International Journal of *Molecular Sciences*

MDPI

Perspective

Analysis of nanomaterials on biological and environmental systems and new analytical methods for improved detection

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Abstract: The advancing field of nanoscience has produced lower mass, smaller size, and expanded chemical composition nanoparticles over recent years. These new nanoparticles have challenged traditional analytical methods in terms of qualifying and quantifying. Such advancements of nanoparticles and nanomaterials have captured the attention of toxicologists with concerns regarding the environment and human health impacts. Given that nanoparticles are only limited by size (1 - 100 nm) their chemical and physical characteristics can drastically change and thus alter their overall nanotoxicity and in unpredictable ways. A significant limitation with the development of nanomaterials is that traditional regulatory and scientific methods used to assess biological and environmental toxicity of chemicals do not generally apply to the assessment of nanomaterials. Significant research effort has been initiated but much more is still needed to develop new and improved analytical measurement methods for the detection and quantitation of nanomaterials in biological and environmental systems.

Keywords: nanoparticles; engineered nanomaterials; nanotoxicity; *in vitro*; *in vivo*; analytical chemistry; method standardization

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Int. J. Mol. Sci.* **2022**, *23*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

1. Introduction

Nanoscience has consistently been a developing and advancing field with a great diversity of applications in medicine, energy, electronics, biotechnology, materials, etc [1]. Engineered nanomaterials (ENMs) and nanoparticles (NPs) are simply defined as material with at least one dimension of 1 - 100 nm in size [2]. This definition means that their various chemical and physical properties allow them to be altered and changed in order to perform their targeted functions and tasks [2-4]. Additionally, due to their vast diversity in multiple fields of research, nanoparticles have now been incorporated into common everyday products such as food preservatives, cosmetics, clothes, etc [4]. This constant unseen contact with nanoparticles has promoted the field of nanotoxicology in order to study the greater impact these ENMs have on both biological and environmental systems (Figure 1) [5]. However, due to limitations in analytical instrumentation and analytical test methods directly applicable to measure ENMs in the environmental and biological matrices, nanotoxicity remains an underdeveloped field as it struggles to keep up with the advancing research and development of nanoparticles and nanoparticle-based materials actively being developed [6,7].

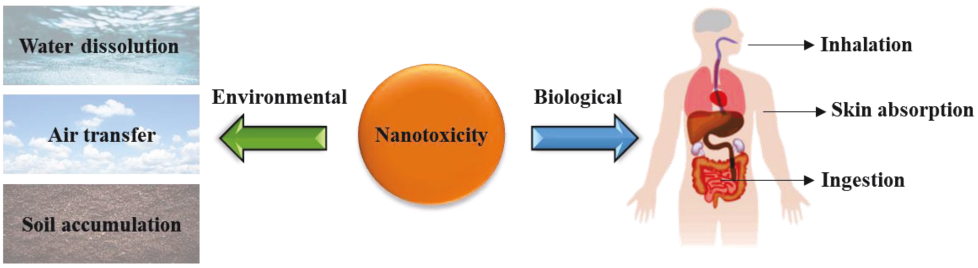

Figure 1. Nanotoxicity exposures for environmental and biological matrices.

One differentiating characteristic is the size of the nanoparticles, which can make them chemically different from larger particles and bulk materials (e.g., diffusivity across cell membranes) [2,4-8]. Additional unique biological cell interaction comes in the form of ENM surface charge, with anionic and neutral ENMs generally having a lower toxicity than cationic materials. ENM surface charge may also have an additional influence on the particles' overall shape and the shape of ENMs can alter cell membranes as well, thus heavily influencing the cellular uptake mechanism [5,8,9]. Surface coatings of ENMs can alter their toxicity by providing additional electrostatic forces, molecular adhesion, and atomic layer deposition which have contributed to cell death [9]. Furthermore, the elemental composition of ENMs contributes to their overall toxicity to both biological and environmental systems [10]. Such elements can range from transition metals (gold, silver, copper, iron, etc.) to non-metals (silica, carbon) and can greatly alter the previously listed properties of size, morphology, coating, physical, and chemical properties.

NPs and ENMs are primarily introduced into the environment through consumer products [11]. This problem has many arising concerns due to low concentrations of detection, usually ng/L, and the current limits of detection of analytical instruments [12]. NPs can also be integrated into the human body by a multitude of ways, but most commonly through inhalation, ingestion, and skin absorption, while environmental exposure is usually through the air, water, and soil integration [4]. For biological matrices, ENMs can impact the mitochondrial function of cells in addition to producing reactive oxidative species (ROS). The analytical measurement of mitochondrial function, damage, and ROS levels in biological systems remains a primary tool in the assessment of toxicity [13]. Cell metabolism is greatly impacted by ROS levels as they are natural byproducts in cell metabolism and contribute to cell survival, death, signaling, inflammation, and differentiation [13]. An imbalance of ROS leads to disrupted redox homeostasis in cells which ultimately interferes with the cell's overall function in relation to DNA/RNA breakage, membrane destruction, protein carbonylation, and other means [14]. However, ROS compounds have been looked at previously as an alternative to chemotherapy for cancer treatment [15]. Radical compounds such as superoxide ($O_2^{\bullet -}$), hydroxyl ($HO^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$), peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), carbon dioxide ($CO_2^{\bullet -}$), carbonate ($CO_3^{\bullet -}$) and singlet oxygen ($^1O_2$) are involved in key cell reactions that revolve around signaling and homeostasis processes [13-15]. However, high levels of ROS compounds can result in oxidative damage to healthy cells and interfere with cell metabolism with accumulation of ROS contributing to normal cells turning into cancer cells [16,17]. NPs introduction into biological systems can interfere with ROS generation in several ways depending on the characteristics of the NPs [13].

With the field of nanoscience and nanotoxicity expanding, the purpose of this perspective is to investigate what analytical techniques are used in toxicology assessments to effectively measure ENMs and NPs toxicity. Furthermore, this perspective will also include advancing analytical techniques to better detect and evaluate ENMs in biological and environmental matrices and what future methods could be introduced to better detect

ENMs toxicity. However, there is no current federal or state legislation in the United States specific for nanomaterials. Regulatorily, there are agencies such as the intergovernmental Organization for Economic Co-operation and Development (OECD), the American Industrial Hygiene Association (AIHA), and the US Environmental Protection Agency (USEPA) that investigate ENMs and their greater impact on human health and the environment as well as regulate analytical methods of testing ENMs. Given~~Given~~ the increasing amount of ENMs integrating into consumer and industrial products, the OECD has identified a greater need than ever to have accurate testing methods since the potential risks and impacts of nanomaterials is not well developed. To date, the OECD has documented over 780 studies on specific physiochemical properties of nanomaterials that contribute to plant/animal toxicity as well as ecotoxicity [18]. Additionally, with the evolving field of ENMs, the OECD has continued to modify current methods and promote new ones as a means of keeping up with the advancing technology around ENMs [18].

2. Current methods and concerns for *in vitro* nanotoxicity determination

Due to the variety of factors that impact ENMs toxicity, as previously mentioned, there is not a singular method for accurate detection of ENM toxicity. Rather, there are several methods that are commonly used in conjunction to help identify the characteristics of ENMs and their overall toxicity [19]. Dynamic light scattering (DLS) is most commonly used to determine particle hydrodynamic size and zeta potential (also determined by the DLS instrument) determines particle surface charge, while methods such as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) allow for the visual detection of ENMs that can then be measured for their size distribution. Although these methods give insight on the characteristics of the ENMs, they do not give toxicity analyses. For this, researchers turn to *in vitro* and *in vivo* examinations.

To examine *in vitro* toxicity first, one standard measurement technique to measure ENM toxicity in *in vitro* studies is by MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) assays, which assess the cells' mitochondrial function by detecting mitochondrial dehydrogenase through an enzymatic reduction. Another standard measurement technique for toxicity determination is by examining ROS formation within the cells, which indicates oxidative stress and interference in cell function. In order to measure intracellular ROS, a fluorescent ROS indicator is typically utilized. This indicator, when in the presence of ROS, will chemically change and thus yield a different fluorescent signal. This signal can be observed through fluorescence spectroscopy [20,21] or through confocal microscopy [22]. The most common *in vitro* assays are summarized in Table 1.

Table 1. *In vitro* assay types used for the analysis of nanotoxicity.

Assay Type	**Cell Toxicity Investigation**
Proliferation	Cell metabolism
Apoptosis	DNA, protein, and lipid damage
Necrosis	Membrane integrity
Oxidative Stress	DNA/RNA damage, lipid peroxidation, protein oxidation/nitration, ROS generation, antioxidant counterbalance

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However, these two methods are not completely accurate means of determining toxicity. For example, an interesting study was performed by Dönmez Güngüneş et al. where three different kinds of the NPs (Fe_3O_4, fullerenes (C_{60}), and single walled carbon nanotubes (SWCNT)) were tested in two different cell lines (human periodontal ligament fibroblasts (hPDLF) and mouse dermal fibroblast (mDF)) [23]. Although the MTT assay and ROS analyses of the three different kinds of NPs showed that the hPDLF cells, compared to the mDF cells, were more susceptible to all three NPs (showing higher ROS levels and larger MTT decrease in cell viability), Dönmez Güngüneş et al. utilized a relatively newer analytical method known as xCELLigence where a gold microelectrode is labeled with an antigen to which the test cells are exposed. The current between the gold and reference electrode will increase as the cells neutralize the antigen blocking the signal on the gold surface which allows for a real-time kinetic measurement of cell health and behavior. Dönmez Güngüneş et al. found that although the human cells were more susceptible to the NPs in terms of raw cell viability in the MTT assays and mitochondrial failure due to ROS formation, the internal mechanism of the cells remained unchanged with all three types of NPs tested. The mouse cells, however, showed internal failure as most of the cells no longer performed as they should, an indication of some toxic effects within the cells. Without this third analysis in xCELLigence, one could have concluded that the mouse cells were relatively unaffected by the NPs solely based on the MTT and ROS analyses without knowing the true impact on the intercellular mechanisms that were impacted to a larger degree compared to the human cells.

3. Current methods and concerns for *in vivo* nanotoxicity determination

Furthermore, there are concerns around unintended particle accumulation in organs, and thus induced toxicity, for *in vivo* studies, where the potentially hazardous NPs are directly or indirectly introduced into living organisms with measurements of toxicology endpoints. Several nanoparticles, such as gold-based and other metal-based NPs, have shown to display toxic effects and organ accumulation, however, the toxicity pathways are not fully understood [24,25]. The most common organs tested for, and impacted by, NPs accumulation are the liver, heart, kidney, spleen, lungs, intestine, and stomach with the liver and organs with high blood flow being the most unintended accumulation sites [26,27]. Which organs are impacted more depends on the elemental composition and size of the NPs [28]. For instance, carbon-based NPs show the most unintended accumulation in the liver [29], however, smaller carbon-based particles less than 20 nm, such as quantum dots (QDs), showed increased accumulation in the brain parenchyma [30]. The QDs can pass through the BBB pathway and through the trigeminal nerve or olfactory epithelium, which can cause additional problems when investigating *in vivo* toxicity [31,32]. However, despite the accumulation of carbon-based particles in organs, due to their chemical makeup, carbon-based NPs typically display little to no significant increase in toxicity when examining *in vivo* [33], however, some toxic effects have been recorded [34].

Silica-based NPs show similar low toxicity when accumulated in organs compared to carbon-based NPs; although some uterine metabolic issues have been discovered in mice [35]. Silica NPs appear to accumulate the most in the liver, lungs, and spleen [36], with some kidney accumulation also being observed [37]. Histological studies of silica-based NPs showed no ill effects in organs when the NPs are cleared from the organs within a few months [38,39]. For NPs composed of less harmful chemicals such as carbon and silica, their size plays a much greater role in their toxicity in addition to their chemical composition. Generally, smaller particles are more toxic due to their size allowing them to better interact with cellular components such as proteins, fatty acids, and nucleic acids [40]. However, larger silica NPs have also been shown to possess greater toxicity than smaller silica NPs [39]. Polymer- and metal-based NPs with low clearance rates generally showed the greatest toxicity and organ accumulation [41,42] and sometimes containing greater metabolic disturbance [43].

However, there remains several limitations to *in vivo* analysis of NPs organ accumulation and long-term toxicity. For some studies, a lack of macrophage uptake and blood circulation suggests the need for better assays [44]. Additionally, *in vivo* studies with animas do not necessarily carry over to human studies as many nanoparticles never reach their intended site and are cleared from the bloodstream quickly [45], adding to the difficulty of detecting *in vivo* toxicity and attributing it to NPs. Furthermore, most *in vivo* studies examine toxicity on week- or month-long analyses. Year-long analyses are rarely examined in research primarily due to time constraints despite being informative and essential [46]. Nonetheless, these are all considerable parameters when examining *in vivo* nanotoxicity, with several current methods needing improved testing parameters and animal models for more accurate assessments of toxicity, especially when examining the complexity of human health [47]. The most commonly used *in vivo* analysis methods are listed in Table 2.

Table 2. *In vivo* methods for nanotoxicity determination.

Methods	**Toxicity Analysis Examination Types**
Radiolabeling	Radioisotope tracking of NPs through biological systems examined for biodistribution
Clearance	Excretion and metabolism of NPs examined after various exposure times
Serum Chemistry	Enzymes, lipids, hormones, proteins in serum examined for metabolic interferences
Histopathology	Cells, tissues, and organs examined for disease manifestation
Hematology	Red and white blood cells, platelets, and coagulation system examined for disorders

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To specifically measure nanotoxicity *in vivo* several factors are taken into consideration. Immune system response compounds such as globulin, TNF-alpha, and KC-GRO are typically analyzed in order to determine toxicity. However, correlation is not necessarily causation, as simply analyzing these markers after the introduction of ENMs is not an accurate means of analyzing toxicity. A far more exact means of determining ENM toxicity *in vivo* is by utilizing ICP-MS and microwave digestion; where tissue and organic samples are prepared via microwave digestion before being subjected to ICP-MS. This is a more accurate way of confirming ENMs integration and concentration within key organs, as shown in a study by Weaver et al. [48]. This is because there needs to be a way to confirm that the amount of ENMs or NPs injected into the animal remained in the organs and did not pass through the bloodstream without interacting. Unfortunately, ICP-MS is limited to metal-based NPs and cannot be used for NPs that are, for example, polymer-based. For these NPs, it is much more difficult to accurately determine their concentration and integration *in vivo*. Therefore, methods previously mentioned (such as fluorescence, bioluminescence, microscopy, and spectroscopy) are utilized as accurately as possible [49,50]. Additionally, smaller sized NPs concentrations tend to be more difficult to determine [51]; with the importance of concentration control for *in vitro* and *in vivo* it is vital to accurately determine biological target tissue the concentration for nanotoxicity tests [52]. However, despite the extensive means to confirm ENMs integration and concentration, *in vitro* and *in vivo* toxicity tests have been known to be highly inconsistent and sometimes do not agree with each other [53,54], possibly due to variation between cell culture lines and animal species [55] and issues with testing method accuracy [56]. The OECD has made several adjustments to their nanotoxicity testing protocols to combat this variation problem; however, to this day, no singular method has proven to be the golden standard for testing nanotoxicity in ENMs, and methods continue to be tested and enhanced/modified to keep up with the accelerating ENMs development field [57].

For example, the OECD has implemented newly revised inhalation toxicity testing guidelines, 412 and 413, for 28-day and 90-day inhalation toxicity studies, respectively, for carbon based ENMs. These methods focus on inhalation since it is a primary route of ENM exposure to humans [58]; with employees of carbon-based ENM manufacturing being the largest group at risk of physical contact and, due to an increase in number of applicational fields, there is a growing concern about overall hazardous potential when it comes to inhalation [59]. However, according to a study performed by Kim et al., there is a significant lack of data for the toxicity regarding multi-walled carbon nanotubes (MWCNTs) [60,61]. Nonetheless, following the OECD guideline [62], Kim et al. investigated the 28-day inhalation toxicity study by exposing rats to MWCNTs at 0, 0.257, 1.439, and 4.253 mg/m^3 for 28 days [60]. They generated their MWCNT aerosols by using an acoustic dry aerosol generator and made sure to test the aerosol chamber concentrations using OC/EC and field emission-transmission electron microscope (FE-TEM) to confirm MWCNT exposure. Cytotoxicity markers lactate dehydrogenase (LDH), micro-albumin (mALB), and micro-total protein (mTP) were examined in bronchoalveolar lavage fluid (BALF) for toxicity analysis as proposed by the OECD method. Samples of the rat lungs were taken after 1-day, 7-day, and 28-day post exposure (PEO-1, PEO-7, and PEO-28). They noticed that the alveolar macrophages of the lungs contained MWCNT material in all the samples. Low concentration (0.257 mg/m^3) did not show any pneumocyte damage or cell inflammation. Agglomerated MWCNTs, however, were seen in throughout the lungs including the bronchi, alveolar ducts, and alveoli [60]. Additionally, the moderate and high concentration samples (1.439 and 4.253 mg/m^3) showed granulomatous lesions filled with MWCNT in all PEO sampling days. It is of note that Kim et al. did not find any significant organ weight changes after exposure for all time periods, demonstrating that solely relying on organ weight as an indication of toxicity is inadequate, which was also pointed out in the study by Weaver et al. However, toxicity results varied drastically between similar studies and studies that used different types of MWCNT [28,63,64]. Although the new OECD method significantly improved their previous 412 method, the lack of data available on MWCNTs proved to still be a major setback with general testing methods.

4. New and enhanced methods of nanotoxicity determination and particle detection

Although the above methods help detect ENM and NP toxicity, there remains an issue with the accuracy of the methods [56,65]. Part of the reason is due to the lack of technology applied to analytical methods. With the ever-advancing field of nanomaterials, analytical methods lag behind or suffer due to lack of data, as shown previously with the OECD 412 method. Additionally, if there are no measures taken to confirm the successful integration of the ENMs or NPs into the cells/organisms being tested for toxicity, then there cannot be an accurate follow-up evaluation/conclusion that the concentration of ENMs/NPs injected caused toxicity. Furthermore, research articles do not necessarily expand beyond their target application when it comes to the ENM degradation and integration into other matrices and systems, particularly their environmental exposure/fate [66], leading to many published articles in the nanotoxicity field to be limited and questionable [67]. In fact, most published ENM test methods for environmental or biological testing applications have not been validated following the procedures set by the USEPA or other regulatory bodies [56]. Although the regulatory guidance for the testing of chemicals set by the USEPA, OECD, and AIHA provide extensive guidelines that help protect both humans and the environment, there remains limited guidance for analytical test methods or toxicity assessment procedures for direct measurements of ENMs, rather than indirect measurements [65]. This exemplifies the need to further advance analytical instrumentation and test methods in order to better directly qualitatively and quantitatively evaluate ENMs, especially in complex biological matrices and environmental matrices (e.g., air, sludge, and water) [68]. In many cases with environmental and biological testing, there is a lack of sufficient standards to compare with real-time measurement analyses [69,70]. Ultimately, analytical methods need to be improved or new methods developed in order to counter the detection and accuracy problems seen in nanotoxicity assessments [71].

Such advancements in both analytical instrumentation and test methods can be seen across several recently published articles. The first being by Mader et al., who applied the USEPA analytical test method validation guidance in the development of a new test method for the quantitation of engineered NPs in water matrices [65]. The validated ENM analytical test method for water is not limited to metal containing NPs and was applied to two OECD ecotoxicity test methods for both Daphnia and algae; by direct measurement of nanoparticle size distribution and concentration in the ecotoxicity test matrix. Analytical NP measurement was performed on a Liquid Nanoparticle Sizer [72], Differential Mobility Analyzer [73], and Nano Water-Based Condensation Particle Counter [74]. The role of the Liquid Nanoparticle Sizer was crucial as it quantitatively diluted sample solutions using ultrapure water by a 20:1 to 20,000:1 ratio prior to nebulization. The nebulizer was adjusted to produce an aqueous aerosol with a droplet size of 300 nm with the sample dilution ensuring that only one particle was present in each droplet. The resulting nebulized aerosol was then dried, classified and counted. This combination of the two instruments allowed for the measurement of the number of each size of particles in a volume of air. Additionally, by scanning a range of particle mobility in the differential mobility analyzer the number-weighted NP size distribution could be determined. The validated Mader et al. method quantified both the NP size distribution and dose level verification concentrations in the daphnia and algae ecotoxicity test matrices. The most important factor in accurate quantitative measurements for the method was the application of matrix-matched NP standard calibration curves to minimize analytical response factor difference between standards and test matrices [62]. The analytical method requires the use of certified NP reference materials for calibration standard preparations and, because of the availability of other certified metal and nonmetal NP materials, it is possible to adapt their EPS guidance validated test method for other ENMs and in other water ~~matricies~~matrices (e.g., drinking water, wastewater, groundwater) [65].

Additionally, Savić-Zdraković et al. [75] also utilized an OECD testing guideline, in this case guideline 218 [76], for the examination of CeO_2 NP uptake in relation to oxidative stress parameters, *in vivo* genotoxic effects, larvae, and life-trait toxicity parameters, using ICP-MS analysis. Through this study, the importance of establishing a standardized methodology for larvae lethality and sub-lethality cutoffs was established as their results indicated that the larvae were not at risk of CeO_2 NP toxicity; however, accumulation of these particles could impact organisms that consume the larvae. Therefore, much like the Kim et al. study, the value of the OECD guideline is greatly impacted by the lack of data surrounding CeO_2 NP toxicity testing which also contributes to these NPs being listed on the OECD priority list of environmental impact assessment [75].

Another method that advanced the analytical side of NP detection was by Hadioui et al. who discussed detecting NPs in the environment by inductively coupled plasma mass spectrometry (ICP-MS), due to it being one of the best analytical techniques for detecting ENMs [77,78]. In addition to ICP-MS being limited to metal-based NPs, it is also limited by particle size detection limits, with many NPs and their oxides being out of detection range. Hadioui et al. helped to improve this detection limit by adjusting the kind of aerosols introduced into the ICP-MS. They examined different nebulization desolvating techniques; distinguished as "dry" and "wet" aerosols. Hadioui et al. noted an increased number of counts in the dry mode, and for smaller particles (9 nm Ag) more ions were extracted using the desolvator for both wet and dry aerosols. Both desolvating systems lead to an increased signal intensity of the 9 nm Ag and 25 nm TiO_2 NPs. Thus, dry aerosols had a better detection and resolved peak intensities for small NPs [77]. Additionally, an increase in sensitivity was also noted for the 5 nm Ag NPs. Injecting 2.3 ng/L, the dry aerosol compared to the wet aerosol showed a drastic increase in the particles that were detected; and no particles were detected when using the ICP-Q-MS (the quadrupole ICP-MS used to evaluate instrument sensitivity) [76]. The size detection limit for ICP-Q-MS was 17 nm while single particle sector field ICP-MS (ICP-SF-MS) was 5 nm, which could further be reduced to 3 nm using the desolvating nebulizer. Thus, by using dry aerosols for ICP-MS, Hadioui et al. successfully improved sensitivity and enhanced ion extraction [77].

Cui et al., in their study, helped examine the fate and improved detection of TiO_2 NPs in the environment using ICP-MS by changing synthesis parameters, utilizing Ho as a chemical marker in their NPs and thus designing $NaHoF_4@TiO_2$ NPs. By using an $Al(OH)_3$ layer around Ho in $NaHoF_4$, the added colloidal stability and hydrophilic surface helped TiO_2 deposition and coating when synthesizing the NPs [79]. The goal of the Cui et al. study was to be able to detect engineered TiO_2 NPs in the environment without Ti background interference. Using their unique synthesis, the addition of Ho as a tracer significantly helped detect the engineering TiO_2 NPs in the environment, despite being in low concentrations (100 million-fold dilutions or 5000-200,000 particles/mL) [79].

As for biological fates of nanoparticles, studies such as Turco et al. [80] and López-Serrano Oliver et al. [81] provide valuable insights into biological nanotoxicity testing. Turco et al. utilized a sputtering-enabled intracellular X-ray photoelectron spectroscopy (SEI-XPS) method in which metallic NPs were cultured in media and cells before being directly measured for their internalization, stability, and oxidation state. Utilizing this technique, Turco et al. provided a possible method to help assess NPs integration, accumulation, and longevity and thus provide valuable insight into nanotoxicity. López-Serrano Oliver et al. also looked at metal-based nanoparticles, in the form of silver, and focused on developing a new method of mass cytometry that can quantify NPs numbers per single cell [81]. Although they were able to make some interesting and important discoveries for new nanotoxicity analyses of NPs, there remains an issue with NPs monitoring and intracellular uptake of NPs out of the mass range of mass cytometers.

Lastly, a fairly recent method to determine nanotoxicity is through *in silico* analyses. These methods have proven to be advantageous as they bypass the costs of extensive *in vitro* and *in vivo* experiments and remove the need for animal experiments [82]. These *in silico* studies helped provide insight into potential alternative testing methods for nanotoxicity as existing experimental data surrounding the tested NPs and ENMs confirmed the results of the computational data obtained [83,84]. However, the main issue with *in silico* analyses is a similar issue as the *in vitro* and *in vivo* experiments, which is a lack and inconsistency of data. *In silico* methods are dependent on existing nanotoxicity data to confirm the model's accuracy [85] and therefore the future accuracy of *in silico* is also dependent on advancing current analytical methods to better analyze nanotoxicity experimentally.

Through all of these studies it is seen that analytical methods and the increased availability of certified analytical NPs standards are vital in the measurement of ENMs and NPs in toxicity assessments as these materials need to be accurately identified and quantified in both biological and environmental matrices [~~82~~86]. Although progress has been made in developing toxicity assessments and testing methods, the accuracy of these methods have been called into question on more than one occasion [~~83~~87]. Agencies like OECD and AIHA help provide guidelines for nanotoxicity testing, however, a repeated problem across studies is the lack of data and certified analytical standards surrounding the NPs and ENMs, particularly the newer ones that are being produced at a rate that is not matched by development of the analytical instrumentation, even with enhanced methods and new analytical techniques [~~84~~88]. There is also the problem with long-term ENMs and NPs exposure/integration, with toxic degradation particles potentially never being assessed as NPs and ENMs production continues. Generational and long-term biological and environmental impacts of ENMs and NPs nanotoxicity is an underdeveloped field, but one that should be considered for exploration [~~85~~89].

5. Conclusions

When it comes to the world of nanoscience, its vast diversity in engineered materials makes this area of research rich in possibility but ultimately leads to uncertainty in the health of humans and the environment they come into contact with. Truly, there is an abundance of nanotoxicity data among research articles, but small groups of ENMs and NPs tend to be studied and their long-term environmental fate or chemical/physical changes over time are not analyzed [~~86~~90]. Even with several agencies and organizations across multiple countries pooling their information together on the topic of nanotoxicity safety policies [91~~87~~] the problem still remains; as the field of nanoscience advances, analytical instrumentation and methods struggle to find applicability when it comes to accurately measuring ENMs' and NPs' harmful effects and possible integration into alternate media. Although traditional biological testing such as *in vitro* and *in vivo* help glimpse the impact NPs might have on chosen cells and living organisms, the methods tend to be indirect measurements of ENMs, by analyzing organ weight, mitochondrial function, and ROS levels. Indirect methods tend to only analyze the cell's or organism's response to the ENMs without confirming the exact amounts of ENMs that were involved in the negative or positive response to the ENMs. As a result, there has been a movement to develop direct methods of measuring ENMs in the environment and in biological systems as it remains a challenge to multiple national and international agencies to accurately assess the toxicity of ENMs. As new ~~nanoparticles~~ NPs and ~~nanomaterials~~ ENMs are synthesized, many existing protocols and methods for their detection have been amended by researchers outside of the agencies that designed them, still with no one method being suitable for all ENMs or NPs. A significant limitation with the development of nanomaterials is that traditional regulatory and scientific methods used to assess the biological and environmental toxicity of chemicals do not generally apply to the assessment of nanomaterials. This limitation is directly related to the need for advancements and further developments of analytical instrumentation, analytical test methods, and analytical standards for the direct measurement of ENMs and NPs in biological and environmental matrices.

There is no doubt that NPs and ENMs pose potential risks environmentally and biologically [92]. Without the issues of accurate and consistent nanotoxicity determination being addressed, unknown degradation products, accumulation, and induced toxicity are of increasing concern. Given that ENMs and NPs are continually being advanced and incorporated into commercial products and medical treatments, the potential risk of undetected toxicity short- or long-term is also increasing. Particularly for the use of NPs in medicine, it is of utmost importance that the treatment NPs are precisely characterized, detected and traceable, that their degradation products are not harmful, and that short- and long-term toxicity is not induced in the treated patient, especially for human trials. Similarly, the environmental fate of NPs and ENMs needs to be heavily considered as they can accumulate in soil, water, plants, and animals. If not properly detected, quantified, and removed as contaminants, NPs and ENMs both whole or degraded can pose increasing environmental rick and potentially cause irreversible harm to the ecosystem. With 9806 products [93] currently incorporation nanomaterials it is vital to address the problem of detection limits and improved analytical testing methods.

Acknowledgments: This work was partially supported by the National Science Foundation grant CHE 1709160 and Cooperative Agreement Award OIA #1946202.

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