CHAPTER 22

Advanced Approaches for Efficacy Evaluation and Risk Assessment of Nanomaterials in Food

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Abstract

While nanomaterial applications in the agrifood value chain are rapidly increasing, concerns related to their safety must be carefully considered. For marketing authorization, regulation requires specific or suitable methods to demonstrate safety of nanobased products. The safe-by-design approach extends this concept by considering safety implications as early as possible in the innovation process, to reduce risks and uncertainties while balancing benefits and costs. The European Food Safety Authority supports this approach by providing an operative definition of nanomaterial and dedicated guidelines for risk assessment. From this perspective, the chapter discusses the current approaches for safety evaluation of nanomaterials in food-related applications, ranging from the regulatory framework and its future evolution to the currently available methodological tools for nanomaterial physicochemical characterization and *in vitro* testing.

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22.1   INTRODUCTION

The use of nanomaterials in agrifood value chain is increasing, as reported in the Consumer Products Inventory (Project on Emerging Nanotechnologies 2016) and the French Registry of nanomaterials placed on the market (Ministère de l'Environnement de l'Énergie et de la Mer 2015). In the food and beverage category of the Consumer Products Inventory including cooking, food, storage, and supplements, nanomaterial-containing products currently on the market passed from 64 in 2007 to 72 in 2014 (Vance et al. 2015), and 118 in 2016 (Project on Emerging Nanotechnologies 2016). With 70 commercially available products, food supplements represent the main application of nanomaterials in food and beverage category (Project on Emerging Nanotechnologies 2016). Similar trend emerges from the last French Registry report, which shows a steep increase of

nanomaterial registration in the food and feed category. In 2014, the registration of nanomaterials in the "agriculture, forestry and fishery" sector covered 10,000 of the total 15,000 declarations, with an increase from 3% in 2012 to 70% in 2014 (Ministère de l'Environnement de l'Énergie et de la Mer 2015).

According to a recent survey carried out by European Food Safety Authority (EFSA), 55 different nanomaterials find currently employment in 12 main types of application related to the food value chain (Peters et al. 2014). Based on the chemical composition, nanomaterials can be classified as inorganic, organic, or composite materials (Table 22.1). Inorganic nanomaterials, namely metals and metal(loid) oxides including titanium dioxide, silica, zinc oxide, gold, and silver, cover 55% of the applications, while clays and carbon-based nanomaterials (fullerenes and nanotubes) represent 13% of uses (Peters et al. 2014, 2016). In agriculture, inorganic nanomaterials are used as insecticidal, antimicrobial, or antifungal products, as well as fertilizers (Rossi et al. 2016). In feed, their application as antibacterials, mycotoxin adsorbents, and nutrients is mostly under development. In food and food supplements, inorganic nanomaterials are used as additives (e.g., E 171), while in food contact materials, they are used to increase shelf life, for filtering food during processing, in nanosensors, and in active and intelligent packaging.

Organic nanomaterials currently cover 26% of applications in the food sector, and they are gaining increasing attention (Peters et al. 2016). They include lipid, protein, and polysaccharide-based systems, most commonly micelles, liposomes, and dendrimers. Such organic nanomaterials found major applications in food and food supplements as functional nanoparticles and nanocarriers of nutrients or additives (Kumar and Lal 2014). They are also used as sensors and additives for improving food shelf life, as compound protection from extreme pH, ultraviolet (UV) radiation, and temperature (Pan and Zhong 2016).

Nanocomposite materials account for the remaining 7% of applications and include inorganic

nanostructures whose surface has been modified or functionalized with (in)organic components or polymeric materials with nanosized organic or inorganic fillers. The latter are widely employed in the food-packaging sector to lend antibacterial effects or to improve gas barrier properties and physical performances (Huang, Li, and Zhou 2015; Mihindukulasuriya and Lim 2014).

European regulation requires safety-based authorization before placing products into the market. Specific or suitable methods are therefore required to demonstrate the safety of new nano-based products, also taking into account *in vivo* fate. Safety assessment along the whole life cycle is not required, but it well fits with a responsible research and innovation approach as safe-by-design (SbD). SbD is an approach aimed at including safety concerns as early as possible in the innovation process to reduce risks and uncertainties while balancing benefits and costs. The SbD is covering all potential targets (workers, environment, and consumers) and all life cycle stages of nanomaterials, also including prenormative and normative procedures.

22.2 CURRENT APPROACH TO SAFETY ASSESSMENT: LESSON LEARNED

Concerning the use of nanomaterials in food, the European Union (EU) has one of the most developed frameworks for regulatory safety assessment, as for example, regulations and directives on novel food, food supplements, and food additives. The European food legislation includes provisions both at the European level (e.g., food additives) and at the national level (e.g., botanicals). In both cases, the regulation is based on positive lists of authorized ingredients. The addition of ingredients in the positive list is granted by the European Commission (EC) or by the National Ministry on the basis of the scientific opinion and policy considerations by the respective competent authority.

This legislative framework is also valid for nanomaterials, which are explicitly considered in some

Regulations such as novel food Regulation (European Parliament and of the Council of the European Union 2015) and food additives Regulation (European Parliament and of the Council of the European Union 2008). In food legislation, the safety of nanomaterials generally requires a precautionary approach. For example, the authorization of food additives in nanoforms has to be explicit and expressly requested, since it is not included in the authorization of bulk forms (European Parliament and of the Council of the European Union 2008). In this regard, some potential inorganic nanomaterials such as titanium dioxide, silica, gold, silver, and iron oxides are currently under reevaluation as food additives in Europe (EC 2010). In the revised European Novel Food Regulation (European Parliament and of the Council of the European Union 2015), nanoforms are always considered novel foods and, thus, subjected to a new authorization to be placed on the market. Novel nano-based formulations and nanoforms used in food supplements fall within the novel food Regulation provisions. Therefore, they will need to be authorized at the EU level before using them at the national level.

The application of the European regulatory framework on nanomaterials is based on a regulatory definition of *nanomaterial*. The EU is the only political entity having a regulatory definition of nanomaterial based on the number of primary particles (also in aggregates and agglomerates) having size from 1 to 100 nm (EC 2011a). Materials with more than 50% of primary particles within this dimensional range are considered nanomaterials, and the ongoing revision of the recommendation will keep this approach. Therefore, the recommendation implementation through the identification of appropriate size distribution assessment methods is one of the main topics in the current regulatory research (Barahona et al. 2016).

Once a food ingredient is classified as nanomaterial, its safety has to be specifically addressed following EFSA risk assessment guidance (EFSA Scientific Committee 2011), currently under revision (Statute deadline: July 31, 2018). This is an exposure-based guidance, and risks are only

considered if ingredients maintain their nanomaterial nature during ingestion. Therefore, the physicochemical characterization of nanoingredients at different stages (e.g., food, stomach, and intestine) is crucial. Based on the exposure level and available hazard knowledge, the guideline defines an incremental testing strategy going from *in vitro* toxicity to specific *in vivo* testing. Recommended additional toxicity tests include *in vitro* mutagenicity and *in vivo* repeated exposure absorption, distribution, metabolism, and excretion (ADME) and genotoxicity.

To understand how the regulatory bodies apply regulations and directives, it is important to consider the EFSA scientific opinions on nanomaterial safety. Since there is no opinion on new additives in nanoform yet, additive reevaluation opinions were considered. In particular, food additives in powder or particulate form such as silver (E174), gold (E175), iron oxides and hydroxides (E172), and titanium dioxide (E171) were recognized by EFSA as possibly including a nanofraction. The reevaluation highlights the need to assess additive safety, although the nanofraction is below the 50% threshold. Since nanoscale may affect material behavior and hazard, unknown particle size distribution together with lack of data on specific nanofraction toxicity hampers the safety evaluation of food additives. This is the case of silver, in which the Panel concluded that the relevance of the available toxicological studies to the safety evaluation of silver as a food additive E174 could not be established (EFSA Scientific Committee 2016). The panel also recommended that E174 characterization should include particle size and particle size distribution, as well as the frequency of particles having at least one dimension below 100 nm. Additional data on E174 safety would also be required in compliance with EFSA guidance document. On the contrary, available data on titanium dioxide (E171) were sufficient to establish that the nanofraction does not pose any additional or specific toxicity since ADME and effects are comparable to the nonnanofraction. Therefore, the characterization of the additives in batch as well as in food and testing environments becomes extremely relevant to further evaluate additive safety in the presence

of a nanofraction. The size distribution determination of pristine food additive should be carefully evaluated with two independent methods (EFSA Scientific Committee 2011), such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM). According to the EFSA risk assessment guideline, to evaluate the real exposure to nanomaterials, it is very important to detect them in complex matrices, such as food, digestion simulants, and cell growth media used in *in vitro* toxicity testing (Bouwmeester et al. 2014). The fate of both inorganic and organic nanoparticles during digestion and absorption is indeed essential to define risks associated to the use of nanomaterials in food (Esfanjani and Jafari 2016). While some techniques for detecting inorganic nanomaterials in complex matrices exist, the detection of organic nanoparticles mainly composed of polymers, lipids, proteins, and polysaccharides is still challenging (Pan and Zhong 2016).

Regarding hazard assessment, the current regulatory approach is based on standard *in vivo* studies (Bouwmeester et al. 2014). Due to the large number of nanoformulations, it is neither ethically nor economically feasible to work only on animal studies. As proposed in the EFSA guideline, tiered approaches incorporating *in silico* and *in vitro* studies are necessary (EFSA Scientific Committee 2011). Within tiered approaches, *in vitro* digestion models play a significant role to assess both nano-formulation efficacy and safety.

Nanomaterials in food are derived from an intentional use of nanoingredients and an unintentional transfer of nanomaterials in food along the value chain (e.g., nanopesticides and nanopackaging). To this regard, there are regulations in place to assure the health safety of nanomaterials used in packaging (EC 2011b) and in pesticides (European Parliament and of the Council of the European Union 2005) on maximum residue levels. Some aspects are not satisfactorily tackled, such as the applications of sensors in the value chain (Bülbül, Hayat, and Andreescu 2015), which are not effectively covered by any regulation (e.g., Registration, Evaluation, Authorisation and Restriction of Chemicals, REACH [European Parliament and of the Council of the European Union 2006).

Food packaging and food-contact materials are regulated within the food legislation (EC 2004), which sets general rules concerning safety, labeling, and traceability. Specific regulations contain measures for specific food contact materials, such as plastics (EC 2011b) and active and intelligent packaging (EC 2009). Both regulations foresee only the use of authorized nanomaterials, as for silica and carbon black (all uses) and titanium dioxide (only specific uses) indicated in Annex I of plastics Regulation. After positive opinions by EFSA, the EC amended the Annex including other nanomaterials such as kaolin, nanocopolymer, and titanium nitride (EFSA Scientific Committee 2007, 2012, 2015). To be authorized for integration into food-contact materials, any other substance in nanoform requires submitting an application with specific data on exposure and toxicology. In the United States, food contact materials are regulated by the Food and Drug Administration (FDA) through the process of Food Contact Notification (FCN) (US Department of Health and Human Services, FDA, and CSFAN 2007). While the guidelines for FCN do not make specific reference to nanomaterials, the chemistry guidance contemplates a dependence of substance properties by the size and recommends specific physicochemical data. Beside these indications, the safety assessment for nanomaterials by FDA is addressed on a case-by-case basis.

22.3 PHYSICOCHEMICAL CHARACTERIZATION OF NANOMATERIALS IN FOOD APPLICATIONS

As previously mentioned, the EFSA risk assessment guidance is an exposure-based document raising the importance of knowing physicochemical properties of pristine nanomaterials as well as their matrix-dependent physical and chemical transformations. This requires analytical methodologies able of identifying, characterizing, and quantifying specific nanomaterials in a wide variety of feed and food matrices, from raw materials to commercially available products. On the

other hand, the high composition variability of matrices makes investing in the development of standardized protocols for sample selection and preparation essential to maximize reproducibility and extendibility of results.

22.3.1 Priority Properties for Characterization of Nanomaterials in the Food Chain

The behavior of nanomaterials in biological matrices depends on their physicochemical properties such as size and size distribution, agglomeration/aggregation state, concentration and total amount, chemical composition (bulk and surface), shape, crystalline structure, charge, and surface area (Grieger, Harrington, and Mortensen 2016). Since these properties are interdependent and influenced by the surrounding matrix composition, they should be taken into consideration when assessing nanomaterial safety. An exhaustive characterization is often operatively complex and expensive; therefore, a priority ranking is reasonable to face the issue in a decision-making framework (Grieger, Harrington, and Mortensen 2016). As reported in Section 22.2, nanomaterials are defined on the basis of dimensional criteria (EC 2011a). Estimating the size distribution of materials allows to classify them as nanomaterials from a regulatory point of view and to assess risks according to a dedicated procedure (EFSA Scientific Committee 2011). Agglomerates and aggregates composed of nanometric primary particles also represent an important challenge in classifying materials as nanomaterials or nanostructures. Surface area is another useful parameter to identify some of them, especially nanostructures and porous materials. Shape is an important property to be considered for selecting the most appropriate methodology for size distribution determination and nanomaterial classification. Most techniques, such as dynamic light scattering, indeed provides hydrodynamic dimensions, useful for spherical particles but unreliable for anisotropic objects. Once a nanomaterial has been identified, its concentration and chemical composition are fundamental properties for further toxicological testing or product/process optimization. Measuring nanomaterials within food matrices as well as in their pristine form permits

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the identification of both intentionally and unintentionally added nanomaterials, and it makes possible to monitor their transformations at different stages of food processing. The other aforementioned properties are less important from a regulatory point of view.

A wide range of analytical techniques is potentially suitable for determining one or multiple properties of nanomaterials in foods and food-related matrices. Figure 22.1 schematically represents the possible combinations and corresponding performances of currently available analytical techniques.

22.3.2 Sample Preparation

Although techniques are available to detect or characterize nanomaterials directly in solid samples, a wider range of methodologies and improved analytical performances are typical for liquids.

Nanocomposites and raw nanomaterials, such as nanostructured materials and powder nanoparticles, can be conveniently analyzed in solid form, while homogenization is generally the first step for complex soft solid and semisolid specimens such as foods. Homogenization ranges from manual mixing or agitation to heating or sonication (Mattarozzi et al. 2016).

Nanomaterial extraction is commonly the second step of analysis. Transferring of nanomaterials into a liquid phase is often advantageous for food matrices to minimize interferences, access additional methodologies, and/or improve the analysis. The liquid phase (medium) and conditions are therefore determined by analytical rationales. Water extraction, possibly combined with sonication, is the simplest strategy but potentially subject to low recovery (Loeschner et al. 2013). To extract inorganic nanomaterials, chemical (acid or alkaline) digestion can be used, paying attention to preserve the nanomaterial of interest (Mattarozzi et al. 2016). The enzymatic digestion of the matrix is a soft and general approach to extract nanomaterials from foods. For organic nanomaterials such as micelle-like structures, sample preparation is quite limited due to their fragile nature.

For food-contact materials, the evaluation of nanomaterial release into food is a major issue. This concerns both (a) migrating systems, where nanomaterials or their transformation products (e.g., ions) are intentionally released to obtain a specific action (e.g., antimicrobial), and (b) nonmigrating systems, where immobilized nanomaterials or their derivative can be released accidentally. Four main types of release mechanisms can characterize the life cycle of food-contact nanocomposites: desorption, diffusion, dissolution, and matrix degradation. These phenomena are determined by the location of the nanocomponent into/onto the material, its ability to migrate through it or to detach from the surface, and the physicochemical interactions with the medium (Noonan et al. 2014). Migration into food resembles an extraction process, although medium characteristics and experimental setup are defined by the existing legislation to mimic real usage conditions (Wyser et al. 2016). Although real foods can be used in migration testing, food simulants are usually preferred to simplify and standardize the experimental approach. Typical media (see Table 22.1) can include water, dilute acetic acid (acidic foods), olive oil or isooctane (fatty foods), and various concentrations of ethanol in water (Noonan et al. 2014). For hydrophobic media, subsequent liquid–liquid extraction to an aqueous phase may be required to allow compatibility with the detection technique.

After extraction or migration, an additional step of purification and/or preconcentration is often required, including off-line centrifugation, ultracentrifugation, membrane filtration, or selective precipitation (Kowalczyk, Lagzi, and Grzybowski 2011). A final stabilization step is also suggested to avoid dissolution or aggregation phenomena. Stabilization can be achieved by adjusting the pH and ionic strength to the original conditions or by adding detergent-containing solutions to form stable complexes and/or micelles (Mattarozzi et al. 2016). Surfactants may also be added to the extraction medium for improving extraction efficiency and stabilizing nanomaterials.

Due to the high reactivity, nanomaterials may interact with the surrounding matrices and sample

preparation conditions, changing their physicochemical properties and potentially leading to artifacts (von der Kammer et al. 2012). Reducing the number and duration of preparation steps, as well as storage time before analysis, helps in preserving the native state of nanomaterials in food. The careful optimization of sample processing is then recommended, establishing a number of quality check criteria to assess particle size stability and recovery efficiency during the main steps (Mattarozzi et al. 2016).

22.3.3 Analytical Techniques

22.3.3.1 *Size and Size Distribution*

For nanomaterials and nanoparticles, size definition has multiple meanings depending on the analytical technique used for determining it (Linsinger, Roebben, and Gilliland 2012). In light of the EC definition, nanomaterial size distribution must be expressed as the number of particles. Depending on analytical method, size can also be expressed as mass or signal intensity, suggesting that size data should report the specification of adopted methodology since conversion between physical quantities and measurement units is possible but often insidious (Contado 2015).

The analytical techniques for sizing nanomaterials can be classified in three main groups: (a) counting; (b) ensemble; and (c) separation techniques. In counting and ensemble methods, the nanoobject size distribution is determined directly and selectively by measuring the particles individually/sequentially (counting) or simultaneously (ensemble). Separation techniques provide a size-based fractionation of nanomaterials, with size measurement achieved by either off-line combination with- or online coupling to- a selective or unselective detector.

With the exception of electron microscopy and X-ray diffraction (XRD) techniques, the methods for size determination are unable to distinguish aggregated/agglomerated particles from individual objects of the same size. They can be identified only knowing primary particle size or by comparing samples before and after agglomerate dissociation into primary particles (e.g., by sonication)

(Linsinger, Roebben, and Gilliland 2012).

22.3.3.1.1 Counting Techniques

SEM and TEM are the most used and recommended techniques for determining the size and size distribution of nanomaterials (Grieger, Harrington, and Mortensen 2016; Linsinger, Roebben, and Gilliland 2012). The high accuracy of these techniques makes them the reference for regulatory testing of nanomaterials (Blasco and Picó 2011). Individual particles are visualized at high magnification (resolution of ~3 and ~0.07 nm for SEM and TEM, respectively) and characterized for morphology and geometrical size, agglomeration/aggregation state, and location in solid matrices (Dudkiewicz et al. 2015a). In focused ion beam (FIB) -SEM, an ion beam can be used for direct sectioning of a solid specimen and performing three-dimensional (3D) imaging with a resolution of ~10 nm. By using a conventional setup, observations are performed under high vacuum upon sample fixation, dehydration, or drying with potential formation of artifacts. Alternatively, hydrated samples can be encapsulated within thin electron-transparent membranes under relative vapor pressure and analyzed by environmental SEM or TEM systems with minimal loss in resolution, as well as in cryogenic conditions (cryo-SEM/TEM) using a specifically designed stage (Dudkiewicz et al. 2015a).

Due to the high magnification required for visualization, only low-volume samples can be analyzed, particularly for TEM (~3 µL for liquids, <100 nm ultrathin sections for solids), raising some concerns on sample representativeness (Blasco and Picó 2011). The low electron density of organic nanomaterials, similar to the matrix, represents a challenge for their detection in foods.

Atomic force microscopy (AFM) is another imaging technique (3D) used to determine the size, morphology, and agglomeration/aggregation state, with lateral resolution of ~1 nm and vertical resolution down to ~0.1 nm (Tiede et al. 2008; Yang et al. 2007). AFM images are obtained by using a laser beam to measure changes in the magnitude of the interaction (commonly van der

Waal's forces) between the probe and sample surface, in response to its topography during a scan. This technique allows direct observation of hydrated liquid and solid specimens without sample pretreatment, but with limitations similar to the other microscopy techniques for the detection of nanomaterials in complex matrices.

Single particle (sp) inductively coupled plasma–mass spectrometry (ICP-MS) is a completely different approach to estimate the size of inorganic nanoparticles in liquid suspensions. By introduction into a 8000 K plasma, atoms in the sample are excited up to generate ions with element-specific mass-to-charge ratio for spectrometric detection in mass spectrometry (MS). Differently by conventional ICP-MS, in sp mode, extremely diluted suspensions (10^8 L^{-1} or lower) are analyzed with very high data acquisition frequencies (10^2–10^5 Hz) in order to detect nanoparticles as individual events sticking out over a continuous background signal generated by dissolved species (Laborda et al. 2016). The size distribution of particles can be calculated based on the quantitative determination of their elemental mass content, assuming that shape, composition, and density are known (Montaño et al. 2016). Information about the number and mass concentration of nanoparticles can also be achieved in the same analytical run. spICP-MS is a useful tool for tracking nanoparticle dissolution since it allows the detection of both nanoparticles and dissolved forms of the nanoparticle constitutive element simultaneously (Linsinger, Peters, and Weigel 2014). Number concentration detection limits in spICP-MS can reach 1000 particles per milliliter (Dudkiewicz et al. 2015b), whereas size detection limits range from 10–20 nm for monoelemental nanoparticles to 80–400 nm for oxides (Lee et al. 2014).

Nanoparticle tracking analysis (NTA) uses laser light scattering microscopy to calculate the hydrodynamic diameter of individual nanoparticles in liquid suspension by tracking their Brownian motion (Filipe, Hawe, and Jiskoot 2010). This technique provides good resolution for sizing monodisperse and polydisperse nanoparticles in the range of 30–1000 nm, but with lower accuracy

compared to the other counting techniques.

22.3.3.1.2 Ensemble Techniques

Dynamic light scattering (DLS), also named photocorrelation spectroscopy (PCS) or quasi-elastic light scattering (QELS), determines the hydrodynamic diameter of liquid-suspended particles (both inorganic and organic) measuring the time-dependent fluctuations in scattering intensity of a laser light beam due to their Brownian motion (Luykx et al. 2008). The technique provides size distribution in terms of both signal intensity and number over a wide dimensional range. However, it has some limitations for polydisperse systems and complex matrices because large particles or agglomerates mask and/or hide smaller ones (Grombe et al. 2014). Since DLS is a cheap, fast, and easy to use technique, it is often employed as a screening method to provide preliminary indications on the presence of nanoparticles.

Multiangle laser light scattering (MALLS) and small-angle X-ray scattering (SAXS) are affine techniques measuring the intensity and angle of radiation scattered by suspended nanoparticles to extract information on their size (as gyration radius) and shape (Li, Senesi, and Lee 2016; Wagner et al. 2015). These techniques provide good accuracy, precision, and reproducibility for relatively monodisperse samples, carrying out fast and simply to perform analyses.

XRD is a widespread tool used to characterize crystalline nanomaterials. Diffraction pattern originated from the interaction of specimens with the incoming beam is elaborated to extract the orientation of particles according to the Bragg equation and size according to the Sherrer equation. Due to its low sensitivity, highly concentrated nanoparticles can be characterized (>1–3% by weight) (Tiede et al. 2008); thus, the method is more suited for raw materials and products (e.g., food-contact materials), rather than real food specimens.

22.3.3.1.3 Separation Techniques

Size-exclusion chromatography (SEC) and hydrodynamic chromatography (HDC) are liquid chromatographic techniques useful to fractionate suspended nanoparticles. Both separate nanoparticles according to their hydrodynamic diameter based on a physical retention mechanism, assuming no chemical interactions with a stationary phase. In SEC, the column is packed with rigid particles having controlled porosity. Smaller nanoparticles traveling through the column can penetrate the pores deeper than bigger nanoparticles, resulting in increased retention times. HDC columns are packed with inert rigid nonporous particles (e.g., glass spheres) or consist of open/packed capillaries, conceived to maximize the flow rate gradient. Smaller nanoparticles have the ability to travel statistically closer to the surface of packing particles or capillary, resulting in higher retention time. SEC and HDC are robust and potentially rapid (separations might be achieved in less than 10 min) separation approaches, and have high compatibility with dispersion/extraction solvents (Rakcheev, Philippe, and Schaumann 2013; Striegel 2012; Robertson et al. 2016). These techniques allow the effective separation of nanoparticles larger than 20 nm of size (to 1 µm) with relatively low resolving power. They are mostly applied as a step of preliminary partial fractionation.

Field-flow fractionation (FFF) is a family of techniques for the separation of liquid-suspended nanoparticles based on the same physical principle of HDC (transport into a laminar flow). It combines a simultaneous size/density-dependent process of the diffusion of particles with an additional perpendicular field force (Schimpf, Caldwell, and Giddings 2000). The configuration of separation chambers and the type of additional field defines individual techniques belonging to this family. In asymmetric flow FFF (AF4), separation occurs over a semipermeable membrane in a thin flat chamber applying a unidirectional cross-flow, while in hollow fiber flow FFF, the membrane encloses a cylindrical chamber and the cross-flow is radial. In thermal FFF, the separating force is

generated by a thermal gradient between the upper and lower facets of a flat chamber, while in centrifugal or sedimentation FFF, separation is operated by centrifugal force in a rotating flat circular chamber. Overall, FFF methods are reliable for high-resolution separation of particles with sizes ranging from a few nanometers to several micrometers in 20–60 min.

SEC, HDC, and FFF techniques fractionate nanoparticles in liquid suspensions according to their size or size/density, but the actual measurement of such property requires a combination with or coupling to an appropriate external detector. To this purpose, a wide variety of detectors is available. The presence and concentration of nanoparticles in a given fraction can be traced by using unselective detectors (e.g., measuring elemental mass or light absorption/emission), while the corresponding size is obtained by calibrating the separation system (elution time) with external standards. Additional information concerning concentration and/or chemical composition can be obtained combining unselective detectors, as is addressed in Section 22.3.3.2. As an alternative approach, SEC, HDC, and FFF can be combined/coupled to selective detectors, which measure the size of particles in their own right, improving analytical performances and ensuring that the suspension passing through the detector contains almost monodispersed particles rather than complex polydisperse mixtures.

Centrifugal liquid sedimentation (CLS), also known as differential centrifugal sedimentation (DSC), is a technique in which suspended nanoparticles are injected into a hollow, optically clear, rotating disk, containing a liquid. Their separation occurs as function of corresponding size/density-dependent stabilization condition in a slight density gradient formed within the liquid, while detection is achieved by measuring the decrease in transmission of a light beam when they approach the outside edge of the disk. CLS is effective for particles in the 10 nm–50 µm range, possibly extended from a few nanometers to 120 µm or more depending on the density of the particles. The time for analysis spans from 3 min up to hours, depending on the sample characteristics. CLS has a

very high sensitivity and provides highly reproducible results with extremely high size resolution power (Cascio et al. 2014). Differently from SEC, HDC, and FFF, it does not allow coupling to other detectors or fraction collection for further off-line characterization.

22.3.3.2 *Concentration and Chemical Composition*

Except electron microscopy, all the other analytical techniques suitable to determine the size distribution also provide some quantitative or at least semiquantitative (relative) information on nanomaterial concentration. Most detectors are also selective for specific chemical components (inorganic or organic) of nanomaterials; thus, data on chemical composition can be extracted.

Among the detectors combinable with separation techniques, ICP-optical emission spectrometry (OES) and ICP-MS (used in both conventional and sp modes) offer high sample throughput, multielemental analysis, wide linear dynamic range (10^6 or more), high selectivity and accuracy, and detection limits between 0.01–10 parts per billion (ppb) and 0.0001–1 ppb, respectively. With the typical setup, ICP-OES/MS can analyze liquid samples, such as those processed by separation techniques, but direct analysis of solids is also possible using dedicated sample introduction systems, namely, electrothermal vaporization (ETV) and laser ablation (LA) and with a potentially minimum sample preparation. LA-ICP-MS and particle-induced X-ray emission (PIXE) can also be used for quantitative elemental imaging of solids. All these instruments perform element-specific, possibly multielemental, measurements on a spectral base, providing direct data on the chemical elemental composition of the specimen. They do not distinguish the chemical form (species) of elements (e.g., nanoparticulate or nonnano), and they are not selective for nanomaterials with respect to the matrix. Therefore, the efficiency of sample preparation and the coupled/combined fractionation could be critical to guarantee reliable results. This problem does not occur when coupling an energy-dispersive X-ray spectroscopic detector to TEM and SEM instruments. It takes advantage of the high spatial resolution proper of electron microscopy, to perform semiquantitative

elemental analysis of the surface of individual nanometric objects, even embedded into a complex matrices. Detectors based on differential refractometry (DRI) or UV-visible absorption spectroscopy (working as mass-turbidity detector) can also be coupled/combined to separation techniques to achieve quantitative data, with higher detection limits compared to the preceding methods, but the advantage of being applicable to both inorganic and organic nanomaterials.

Identifying the constituents of organic nanomaterials is a major analytical challenge, due to the difficulty of discriminating their characteristic components from those of the matrix, themselves organic. For this reason, even if a great variety of analytical techniques is theoretically available for organic chemical characterization, such as matrix-assisted laser desorption (MALDI) and liquid chromatography coupled to MS, often their application cannot disregard complex sample preparation steps (extraction, purification), resulting in the high potential of alteration of the analytes and artifacts formation.

Light scattering techniques (DLS and MALLS at 0° angle) also provide semiquantitative information derived from the intensity of scattering, although it is mostly limited to monodisperse particles.

22.3.3.3 Surface Area

The Brunauer, Emmett, and Teller (BET) method is the standard technique for determining the surface area of nanomaterials (Linsinger, Roebben, and Gilliland 2012). The method is applicable only to dry solid materials (nanopowders and nanostructured materials) and bases on measuring the amount of gas (typically nitrogen) adsorbed on the surface at controlled temperature and pressure. Absorbed gas is proportional to the absolute area of the material and can be used to calculate the volume-specific surface area. BET does not provide information on the aggregation state, and it is unselective with respect to the matrix. It is suitable to characterize raw nanomaterials or finished products such as food-contact materials.

22.3.4 Analytical Approaches

Food analysis may be targeted to the detection, identification, characterization of nanomaterials, or any combination of them, depending on application and regulatory requirements. An intelligent testing strategy should guarantee the highest analytical quality of data according to the priority ranking of properties, minimizing steps and costs and allowing the analysis of complex and heterogeneous matrices (Tiede et al. 2008). An example of such tiered approach is proposed in Figure 22.2.

Detecting nanomaterials in food matrices requires sample preparation optimized for specimen typology (i.e., solid, semisolid, or liquid). Optimal sample preparation should preserve nanomaterials in their original state. Separation from matrix minimizing artifact generation can be achieved by balancing the extraction conditions with a possible purification step and the choice/optimization of the analytical technique(s). For detection and identification purposes, relatively low recovery may be accepted as long as procedures are reproducible and unselective with respect to the size of the particles. Since a preconcentration step may significantly affect nanomaterial dispersion state, it should be only introduced once it is verified that nanomaterial amount is not sufficient for testing.

An effective analytical approach could consist of a preliminary screening by DLS followed by a coupled separation technique as the main method. Among the FFF methods, AF4 is one of the most effective methods for the entire size range of interest (1–100 nm), and it can be coupled online to multiple detectors (Figure 22.2). Coupled methods allow to obtain many information within a single run of analysis, such as nanomaterial detection, size distribution, chemical characterization, and quantification. They can be complemented or alternatively replaced by a wide variety of combinations among techniques based on the principle of separation, fractions collection (possible preconcentration), and subsequent off-line detection and characterization.

22.4 HAZARD IDENTIFICATION

The procedure for assessing risks posed by nanomaterials in food applications is described by EFSA in its *Guidance Risk Assessment of NMs in Food and Feed Chain, including Food Additives, Enzymes, Flavourings, Food Contact Materials, Novel Foods, Feed Additives and Pesticides* (EFSA Scientific Committee 2011). The guidance is under revision to take into consideration both the novel scientific developments, including methods for detection and characterization in food, and the novel food regulation, which is going to enter into force at the beginning of 2018.

In vitro and *in vivo* testing strategy devised by EFSA is exposure based, with six general cases organized in tiers, with an increasing demand of data. Exposure assessment must be considered for food-contact materials where nanomaterials could migrate into food. In the first three cases, there is the complete degradation of nanoingredients to nonnanoform materials, or no migration into food occurs. In these cases, risk assessment can be conducted according to the conventional EFSA procedure. The remaining cases (4–6) concern the exposure of consumers to nanomaterials through food. When nanomaterials are persistent in gastrointestinal fluids, EFSA guidelines suggest a list of additional relevant tests to be performed, for comparing data with those obtained on the nonnanoform, when available or pertinent. In the worst exposure case, the main endpoints requested in the guidelines include *in vitro* tests (e.g., digestion, genotoxicity, cytotoxicity, oxidative stress, potential for inflammation and immunotoxicity, barrier integrity/permeability) and *in vivo* studies (e.g., ADME, oral toxicity, reproductive, and developmental toxicity).

Risk assessment has to consider nanomaterial digestive process, which is often very aggressive, and it causes significant physicochemical transformations such as degradation, dissolution, and solubilization (Noack et al. 2012; Peters et al. 2012; Walczak et al. 2012, 2015; Böhmert et al.

2014; Yi et al. 2014;).

Several *in vitro* models are being developed to simulate human digestion processes and intestinal epithelia to evaluate hazard posed by nanomaterials in food. *In vitro* models have several advantages: reduction of costs and time, control of experimental conditions leading to good reproducibility and repeatability, and the possibility to measure kinetics. On the other hand, they often lack complexity, and it is difficult to link *in vitro* results with *in vivo* outcomes. Growing datasets obtained in research projects and focused on the *in vitro* test validation, as well as the increasing use of the adverse outcome pathways concept (Gerloff et al. 2017), may support a more robust validation in the future. Concerning *in vitro* digestive models, they can simulate different physiological conditions by modifying temperature, peristalsis, digestive fluids, and transition time. They are applicable to study the behavior of nanocarriers during digestion and for collecting data on the release of active ingredients. The definition of the model parameters is often supported by standards: for example, the US Pharmacopeia 33-28NF (US Pharmacopeial Convention 2010) and European Pharmacopeia 7.0 (Council of Europe 2010) provide the chemical composition of gastrointestinal fluids in different physiological conditions. *In vitro* intestinal epithelia using 3D cell cocultures in place of single cell cultures is a way to have a model closer to real conditions.

While EFSA recommends the use of *in vitro* methods, to date, there are no validated approaches (Park et al. 2009). In particular, several issues must be addressed before *in vitro* testing, as interferences of nanomaterials with *in vitro* assays leading to negative/positive false. Interferences may happen through three main mechanisms: optical activity (e.g., autofluorescence), adsorption of assay or culture media components, and the reactivity with test molecules (Bregoli et al. 2013). Taking into account these factors, it is possible to adapt existing *in vitro* assays to nanomaterials (e.g., US Nanotechnology Characterization Laboratory) for performing a screening phase and providing information on nanomaterial hazard compared to the nonnanoform. *In vitro* assays are

also useful to disclose nanomaterial mode of action. If *in vitro* results highlight changes in cell viability, epithelial permeability, release of inflammatory mediators, and immune response, *in vivo* studies should be considered.

Nanomaterial internalization is an important issue for risk assessment, so the evaluation of gastrointestinal barrier permeability/integrity is very important. To this aim, several *in vitro* models can be used, such as the differentiated human colorectal adenocarcinoma (Caco-2) cells cultured on semipermeable support (James Kirkpatrick et al. 2007; Kandárová and Letašiová 2011). Caco-2 models are considered the gold standard to assess oral drug adsorption (Hidalgo, Raub, and Borchardt 1989), and both FDA and EFSA are recommending this model, also for the evaluation of nanomaterials safety in food (Löbenberg and Amidon 2000; Committee for Proprietary Medicinal Products 2001; Yu et al. 2002; EFSA Scientific Committee 2011). This system allows the measurement of barrier integrity/permeability through different endpoints such as cell viability (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT, assay), membrane damage (lactate dehydrogenase, LDH, assay), transepithelial electrical resistance (TEER), paracellular flux, inflammatory mediators, and radical species generation. Caco-2 cell culture can be improved by adding goblet cells (i.e., mucus secretion) and microfold (M) cells to measure immunological responses. Mucus is important for the nanomaterial uptake, while M cells are the main uptake gateway for nanomaterials in the gut. In addition to safety assessment, this model can be used to evaluate the efficacy of nanoformulations through the investigation of apical-to-basolateral translocation and the identification of absorption and secretion molecular mechanisms such as paracellular, transcellular, and persorption uptake and P-glycoprotein-mediated efflux. This feature is important because nanotechnology (e.g., phytosomes) is often used to improve stability and bioavailability of active ingredients.

While immune response can be assessed by using cocultured Caco-2-based model, also the whole

blood assay can be applied to get immunostimulation/immunosuppression responses upon exposure to nanomaterials (Langezaal, Coecke, and Hartung 2001; Langezaal et al. 2002). A number of *in vitro* tests with good or acceptable predictivity have been recently developed to assess the immunotoxic effects of nanomaterials (Dobrovolskaia and McNeil 2016), and some of them have been standardized by the American Society for Testing and Materials International (ASTM 2013a, 2013b). Immunostimulation is usually studied in whole blood, peripheral blood mononuclear cells, and some human-derived cell lines with enzyme-linked immunosorbent assay (ELISA) for detecting both pro- and anti-inflammatory cytokines, by using cell-based monocyte-derived dendritic cell maturation assay for proinflammatory cytokines and analyzing differentially expressed genes by microarray (Stone, Johnston, and Schins 2009). Since nanomaterials have a strong potential of crossing biological barriers, hemolysis (Dobrovolskaia and McNeil 2013), complement activation (Neun and Dobrovolskaia 2011), and thrombogenicity testing (Myerson et al. 2011) are also highly recommended. Conversely, immunosuppressive effects (Bregoli et al. 2009), such as myelosuppression and cytotoxicity of immune cells, have been demonstrated for nanomaterials. They can be traced by assessing the suppression of colonies formation in both erythroid– and granulocytic–monocytic precursors in primary cultures of human hematopoietic progenitors cell (Ciappellano et al. 2016).

Genotoxicity is another important endpoint for nanomaterial hazard identification. Two *in vitro* assays—Organisation for Economic Co-operation and Development (OECD) test guideline 476 (*in vitro* mammalian cell gene mutation test, preferably the mouse lymphoma tk assay with colony sizing) and OECD test guideline 487 (*in vitro* mammalian cell micronucleus test) (OECD 2014a)— are recommended. Three *in vivo* follow-up tests are recommended for nanomaterial genotoxicity: (a) mammalian erythrocyte micronucleus test (OECD test guideline 474) (OECD 2014b), (b) *in vivo* Comet assay, and (c) transgenic rodent gene mutation assay.

If nanomaterials are able to cross the intestinal barrier and enter into the blood circulation, it is important to study the ADME. Nanomaterials may possess different toxicokinetics and tissue distribution compared to the nonnanoforms. OECD test guideline 417 (OECD 2010) is generally used to study the toxicokinetics of nanomaterials, by using at least two low toxic doses identified with a pilot study. The persistence of nanomaterials in the body may correlate with toxic chronic effects, while prolonged localization in mononuclear phagocytes systems (e.g., liver, spleen, lymph nodes) may have implications for the immune system.

In vivo oral toxicity can be assessed by adding nanomaterials to feed or drinking water or by gavage. Among relevant limitations of the three methods, it is important to stress that gavage is not the best solution: while allowing a better experimental conditions control, it allows nanomaterials to skip the first part of the digestive process.

There are several *in vivo* test guidelines that can be used to study acute and chronic toxicity. The basis for the determination of benchmark dose lower confident limit (BMDL) or a no-observed-adverse-effect level (NOAEL) *in vivo* is the 90-day study in rodents with repeated dose (OECD test guideline 408 [OECD 1998]), modified to incorporate endocrine-related endpoints (OECD 2008). The OECD test guideline 453 (OECD 2009) can be applied to evaluate the long-term toxicity (12 or 24 months of exposure).

Finally, studies on the reproduction and development effects are described in OECD test guidelines 414, 415, and 416 (OECD 1983, 2001a, 2001b).

The increased bioavailability of active ingredients due to nanotechnology-based delivery systems also requires a toxicological evaluation. Therefore, the assessment should include the analysis of the entire system both with and without active ingredient, as well as the active ingredient in free form.

22.5 CONCLUDING REMARKS

Nanotechnology is used in the food industry to improve ingredient shelf life and properties such as taste, color, texture, as well as absorption and bioavailability of nutrients and active ingredients. Common sense and legislation requires the safe use of new ingredients and formulations (i.e., novel food) before usage. Data to be included in the safety dossier for being evaluated by competent authorities are generally defined in regulations and related guidelines. In the EU, the Novel Food Regulation, entering into force on January 1, 2018, considers engineered nanomaterials as novel foods and are thus subjected to a European authorization process. Safety assessment is carried out by using specific EFSA risk assessment guidelines, which are currently under revision to address technical developments and environmental aspects. A main aspect of the regulatory implementation is an operative definition of nanomaterial, which, in Europe, was proposed by the EC. The current process of EC recommendation revision will lead to a harmonization of nanomaterial definition in different legislations, simplifying the safety assessment process. The physicochemical characterization of nanomaterials is at the basis of their regulatory identification. While characterizing raw materials is simple, investigations of nanomaterials in complex matrices is quite difficult since it requires (a) sample preparation procedures, (b) methods for size distribution measurement in complex matrices, and (c) methods to detect and characterize inorganic and organic nanoparticles. Physicochemical characterization is essential for both exposure and hazard assessment.

EFSA hazard testing strategy is based on exposure: the higher the likelihood of nanoforms uptake, the more specific testing is needed. A combination of *in vitro* and *in vivo* testing is recommended at each stage. *In vitro* testings are useful to model human digestive process and assess at the same time

the stability (as nanoforms), efficacy (as bioaccessibility and bioavailability), and safety (as cell viability, inflammatory processes, immunotoxicity, genotoxicity) of nanoforms. The process of validation of *in vitro* studies is currently ongoing in several research projects, and it could lead to prevalidated methods in the next future. *In vitro* tests provide a screening stage, to identify relevant hazardous properties to be further investigated in *in vivo* studies. Validated *in vitro* methods are necessary to reduce the number of costly *in vivo* investigations, so increasing the pace of sustainable innovation in many areas such as food industry.

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Tables

Table 22.1 Main Types of Nanomaterials Employed in the Agri/Feed/Food Sector and Related Matrices of Analytical Interest

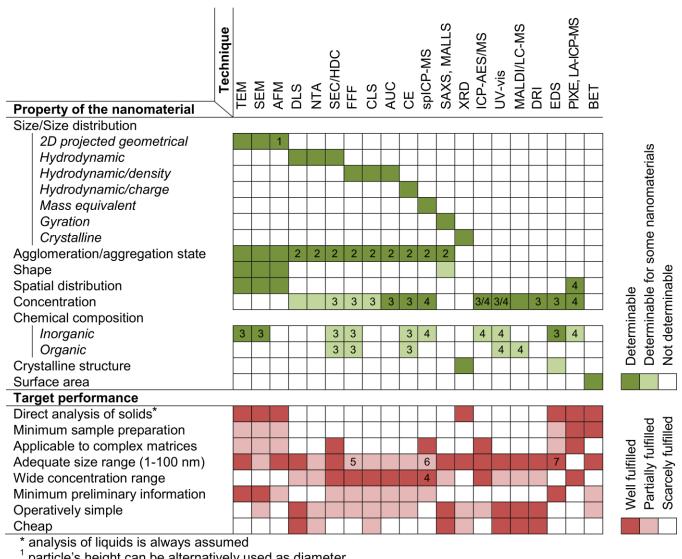| Nanomaterials		Matrices	
Inorganic		**Feed (any)**	
Titanium dioxide, TiO_2	E171	Feed supplements (any)	
Iron oxides, Fe_2O_3/Fe_3O_4	E172	Food (any)	
Metallic silver, Ag	E174	Food supplements (any)	
Metallic gold, Au	E175	Food simulants	
Amorphous silica, SiO_2	E551	(migration from food-contact materials)	
Aluminum oxide (alumin), Al_2O_3		A	Ethanol 10%
Zinc oxide, ZnO		B	Acetic acid 3%
Cerium oxide, CeO		C	Ethanol 20%
Nanoclays		D1	Ethanol 50%
Fullerenes and nanotubes		D2	Vegetal oil
Organic		Subst. D2	Ethanol 95%, isooctane
Nisin	E234	E	Poly(2,6-diphenylphenylene oxide)

Chitosan

Nanoencapsulated (micelles, lyposomes,
and dendrymers)

Composites

Functionalized nanoclays

Functionalized metallic nanoparticles

Polymeric matrix nanocomposites

Technique

Columns (left to right): TEM, SEM, AFM, DLS, NTA, SEC/HDC, FFF, CLS, AUC, CE, spICP-MS, SAXS MALLS, XRD, ICP-AES/MS, UV-vis, MALDI/LC-MS, DRI, EDS, PIXE LA-ICP-MS, BET

Property of the nanomaterial

Property	TEM	SEM	AFM	DLS	NTA	SEC/HDC	FFF	CLS	AUC	CE	spICP-MS	SAXS, MALLS	XRD	ICP-AES/MS	UV-vis	MALDI/LC-MS	DRI	EDS	PIXE, LA-ICP-MS	BET
Size/Size distribution																				
2D projected geometrical	■	■	1																	
Hydrodynamic				■	■	■														
Hydrodynamic/density							■	■	■											
Hydrodynamic/charge										■										
Mass equivalent											■									
Gyration												■								
Crystalline													■							
Agglomeration/aggregation state				2	2	2	2	2	2	2	2	2								
Shape	■	■	■																	
Spatial distribution	■	■	■																4	
Concentration				■		3	3	3	3	3	4			3/4	3/4		3	3	4	
Chemical composition																				
Inorganic	3	3				3	3			3	4			4	4			3	4	
Organic						3	3			3					4	4				
Crystalline structure													■					■		
Surface area																				■

Target performance

Performance	TEM	SEM	AFM	DLS	NTA	SEC/HDC	FFF	CLS	AUC	CE	spICP-MS	SAXS, MALLS	XRD	ICP-AES/MS	UV-vis	MALDI/LC-MS	DRI	EDS	PIXE, LA-ICP-MS	BET
Direct analysis of solids*																				
Minimum sample preparation																				
Applicable to complex matrices																				
Adequate size range (1–100 nm)							5				6								7	
Wide concentration range											4									
Minimum preliminary information																				
Operatively simple																				
Cheap																				

Legend (Property, green scale): Determinable / Determinable for some nanomaterials / Not determinable

Legend (Target performance, red scale): Well fulfilled / Partially fulfilled / Scarcely fulfilled

* analysis of liquids is always assumed
1 particle's height can be alternatively used as diameter
2 assumptions or complementary information needed
3 requires coupled/combined technique
4 depends on matrix interference
5 depends on the type of FFF
6 depends on sample composition
7 depends on the coupled technique

Figure 22.1 Main properties of nanomaterials and available analytical techniques for their determination in foods, beverages, food-related matrices (simulants), and food-contact materials, with qualitative indication of the corresponding expected performances.
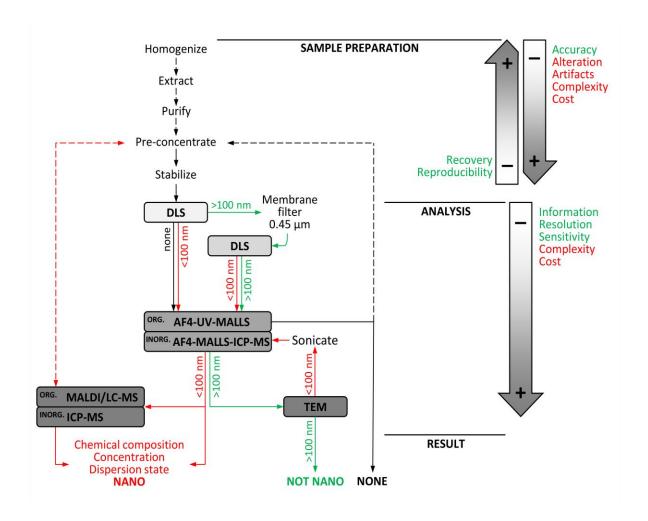
Figure 22.2 Generic analytical strategy for detection, identification, and characterization of a nanomaterial in foods, beverages, and food-related matrices (simulants). According to the definition of nanomaterial, < or > 100 nm refers to the >50% of particles.