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Mass spectrometry-based proteomics for systems-level characterization of biological responses to engineered nanomaterials

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Abstract

The widespread use of engineered nanomaterials or nanotechnology makes the characterization of biological responses to nanomaterials an important area of research. The application of omics approaches, such as mass spectrometry-based proteomics, have revealed new insights into the cellular responses of exposure to nanomaterials, including how nanomaterials interact and alter cellular pathways. In addition, exposure to engineered nanomaterials often leads to the generation of reactive oxygen species and cellular oxidative stress, which implicates a redox-dependent regulation of cellular responses under such conditions. In this review, we discuss quantitative proteomics-based approaches, with an emphasis on redox proteomics, as a tool for systems-level characterization of the biological responses induced by engineered nanomaterials.

Graphical Abstract

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Compliance with Ethical Standards

The authors declare no conflict of interest.

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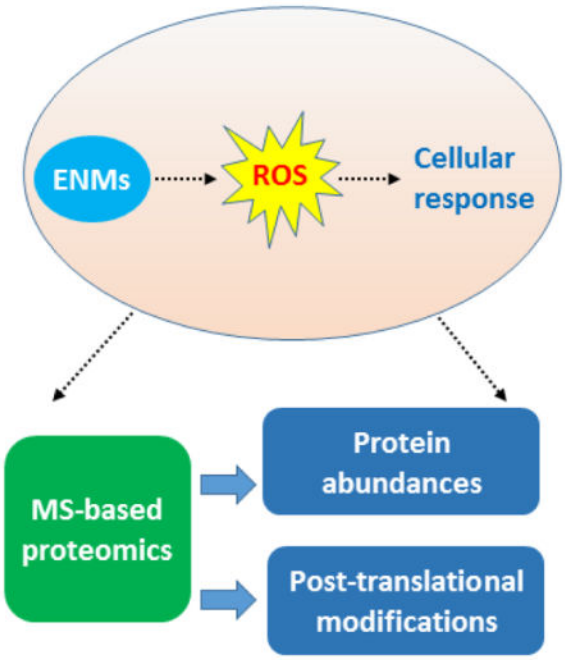

Keywords

Engineered nanomaterials; Proteomics; Post-translational modifications; Redox proteomics; Thiol; Oxidative stress

Introduction

Advances in nanotechnology have resulted in the production of an ever increasing number of engineered nanomaterials (ENMs), diverse in their properties, enabling a broad range of applications in science, industry, and medicine. For example, metal oxide ENMs have been used in food packaging due to their anti-microbial activities [1,2]. Applications of ENMs in diagnostic assays and drug delivery systems for treatment of human diseases have also increased substantially in the last decade [3–5]. However, the potential risk to human health due to the prevalent use and potential exposure to many kinds of ENMs through ingestion, inhalation, or even penetration via the skin is also an important concern [6,7].

A better understanding of the health impact and biological responses to ENMs requires the knowledge of ENM-cellular interactions occurring both extracellularly and intracellularly. Numerous studies have shown that various physical properties such as size, shape, charge, and surface characteristics affect the cellular uptake process [8,9]. Within a given biological milieu such as cytoplasm, ENMs typically adsorb proteins dynamically to form a protein corona [10,11]. Consequently, the physicochemical properties of ENMs are significantly affected by the associated proteins [12]. The identities and nature of proteins forming the corona have been under intense investigation, especially through mass spectrometry (MS)-based approaches [13–18]. Moreover, the ability of various ENMs in initiating cellular oxidative stress or generating reactive oxygen species (ROS) has been suggested as a major

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paradigm of adaptive and toxic cellular responses to ENMs [19–22] (Figure 1a). Increased oxidative stress is closely associated with various cellular responses such as DNA damage, endoplasmic reticulum (ER) stress, lipid peroxidation, mitochondrial dysfunction, immune dysfunction, and apoptosis [23–26]. Despite these advances, the biological impact and molecular mechanisms governing ENM-cellular interactions remain challenging to predict.

Omics-based approaches such as transcriptomics have been applied extensively for profiling gene expression changes upon ENM exposure [25,27–30]. Although these studies provide valuable information on how ENMs alter gene expression, the changes at the mRNA level do not necessarily correlate with changes at the protein level. Furthermore, ENM exposure can generate ROS by both direct and indirect mechanisms, which may induce protein post-translational modifications (PTMs) that affect cellular functions [24,31,32]. MS-based proteomics is an ideal tool for not only large-scale quantitative profiling of thousands of proteins, but also for extensive profiling of different types of PTMs, which play a critical role in cellular signaling and regulation. Thus, MS-based proteomics represents a highly promising tool for elucidating potentially ENM-specific mechanisms of actions (e.g., by oxidative stress) by identifying altered cellular pathways through broad quantification of cellular protein expression and PTMs. Such measurements serve as a more direct readout of signaling and regulation than transcriptomics. Herein we briefly review MS-based proteomics approaches in the context of characterizing biological responses of ENM exposure at the proteome and PTM levels with an emphasis on thiol-based redox modifications. We also highlight biological pathways that are responsive to ENM exposure as revealed in recent proteomics studies.

MS-based global proteome profiling

MS-based proteomics workflows typically consist of a number of steps that include protein extraction, enzymatic digestion, separations at protein or peptide levels, liquid chromatography–tandem mass spectrometry (LC-MS/MS) analyses, and bioinformatics data analysis and interpretation. Due to the high complexity of the proteomes of biological systems, protein/peptide separation is a key step prior to MS analysis. Traditionally, two-dimensional gel electrophoresis (2D-gel) was the method of choice to separate proteins. Over a thousand protein spots can be resolved and proteins of interest are selected, excised, and subsequently identified by MS. However, due to its laborious nature, the 2D-gel approach has been largely replaced by more automated LC-MS/MS methods. The current LC-MS/MS approaches have enabled deep profiling of proteomes (e.g., quantification > 10,000 of proteins) with great dynamic range [33].

Gel-based proteomics

2D-gel is a classic method for protein separation that can be easily coupled with MS analysis, and most of the earlier proteomics data on the biological responses to ENMs were based on this approach. Using this method, protein samples from control and ENM-treated tissues or cell lines are first separated on a gel by isoelectric point. A second dimension of electrophoresis is performed perpendicularly to the first dimension to separate the proteins by molecular weight. An image of the resulting gel is then captured and the intensity of

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individual gel spots is quantified by imaging software. Protein spots showing significant differences in intensity are excised from the gels and the identities of these proteins are subsequently determined by LC-MS/MS analysis. It is noteworthy that gel-to-gel variation may result in a poor reproducibility in protein quantification. To overcome this, a two-dimensional fluorescence difference gel electrophoresis technique has been developed [34].

To study the response of mouse fibroblast cells to ENMs, Gioria et al. employed 2D-gel to profile protein expression patterns under gold ENM exposure [35]. Pathway analysis of 143 significantly changed proteins revealed that exposure to gold ENMs results in the alterations in cellular processes such as cell growth and proliferation, cell morphology, and oxidative stress responses. More recently, Ge et al. used a two-dimensional difference gel electrophoresis (2D-DIGE) to profile the proteomes of human bronchial epithelial cells following exposure to titanium dioxide (TiO_2) ENMs [36]. They found that the altered proteins included some key proteins involved in cellular stress responses, cytoskeletal dynamics, metabolism, adhesion, cell signaling, and cell death. Using a similar approach, proteome changes in human monocyte (THP-1) derived macrophages exposed to TiO_2 ENMs with and without silica coating were investigated [37]. Again, the altered proteins were linked to metabolic homeostasis, cytoskeleton remodeling, and oxidative stress.

Gel-free LC-MS/MS-based proteomics

Advancements of automated LC-MS/MS-based approaches over the last decade have largely replaced the gel-based techniques. In a typical LC-MS/MS-based proteomics workflow (Figure 1b), proteins are first digested to peptides, which are then fractionated by chromatographic techniques such as strong cation exchange (SCX) or reversed-phase (RP) LC prior to final stage of LC-MS/MS analyses. Thus, sample complexity is greatly reduced by fractionation at the peptide level, rather than at the protein level as in the 2D-gel approach. The orthogonality of multidimensional LC strategies, such as SCX [38] or high pH RPLC [39], followed by low pH LC-MS/MS has greatly enhanced proteome coverage. To enable more accurate quantification of protein abundances, stable isotope labeling strategies have often been employed. Typically, either metabolic labeling strategies such as stable isotope labeling by amino acids in cell culture (SILAC) [40] or isobaric labeling strategies such as isobaric tag for relative and absolute quantitation (iTRAQ) [41] or tandem mass tag (TMT) [42] can be incorporated into this workflow to facilitate quantitative analysis.

While MS-based proteomics have made significant advancements, studies on biological responses of ENMs are often limited by the overall proteome coverage. For example, in one study applying iTRAQ-based 2D-LC-MS/MS approach, the abundance of 46 proteins were observed to be altered significantly following exposure to zinc oxide (ZnO) ENMs in rat bronchoalveolar lavage fluid [43]. Gene ontology analysis of these proteins suggested that immune responses and inflammatory processes were affected by such exposure. More recently, a similar iTRAQ-based 2D-LC-MS/MS approach was applied to investigate the cellular responses of human LoVo cells to silver ENMs [23]. A deeper coverage of ~3,000 proteins was achieved in this study with hundreds of proteins observed with altered expression. The data revealed some unique cellular processes based on the sizes of ENMs.

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The larger 100 nm ENMs exerted more indirect effects via several kinase/phosphatase signaling pathways, while the smaller 20 nm nanoparticles had a direct effect on cellular stress [23]. These results were supported by the observation that 20 nm particles were internalized by the cells while 100 nm particles were not.

Besides isobaric labeling, label-free approaches have also been used in MS proteomics. Generally speaking, there are two common methods used with this approach [44]; one relies on intensity of the MS precursor ions while the other uses MS/MS spectral information (spectral counting, for example). Technical details of each method, as well as their relative strength and weakness, is reviewed elsewhere [44]. A recent application of label-free proteomics was performed in the marine bacterium *Pseudomonas fluorescens* BA3SM1, where cadmium selenide treated cells were compared with the control at the proteome level [45]. Among the 996 proteins quantified based on MS/MS spectral count, the abundance of 31 were found to be significantly altered after treatment. These differentially expressed proteins were involved in a number of biological processes such as tricarboxylic acid (TCA) cycle, metal resistance, and oxidation-reduction processes. Notably, a label-free approach can also be coupled with traditional gel electrophoresis for protein quantification. For example, proteins from A549 lung epithelial cells treated with SiO_2 ENMs were first fractionated by 1D-gel and slices of the gel were subjected to in-gel digestion and LC-MS/MS analysis [46]. MS-based intensity was used to determine protein abundances, showing 47 proteins with significant changes upon treatment. These proteins were involved in processes such as apoptosis, ER unfolded protein response, and protein synthesis.

Overall, global proteomics clearly demonstrates its utility for the purpose of profiling proteome changes and for identifying cellular processes upon ENM exposure (Table 1). Many altered cellular pathways, due to changes in protein expression patterns and abundances, are associated with general stress responses such as oxidative stress, cytoskeleton remodeling, and metabolism. A deep proteome profiling with detailed quantitative studies on the doses and types of ENMs has the potential to provide unique systems-level insights into the signaling pathways governing the cellular responses to ENMs.

MS-based PTM profiling

While quantitative profiling of protein expression is important, protein function, activity, or cell signaling is often dynamically regulated through the levels of PTMs. PTMs greatly expand the diversity of proteins and can drastically modulate functional changes of proteins in signaling transduction or enzymatic activities. The ability to measure a variety of PTMs is a unique aspect of advanced proteomics technologies. Currently, more than 200 biological relevant PTMs have been reported [47] and several types of them, such as phosphorylation, acetylation, glycosylation, and thiol-based redox modifications, have been studied extensively [24,32,48–50].

Thiol-based redox modifications

Of particular relevance is the potential of cells to respond to ENM-induced oxidative stress through a redox-dependent mechanism. This is due to the fact that many ENMs are known

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to induce ROS across different species from bacterial cells [1], plant [51], to mammalian cells [19,22]. It has been hypothesized that the adverse effects of ENMs- can be predicted from the level of oxidative stress they cause [19,52]. In this oxidative stress paradigm, a hierarchy of cellular responses including anti-oxidant defense, pro-inflammatory responses, and cytotoxicity are activated upon different level of oxidative stress. In addition to ROS, reactive nitrogen species (RNS) have also been implicated in ENM-induced toxicity [12]. ROS/RNS are known mediators of thiol-based redox modifications [32], which include several types of reversible modifications such as S-nitrosylation (SNO), S-sulfenylation (SOH), S-glutathionylation (SSG), and redox-sensitive disulfide formation, and irreversible modifications such as sulfinic acid (SO_2H) and sulfonic acid (SO_3H). These modifications could either play a role in the signaling and regulation, or represent pathological signatures of cellular toxicity (Fig. 2a).

Although redox regulation has been studied for decades, identification of redox-sensitive proteins and mapping of redox PTMs are far from routine. Given the labile nature and relatively low abundance of redox PTMs, several technical considerations are critical. As cysteine thiols can assume different redox states, free thiols are typically blocked with N-ethylmaleimide (NEM) or iodoacetamide during cell lysis and protein extraction. Subsequently, PTMs of interest are selectively reduced by specific reagents to generate new free thiol groups. For example, sodium ascorbate, a glutaredoxin enzyme cocktail, or dithiothreitol (DTT) were used to selective reduce SNO-, SSG-, or all reversible oxidative modifications, respectively [53]. Those proteins with newly formed free thiols could be then captured with thiol-reactive biotin, which allows for affinity enrichment of modified proteins/peptides with streptavidin [54,55]. Termed as biotin switch technique, this method has been broadly applied and a number of variations have been developed to study multiple redox modifications [56]. More recently, a much improved strategy was developed for direct enrichment of thiol-containing proteins/peptides through co-valent capture by a thiol-affinity resin (Thiopropyl Sepharose) [53,57]. This resin-assisted capture approach significantly improves the enrichment specificity and simplifies the overall workflow. For quantifying redox PTMs, multiplexed isobaric labeling strategy such as TMT can be easily coupled with the resin-assisted capture approach (Figure 2b).

As an example of the role of redox PTMs in ENM-induced biological response, a recent study from our group investigated protein SSG as an underlying regulatory mechanism by which ENMs may alter macrophage innate immune functions using the redox proteomics approach [24]. The impact of three high-volume production ENMs (SiO_2, Fe_3O_4, and CoO) were investigated for their impacts on macrophage function. In total, 2,494 unique SSG-modified Cys sites were identified in RAW264.7 macrophage cells treated with ENMs. The increased SSG modifications (Figure 2c) were found to correlate well with the overall level of cellular redox stress and impairment of macrophage phagocytic function (CoO > Fe3O4 ≫ SiO2). Moreover, the data also revealed pathway-specific differences in susceptibility to SSG between ENMs which induce moderate versus high levels of ROS. The study provides insights into the protein signatures and pathways that serve as ROS sensors and may facilitate cellular adaption to ENMs.

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Other PTMs

Besides redox modification, phosphorylation is another important type of PTM for gaining mechanistic understanding of cellular responses to ENMs. Traditionally, phosphorylation was profiled by Western blot. Using this method, Rinna et al. showed that silver ENMs activate mitogen-activated protein kinases such as ERK1/2 and JNK1/2 human epithelial embryonic cells [58]. Similarly, an increase in total tyrosine and threonine phosphorylation has been found in human small airway epithelial cells under exposure to both CoO and La_2O_3 ENMs [59]. With the advances of MS-based proteomics, global phosphoproteome profiling now allows quantifying > 10,000 site-specific phosphorylation events in one experiment. Various enrichment techniques such as immobilized metal affinity chromatography (or IMAC) have been developed to overcome the low-abundance nature of phosphorylation [60]. For instance, in one study using IMAC-enrichment of phosphopeptides, differential phosphorylation on 32 proteins were observed in human lung cells treated with CuO [61], and many proteins were involved in multiple signaling pathways such as lipid antigen presentation and telomerase signaling.

Although a number of studies have reported that the abundances of proteins potentially involved in other types of PTMs are altered under ENM exposure, most of the studies have not identified the exact protein modifications. For instance, protein abundances of the phosphorylation-based p70S6K signaling pathway and protein ubiquitination pathway were reported to be altered with selenium quantum dots treatment [62]. In addition, proteins involved in ubiquitination were found altered in human lung cells treated with copper-oxide ENMs [61], Balb/3T3 mouse fibroblast cells treated with gold ENMs [35], and mice that inhaled silver ENMs [63]; however, the identity of modified proteins has not been reported.

One of few examples of ENM-induced PTMs being investigated is protein carbonylation, an irreversible PTM indicative of loss of protein function induced by oxidative stress [64]. Rainville et al. found that the carbonylation levels decreased in proteins such as 14-3-3 in response to silver ENMs [65]. The authors used fluorescein-5-thiosemicarbazide to label protein carbonyls, which were later separated and visualized on a 2D-gel. However, an overall increase of carbonyl groups was observed in MRC-5 human lung fibroblast cells following exposure to SiO_2 ENMs [66]. In a more comprehensive study, the effects on protein carbonylation of a panel of 24 representative ENMs, including amorphous silica, metal oxide, carbon nanotubes, and silver, were assessed in NRK-52E cells [31]. Briefly, protein carbonyls were probed with 2,4-dinitrophenylhydrazine and then detected by immunoblotting. The identity of the proteins were then determined by MS. The results showed that 11 out of 24 ENMs induced an increase in protein carbonylation and that the modified proteins cover a broad range of functional categories from enzymes involved in central metabolism to proteins involved in stress response.

It should be noted that the effect of ENMs on PTMs should be assessed in a case-by-case manner. Although ENM-triggered ROS burst has been observed in many cases, ENMs that can switch between different oxidation states may also act as ROS scavengers. For example, cerium-oxide nanoparticles deposited in rat lung protect the animals against oxidative stress by limiting ROS production, glutathione oxidation, and lipid peroxidation [67]. Thus, the anti-inflammatory capacity of these ENMs may prevent oxidative PTMs on proteins in vivo.

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Cellular responses to ENM exposure

To assess cellular responses to ENM exposure, both in vitro and in vivo studies have been utilized to identify adaptive, pathophysiological, and cytotoxic responses [19,21,22,68]. Adaptive responses such as decrease in biosynthesis of proteins and DNA and increase in biosynthesis of heat shock proteins and antioxidant enzymes can be activated by certain ENMs under low dosage [69]. However, many ENMs are known to trigger toxic responses that lead to membrane damage, protein degradation, and apoptosis [20]. The ability of quantitative proteomics to globally profile protein abundances and key PTMs has proven to be a useful tool to shed new insights into the molecular regulatory mechanisms, including post-translational regulation of cellular responses to exposure of ENMs. While the outcome would be largely dependent on the cells, tissues, or animal models being used, as well as on the nature of ENMs and exposure parameters, in this section we briefly summarize several common biological responses and pathways that have been reported to be altered with ENM exposure based on proteomics data (Table 1).

Oxidative stress

Many studies have highlighted the role of ROS in cellular responses to ENMs. For example, the ROS level in MRC-5 human lung fibroblast cells increased after 24, 48, and 72 h exposure to SiO_2 ENMs. On the other hand, the levels of glutathione (GSH) decreased at all time points measured [66]. Likewise, the free GSH levels in macrophages were also decreased under the treatment of copper-based ENMs, indicative of a shift in redox homeostasis [70]. However, low levels of ROS could also activate antioxidant responses to restore intracellular redox homeostasis. For instance, Gioria showed an increased level of GSH in human colon cells treated with gold ENMs for 72 h [71]. The activation of GSH pathway in Caco-2 cells exposed to gold ENMs was also investigated using metabolomics [71], supporting the proteomics findings that ENMs induce the expression of enzymes in GSH biosynthesis such as glutamate-cysteine ligase. In another recent quantitative proteomics study of the effects of silver nanoparticles in human LoVo cells, cellular ROS was also clearly increased upon exposure of both particle sizes (20 and 100 nm) [23]. Moreover, a shift in redox homeostasis towards oxidation was also clearly demonstrated in our recent redox proteomics study of RAW264.7 macrophage cells exposed to three different metal oxide ENMs (SiO_2, Fe_3O_4, CoO) (Figure 2c) [24]. Oxidative stress has been closely linked to ER stress, and dysregulated protein translation and immune suppression in response to ENMs that induce subcytotoxic levels of oxidative stress [24]. Together, all these lines of evidence lend support to oxidative stress as a major paradigm of cellular responses to many ENMs [19,22], and proteomics has made some unique contributions to the mechanistic understanding.

Immune response

The interaction between immune cells and ENMs is also of particular interest because many ENMs are engineered to either avoid immune recognition or specifically inhibit or enhance the immune responses [72]. Identification of key proteins and pathways is critical for a better understanding of ENM-induced immune responses or suppression. In a recent study using label-free proteomics, the effects of Au, CuO, and cadmium telluride (CdTe) ENMs on the

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innate immune system was assessed using the human monocyte cell line THP-1 as a model [73]. Despite a similar overall toxicity effect, the three ENMs induced distinct proteomic signatures, with the strongest effect being induced by CdTe, followed by CuO and gold ENMs. The gold ENMs induced upregulation of the key inflammatory mediator, NF-κB, by directly targeting its inhibitor TIPE2 [73]. Furthermore, gold ENMs triggered activation of NF-κB as shown through phosphorylation of the p65 subunit. In our recent redox proteomics study, exposure of RAW264.7 macrophage cells with metal oxide ENMs (Fe_3O_4, CoO) significantly disrupted macrophage function by decreasing their phagocytic activity. In addition, we observed that proteins involved in phagocytic processes, such as actin-binding protein, were generally more oxidized following ENM treatment [24]. These studies illustrate that quantitative proteomics approaches for profiling protein abundances and PTMs can be used as effective tools for characterizing the effects of ENMs on immune cells and their underlying molecular mechanisms.

Energy metabolism

ENMs are also known to have an impact on cellular metabolic processes. Alterations of key enzyme activities in metabolic pathways could be one mechanism that ENMs exert to affect metabolism. For example, protein abundances of both α-enolase and malate dehydrogenase, two enzymes involved in glucose metabolism, were decreased in human lung epithelial cells under long-term exposure to TiO_2 ENMs [74]. Similarly, glycolysis was observed as most impacted pathway in RAW264.7 macrophage cells exposed to CoO, an ENM inducing a relative high level of ROS [24].

Moreover, perturbation in oxidative phosphorylation and fatty acid metabolism were also observed in mouse neuroblastoma N2a exposed to silver ENMs [7]. A similar observation was made in human monocyte THP-1 cells treated with gold ENM, in which proteins involved in energy metabolism such as glucose-6-phosphate dehydrogenase and long-chain fatty acid-CoA ligase 1 were up-regulated [73].

As a hub for energy metabolism, mitochondria are also sensitive to exposure to ENM. Indeed, several proteomics studies have identified mitochondrial proteins as being significantly altered in response to ENMs [35,74–76]. Proteins involved in key mitochondrial activities, such as electron transfer in the respiratory chain, TCA cycle, and β-oxidation of fatty acid, decreased in abundance upon exposure to ENMs. The down-regulation of mitochondrial enzymes was further confirmed by measuring the membrane potential to indicate the decrease in mitochondrial activity [74,76].

DNA damage and cytoskeletal remodeling

Disruption of redox homeostasis has been connected with DNA damage and cytoskeletal remodeling [77,78]. In the case of ENMs, proteomics data support that they can alter the abundance of proteins important in DNA replication and repair and maintenance of cytoskeleton [73–75,79]. For example, a recent proteomics study discovered that the toxicity mechanism of CdTe ENMs involves down-regulation of topoisomerases, suggesting that CdTe ENMs may inhibit cellular DNA repair mechanisms [73]. In another study, the abundance of serine-threonine kinase receptor-associated protein, an activator of P53 for

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mediating DNA damage response, was observed to be increased in A549 cells exposed to TiO2 ENMs [74]. Alternatively, other ENMs were observed to induce upregulation of proteins in the ubiquitin-proteasome system, a major player in DNA repair [23].

Cytoskeletal remodeling is another commonly observed pathway in response to ENM exposure. This is supported by studies that find ENMs can impact the fundamental structure of the cytoskeleton system [80]. Ng et al. observed the expression profiles of a number of proteins involved in cell adhesion and F-actin stress fiber arrangement in the MRC5 lung fibroblasts were altered in response to exposure to gold ENMs [79]. Overrepresentation of cytoskeleton-related proteins from proteome profiling also prompted Triboulet et al. to examine the changes in cytoskeleton induced by copper and copper-oxide ENMs. Interestingly, only actin-myosin cytoskeleton, not tubulin, was altered [70]. Similarly, we observed increased SSG modifications on a number of actin-binding proteins, which is closely associated with phagocytic regulation [24].

Future Perspectives

Proteomics, compared to transcriptomics, has the clear advantages of directly measuring the levels of protein expression and PTMs, providing more accurate information regarding protein activities and signaling. While the applications of quantitative proteomics in the investigation of cellular responses to the exposure of ENMs have been rapidly increasing, such technique is still not established in toxicological screening of nanoparticles. Moreover, the majority of previous studies have been limited by the overall depth of proteome profiling. In particular, quantitative profiling of PTMs in this field is still very limited. As advanced proteomics technologies become more mature and commonly available, we anticipate the systematic assessment of cellular responses and health impacts of a variety of ENMs should be feasible by applying quantitative proteomics approaches. With the integration of quantitative proteomics along with other phenotypic assays in toxicological screening, we shall be able to learn many more details regarding regulatory mechanisms of cellular responses. The advantages of applying proteomics in toxicity screening of ENMs have also been recognized in several recent reviews [81,30]. Proteomics can not only identify cellular pathways that are typically targeted by traditional phenotypic assays, but also reveal novel pathways relevant to cellular responses to exposure. In some cases, proteomics are sensitive enough to reveal changes at protein expression or PTM level at low-dose of exposure where no apparent cytotoxic effects can be observed.

One challenging aspect of studying the biological impact of ENMs by proteomics is the large number of ENMs that may pose health risks and the relatively limited sample throughput of proteomics approaches along with the generally high cost of proteomics assays. To date, only a handful of ENMs have been investigated in a limited number of cell models using proteomics to provide a first glimpses of altered proteomes. It would be of great interest to systematically screen a relatively large number of diverse ENMs in a single study to discover common pathways and pathways specific to individual ENMs that are affected. In order to integrate proteomics approaches with toxicological screening of ENMs, the proteomics approaches including sample processing procedure need to be standardized and quality assurance or quality control practices need to be implemented to ensure

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reproducibility. Such standardization and large-scale studies are feasible since they have already been demonstrated in other fields such as the large-scale tumor proteome analyses [82,83].

It is also anticipated that quantitative profiling of PTMs such as redox modifications and phosphorylation will allow a better understanding of regulatory or signaling mechanisms in response to ENMs. For example, although numerous studies have demonstrated the disruption of redox homeostasis upon exposure to ENMs, the extent to which redox imbalance leads to changes in PTMs has not been extensively studied. Similarly, the relevance of other PTMs such as phosphorylation, acetylation, and ubiquitination in biological responses to ENMs has been largely unexplored. Ideally, further investigation of the biological impacts of various ENMs will not only involve global proteome profiling, but relevant PTMs such that more detailed regulatory mechanisms will be revealed.

There is also an emerging interest to integrate multiple omics platforms or data to better understand molecular networks in complex biological systems. Integrative multiomics profiling also represents an interesting option for assessing cellular responses to ENMs. Among different omics techniques, metabolomics aims to profile a broad set of metabolites within a cell or tissue. Since many biochemical pathways can be measured directly from the composition and concentration of various metabolites, metabolomics profiling provides a direct readout of cellular activities. During the last several years, applications of metabolomics in the field of ENMs in combination with proteomics have been reported. For instance, metabolomics and proteomics have been integrated to investigate the responses of Caco-2 human colon cells to gold ENMs [71]. The combination of two datasets enabled cross-validation of changes in multiples cellular activities such as growth and proliferation, DNA and protein biosynthesis, and defense against oxidative stress [71].

Conclusions

Given the prevalent use of ENMs and the potential health risk of human exposure, there has been a growing interest in investigating the underlying molecular mechanisms of cellular responses to ENM exposure. Quantitative proteomics approaches offer the unique advantage of directly quantifying protein abundances and various PTMs such as redox modifications, which provide new insights into post-translational regulation of protein function/activity triggered by ENMs. To date, proteomics studies have made a significant contribution in revealing the mechanistic details of cellular responses such as oxidative stress, immunity, energy metabolism, DNA damage, and cytoskeletal modeling to ENMs. Moreover, quantitative proteomics, especially at the level of PTMs as used in a recent redox proteomics study [24], enables delineation regulatory pathways linked with adaptive stress responses and toxicity-driven responses by quantitatively profiling the proteome or PTMs of cells exposed to various ENMs across doses. With the advancement of proteomics technologies, a systems-level mechanistic understanding of cellular responses to ENM exposure and their potential health impact seems to be highly attainable.

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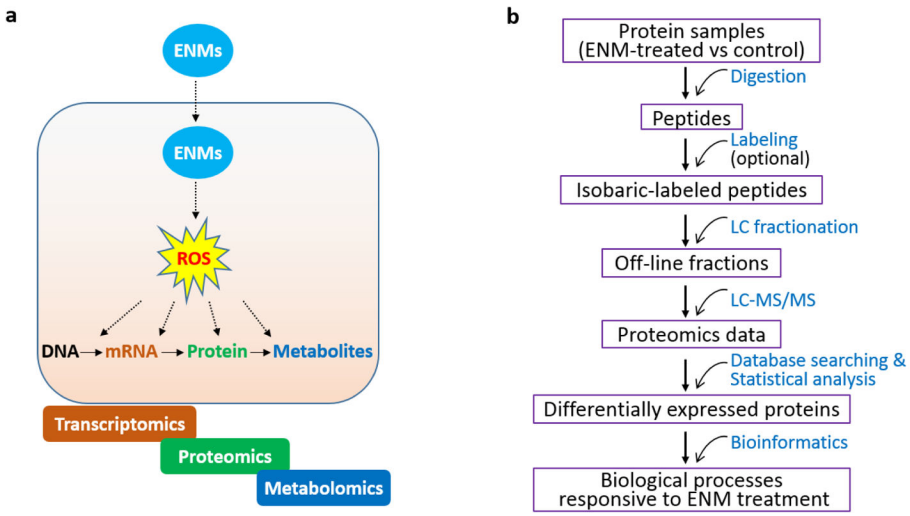

Figure 1.

Proteomics approaches for studying the biological effects of ENMs. (a) Interaction between ENMs and biological systems. (b) Schematic of a typical shotgun proteomics workflow.

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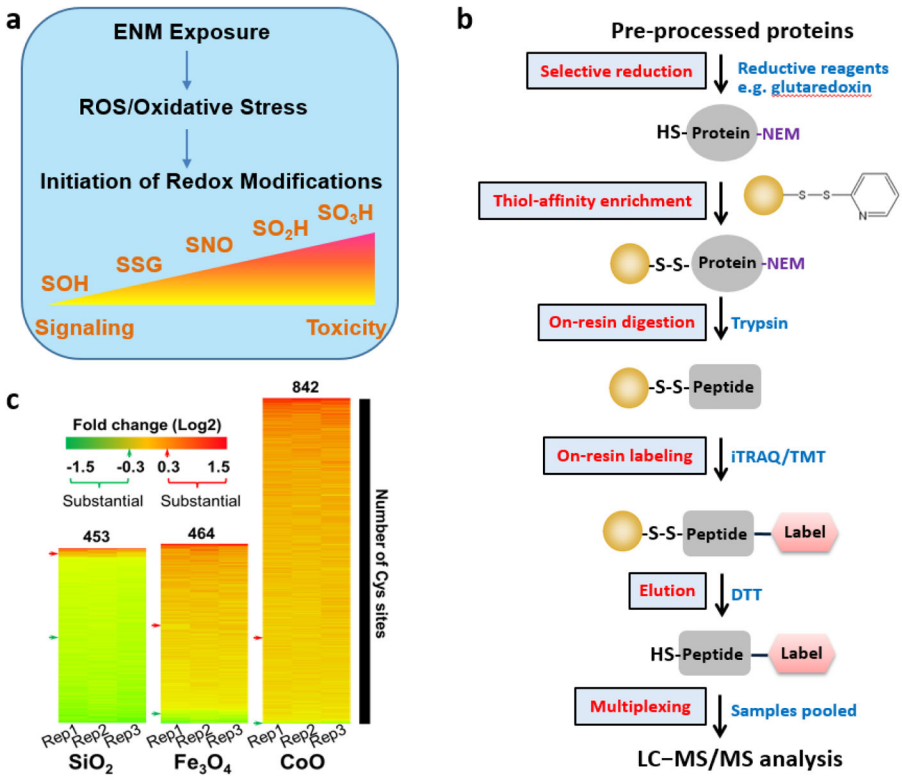

Figure 2.

Profiling of ENM-induced redox modifications by quantitative redox proteomics. (a) Various redox modifications on protein cysteine residues upon exposure to ENMs. (b) A typical workflow for quantitative characterization of SSG modification. (c) Differential levels of SSG modifications induced by three types of ENMs (SiO_2, Fe_3O_4, and CoO). Adapted from Duan et al [24]. Copyright (2016) American Chemical Society.

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Table 1

Summary of recent proteomics studies on cellular responses to ENM exposure

ENMs	Size (nm)	Biology system	Conditions	Physiological observations	Method	Affected biological processes	Refs
Abraxane	10–100	A549 human lung cells	100 nM; 4 h	No significant change in cell morphology	LC-MS/MS	Lipid metabolism, cell cycle, cytoskeleton, apoptosis	[84]
Ag	3–5	mouse neuroblastoma N2a	4 μg/ml;24 h	Permeability increased	LC-MS/MS	Oxidative phosphorylation, fatty acid elongation, ribosome biogenesis, and neuronal diseases	[7]
Ag	20/100	human colon LoVo cells	10 μg/mL; 24 h	Only small ENMs can enter the cell	LC-MS/MS	Oxidative stress, DNA damage, translational initiation, ubiquitination, and mRNA splicing	[23]
Ag	20/200	human colon Caco-2 cells	1 mg/L; 24 h	Generation of ROS and release of IL8	2D-gel proteomics	Ubiquitination, glutathione biosynthesis, metabolism, oxidative stress, and immune response	[85]
Ag	33	mice	3.3 μg/ml; 6 d	Significant allergic responses	LC-MS/MS	Immune response, ubiquitination, cytokine signaling, and TCA cycle	[63]
Ag	14	human colon Caco-2 cells	2.5, 25 μg/mL; 24 h	No cytotoxicity based on cell viability	2D-gel proteomics	Protein synthesis and folding, cellular assembly, and fatty acid and energy metabolism	[76]
Au	5/15	mouse fibroblast cell	300 μM; 72 h	mitochondria and ER dilated	2D-gel proteomics	Oxidative stress, cellular growth and proliferation, and inflammatory response	[35]
Au	5/15	human colon Caco-2 cells	300 μM; 72 h	Reduced cell growth	2D-gel proteomics	Cytoskeleton organization, cell adhesion, cell growth and proliferation, DNA damage, and oxidative stress	[71]
Au	20	human lung fibroblasts	1 nM; 72 h	altered F-actin arrangement	LC-MS/MS	Cell adhesion and extracellular matrix/ cytoskeleton remodeling	[79]
CdTe/CuO/Au	<4/10–20/10–20	human monocyte THP-1	5/22/15 μg/ml; NA	50% cell death	LC-MS/MS	Oxidative stress, DNA damage, inflammatory response, and energy metabolism	[73]
CuO	30–50	BEAS-2B human lung cells	0.01 μg/cm^2; 24 h	7% cell death	LC-MS/MS	Protein synthesis, cytoskeleton maintenance, and cell death	[61]
CuO	<50	mouse RAW264.7	10 μg/mL; 24 h	20% cell death	2D-gel proteomics	Oxidative stress, glutathione biosynthesis, cytoskeleton, and oxidative phosphorylation	[70]
NiO,	<10	rat lung	20 μg/animal; 24 h	pulmonary injury and inflammatory responses	LC-MS/MS	Glutathione metabolism, focal adhesion, endocytosis, and immune responses	[86]
Se	22	human colon Caco-2 cells	100 nmol; 2 h	stimulated Caco-2 cell proliferation	2D-gel proteomics	Ubiquitination, glutathione synthesis, energy metabolism, and immunity responses	[87]
Si	26	Murine macrophage	10,20 μg/mL 24 h	20% cell death at 20 μg/mL	2D-gel proteomics	DNA damage, cytoskeleton, energy generation, and AMPK pathway	[75]

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ENMs	Size (nm)	Biology system	Conditions	Physiological observations	Method	Affected biological processes	Refs
$SiO_2/Fe_3O_4/CoO$	<100/15/13	mouse Raw 264.7 cells	12.5 μg/ml; 24 h	Different levels of ROS stress	LC-MS/MS	Oxidative/ER stress, translation initiation, immune suppression, glycolysis	[24]
TiO_2	25	A549 human lung cells	2.5, 50 μg/mL; 2 m	DNA damage	2D-gel proteomics	Glucose metabolism, mitochondrial function, proteasome activity, and DNA damage	[74]
TiO_2/CuO	25/15–70	mouse macrophages	125 μM; 24 h	Oxidative stress	2D-gel proteomics	Glutathione synthesis and oxidative stress	[88]