and use patterns) will continue to be important for informing exposure estimates and for ultimately linking ENM properties with fate and effects. However, we do not have to mechanistically understand how material and system properties govern transformation processes, for example, in order to quantify the occurrence and rate of such processes in given systems for given materials. Instead, FAs can directly measure the phenomena that cause nanomaterials to form, dissolve, change, move, or impart chemical changes in neighboring constituents. Parameters derived from FAs incorporate complexities in environmental interactions. For example, particle size has been shown to effectively predict Ag NP solubility(Zhang, Yao et al. 2011, Ma, Levard et al. 2012, Dobias and Bernier-Latmani 2013), but even small degrees of sulfidation in environmental systems alter solubility(Levard, Reinsch et al. 2011). Measurement of dissolution rate in a prescribed system that is pertinent to the risk evaluation under consideration takes into account the interactions between these two processes as well as other processes that potentially affect dissolution rate (e.g. interaction with NOM)(Gondikas, Morris et al. 2012). These measurements provide immediate and valuable empirical information to predict exposure potential and consequent bioactivity. Later, as data and meta-data are consistently amassed describing the scenarios, materials and systems associated with these functional assay measurements, the data can be interrogated for more fundamental relationships. Standardizing these assays and the conditions and methods by which they are measured offers a path toward amassing data that can support near-term, realistic assessments of potential exposures and hazards.

179 Though many of these concepts are independently well understood, they have yet to be adopted
180 by the nanoEHS community in a systematic way that enables a cohesive and tractable approach
181 to amassing evidence for informing risk-based (or performance-based) decisions on the
182 sustainable development of nanotechnology.

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184 The three components of a functional assay are standardized protocols for parameter
185 determination and reporting, reference systems, and a theoretical context for parameter
186 application. Each of these will be addressed here. Though explicit handling of uncertainty is not
187 a topic of this manuscript, it is important to note the existence of inherent uncertainty with regard
188 to identification of the optimal parameters for forecasting behavior, judgment of relevant
189 reference systems, development of measurement and reporting protocols to measure, forecasting
190 exposures and effects of nanomaterials from FA measurements, and the hope that capturing FA
191 measurements with consistent meta-data will ultimately provide the ability to link between
192 nanomaterial and system properties and observed behaviors. Increased awareness and adoption
193 of FA approaches will allow systematic investigation into these uncertainties.

Figure 1a. Nanoparticle property-rooted approach **Figure 1b.** Functional assay-rooted approach

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Protocols for parameter determination: Intrinsic/extrinsic nanomaterial properties and system properties

Many lists of physical-chemical characteristics (e.g., (Thomas, Aguar et al. 2006, Stone, Nowack et al. 2010, OECD 2012)) of nanomaterials found in risk assessment gap analyses or recommended characterization protocols include a mix of properties that are both intrinsic to the material identity as well as those that exist *solely* as a function of the environmental systems that the nanomaterial passes through. In characterizing nanomaterials in reference systems, there is an increasingly recognized need to separate intrinsic system-independent properties of the materials from those properties that are extrinsic, or system-dependent (2008, Maynard 2009, Kandlikar, Satterfield et al. 2013, Lynch, Weiss et al. 2014), and to explicitly consider system parameters (2008, Klaine, Alvarez et al. 2008, OECD 2012, OECD 2014).

Some properties are intrinsic to the identity of a nanomaterial; if they change, one could argue it means the nanomaterial has a new identity (Figure 2). Other properties of that material are system-dependent, or extrinsic, properties. Making distinctions between intrinsic and extrinsic properties is insufficient without critical information describing the surrounding systems that influenced or induced these extrinsic states of being. The influence of the system on extrinsic nanomaterial properties (Levard, Reinsch et al. 2011) and the role of the physiological properties of the receptor (Judy, Unrine et al. 2011, Unrine, Shoults-Wilson et al. 2012) have been repeatedly demonstrated to be more important to predicting a variety of effects than the nanomaterial properties. Though it could be argued that fundamentally, all nanomaterial properties may eventually change via transformations, one can separate these properties into those that, if they change, would effectively confer a new fundamental identity to the material vs. those that for a material of the same identity could easily change as a function of its

surroundings. Moreover, not all systems are equally likely or pertinent to real world scenarios; hence, the importance of intentionally selected standardized reference systems. All three categories of characterizations in this figure must be included explicitly in designing experiments and compiling datasets to examine nanomaterial behavior: intrinsic properties of a nanomaterial, the surrounding system properties, and the extrinsic properties derived from the nanomaterial existing in the system.

Figure 2. Intrinsic vs. extrinsic (system-dependent) properties; a simple ENM example

Intrinsic Properties of a Nanomaterial	Extrinsic Properties or States	Environmental or Biological Systems
Core composition Band gap Particulate diameter	State 1	In a WWTP Secondary Clarifier
	Surface composition 1 Surface charge 1 Aggregation state 1	<ul style="list-style-type: none"> • Roughly neutral pH • Moderate ionic strength • Oxic conditions • Moderate DOC
	State 2	In Fresh Surface Water
Core composition Band gap Particulate diameter	Surface composition 2 Surface charge 2 Aggregation state 2	<ul style="list-style-type: none"> • Roughly neutral pH • Low ionic strength • Oxic conditions • Low DOC
Core composition Band gap Particulate diameter	State 3	Subaquatic Freshwater Sediment
	Surface composition 3 Surface charge 3 Aggregation state 3	<ul style="list-style-type: none"> • Roughly neutral pH • Low ionic strength • Reducing conditions/sulfidic • Moderate to high DOC

The need for reference systems

While the utility of standardized nanomaterials has been widely recognized as a prerequisite to obtaining comparable data across studies, there has been a surprising lack of standardization of the systems in which nanomaterials should be characterized and evaluated. *Reference systems*

constitute a critical element of standardized protocols needed for ENM characterization and for meta-data collection associated with these systems, in order to track how system properties impact materials' extrinsic characteristics (Keller, Wang et al. 2010, NRC 2012). Ideally, functional assays determined in contextually relevant reference systems yield quantitative estimates of parameters that can be used in fate model calculations and to assess bioavailability. Rate constants are one such example that enable theoretical mass balance calculations to inform exposure assessment (Dale, Lowry et al. 2013).

Because extrinsic properties of nanomaterials may vary as a function of the history of systems that the nanoparticles experience, comparison between experimental results requires contextual information that is provided by a reference system. Throughout the nanoEHS community there is agreement that standardized materials and characterization protocols are needed (Tran, Donaldson et al. 2005, Klaine, Alvarez et al. 2008, EC 2009, DEFRA 2011, NRC 2012, OECD 2012, NNI 2014). To date though, even references to clear distinctions between intrinsic and extrinsic nanomaterial characteristics have stopped short of proposing a set of reference systems to correspond to lists of reference nanomaterials (Kandlikar, Ramachandran et al. 2007, Boverhof and David 2010). Some testing protocols include such standardized systems such as EPA Moderately Hard Water (EPA 2007), and many tests are conducted in standard media that may be logical candidates to include in a designated set of reference systems. Ideally, an accepted set of reference systems, or at a minimum, consensus on a set of preferable systems among those already commonly used, will span conditions simulating likely scenarios of interest to regulators and researchers. An associated list of consistent characterization parameters for

various systems aids in comparing measurements made in different systems (e.g., (Keller, Wang et al. 2010, Quadros, Pierson et al. 2013)).

Only a finite number of the vast potential combinations of materials and systems can be tested, and not all combinations will be equally relevant to either 1) represent realistic scenarios or 2) allow the prediction of conservative upper bounds of potential effects. Different environmental and biological systems may be useful to evaluate based on their relevance to specific exposure pathways or regulations; considering these reference systems with explicit linkage to their intended risk forecasting context will provide a link in the risk information supply chain that is currently not clear.

In defining systems of interest, the opportunity also exists to connect nanoEHS research with industry and nano-application research and development communities. Use of common testing systems could help to develop standards and systems that may be relevant to known exposure scenarios, and to link nano-application evaluation practices with nano-implication evaluation practices as well as regulatory categorization and risk management recommendations.

A few examples of commonly used reference systems of environmental relevance that might be considered by the NanoEHS community for adoption are listed in Table 1, along with some representative parameters that define these systems. To enable the goal of comparing nanomaterial behavior across different types of media, more extensive specification and characterization of the media may be required beyond those required for the uses initially

envisioned for these media (e.g. ionic strength, pH, UV254, conductivity, temperature, flow regime, ionic composition, redox state).

Table 1: Examples of standardized systems and key associated parameters

Samples of system types	Examples from literature and current standards	Parameters specified in current standard methods
Seawater	EPA artificial seawater(EPA 2007) Santa Barbara seawater(Keller, Wang et al. 2010) Bodega Bay seawater(Keller, Wang et al. 2010) Instant Ocean(Park, Tuttle et al. 2013)	Temperature, ionic and mineral composition
Freshwater	EPA reconstituted fresh water (5 variants ranging from very soft to very hard)(EPA 2007) Hoagland's hydroponic medium(Hoagland and Arnon 1950)	pH, mineral composition, hardness, alkalinity, proportion of mineral water Specified mixture of macro and micronutrients: N, K, Ca, P, S, Mg, B, Fe, Mn, Zn, Cu, Mo
Groundwater	ASTM groundwater sampling guide(ASTM 2013) (Establishes parameters to be characterized but no prescribed value ranges)	Flow rate, water level, pH, alkalinity, hardness, specific conductance, turbidity, TSS, specified elemental and common contaminant concentrations
Synthetic raw Wastewater	e.g., Boeiji et al.(Boeije, Corstanje et al. 1999)	COD, N, P, ionic strength, trace metals
Biosolids/ Sludge	Synthetic WWTP biosolids(Ma, Levard et al. 2013), NIST domestic(NIST retrieved July 23, 2014) and industrial(NIST retrieved July 23, 2014) sludges	Specified elemental concentrations (mg/kg)
Human cell growth media	e.g., RPMI media(Moore and Woods 1977), Dulbecco's Modified Eagle Medium (DMDM)(Ausubel, Brent et al. 2002), etc.	Nutrients required for growth (e.g., protein, sodium chloride, N,P, enzymes, growth factors...)
Microbial growth media	e.g., Lysogeny broth(Bertani 2004), MacConkey's Agar, Mannitol Salt Agar, etc.	Nutrients required for growth (e.g., protein, agar, sodium chloride, N, P, lactose...)
Synthetic saliva	SAGF medium(Gal, Fovet et al. 2001)	pH, ionic and chemical composition, ionic strength
Synthetic sweat	AATCC, ISO and EN standards (as consolidated in (Kulthong 2010))	pH, chemical composition, temperature
Synthetic urine	S-urine formulation(Mayrovitz and Sims 2001)	pH, chemical composition

Key functional assays for predicting exposure

A generic mass balance describing the change in concentration of nanoöbjects of a given size and speciation over time in a system includes source terms (e.g., discharge, biogenesis, or precipitation), reactive transformations (e.g., sulfidation or changes in redox state), reactive losses (e.g., dissolution or biodegradation), and transport (e.g., deposition, aggregation,

advection and settling). In addition, nanomaterials may be taken up and depurated by biota. Reactive transformations, transport, reactive losses, biouptake, and transport are all potentially amenable to description using function assays. The specificity of chemical reactivity and biology present a challenge to identifying generalizable functional assays for reactive transformations and biouptake, which perhaps may be ultimately addressed through the use of indicator species for biouptake, or chemical groupings (e.g., redox potential) for reactive transformations.

By way of examples, we focus here on functional assays to determine dissolution rate and surface affinity. Assays to determine these parameters may be immediately applied, are easily executed, and ultimately characterize most of the dominant processes dictating the fate of nanomaterial mass in environmental systems. Self-descriptive, the dissolution rate (or leaching rate) characterizes the release of elements contained in nanoobjects to solution, representing both a loss of nanoparticle mass and a potential source of toxicity. Measurements of dissolution rate in a representative matrix can ultimately be correlated with e.g. bioavailability, toxicity, and persistence in the environment. System properties that, in concert, decrease dissolution rate will all be captured in the measurement without the need to explicitly understand the mechanisms of dissolution. The transport processes of particle aggregation, deposition, and settling are determined in part by the affinity of interacting surfaces. Particle aggregation involves the transport of one particle to the vicinity of another where, if surface affinity is favorable, attachment may occur and aggregates (or agglomerates) may form. Larger aggregates will tend to have faster settling rates. When surface affinity (attachment efficiency) is high, aggregate structure will also tend to be more porous, leading to reduced drag coefficients and faster aggregate settling rates. Similarly, deposition involves the transport of particles to the vicinity of

a stationary surface (e.g., leaves of rooted aquatic plants or aquifer material) where if surface affinity is favorable, deposition may occur.

Surface Affinity

Surface affinity (α) describes the probability of particle attachment when particles collide with another particle or a stationary “collector” surface. Surface affinity determines the mobility of nanomaterials (and other small particles) in environmental matrices such as porous media (Lecoanet, Bottero et al. 2004) their propensity to heteroaggregate (Brant, Labille et al. 2007) (Therezien, Thill et al. 2014) and, in some cases, the reactivity of nanoparticle aggregates (Jassby, Budarz et al. 2012). Surface affinity is a function of numerous phenomena including van der Waals forces, electrical double-layer interactions, steric interactions, hydration forces, and particle/surface hydrophobicity. It is therefore affected by surface treatments engineered into nanoparticles in the form of charged functional groups or macromolecules. Surface affinity also reflects changes in nanoparticle surface properties due to the adsorption of natural organic matter, or proteins (e.g., protein corona), the conditions of solution chemistry such as pH, ionic strength, specifically sorbing ions, surface heterogeneity, and myriad other factors determined by the system containing the nanoparticle. The value of α is unity when there are no barriers to particle deposition and attachment is favored but may exceed 1 if phenomena at small separation distances draw particles to the surface (Elimelech and O'Melia 1990). All nanoparticles can be characterized with respect to their surface affinity, which will vary as a function of the medium in which nanoparticles are dispersed or embedded.

The affinity of a given type of nanoparticle (as manufactured or following modification in the environment) for a single surface or complex mixture of surfaces (such as a soil, sediment, or

activated sludge) can be determined by introducing the nanoparticles to a suspension of the “background” material of known concentration (Barton, Therezien et al. 2014). Following a programmed period of heteroaggregation, background particles are separated, typically by settling, from the suspension. The surface affinity is determined from measurements of the concentration of nanoparticles remaining in suspension, n , in the context of a simplified Smoluchowski equation ((Smoluchowski 1917)) describing heteroaggregation between nanoparticles and background particles of concentration B . The heteroaggregation rate is given as the product of the second order collision rate constant, $\beta(n, B)$, the concentration of un-heteroaggregated nanoparticles, n , the concentration of background particles, B (which remains constant), and the affinity coefficient between nano- and background- particles, α_{NB} . A reverse reaction due to break-up can be assumed to be proportional (by the rate constant k_B) to the number of nanoparticles that have heteroaggregated ($n_0 - n$) such that:

$$\frac{dn}{dt} = -\alpha_{NB}\beta(n, B)nB + k_B(n_0 - n) \quad (1)$$

Initially, breakup can be assumed to be negligible and the rate equations describing aggregation between nanoparticles and background particles can be solved to yield a predicted linear relationship between the natural logarithm of n_0/n and mixing time:

$$\ln\left(\frac{n_0}{n}\right) = \alpha_{NB}\beta(n, B)Bt \quad (2)$$

Linear regression of the linear portion of the graph will provide a trend line where the slope of the line will be $\alpha\beta B$. Dividing by the number concentration of the background and a calculated collision frequency yields a value for α_{NB} . Alternatively, α_{NB} can be obtained by normalizing

values of $\alpha\beta B$ to the slope obtained when conditions are manipulated to yield a surface affinity that approaches unity (such as high ionic strength in the absence of steric stabilization). This method has been applied to determine surface affinities for a series of metal and metal oxide NPs heteroaggregated with activated sludge (Barton, Therezien et al. 2014). We have further demonstrated that the trends in surface affinity for idealized reference systems of nanoparticles and glass beads follow well-established behaviors with respect to pH, ionic strength, and the concentration of natural organic matter known for colloidal systems.

Dissolution rate, k_d (md^{-1})

Some NPs will undergo dissolution in the matrix that they are in (e.g. lake water, lung fluid, sediment porewater). Dissolution rate describes the speed at which the NP forms a solution in a given solvent, and may be critical to predicting both the persistence and fate of and hazard posed by soluble NPs. In many cases it is the release of metal cations from the NPs that promotes bioavailability and toxicity, though this may not always be the case (e.g. filter feeders or other organisms that ingest particles)(Luoma 2008, Fabrega, Luoma et al. 2011). Many environmental factors can influence dissolution rate, e.g. redox conditions, presence of sulfide or other strong ligands for the NPs. Despite the complexity surrounding the interactions of various ligands and redox conditions, a simple assay in relevant environmental media can be used to measure the dissolution rate. We propose that developing a class of functional assays to measure dissolution rates for particular systems of interest could enable forecasting of their fate and effects in the environment.

It is important to distinguish between dissolution rate and solubility in a functional assay. The former is a reasonable surrogate for the delivery rate of metal cations under dynamic conditions where the released ions are carried away from the particles, while the latter is an equilibrium measurement that does not provide any rate information. Under most conditions, NPs will be present at low concentration and in a dynamic environment such that equilibrium is not established, so the rate of dissolution is the important parameter to consider. Thus, a functional assay should be designed to provide an estimate of the dissolution *rate* under representative environments.

Three types of dissolution rate functional assays will likely be necessary. The first is dissolution rate measured in a liquid, e.g. freshwater, seawater, or porewater environments. The chemical composition of the solution (e.g. presence/absence of ligands) and redox conditions must reflect the environmental conditions of the environment being considered; the basic measurement will be the same for each type of matrix sample. The dissolution rate can be measured at a low NP to solution ratio, and over a time period long enough to assess the rate of dissolution and any changes in rate that may result. The important consideration for most NPs would be to ignore transient behavior at the beginning of the measurement and focus on the longer-term dissolution rate. The transient behavior could include rapid initial dissolution due to release of adsorbed ions (Dobias and Bernier-Latmani 2013), or slower dissolution due to a protective coating (Sotiriou, Watson et al. 2014). However, if these are relatively short-term effects (e.g. <1d), they may not be important when considering the long-term dissolution behavior of the material.

There are many potential functional assays to measure dissolution rate. One can follow the procedures used by many researchers: addition of NPs to the solution, brief sonication as needed, mixing on an orbital shaker for the duration of the test to minimize mass transfer resistance, and periodic measurements using centrifugal filtration with a 3 or 10 kDa filter to separate ions from NPs (Liu, Sonshine et al. 2010, Ma, Levard et al. 2012). Testing may eventually lead to the need for a larger MWCO filter that includes metal-macromolecule complexes in the “filterable/bioavailable fraction” to be measured in the test (Ma, Stegemeier et al. 2014, Li, Hu et al. 2015). These are simple batch tests adaptable to any type of solution that best represents the system of interest (e.g. freshwater, sediment porewater, wastewater treatment activated sludge), and can eventually be automated. One potential problem with this batch approach is that redox conditions may change during the test, or the ligands present in the solution that promote dissolution may become depleted. This can likely be avoided by using a low concentration of NPs. However, more complex flow-based systems may also be used for this purpose. For example, Kent et al., 2012 used a flow through cell containing deposited NPs for such measurements. In their experiments, the concentration of ion was monitored in the cell outlet and the inlet conditions (ligands, redox, pH, flow, etc.) were easily controlled (Kent and Vikesland 2012). One could imagine a standard method for depositing particles in a flow cell would enable monitoring of dissolution rates under a variety of conditions. It would also be possible to expose deposited NPs to representative conditions, e.g. WWTP activated sludge, prior to making dissolution measurements. The flow through system would have better control, but would be more complicated to implement. For comparability with other data, all dissolution rate data should be reported as a surface recession rate, m^3 reacted per m^2 of surface area per d (Liu, Sonshine et al. 2010).

A second dissolution assay is needed for nanomaterials in soils and sediments. In soils, moisture content, heteroaggregation, pH, and organic constituents can all affect the solubility of NPs in soils. Soil systems may range from fully water saturated (subaquatic sediment) to partially saturated soils. Sorption-desorption and leaching studies (e.g. OECD test guidelines 106 and 312) are not necessarily suitable to determine the solubility of NPs in soils. A functional assay that may be appropriate for soils is a measurement using DGT (Harper, Davison et al. 1998, Ernstberger, Zhang et al. 2005). DGT devices can be emplaced in soils, recovered, and the mass of metal released from the NPs is measured in the gel. Dialysis coatings placed over the gel surface can be used to eliminate the uptake of NPs (Sekine, Brunetti et al. 2015). Simple models and an equilibrium measurement between the metal of interest and the soil are used to calculate the “delivery rate” of metal ions derived from the NPs (Harper, Davison et al. 2000, Ernstberger, Davison et al. 2002). This rate of delivery of metal may ultimately be correlated to the bioavailability and/or toxicity (Tandy, Mundus et al. 2011). These devices are versatile and can be used under a range of moisture contents and redox conditions. A simpler method may be a modification of TG 106 where extracted pore water is recovered, filtered with an appropriate MWCO filter, and the soluble fraction determined at a given time point (Whitley, Levard et al. 2013). Monitoring the increase in soluble fraction with time can give you the required estimate of dissolution rate. Care must be taken here to avoid reaching the solubility limit of the NPs in the porewater. Careful choice of reference soils and soil conditions is also needed to best represent the range of soil conditions (pH, organic content, etc.) that may be encountered. A dissolution functional assay may be needed for measuring the dissolution rate of NPs that are incorporated into commercial or consumer products. The encapsulation of NMs into polymeric

matrices, or adhered to surfaces, can alter their dissolution rate. There is a large range of polymeric matrices and potential methods to encapsulate those NPs. However, a FA that measures the rate of leaching of NPs and ions from those products may be sufficient to predict their toxicity potential from metal and/or NP exposures downstream of the devices. Once again, careful choice of the matrix (e.g. artificial saliva, fresh water, saltwater, landfill leachate) will be required to provide results that are predictive of that product in the matrix of interest and via the exposure route of interest.

Conceptual demonstration of the role of α and dissolution rate in forecasting nanomaterial fate, effects, and relative risk:

Figure 3 reprises the graphical abstract for this paper to present a hypothetical example of how two functional assays, surface affinity (α) and dissolution rate (k_d), predict relative risks for two different use and exposure scenarios for PVP-coated Ag NPs.

Figure 3: Hypothetical example of applying 2 functional assays to forecast Ag NP behavior under different scenarios.

Scenario A is based on the direct application of Ag NPs to soil as a fungicide, which has been described as a possible use scenario (Lamsal, Kim et al. 2011, Sawinska, Khachatryan et al. 2014). In this scenario the particles are exposed to aerobic conditions with low sulfide concentrations that would result in limited sulfidation but relatively higher oxidative dissolution (Levard, Reinsch et al. 2011). Scenario B is based on the release of Ag NPs to wastewater from antimicrobial textiles and subsequent processing in a wastewater treatment plant where Ag particles are typically extensively if not completely sulfidized (WWTP) (Kaegi, Voegelin et al.

2011, Lombi 2013, Ma, Levard et al. 2013, Ma, Stegemeier et al. 2014). In this scenario, surface affinity (α) of sulfidized PVP-coated Ag NPs to glass beads in a packed column is approximately two orders of magnitude smaller than that for unsulfidized PVP-coated Ag NPs (O'Brien unpublished data; see Supplemental Materials). Dissolution rate in moderately hard water (as a proxy for soil pore water) is two also orders of magnitude lower for the sulfidized PVP coated Ag NPs compared with the unsulfidized particles (Starnes, Unrine et al. 2015). Qualitatively, these differences in dissolution rate and α are consistent with our observations that the sulfidized Ag NPs resulted in decreased Ag bioaccumulation and toxicity in a model soil invertebrate, *Caenorhabditis elegans*, than for unsulfidized PVP-coated Ag NPs (Starnes, Unrine et al. 2015). Ag NPs attachment to surfaces is favored and, once attached, these unsulfidized NPs more readily deliver dissolved Ag to the organism. The differences in FA values would have also predicted decreased attachment to soil solids, and increased mobility and decreased dissolution in soil for sulfidized Ag NPs in a sandy loam as compared to unsulfidized particles, as has been observed over the short term by Whitley et al. (Whitley, Levard et al. 2013). In terms of Ag mobility, bioavailability, and toxicity in soil invertebrates, these two functional assays would have been predictive of the higher relative risk seen in Scenario A as compared to Scenario B. While based on experimental data, this is a hypothetical example with embedded assumptions (e.g. that the loading of Ag is similar in both scenarios. The power of these functional assays would be increased if parameters measured in controlled reference systems (e.g., glass beads) can be shown to be predictive of measurements obtained in more complex systems of variable pore water, soil composition and ecological receptor species.

Conclusions

Orienting risk assessment research around the identification of functional assays and the systematic generation of measurements for those functional parameters that include consistent meta-data on both material AND system characterizations will allow the nanoEHS community to work the problems backwards, holding endpoint responses constant and querying which functional assays appear to be most predictive, and ultimately to identify which material and system parameters matter – or do not matter – in affecting outcomes of interest. This focus is important in searching for functional assays that ideally allow for generalization between systems. The measurements might be adapted for distinct systems, but we should be searching for those parameters that, like rate constants for transformation reactions or equilibrium constants like K_{ow} for neutral organic compounds, could be applied to predict material fate in many different systems. Systematic approaches must be developed as a community for making the types of critical choices presented in our example FAs of surface affinity and dissolution, such as the relevant environmental matrices in which they are measured and the associated characteristics that must be reported. This will be equally important for reducing uncertainties of FA applications as well as for appropriate interpretation of FA results in providing guidance (e.g. categorizing nanomaterials for tiered testing strategies, determining subsequent experimental designs).

To summarize, we underscore the following proposals of factors to be taken into account in advancing nanoEHS work to achieve near-term predictions of nanomaterial risks:

1. Nanomaterial characterization that clearly distinguishes between intrinsic and extrinsic, i.e. system-dependent, characteristics.

2. Adoption of reference systems – and associated minimum criteria for characterizing these systems – to represent environmental and biological systems that are expected to be of particular importance across the value chain and life cycle of nanomaterials and their transformation by-products. Such reference systems will allow the community to amass interoperable data across a relevant spectrum of system properties.

3. Identification of key functional assays and associated parameters that will improve our power to predict nanomaterial fate and impacts, and generation of data on these parameters (with consistent metadata as described above) in systems of interest to forecast outcomes of interest. Benchmark data generated by applying these assays to reference nanomaterials and reference systems can then be compared with results obtained from more complex systems. Serving as a link between completely empirical observations and first principles mechanistic theories, FA parameters may allow us to group material-system combinations that impart similar results.

Basic science is unfolding as we better understand matter on the nano scale. This scale itself has always existed; our ability to manipulate, characterize, and harness the novel advantageous properties is new. We are learning what mechanisms govern the behavior of engineered, naturally occurring, and incidental nanomaterials, and their subsequent interactions and impacts on the environment and health. Functional assays provide a systematic path forward that will be critical for planning research rationally, collecting the resulting data coherently to allow for post-analysis, and aligning databases with the targeted analyses they will support. The integrated research strategy enabled by community-wide adoption of these calls, both in terms of exploring applications and predicting implications of ENMs, is in line with the visions put forth by both the National Nanotechnology Initiative (NNI 2014) and the National Research Council (NRC 2013)

in the US and the NanoSafety Cluster in the EU (Savolainen, Backman et al. 2013). Distinguishing and consistently capturing material and environmental system data in conjunction with generating functional assay parameters will help realize the promise of nanoEHS research to inform near-term decisions, while laying the foundation for fundamental first principles discovery.

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