Measurement Methods for the Oral Uptake of Engineered Nanomaterials from Human Dietary Sources: Summary and Outlook

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Abstract: This article is one of a series of 4 that report on a task of the NanoRelease Food Additive (NRFA) project of the International Life Science Institute Center for Risk Science Innovation and Application. The project aims to identify, evaluate, and develop methods that are needed to confidently detect, characterize, and quantify intentionally produced engineered nanomaterials (ENMs) released from food along the alimentary tract. This particular article offers an overview of the NRFA project, describing the project scope and goals, as well as the strategy by which the task group sought to achieve these goals. A condensed description of the general challenge of detecting ENMs in foods and a brief review of available and emerging methods for ENM detection is provided here, paying particular attention to the kind of information that might be desired from an analysis and the strengths and weaknesses of the various approaches that might be used to attain this information. The article concludes with an executive summary of the task group’s broad findings related to the 3 topic areas, which are covered in more detail in 3 subsequent articles in this series. The end result is a thorough evaluation of the state of ENM measurement science specifically as it applies to oral uptake of ENMs from human dietary sources.

Keywords: characterization, detection, food safety, measurement methods, nanomaterials, nanotechnology, nanotoxicology

Introduction

Owing to the unique physical and chemical changes that materials possess at the nanoscale, engineered nanomaterials (ENMs) are increasingly being used to improve the function of a wide range of consumer products. Some of this research and development is occurring in the food sector, where the use of nanoscience in food processing, packaging, and ingredients is an area of intense exploration. Many scientists anticipate that the application of nanotechnology to food science could give rise to a wide range of benefits, including foods that are more nutritious, more plentiful, more environmentally friendly, better tasting, and less expensive.

Unfortunately, the very changes in physical and chemical properties that are the basis for these envisioned benefits also give rise to a number of uncertainties with respect to the risks these materials may pose to human health and the environment. In particular, there is an urgent need to understand how the nanoscale impacts the toxicological properties of materials, as well as the likelihood of exposure. The magnitude and impact of these uncertainties is exacerbated by the fact that the experimental techniques that would be utilized to address them for conventionally sized materials may not be useful for matter at the nanoscale. Recent public opinion surveys and commentaries (Duncan 2011a) concluded that a proactive approach toward understanding and communicating the risks of nanotechnology-enabled food and food packaging is the best way to ensure that this emerging technology does not succumb to a crisis of negative risk perception. Therefore, it seems imperative that the present gaps in our ability to evaluate the risks of nanotechnologies in foods be addressed immediately.

The NanoRelease Food Additive (NRFA) project is an international, multistakeholder endeavor that brings together dozens of scientists across academia, industry, government, and nongovernment organizations (NGOs) with a shared interest in the future application of ENMs in the food industry (Szakal and others 2014). The aim of this project is to identify, evaluate, and develop methods that are needed to confidently detect, characterize, and quantify intentionally produced ENMs released from food along the alimentary tract. The ENMs considered include those occurring through either direct addition or through indirect incorporation in food via environmental contamination or migration from food contact materials. As part of its initial attempt to identify knowledge gaps in this area, NRFA established 5 groups of experts to...
gather and evaluate information on the following: 1) the ENM and food matrix characteristics relevant to ENM absorption, 2) the alimentary canal environment as relevant to the behavior/uptake of relevant ENMs, 3) alimentary canal models as relevant for use in nanomaterial measurement methods, 4) potential and existing measurement methods, and 5) risk management aspects relevant to these issues in a global perspective.

This article is the first in a series of 4 articles related to the 4th task identified above. The article’s goal is to fully describe the project’s charge and scope, give an overview of the problem of ENM detection in foods and other complex matrices, and provide an executive summary of the task group’s overall findings. The other articles in this series cover individual topic areas in the task group’s scope (see below) in a high level of detail: the 2nd, 3rd, and 4th articles, respectively, describe methods to characterize and detect ENMs released into foods from food contact materials (Noonan and others 2014), characterize and detect ENMs in foods (including sample preparation) (Singh and others 2014), and characterize, detect, and study the behavior of ENMs introduced into the alimentary tract through food ingestion (Alger and others 2014). While this particular article is capable of standing alone, due to the fact that some experimental methods may have utility in multiple areas relevant to the project’s overall scope, some methods discussed within this article may have additional descriptive detail offered in other articles in this series.

Objectives of This Project

Ensuring the safety of food nanotechnology is predicated on answers to many questions. We have to know what kind of ENMs are being put in foods and how much. We need to know what these ENMs look like, what size and shape they are, what they are made of, and what happens to them over time. Once ENMs in these foods are eaten, we need to know how they behave in the mouth, stomach, and intestine. We also need to know where they go after they are digested, and, when they get there, we need to know what kind, how much, what they look like, what they are made of, how long they stay there, and how and when they leave.

In some respects, these are the same questions that risk assessors must answer for any new chemical or substance that is added to a food. ENMs, however, present a more challenging case because the aforementioned questions often give ambiguous answers when they are framed in a way that is designed for more conventional materials. The question “How much is there?” for example, has a fairly well-understood meaning for a traditional chemical. Transitioning between a per molecule paradigm to a per molecular attribute paradigm is effortless because each molecule is expected to have identical attributes. As such, we can easily express “How much is then?” in terms of concentration on a per molecule basis (moles per liter or parts per million) or a per mass basis (milligrams per liter) if 1 metric is provided and we desire the other. We encounter problems, however, if we are provided a concentration of ENMs in milligrams per liter, because the mass of every ENM with a given composition is not identical. Therefore, it is necessary to know other properties of the ENMs (for example, size, shape, and density) in order to compare values of mass-based concentration between different studies. Even with this information, making comparisons is not always straightforward. Moreover, this approach still ignores the very important consideration that “How much is there?,” which is motivated by the expectation that the dose makes the poison, may not mean what we think it means for an ENM. If the metric being used to assess safety is “How much mass is there?,” and what is really critical to know is “How much surface area is there?”, then molecular attributes may be misinterpreted from the available data.

The methodological challenge of measuring ENMs in foods and their possible adverse (or beneficial) effects on human health creates additional difficulties. Even if we can properly identify what questions are the most appropriate to answer, and develop a consistent understanding of what is the most appropriate way to frame the answers, we are left with the challenge of actually answering them. To do so requires new modes of thinking about chemical analysis, in which detecting an ENM analyte by its composition alone is not necessarily sufficient to determine whether it is present, and quantifying the amount of mass is not necessarily sufficient to determine how much is there. These new modes of thinking must be accompanied by new methods of measuring—new techniques to acquire additional types of information that were never needed before, on materials that are novel and prone to chemical and physical processes we do not fully understand, in some of the most complex matrices (foods and biological tissues) that analytical chemists ever have to deal with. As such, this is the challenge in scope for those involved in nanocharacterization efforts within the food science arena.

One of the early steps toward solving this methodological puzzle is assembling a detailed picture of what is known about measuring the properties of ENMs, both in matrices relevant to safety evaluations but also in pristine states (simple aqueous media). Unfortunately, while many studies have been published that incorporate some form of ENM detection, quantification, and/or characterization, most of these studies are focused on results and devote far less attention to how the results were obtained. Therefore, it is difficult to arrive at a general understanding of the state of ENM measurement science.

The NRFA project Steering Committee charged our task group to provide an analysis of current progress by physicists, chemists, biologists, and toxicologists toward developing the methodological toolset needed to meet the challenge of ENM detection in foods, food contact materials, and the alimentary tract. In particular, the task group was directed to 1) provide an evaluative overview of the analytical methods with respect to detection of ENMs and characterization of attributes of significance for potential uptake of ENMs by the body from the alimentary tract and 2) to identify gaps in the methods or methods development needs with respect to such measurement methods.

To this end, the objectives of this series of articles are as follows:

- Identify the requirements for useful ENM characterization methods, both as applied to pristine and complex matrices, including the questions of ENM detection and characterization methods needing answers, the types of information these characterization results should provide, and the types of limitations these characterization methods need to overcome.
- Offer a prescriptive means to determine what method, or combination of methods, can be used for a given ENM type, food type, or informational need, in support of our understanding of ENM uptake by the alimentary tract.
- Evaluate measurement methodologies currently being used for understanding risk-relevant exposures to ENMs in foods, including relative benefits and limitations, and review the body of literature to which they have been applied (here, “methodologies” includes analytical techniques, simple preparation procedures and other experimental approaches.
toward evaluating ENM exposure, uptake, or biological effects).

- Identify knowledge gaps where additional methods development is needed, supported by an analysis of the limitations of literature related to ENM detection and characterization in complex matrices.

**Strategy for addressing objectives**

In pursuit of these goals, the task group, which was composed of numerous individuals drawn from academia, government, industry, and NGOs, with expertise across a broad range of scientific disciplines, considered 3 general areas or control points where ENM detection methods are critically valuable for evaluating the likelihood of consumer exposure to ENMs as well as the potential pharmacological and toxicological effects of such exposure: in food contact materials, in foods, and in the alimentary tract. For each of these focus areas, which comprise focus topics of the 3 companion articles in this series, the task group members performed a critical analysis of the literature, looking especially at which questions each published study tried to answer, which methodological tools the study’s authors used to answer those questions, and the general usefulness of the answers. For the most part, the authors of the articles in this series performed their tasks and drafted their manuscripts autonomously under the guidance of the task group chairpersons. However, recognizing that there are numerous methods that may be applicable to more than one of these target areas, the task group decided to designate an additional focus area related to the general problem of ENM detection that could offer a basic summary of currently available and emerging ENM detection and characterization methods, and which would also focus on an identification of knowledge gaps that extend across the entire breadth of the project’s scope. This motivated the inclusion of an overview of ENM detection and characterization technologies and the challenge of how best to choose an analytical method for a particular application, which is the content of the next section.

With respect to depth of coverage, the task group chose to primarily focus on ENM detection and characterization methods that have a high degree of practicality and that are currently being used by scientific professionals, although some attention is paid toward methods in development, or methods that are not currently being used for ENM detection and characterization but may be worth investigating for these purposes. The methods discussed herein have been considered relevant to food packaging sources, direct food additives, dietary supplements, and environmental sources of ENM to food, including drinking water. Furthermore, in deciding what ENM analysis methods to focus on, it was determined that detection limits of methods should be sufficient and characterization methods should be appropriately quantitative with respect to understanding the relevant ENM dose for exposure assessment purposes. Further context for the task is that measurement of migration of materials from food contact materials to food constitutes a critical step in the determination of food safety under a variety of risk management or sustainable product design decisions.

**Overview of ENM Detection and Characterization Methods**

Current scientific ideology regarding ENMs has advanced to a common understanding that such small forms of matter have unique physicochemical characteristics with respect to the same materials in non-nanosized ranges. In part, the large surface area-to-volume ratios of ENMs increase their general reactivity and reduce the bulk/core interactions with their surrounding materials in favor of the sometimes unexpected constituents of the “ENM surface.” This section aims to discuss some of the requirements and challenges associated with ENM detection and characterization, particularly in complex matrices such as food and the human digestive tract. As with any scientific endeavor, precise identification of the problem being asked will often dictate which detection methods are employed for ENM analysis, including whether to rely on existing technology or to delve into the risks of using emerging methods.

When identifying the specific ENM-related problem being asked, as much information about the starting material, the direct contact materials, the matrix surrounding the material, and any expected modifications to the material should be evaluated. This includes core atom chemistry, any expected oxides or salts associated with the ENMs, shape, size distributions, and what the final state of the ENM is expected to be in the target matrix. For example, if 20-nm silver nanoparticles (AgNPs) are added to materials that directly contact food to take advantage of their antimicrobial properties, a variety of different detection methods could be utilized for identification of silver in the food matrix. However, the detection methods will differ based on the following: 1) if the AgNPs persist as AgNPs after release into the food or if they have dissolved into silver cations, 2) if the AgNPs are still isolated 20-nm spheres or if they have agglomerated/aggregated into larger sized species (with or without incorporation of matrix materials), 3) if the AgNPs started with an inorganic or organic surface coating and/or if the surface coating is expected to grow or disappear entirely upon food entry, 4) if quantification of AgNPs is warranted (how much?) or if simply detection is suitable (Is it there at all?), and 5) if it matters where/how the AgNPs are distributed in food or in cells (that is, “Is imaging required?”).

It must be stressed that no single method can answer all of the above questions. Therefore, a combination of methods should be considered to optimize the return of suitable and relevant information while considering the accessibility, cost, and ease of use in attaining the perfect data set. A basic decision tree for ENM characterization methods relevant to the section topics is displayed in Figure 1. This figure will be referenced throughout the section and although it is not meant to be comprehensive, Figure 1 provides some insight into the current possibilities and challenges associated with ENM detection in food-based and food contact matrices.

**Overview of available compositional analysis methods**

Inorganic ENMs. Because of its relevance and broad detectability, the above-mentioned AgNP example is an excellent case study for examining which analytical methods to employ for a given question. The same general detection concepts are expected to extend from AgNPs to other inorganic (noncarbon-based) ENMs, such as those made of titanium and zinc (including their corresponding oxides) as well as aluminosilicates and calcium. For compositional analysis of such inorganic-based ENMs, the “Is the ENM there at all?” question is likely the easiest to answer. Presuming that the analysis will allow for the complete digestion of the surrounding matrix (for example, food, biological tissue, or food packaging) and that the elemental signatures of the ENMs are enough to confirm ENM presence, there are a variety of liquid-based analytical methods capable of detecting such materials, as indicated in Figure 1. Techniques such as flame atomic absorption spectroscopy (AAS) (Pipan-Tkalec and others 2010), surface plasmon resonance (SPR) (Raz and others 2012), high-performance...
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Figure 1—Decision tree for choosing measurement methods for the oral uptake of ENM.

Note: This decision tree is based on nanoparticle and nanomaterial analysis either in the pristine state or within simplified matrices and does not take into account differences due to nanomaterial extraction from the surrounding matrix nor the effects of the matrix on ultimate detection limits. Because of the complexities of food, food packaging, and the alimentary tract as an analytical matrix, the resulting utility of the decision tree may need to be augmented. Rather than this being a comprehensive representation of nanomaterial characterization, an emphasis was placed on what methods can yield near-term accomplishments as well as where considerable amounts of additional research are needed.

liquid chromatography (HPLC), and field flow fractionation (FFF) (Luykx and others 2008) can be valuable for different levels of ENM detection, especially if the targeted ENMs are preseparated or extracted from the complex food matrix.

Currently, one of the most common methods for the identification and ultimate quantification of inorganic ENMs involves the use of inductively coupled plasma (ICP) technology (Lin and others 2011; Mitrano and others 2012a,b), usually combined with mass spectrometry (MS) but also employed with optical emission spectroscopy (OES) (also known as atomic emission spectroscopy). ICP-MS is more sensitive to inorganic ENM detection of the 2 ICP-based methods, and as such can help answer the “Is it there?” and “How much?” questions. For example, ICP-MS data are produced in a quantitative manner that can detect ionic silver from nanotechnology-enabled food packaging down to 0.04 ng/mL (0.75 µg Ag per gram of nanocomposite) with a linear dynamic range (LDR) of 0.2 to 500 ng/mL (Lin and others 2011). Despite not having the imaging ability of SEM or TEM for comparison, and the difficulty in differentiating signals attributed to whole AgNPs from those signals of Ag⁺ adsorbed onto the surfaces of other particles that have nonsilver cores, the technique holds promise as one of the future pivotal methods for detecting nanoparticles within complex matrices such as foods and alimentary tract cells/tissue.

The NanoLyse project (www.nanolyse.eu) has made this technique central to the detection of metal-based nanoparticles within food matrices, including experiments for preliminary validation of SP-ICP-MS for such a purpose. In principle, SP-ICP-MS could detect a single nanoparticle in a large sample volume if one is willing to aspirate the entire volume. However, it is worth noting that using current technology, SP-ICP-MS is not sensitive enough to detect whole particles with diameters less than approximately 20 nm (although this limit is highly dependent on isotope abundance, particle composition, and the amount of dissolved ions in the background), which may limit its ultimate utility in some instances. The use of SP-ICP-MS for detection of ENMs in food matrices is described in more detail in the 3rd article of this series (Singh and others 2014).

Organic ENMs. As illustrated in Figure 1, compositional analysis is more limited in the options for detection when the subject ENMs are organic (carbon-based) in nature, including carbonaceous materials like fullerenes, carbon dots, and carbon nanotubes, as well as those encapsulates consisting of lipids, polymers, or...
molecules/poly saccharide blends. Provided that the targeted molecules are separated from the food or cells of interest first, some of the aforementioned liquid-based separation methods such as HPLC and FFF could be effective for organic ENM detection when coupled with ultraviolet-visible (UV-vis) or fluorescence-based detection systems. However, the best methods for such carbon-based ENM hybrids may come from very specifically targeted techniques such as flow cytometry for nucleic acid ENMs within biofluids (van Gaal and others 2010) and enzyme-linked immunosorbent assay (ELISA) screening kits formulated for antibody-based detection of cross-linked gelatin ENMs (Dehalu and others 2012). An interesting twist would be if label/indicator inorganic ENMs are added to the complex matrices, in which case they may actually enhance the organic molecule detection for techniques such as MS, SPR, and fluorescence spectroscopy (Zamborini and others 2012). The decision tree in Figure 1 provides a pointed contrast between the general status of ENM detection for organic ENMs relative to their inorganic counterparts. The information reveals that there is a continuing need to develop detection methods for organic ENMs in the food arena.

**ENM surface coatings.** Many ENMs feature organic coatings or ligand spheres surrounding organic or inorganic cores in which the ultimate surfaces of such materials dictate their behavior in the food packaging, food, or the human body. Characterizing the surface coatings of ENMs and their fates within complex organic matrices such as food and human cells or tissues requires a different set of analytical methods. Provided that other techniques such as SEM and TEM have been used to locate samples rich in ENMs and ENM aggregates (perhaps after cryomicrotoming of the sample of interest), surface-chemistry-based methods such as X-ray photoelectron spectroscopy (XPS), secondary ion mass spectrometry (SIMS), low-energy ion scattering (LEIS), atomic force microscopy (AFM), scanning probe microscopy (SPM), and scanning tunneling microscopy (STM) can be used to provide very specific chemical information at a range of analytical figures of merit for ENM detection (Baer and others 2010). For example, XPS is a surface-sensitive tool that can be used for examining compound-identifying ENM functional groups (Gorham and others 2012), and time of flight (ToF)-SIMS has recently been used in concert with specific sample preparation methodologies to discern subtle differences in ENM aggregate coatings (Szakal and others 2012). For compositional analysis alone, however, it is difficult to envision widespread deployment of surface-chemistry-based methods for ENM detection because of their high learning curves and considerable expense. At the same time, such methods can provide information that is unattainable with other techniques, creating an interesting future for their use moving forward. This is especially true if companies elect to utilize instrument user facilities instead of directing significant amounts of resources into development of these advanced detection technologies in-house.

**Overview of available imaging analysis methods**

An imaging-based analysis provides critical information that addresses the "seeing is believing" issue that many doubters may have with just compositional information alone. For example, results from imaging-based analyses can address the following types of questions: Where are ENMs located within a complex matrix (for example, in food, cells, or tissues)? Have the ENMs aggregated or agglomerated? If so, what is the nanoparticle size? Specific questions can also be answered with tailored methods such as fluorescence imaging and confocal laser scanning microscopy (CLSM) for studying trophic transfer of ENMs in the invertebrate food web (Holbrook and others 2008). However, there is no question that the majority of ENM-related imaging studies have employed SEM or TEM to answer specific ENM-related size information (Powell and others 1996; Luykx and others 2008; Tiede and others 2008; Pipan-Tkalec and others 2010; Powell and others 2010; Huang and others 2011; Szakal and others 2012; Weir and others 2012). Regardless of ENM shape, the appropriate sample benefits from nanometer and subnanometer lateral resolution that can be offered with SEM and TEM, respectively, can answer critical questions regarding core–shell thicknesses, fates of ENM sizes, and extent of integration (for example, "Was the ENM integrated exactly as we expected/planned in a particular matrix?"). With slicing approaches such as cryomicrotomy, focused ion beam (FIB) milling, and TEM-based tilt series analyses, x- and y-axes imaging information can be extended into 3-dimensional (3D) analyses by including the z-axis. Such information can be vital for application areas such as locating nanoclays within food packaging (as described in detail in the 2nd article of this series (Noonan and others 2014) to finding engineered nanoparticles within cell membranes and organelles (as described in the 4th article of this series, Alger and others 2014).

Information on the presence and morphology of ENMs is not always enough and the combination of compositional analysis and imaging analysis is often paramount to knowing exactly what is occurring in a chemical system. For example, combining SEM with energy-dispersive spectroscopy (EDS) gives high-resolution composition maps with percent-level detection of various elements, which is particularly important for inorganic-based ENM analyses (Huang and others 2011). In addition, TEM coupled with electron energy loss spectroscopy (EELS) (Powell and others 1996) or selected area electron diffraction (SAED) (Szakal and others 2012) can provide pertinent information about the imaged ENM. If the lateral imaging resolution can be on the order of 200 nm to 1 μm, such as with aggregated forms of ENMs, then much more chemical information can be provided with a ToF-SIMS analysis, including simultaneous organic/inorganic information. Such an analysis can now be accomplished with 400-nm organic molecule resolution inside of single cells (Szakal and others 2011) in which ENM aggregates could potentially be located in specific organelles or at the cell membranes of the alimentary tract, while ENM-induced cytoplasm changes could possibly be present in the “fingerprint” mass spectra of different cells. Increasing the lateral resolution further from 2 through 25 μm can include much higher mass organics such as the protein-based encapsulates mentioned earlier for analysis within the food packaging, food, or even alimentary tract tissue via matrix-assisted laser desorption ionization (MALDI)–MS (Luykx and others 2008). Both ToF-SIMS and MALDI would have the same analytical figures of merit regardless of alimentary tract cell type, which is an important consideration. A different SIMS variant in which the lateral resolution can be below 100 nm would be a significantly helpful tool for tracking isotopic ratios and elemental signatures (Musat and others 2012).

The fundamental drawback to these more advanced methods is sample preparation for introduction of the samples into an ultrahigh vacuum environment, including any sample changes that could potentially occur with such manipulation (as discussed later in this work). As an alternative, coherent antistokes Raman scattering (CARS) microscopy can image some similar materials to MS without the rigorous sample preparation, including aggregated nanomedicines in tissues at submicrometer resolution (Garrett and others 2012). It must be mentioned though that the chemical imaging methods will not be able to differentiate natural
agglomeration of ENMs compared with accumulation of smaller ENMs for a given location. Hence, this type of characterization will be most beneficial when combined with other techniques to elucidate the state of the ENM in complex matrices.

Overview of emerging ENM detection methods

Most of the previously discussed ENM detection methods are at varying degrees of gaining establishment as routine analysis tools. However, as the questions become harder to answer, the methods available to answer them need to become more advanced. For example, methods that can eliminate the need for ENM separation or extraction from the food or alimentary tract matrices can provide for simpler and more rapid analyses (Singh and others 2014). On the other hand, a more advanced separation or extraction could dramatically enhance the information available about ENMs.

One relatively new ENM detection method involves the use of microchannel resonators, which are devices with microfluidic channels embedded into an AFM style tip. This approach can achieve both mass and size information of ENMs (Coleman and others 2011) once assumptions about the specific types of ENMs have been made. The native frequency change when an ENM passes through the channels of the tip can be directly related back to the amount of material that entered the channel. Coupled with separations via HPLC, FFF, or even with dynamic light scattering (DLS), more information can potentially be provided than with just the resonators alone. In addition, since the channels can allow for just 1 nanoparticle to pass through at a time, the exit of the microchannel resonators could conceivably be connected to SP-ICP-MS to obtain enhanced chemical information. In principle, several different types of ENMs, including both inorganic and organic materials, could be analyzed with this emerging methodology.

A new approach for separating organic-coated nanoparticles from complex matrices has been achieved with precision drop-on-demand inkjet printing technology (Szakal and others 2012). Although it is very recent, this reproducible preparation method could play a role in conjunction with surface chemistry measurements such as SIMS or XPS, along with TEM, SEM, or even ICP-MS. With these surface-chemistry-based methods, it is possible to potentially achieve quantitative information on coating thicknesses and chemical origin, as well as attain depth information of chemical signals throughout the ENMs (Baer and others 2010). For example, these techniques could be useful for determining if the protective chemistry of ENM coatings becomes compromised after exposure to the food matrix.

Another recently emerging method called atom probe tomography (APT) has been capable of atomic-resolution 3D maps of materials science applications (Miller and Forbes 2009). The sample preparation is challenging, but with continued advances, the benefits of both atomic resolution and chemical identification imaging may lead to some interesting uses for analysis of nanomaterials in complex matrices. For instance, an APT sample approximately 20 to 100 nm in diameter could reveal if ENM core atoms have migrated beyond the coating layers after exposure to acidic environments such as foods and the human gut.

In the event that ENMs cannot be separated from the complex matrix for the question being studied (for example, how or where nanomaterials intercalate into food, packaging, and/or single cells and tissues) and ultrahigh vacuum conditions could perturb the information content, new avenues are possible to obtain this information. Ambient ionization mass spectrometry (AI-MS) represents a class of recently developed methods that reduces the costs of many MS-based chemical analyses and minimizes any sample preparation. Because the analyses take place in the ambient environment, and hence in front of an MS inlet, it is possible to collect chemical signatures from a wide range of sample targets, including food packaging, biological tissue, and wet samples. The methods are related to SIMS and matrix-assisted laser desorption/ionization (MALDI) in that material must be desorbed from the sample first. In AI-MS, this is accomplished by either submillimeter–diameter liquid-based sprays such as desorption electrospray ionization (DESI) (Takats and others 2004), gas sources such as direct analysis in real time (DART) (Cody and others 2005), and low-temperature plasma (LTP) (Harper and others 2008) and photon sources such as atmospheric pressure (AP)-MALDI (Laiko and others 2000), or combinations of multiple source types. Although individual ENMs would not be analyzed or imaged with these methods, it is plausible that a wider chemical range of ENM aggregates can be analyzed compared to other surface-chemistry-based techniques. In addition, the changes to the surrounding matrix that occur in the presence of ENMs could be probed with the alternative sample introduction methods noted above, as well as an in situ MS-based microextraction technique called a liquid microjunction surface sampling probe (LMJ-SSP) (Van Berkel and others 2009).

Each of the articles in this series covers emerging analytical techniques, such as those mentioned above, that may have particular utility in their respective focus areas. For example, the 3rd article, which offers an investigation of measurement methods for ENMs in foods, provides an overview of such emerging technologies as SPR, atmospheric SEM (ASEM) and SP-ICP-MS, among others (Singh and others 2014). An urgent area of need for emerging methods in all of these focus areas is the identification of sample types for which each of these emerging methods is most relevant, as well as standardization of operating conditions and sample preparation methods.

Challenges and Outlook

The previous sections have offered a brief glimpse into some of the challenges related to detecting and characterizing ENMs in complex matrices, a problem which is ultimately necessary to solve in order to evaluate uptake of ENMs from human dietary sources. Moreover, this introduction has hopefully revealed that there is already a wealth of analytical options available to researchers interested in food nanotechnology and toxicology, even if it is not yet always clear how to choose the best option for any particular situation. The remaining articles in this series provide considerably more detail about analytical methods related to the focus areas of ENMs and their release from food contact materials (Noonan and others 2014), ENMs and their behavior in complex food matrices (Singh and others 2014), and ENMs and their interaction with and uptake from various regions of the alimentary tract (Alger and others 2014).

While detection or characterization of ENMs in each of these focus areas is fraught with their own unique issues and challenges (enumerated in detail in each of the respective focus articles), the task group quickly realized that there were 6 general classes of difficulties that were common across the entire spectrum of topics. As these difficulties also constitute the primary areas of need related to the challenge of measuring oral uptake from dietary sources, we present them below as an executive summary of the task group’s findings in response to the NRFA project steering committee’s central charge.
Pristine particle analysis

Whether the focus is on detecting and characterizing ENMs migrating from food packaging, ENMs intentionally added to foods, or ENMs in various stages of digestion, information about ENM presence, quantity, and characteristics is far less useful without the meaningful context of the original ENMs characteristics (in composition, form, and quantity) before they were added to the packaging or food. That is, the characteristics of the ENM starting materials are likely to inform analytical characterization method choices. The general lack of this kind of information in many toxicological and ENM migration studies is a serious methodological forthcoming because without it, making correlations between the properties of the initial starting material and the ultimate effect of the ENM in the system being studied becomes far more challenging, if not impossible. Such a shortfall slows down the construction of predictive frameworks for ENM behavior in complex systems.

It is interesting to note that the methods currently used for pristine particle analysis are largely the same as those presented above and in the other articles in this series for detection/characterization in food and complex matrices, but must be subjected to more stringent analytical requirements. For example, sizing of starting ENMs should be accomplished with more than 1 method (if possible, at least 3 methods) among TEM, AFM, DLS, and FFF. Using multiple techniques to acquire the same data not only can provide confidence in the characterization, but, where differences are found, can elucidate subtle information about the starting material (for instance, if the particles were expected to be spherical but are more elliptical in nature).

On a more fundamental level, successfully measuring the properties of ENMs in simple matrices is a critical means of validating analytical methods so that analogous measurements in more complex matrices can be trusted. It is only then that comparisons of the ENMs detected in foods, packaging, and in cells can achieve the following: 1) relate to the original starting materials, 2) determine whether commercial test materials are relevant to those used in foods/food packaging, 3) identify predictive behavior of the ENMs in foods based on characteristics (for instance, water- or fat-based), 4) predict properties of the ENMs both after industrial processing and at various food-based and analytical tract-based temperatures and viscosities, 5) distinguish the consistency among the ENM starting materials for large batches, and 6) differentiate between natural nanomaterials and deliberately added ENMs. An area of community need is in the validation of the pristine ENM detection and characterization methods in terms of uncertainties, limits of detection, and potential false detection flaws so that these potential achievements in applied ENM measurement science can be realized.

Therefore, an increase in published studies that explore the application of emerging detection methods, and even well-established detection methods, to a variety of ENMs in their pristine states would be beneficial to the nanocharacterization community. Additional standard reference materials of ENMs both alone and in more complex (food) matrices, such as those being developed as part of the NanoLyse project (www.nanolyse.eu), would be helpful to reach these objectives.

Value and limitations of ICP-MS and TEM/SEM and synergy in analytical method combinations

One fact that seems to be conserved over a majority of the research studies considered by the task group is that ICP-MS and SEM/TEM are often viewed by the scientific community as being the minimum (and often sufficient) tools needed for adequate detection and characterization of ENMs. ICP-MS provides a means to obtain some information on ENM quantity or concentration and also defines a potential route to compositional analysis, and electron microscopy fulfills the need for information on ENM form and location. These techniques work well for ENMs based on the best analytical sensitivity to most elements (ICP-MS) and the best lateral resolution for ENM imaging (TEM).

Even this combination of powerful techniques, however, results in potential information gaps in any analysis, although the method development community is not without potential solutions. One of the classical drawbacks of ICP-MS—in which it has been generally indistinguishable whether the elemental signature originated from whole, isolated ENMs, ENM aggregates/agglomerates, or dissolved species—is being addressed to some extent with the advent of SP-ICP-MS, although the technology is not yet at the state where particles with exceptionally small diameters (<20 nm) can be measured reliably. Similarly, one of the drawbacks of TEM has classically been the solid-phase requirement, which is being averted with special liquid-based flow cells to image ENMs in their native solutions (Klein and others 2011). The expense of TEM instrumentation, significant expertise required, and generally low throughput can also be limitations for TEM as a routine screening tool for large volumes of samples, and the issues of non-representative sampling and a lack of utility at low analyte concentrations will likely forever be an issue with microscopy-based methods.

It is predicted that ICP-MS and TEM/SEM will continue to be at the forefront of ENM research because of their large information yield for ENM detection and morphological imaging. However, gaps do exist for compositional imaging (chemical identification), particularly in more complex matrices such as food packaging, undigested food, and alimentary tract cells/tissues. SEM-EDS and analytical techniques associated with TEM (often called analytical electron microscopy, or AEM) can provide elemental information of ENMs at excellent lateral resolution, but often at reduced detection rates relative to SIMS or MALDI along with general insensitivity to organic species. (Additionally, the SEM-EDS imaging resolution is usually not as good as SEM alone.) On the other hand, the lateral resolutions of the latter techniques do not compare with electron microscopy (EM), rendering them to be most useful for ENM aggregation/uptake studies in cells (SIMS, CARS) and tissue (MALDI, CARS), with perhaps both being potential methods for food matrix or food packaging analyses because of the attainable organic chemical information. Not enough work has been done for any of the aforementioned techniques in complex matrices to determine their ultimate efficacy, which leaves a great opportunity for continued study.

On a broader level, the ubiquitous reliance on multiple analytical techniques by scientists in the field for ENM identification, quantification, and characterization has convinced the task group that there is not now, and will likely never be, a single analytical method that provides all the information needed to fully understand the behavior of ENMs in foods and the way they are taken up by the alimentary tract after ingestion. Successful completion of studies that provide useful knowledge along these lines is contingent upon a robust understanding of which methods are most appropriate in certain situations and, in particular, which methods most expediently provide complementary information. Therefore, great gains can be realized with methods development targeted toward the
elucidation of synergisms between existing or emerging detection techniques to maximize efficiency and ensure that research studies produce meaningful and comprehensive data.

Deficiency in organic ENM analysis

Surveys of available measurement methods consistently revealed that while there are numerous mature techniques available to find, quantify, and measure the properties of inorganic ENMs, the same cannot be said for organic ENMs (see, for example, Figure 1). In large part, this is due to the fact that organic ENMs are composed of the same general “stuff” as the background materials in foods and biological tissues. Solutions to this troubling problem often include labeling organic ENMs in some way (fluorescent tags, radiolabels, and so on), but it is widely suspected, although often not proven, that such modifications to organic ENMs can sufficiently change their chemical or physical characteristics to render them poor models of their unlabeled versions. Methods that can detect organic ENMs or organic surface coatings around inorganic ENMs in an organic matrix without such tags are in very short supply.

Therefore, methods development targeted toward generation of analytical approaches, including sampling methods, which can detect and quantify organic ENMs in food or tissue matrices is of paramount importance. Specifically, methods that depend on tagging organic ENMs should be further investigated to ensure that such procedures do not sufficiently alter the characteristics or behavior of these ENMs in living systems.

The challenge of sample preparation

Before the properties of a food or tissue sample can be measured, it must be prepared in some way so as to be compatible with the analytical instrument of choice. Unfortunately, the task group found that virtually every analytical method being used for ENM characterization requires that ENMs be extracted from their native environment, or that the environment be digested, destroyed, or critically altered so that the ENM is in a state that can be measured. This introduces 2 issues that can compromise the value of analytical results. First, sample preparation methods are generally not standardized, which makes it challenging to compare results from one laboratory to another with confidence. Second, little is known about how the sample preparation technique impacts acquired data on ENM characteristics, so it is difficult to know whether samples that have been prepared in a certain way offer data that are a realistic representation of ENMs in their native environments. Therefore, the task group highlights a need for a better understanding of how sampling impacts detection outputs and a need for analytical methods or combinations of methods that can provide data on composition and form of ENMs in their native environment (in other words, methods that do not require removing ENMs from their environment or the need to destroy or modify the environment).

Lack of validated model systems

Although nothing can truly replace a study conducted in a real organism, a real food, or a real packaging material, real systems such as these feature complexities and uncertainties that can obscure meaningful structure–function relationships. In each focus area within the scope of this project, the task group identified valuable model systems that can be conducive toward systematic evaluations of how ENM characteristics determine the behavior of these materials in more complex environments. Such model systems include theoretical or mathematical models of ENM migration into foodstuffs from packaging and in vitro models that can mimic the conditions of an intestinal organ without the need to handle and sacrifice live animals. Unfortunately, in many of these cases, the model systems still require validation in order to confirm that they are good predictive models of true ENM behavior, even if the models may have been used successfully for some time to predict the behavior of more conventionally sized materials. Therefore, resources allocated toward the development and validation of model systems that can predict the transformative behavior of ENMs in complex chemical or biological systems should be prioritized.

Expense and availability

Analytical equipment is expensive and many methods considered by the task group were judged to be too low in throughput to be useful for rapid screening of samples, which could account for some of the poor characterization of ENMs in many of the studies currently available in the literature, particularly oral toxicity studies. In addition, many analytical methods or sample preparation procedures, especially with respect to EM tissue-sectioning, require significant labor and expertise to carry out properly. Therefore, methods development targeted toward analytical approaches, even those of a qualitative or semiquantitative nature, that are low cost, rapid, and require less expertise to use than existing methods are likely to have significant impact on the nanocharacterization community.

Recognizing that sensitive analytical methods are always going to be expensive and that food manufacturers and research laboratories may be unable or unwilling to expend the significant capital required to set up an analytical laboratory capable of obtaining necessary ENM characterization data, or do not have the experts needed to run such equipment, the task group has identified the need for a database of available user facilities that may provide general access to analytical equipment needed to perform relevant analyses. As part of its activities, the task group is evaluating the possibility of assembling such a database, and will post details online at the NRFA website if they become available (http://www.nrsa.org/ResearchFoundation/RSA/Pages/ FoodAdditiveMainPage.aspx).

Concluding thoughts

In summary, the 6 areas of need discussed above represent those that stood out most to the task group and were generally conserved over all the topic areas that fell under the scope identified in the 1st section. That said, other issues remain. For example, provided that a common set of methods for ENM detection is eventually established, there will still be a major gap in current research for the validation of measurements within a given method and across methods. What biases are introduced by a particular measurement? What units should be employed for comparison of multiple data sets from multiple laboratories? What ENMs are being used for controlled studies and are they stable or reproducibly made? How relevant are the test materials to those used in food packaging or in food? Standardization of results reporting (for example, what is the best way to report concentration when conventional terms like parts per million or molarity can have ambiguous meaning for nanoparticles?) and standardization of method choice is likely to be required as ENM characterization in food and food contact substances continues. These are difficult problems to solve both now and in the future.

One further point that deserves mention is that even with a perfect analytical tool set, useful data about uptake of ENMs by the
alimentary tract cannot be obtained without a full understanding of what we are looking for. Choosing appropriate systems to study is contingent upon knowing what ENM-enabled foods are likely to come on the market, or are on the market now, as well as what kinds of ENMs will be used, what their properties will be, what concentrations they will have, what foods they will be added to, and so forth. This type of knowledge can only be obtained through good communication between industry groups, government agencies, and academic scientists.

Ultimately, developing methods to support studies on the uptake of ENMs from foods is a challenging but not impossible problem that requires cooperation between many stakeholder groups and experts from across the full scientific spectrum. It is, however, a problem that is in everyone’s best interest to solve, so as to fully ensure the responsible development of nanotechnology applications in foods.

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Author Contributions

Carlander serves as the task group chairperson and coordinator. Duncan serves as the task group chairperson and coordinator, contributed text to the article, and performed general document editing. Szakal contributed text to the article, created Figure 1, and performed general document editing. Tsytsikova coordinated and managed NanoRelease Food Additive project meetings and materials.

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