

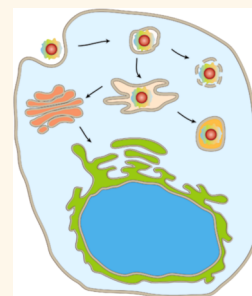
Emerging Techniques in Proteomics for Probing Nano–Bio Interactions

Zon W. Lai,^{†,*,‡} Yan Yan,^{§,‡} Frank Caruso,^{§,*} and Edouard C. Nice^{†,*}

[†]Department of Biochemistry and Molecular Biology, Monash University, Victoria 3800, Australia, [‡]Institute for Molecular Medicine and Cell Research, University of Freiburg, D-79104 Freiburg, Germany, and [§]Department of Chemical and Biomolecular Engineering, The University of Melbourne, Victoria 3010, Australia.

[‡]These authors contributed equally.

ABSTRACT Nanoengineered particles that can facilitate drug formulation and improve specificity of delivery afford exciting opportunities for improved lesion-specific therapy. Understanding and controlling the nano–bio interactions of these materials is central to future developments in this area. Mass-spectrometry-based proteomics techniques, in conjunction with other emerging technologies, are enabling novel insights into the modulation of particle surfaces by biological fluids (formation of the protein corona) and subsequent particle-induced cellular responses. In this Perspective, we summarize important recent developments using proteomics-based techniques to understand nano–bio interactions and discuss the impact of such knowledge on improving particle design.



The convergence of nanoscience and biomedicine is expected to bring momentous advances in the fight against a wide range of diseases. The first generation of nanomedicines, exemplified by Doxil, Abraxane, and Pegasys, has already shown favorable clinical outcomes in the treatment of cancer and infectious diseases.¹ A major goal for second generation nanomedicines is to facilitate formulation and improve drug pharmacokinetics of therapeutics, subsequently reducing side effects and enhancing therapeutic efficacy.² In order to achieve this, nanomedicines have to circumvent an array of biological barriers, such as the endothelium of blood vessels, the epithelium of the gastrointestinal track, and/or the blood–brain barrier. Understanding the complex and dynamic interactions between particles and biological systems is emerging as a rapidly developing scientific frontier that will provide fundamental and conceptual frameworks to improve drug carrier design for safe and effective therapy. For these reasons, there is increasing activity in probing nano–bio interactions. Studies have shown that exposure of nanoparticles to biological fluids (e.g., plasma, interstitial fluid, and cytoplasm) leads to adsorption of proteins on the nanoparticle surfaces, forming a protein corona that significantly influences the particle properties and their biological behavior, such as cellular uptake.³ Once nanoparticles have been internalized by cells, they are subject

to various cellular regulatory machineries allowing them to be processed and transported to specific intracellular locations, such as lysosomes and nuclei.⁴ The ability to characterize and control such intracellular processing is the essence of improving temporal and spatial profiling of therapeutics and facilitating optimal access and delivery to their specific pharmacological targets.

Understanding the complex and dynamic interactions between particles and biological systems is emerging as a rapidly developing scientific frontier that will provide fundamental and conceptual frameworks to improve drug carrier design for safe and effective therapy.

To date, a number of technologies have been used to resolve nano–bio interactions. For example, dynamic light scattering (DLS), UV–vis spectrophotometry, microelectrophoresis (zeta-potential measurements), and electron microscopy have been widely used to characterize and to visualize the protein

* Address correspondence to ed.nice@monash.edu, fcaruso@unimelb.edu.au.

Published online December 07, 2012
10.1021/nn3052499

© 2012 American Chemical Society

corona.⁵ Fluorescence microscopies, including confocal laser scanning microscopy, super-resolution microscopies (e.g., structured illumination microscopy), and single-particle tracking microscopy, have become important tools for studying the cellular processing of nanoparticles, revealing great detail regarding their spatial and temporal dynamics.^{6,7} However, while these technologies have significantly improved our understanding of particle interactions, most protein networks and hierarchical complexes that coordinate the various steps leading to the formation of the protein corona, cellular uptake, and intracellular trafficking remain fragmentary and only descriptive.

In the past decade, significant developments in proteomics, particularly in the areas of resolution, mass accuracy, and speed, which allow both sensitive and specific identification and quantification of proteins and protein complexes, have turned this technology into a powerful platform for investigating cellular organelles and biological networks, such as secretory pathways⁸ and autophagy networks.⁹ The combination of a range of proteomic techniques, including electrophoresis, chromatography, and mass spectrometry (MS),¹⁰ can provide a comparative and quantitative analysis of the protein composition, architecture, and dynamics associated with nanoparticles in various biological environments, resulting in a powerful new toolbox for probing complex nanobio interactions (Scheme 1). For example, these capabilities have recently been harnessed to give new insights into the composition and function of the protein corona on nanoparticles, providing unprecedented molecular insights into biodistribution, transportation, clearance, accumulation, and toxicity.^{3,5} Moreover, owing to the current high resolution of protein separation¹⁰ and sensitive and specific identification, MS-based proteomics techniques can now be used for the detailed analysis of cellular responses to

nanoparticles, facilitating the identification of various cellular mechanisms underlying nanoparticle-enabled delivery and induced cytotoxicity.

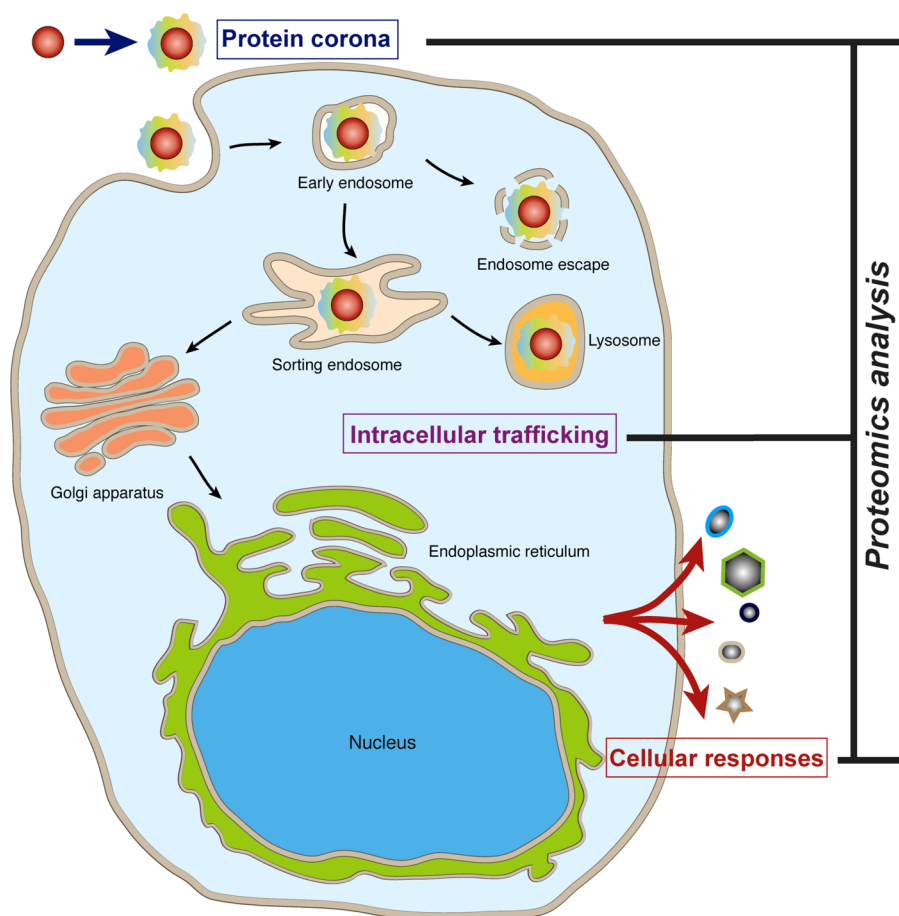
In this Perspective, we provide a brief overview of the evolution of proteomic techniques, focusing on MS-based methods, and highlight several seminal studies that have applied proteomic tools to understand the formation of protein corona and cellular responses to particles. Finally, we discuss potential applications of proteomic techniques in the analysis of particle dynamics at the subcellular level and the current strategies and future perspectives for customizing their biological interactions.

In this Perspective, we provide a brief overview of the evolution of proteomic techniques, focusing on MS-based methods, and highlight several seminal studies that have applied proteomic tools to understand the formation of the protein corona and cellular responses to particles.

Proteomic Techniques. Although the analysis of amino acids and peptides by MS was initially reported more than half a century ago, it was not until approximately 20 years ago that studies of biological proteins and peptides were made possible using MS-based proteomics, assisted by the use of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and the development of revolutionary sample ionizers for MS.^{11,12} Since then, MS has become the pivotal platform for proteomics-related research. This powerful technology can

rapidly identify proteins (through their molecular weight and characteristic peptide mass fingerprints following chemical or enzymatic digestion), peptides, metabolites, and amino acids based on their elemental composition. The basic mass spectrometer configuration comprises an ionizing source, one or more analyzers, and a mass detector. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the two most commonly employed platforms used to ionize molecules such as proteins and peptides into the gaseous state (Scheme 2). These ionized particles are then accelerated into the mass analyzer to determine the precise molecular mass of the resultant ions. There are five types of mass analyzers: time-of-flight (TOF), ion trap, quadrupole, Orbitrap, and Fourier transform ion cyclotron resonance (FTICR).^{11,12} Each of these mass analyzers is distinct in terms of its design and performance, although the different modalities tend to produce complementary mass data. Mass analyzers are now most commonly used in tandem (MS/MS) to achieve higher degrees of ion separation and identification (e.g., triple quadrupoles, QQQ; quadrupole-TOF, QTOF; linear ion trap combined with FTICR; and Orbitrap technologies).^{11,12}

While it is possible to analyze unfractionated samples directly for MS, preliminary separation steps are often beneficial to improve coverage, sensitivity, reproducibility, and throughput for proteomics-based analysis.¹⁰ Some of these steps include depletion of high-abundance proteins (which often mask the detection of biologically significant proteins that are often present in low abundance) and various chromatographic techniques to enrich selectively for specific groups of proteins under investigation.¹⁰ One of the most comprehensive and generally applicable separation methods used to date is two-dimensional electrophoresis (2DE), in which protein-containing samples undergo a first separation based on electrical charge (isoelectric focusing) followed by a second separation based on mass.



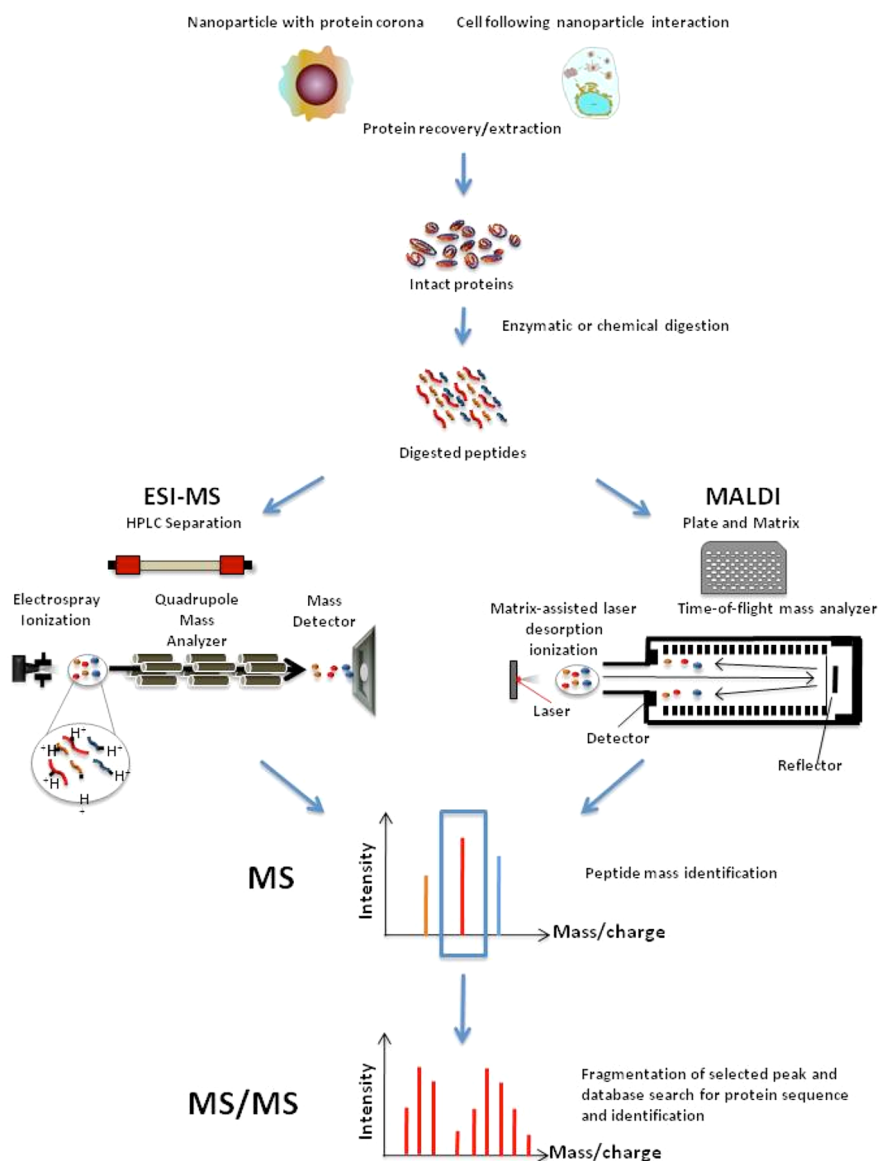
Scheme 1. Proteomics analysis allows molecular insights into the formation of the protein corona, intracellular trafficking of nanoparticles, and cellular responses following exposure to nanoparticles.

Using two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-SDS-PAGE), up to 10 000 distinct protein and peptide spots can be separated from complex biological samples, such as blood plasma, cell lysates, and tissue extracts, in a single gel.¹¹ Advances in gel-based methods for proteomics analysis have enabled comparative measurements of differential protein profiles along with quantitative visualization to be conducted using a parallel methodology called two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), which enables comparison of untreated and nanoparticle-treated samples within the same gel. In 2D-DIGE, samples containing proteins from control and nanoparticle-treated groups are labeled with specific dyes prior to separation by 2D-SDS-PAGE. Protein spots from 2D-DIGE experiments are visualized using a

fluorescence scanner, enabling identification of differentially expressed proteins. The corresponding spots from preparative gels containing an increased amount of sample can be excised poststaining for protein identification using MS-based peptide mass fingerprinting.

Alternatively, accurate protein quantitation for a system-wide approach using MS-based methods is made possible with tagging technologies such as metabolic labeling of proteins in cultured cells using stable isotope labeling by amino acids (SILAC), chemical labeling of proteins and peptides using isobaric tags for relative and absolute quantification (iTRAQ), or label-free isotope-coded protein labeling (ICPL), as well as many other novel strategies that involve the selective labeling of specific reactive groups in proteins with isotope-coded tags.^{11,12} Metabolic labeling for cell cultures using SILAC

involves the incorporation of a specific isotopically labeled amino acid analogue present in the culture medium into all newly synthesized proteins. Proteins containing this particular amino acid will incorporate the isotopically labeled analogue during a few cycles of cell division. Moreover, due to the minimal chemical differences between the natural amino acid and labeled isotope, the cells have properties similar to the control cell population grown in the presence of unlabeled amino acids. This method can be particularly useful for comparative analysis of the cellular effects of nanoparticle treatment *in vitro*. Where metabolic labeling is not feasible (e.g., in *ex vivo* samples), stable isotopes can be introduced *via* chemical labeling (e.g., isobaric tag for relative and absolute quantitation, iTRAQ, and isotope-coded protein label, ICPL).¹¹ Chemical labeling enables multiplexed



Scheme 2. Schematic showing the use of mass spectrometry in the analysis of nano–bio interactions. Electrospray ionization (ESI) and matrix-associated laser desorption ionization (MALDI), which are the most commonly used modes, have been highlighted.

proteomics analysis of samples, including proteins with a diverse range of molecular weights and isoelectric points. Here, samples are digested separately, and the resulting peptide fragments are subsequently labeled with isotope-coded linker fragments. In comparative studies, up to four linker fragments can be used to yield different and unique mass patterns. The labeled samples are then mixed and simultaneously analyzed by liquid chromatography–mass spectrometry (LC-MS) whereby the mass differences of the isotope labels are used to compare the peptide abundance in the different samples. In

addition to iTRAQ and ICPL labels, normal and highly ^{18}O -enriched water can also be used for comparative proteomics analysis of proteins and peptide fragments.¹¹

Protein Corona. When exposed to biological fluids, nanoparticles can bind protein components, forming a so-called “protein corona”, affecting their properties. Typically, the most abundant proteins will bind first but over time will be displaced by those with higher affinity. Identification and quantification of the protein composition of the corona has generally been performed using one-dimensional PAGE (1D-PAGE)

or 2D-PAGE in combination with LC-MS/MS-based proteomics techniques. The initial study using 2D-PAGE to characterize the protein corona on latex particles was reported by Müller and co-workers nearly two decades ago.¹³ In this study, representative protein patterns, comprising albumin, fibrinogen, immunoglobulin G, apolipoproteins, and complement factor B, were identified on three types of latex nanoparticles with varying hydrophobicity. In the ensuing years, understanding the protein corona of nanoparticles has become an area of intense research, and plasma protein adsorption on a

range of different nanoparticles has been investigated by a number of groups. It is increasingly realized that the formation of the protein corona is a complex phenomenon and has fundamental biological consequences.^{14,15} Although absolute correlations of the nature and composition of the protein corona with the physicochemical properties of nanoparticles remain unclear, some important trends are emerging with the improved sensitivity and accuracy of modern MS-based proteomics techniques. We will discuss recent seminal proteomic studies on the protein corona of particles, including polystyrene and silica nanoparticles and liposomes, in order to illustrate several important effects of the protein corona.

Dawson and co-workers performed a systematic study on the protein corona of polystyrene (PS) nanoparticles following incubation with human plasma for 1 h.¹⁶ Using 1D-PAGE and ESI-MS (ThermoFinnigan LTQ iontrap), they compared the protein corona formed on PS nanoparticles with three different surface chemistries (plain PS, carboxyl-modified, and amine-modified) and two particle sizes (50 and 200 nm) and found that the protein corona across all PS nanoparticles comprised a wide range of proteins. Several protein groups were identified according to their function, including immunoglobulins, lipoproteins, complement pathways, acute-phase proteins, and coagulation factors, suggesting significant roles of the protein corona in nanoparticle immunogenicity. Both particle size and surface chemistry showed significant and complex effects on the formation of the protein corona. Size-dependent protein adsorption was more pronounced in amine- and carboxy-modified PS compared to the neutral surface. It was also shown that the protein coronas on particles with similar sizes but different surface chemistry varied dramatically, but the variability did not correlate with either the trends of surface charge or the abundance of proteins, suggesting complex kinetics

and thermodynamics between the proteins and particle surfaces. Interestingly, a recent study investigated the temporal development of protein corona of an equivalent set of PS nanoparticles in human plasma using ¹⁸O-labeling and LC-MS/MS-based quantitative proteomics.¹⁷ In that study, the protein corona was isolated from nanoparticles using “on-nanoparticle digestion” followed by LC-MS analysis on either a Thermo Finnigan LTQ or Orbitrap. Compared to conventional SDS-PAGE sample preparation, which requires multiple LC-MS/MS runs, this approach is simpler and offers higher yield. A total of 153 proteins were identified (88 of which contained two or more unique peptides), of which about 60% were reported in the previous study. Globally, these proteins show ontology similar to that observed previously.¹⁶ Importantly, temporal studies illustrated that protein exchange between the protein corona and plasma is a fast process with equilibrium being reached in less than 5 min following exposure.

Nanoparticle size is a critical parameter affecting the protein corona and has also been investigated using silica nanoparticles. A quantitative proteomic analysis of the protein corona on silica nanoparticles of three different sizes (8, 20, and 125 nm) has been reported.¹⁸ Using a combination of liquid chromatography–mass spectrometry (Waters Q-TOF), 1D and 2D-PAGE, and immunoblotting, 125 proteins were identified from the protein corona, revealing an enrichment of proteins involved in coagulation and the complement pathway. However, despite significant variability in the overall protein corona composition across these silica nanoparticles, the differences in protein fingerprints were not linearly related to the differences in size.

Although there has been an overall general lack of correlation between particle properties and the total protein corona, another recent study demonstrated preferential adsorption for some classes of proteins based on charge density.¹⁹ The protein coronas

of three cationic liposomes (CLs) with different membrane charge densities were analyzed by nanoliquid chromatography–tandem mass spectrometry (Thermo LTQ-Orbitrap XL) and quantified by label-free spectral counting. A total of 177 proteins were identified with 117 proteins (66%) common to the three different CLs. Fibrinogen displayed higher adsorption on the liposomes with high membrane charge density, while apolipoproteins and C4b-binding proteins bound to membranes with lower membrane charge density.

Besides particle size and surface chemistry, the specific nature of the biological environment further influences the formation of the protein corona. Monopoli *et al.* incubated two compositionally different nanoparticles, sulfonated PS and silica nanoparticles (200 and 50 nm, respectively), with various concentrations of human plasma for 1 and 24 h.²⁰ Subsequently, the nanoparticle–protein complexes were characterized by dynamic light scattering, microelectrophoresis (zeta-potential), 1D-PAGE, and LC-MS/MS (Thermo LTQ Orbitrap). The protein corona of sulfonated PS nanoparticles did not vary significantly with increasing concentrations of plasma. In contrast, the protein corona evolved as a function of plasma concentration for the silica nanoparticles. The evolution of the protein corona on nanoparticles has recently been examined in another study.²¹ As part of that study, silica (6 and 9 nm) and PS nanoparticles (50 nm) were incubated initially with plasma (1 h) and then transferred to cytosolic fluid (overnight incubation at 4 °C). The resulting protein coronas were identified and compared using 1D-PAGE and LC-MS/MS (Thermo LTQ iontrap). The change in biological environment led to replacement of some proteins from the original serum protein corona with proteins from the cytosol, suggesting that the formation of the protein corona is a collective and dynamic process whereby proteins are adsorbed in an accumulative and/or competitive manner. In addition, because of the

sensitivity to the local proteome in a given biological environment, probing the protein corona has been employed as a novel tool to identify potential biomarkers.^{22,23} For example, a novel therapeutic target for ovarian cancer, hepatoma-derived growth factor (HDGF), was recently identified by the comparison of protein coronas formed on surface-functionalized gold nanoparticles (AuNPs) after incubation with normal or malignant ovarian cancer cell lysates.²³

While careful control of nanoparticle surface chemistry has been a fundamental consideration in particle design, this is rapidly modified in a largely unpredictable and uncontrollable manner on exposure to a biological environment. One approach to attempt to overcome this has been through modification of the nanoparticle surface with poly(ethylene glycol) (PEG). PEG suppresses protein adsorption by blocking protein-binding sites and creating a thermodynamic barrier to protein diffusion.²⁴ While numerous studies have employed PEGylation, many key design parameters for generating optimal “anti-fouling” properties, such as the choice of PEG length and density, remain unoptimized. A recent proteomic study on the nature of the protein corona on AuNPs with variable sizes (15, 30, 60, and 90 nm) and PEG grafting densities (0, 0.16, 0.32, 0.48, 0.64, 0.80, 1.12, and 10 PEG/nm²) has shed light on some principles for their rationale design.²⁴ Using LC-MS/MS (Thermo Orbitrap Velocite), Chan and co-workers have shown that PEG grafting density modulates the adsorption of over 70 different serum proteins to these nanoparticles. Increasing PEG density decreases the total serum protein adsorption and changes the composition of the adsorbed protein layer. At a fixed PEG grafting density, it was shown that size-dependent protein adsorption is the result of curvature-dependent differences in PEG–PEG steric interactions. The particles were examined for macrophage uptake, which was shown to be PEG-density-dependent, but even high PEG density did not

completely eliminate internalization by the macrophages. These results suggest that an alternative design for nanoparticle surfaces that can selectively adsorb proteins could be helpful for controlling nano–bio interactions. An early stage attempt to exploit the protein corona for enhancing nanoparticle properties has been recently reported.²⁵ It was shown that the protein corona improved the loading capacity of DNA and doxorubicin to cetyltrimethylammonium bromide-coated gold nanorods compared with existing covalent strategies.²⁵

Cellular Responses to Nanoparticles.

Although studies on the protein corona are well advanced, there have been relatively few nanoprotoeomics-related studies utilizing advanced MS techniques to elucidate nanoparticle–protein interactions in different prokaryotic and eukaryotic cells. However, reports to date have confirmed that exposure of cells to nanoparticles results in detectable changes in their cellular responses, albeit often only subtle, requiring the use of sensitive instrumentation.

In an early study, *Escherichia coli* bacteria treated with silver nanoparticles were analyzed using 2DE in combination with MALDI-TOF MS and MS/MS (for details of instrumentation, see Table 1). The particles were shown to interact with proteins of the outer membrane in bacteria (Supplementary Table 1 in the Supporting Information), causing destabilization and collapse of the plasma membrane.²⁶ However, studies on the interaction of nanoparticles with eukaryotic cells (e.g., human and mouse) have shown more prominent effects on multiple biochemical and metabolic pathways that are typically involved in cell growth, protein turnover, and cell death. Using the human lung fibroblast cell line MRC-5, MALDI-TOF/TOF analysis showed that 16 proteins that are related to the regulation of the oxidative stress pathway (see Supplementary Table 1 for a complete list of differentially regulated proteins) were differentially expressed following exposure to

AuNPs.²⁷ Another study has demonstrated that targeted delivery of neural progenitor cells containing magnetic nanoparticles can attenuate subcutaneous melanomas in mice.²⁸ Through the combination of 2DE and MS analyses, the expression of 12 tumor proteins (Supplementary Table 1) was found to be significantly altered following nanoparticle treatment, resulting in tumor cell apoptosis and significant tumor regression.²⁸

Quantitative proteomics using 2D-DIGE in combination with MALDI-TOF/TOF in human bronchial epithelial cells (TiO₂ particles) has shown significant alterations in the expression level of 46 proteins implicated in different toxicity and detoxifying pathways, including stress response, metabolism, adhesion, cytoskeletal dynamics, cell growth, cell death, and cell signaling.²⁹ There was significant overlap between the corresponding proteomic and genomic analyses.²⁹ The cellular toxicity of nanoparticles was also exemplified in the interaction of human epidermal keratinocyte cells (HaCaT) with 15 and 30 nm particles of silicon dioxide (SiO₂). Analysis using 2D-DIGE and MALDI-TOF/TOF showed HaCaT exposure to the SiO₂ nanoparticles induced the differential expression of 16 oxidative-stress-related proteins, chaperone proteins, cytoskeletal proteins, energy-metabolism-related proteins, and apoptosis and tumor-associated proteins.³⁰

Cellular responses to nanoparticles have also been investigated using iTRAQ labeling coupled with liquid chromatography tandem MS (LC-MS/MS) analysis. In studies on human hepatoma HepG2 cells³¹ and human osteoblast hFOB 1.19 cells,³² MS analysis of the resultant protein profiles showed that exposure of these cells to graphene nanosheets (thickness ~0.8 nm), single-walled carbon nanotubes (diameter 1.0–12.0 nm),³¹ or hydroxyapatite nanoparticles (width 10–100 nm)³² induced oxidative stress responses and also affected intracellular metabolic routes, protein synthesis, and cytoskeletal systems, which in turn led to cell death.

TABLE 1. Summary of Nano—Bio Studies Highlighting the Effects of Nanoparticles on Cellular Pathways

ref	particle type	particle size (nm)	target cell	sample preparation mode	MS analysis	no. differentially regulated spots identified	pathways implicated
26	spherical nanoAg	9.3	E. coli	2DE	MALDI-TOF MS (ABI Voyager-DE STR), MALDI MS/MS (ABI QSTAR XL)	8	envelope protein processing, membrane destabilization
27	AuNP	20	MRC-5 human lung fibroblasts	2DE	MALDI-TOF/TOF (ABI 4800)	16	oxidative stress, cytoskeleton, cell cycle regulation, DNA repair
28	Fe/Fe ₃ O ₄ MNP loaded with neural progenitor cells	~25	mouse subcutaneous melanoma	2DE	MALDI TOF MS (Bruker UltraFlexII)	12	tumor invasiveness
29	TiO ₂	12–88	BEAS-2B human bronchial epithelial cells	2D-DIGE	MALDI-TOF/TOF (ABI 4800)	46	metabolism, cellular stress, cell adhesion, cytoskeleton, cell growth, apoptosis, cell signaling
30	SiO ₂	15, 30	HaCaT human epidermal keratinocyte cells	2D-DIGE	MALDI-TOF/TOF (ABI4700)	16	metabolism, oxidative stress, cytoskeleton, molecular chaperones, apoptosis
31	SWCNT	0.8–1.2 (diameter)	HepG2 human hepatoma cells	2D-iTRAQ	2DLC-Q-TOF MS/MS (Agilent 6530)	51	metabolism, redox regulation, cytoskeleton, cell signaling, cell growth
32	needle shape HA, spherical HA	~10–100	hFOB human osteoblasts	2D-iTRAQ	2DLC-Q-TOF MS/MS (Agilent 6530)	30	metabolism, cytoskeleton
36	AuNP	5	blue mussel (<i>Mytilus edulis</i>) digestive gland	thiol Sepharose chromatography 2DE			oxidative stress
33	AuNP	2.2, 5.9, 17	K562 human chronic myelogenous leukemia cells	2DE	ESI-Q-TOF MS (Waters Q-TOF 2)	50	ER stress
35	TiO ₂	14–65	mouse lymph nodes	¹⁶ O/ ¹⁸ O labeling	2DLC ESI MS/MS (Thermo LTQ-Orbitrap)	33	metabolism, immune response, mRNA processing, nucleosome assembly

The combination of different omic approaches further enhances the depth of information generated. Human chronic myelogenous leukemia cells, following treatment with AuNPs of varying sizes (2.2, 5.9, and 17.0 nm), were analyzed by both 2DE-ESI-Q-TOF MS, protein microarrays, and transcriptomic analysis.³³ Growth inhibition, coupled with an apoptotic/necrotic phenotype, was observed. Comparison of proteome and phosphoproteome expression of these cells using system-wide analysis revealed that an unfolded-protein-associated endoplasmic reticulum (ER) stress response was the predominant event.³³ Transcriptomic analysis using mRNA expression and protein microarray analysis, using an antibody-based array to examine the phosphorylation status of 71 human receptor tyrosine kinases, confirmed the ER stress response in these AuNP-treated cells.³³

From the list of the 187 individual proteins identified in the cellular response studies highlighted in this Perspective (Supplementary Table 1), 138 were related to studies on human cells.^{27,29–33} These data allowed investigation of the common proteins dysregulated following interaction with various types of nanoparticles to make general conclusions about the biological pathways implicated. Analysis of the human proteins using STRING (Search Tool for the Retrieval of Interacting Genes/proteins) (Figure 1), which is a database of known and predicted protein–protein interactions,³⁴ identified numerous proteins associated with cellular stress response pathways, including oxidative stress and apoptosis (*e.g.*, glyceraldehyde 3-phosphate dehydrogenase, GAPDH; Thioredoxin 1, Peroxiredoxin 1) (Figure 1B–D), suggesting that nanoparticle exposure triggers a number of intrinsic cell pathways in response to the exogenous materials. Interestingly, a number of proteins have been identified in multiple studies, even though the data have been generated from a wide range of particle types and cellular targets (Supplementary Table 1).

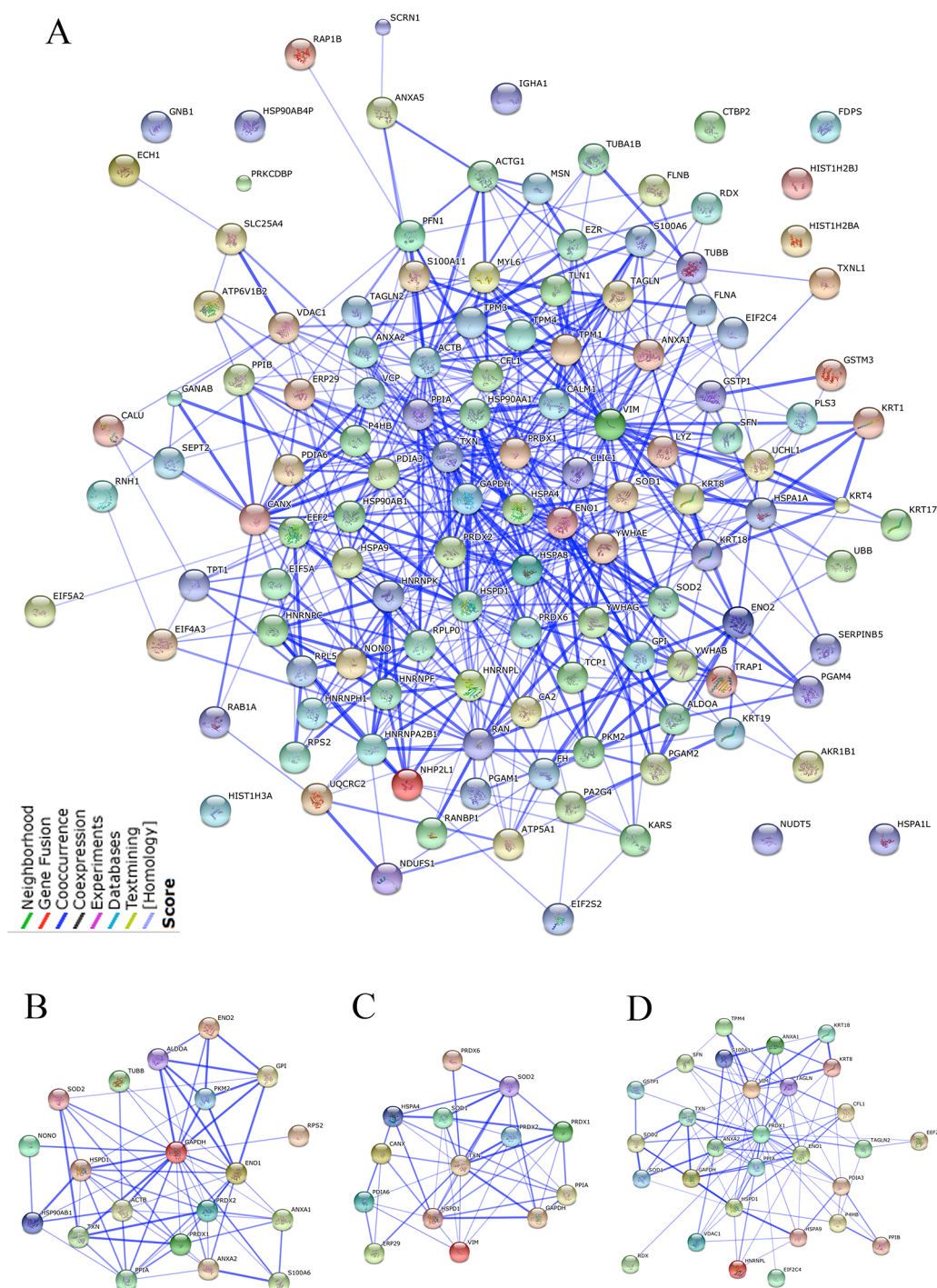


Figure 1. (A) Cluster analysis (STRING) of the proteins identified in the human cellular response studies detailed in this Perspective (Supplementary Table 1). The color code is shown (inset). (B) Glyceraldehyde 3-phosphate dehydrogenase network. (C) Thioredoxin 1 network. (D) Periredoxin network. These proteins are all involved in oxidative stress responses.

For example, changes in Periredoxin 1 (Q06830), which plays a protective antioxidant role in cells, were observed following exposure of Ti, Si, CNT, and AuNPs to human bronchial epithelial cells,²⁹ epidermal keratinocytes,³⁰ hepatoma cells,³¹ and chronic myelogenous

leukemia cells,³³ respectively. Consistently, this protein sits at the hub of one of the signaling networks identified in the STRING analysis (Figure 1) upstream from GAPDH (P04406), which has also been identified in multiple studies.^{29,31,32} Such approaches employing MS-

based techniques and bioinformatics, when compared to non-MS-based analyses, clearly have the potential to provide a deeper understanding of cellular responses following exposure to nanoparticles and of the underlying biological phenomena.

Although the majority of studies to date have used *in vitro* cell culture models to examine nano–bio interactions, system-wide analyses using quantitative proteomics *in vivo* are also possible. Fundamental to such studies is the use of sensitive and specific labeling techniques. Initial *in vivo* studies have already confirmed the effect of nanoparticles in inducing oxidative stress responses, as observed in the aforementioned human and mouse cell-based studies. In one example, proteomic analysis of lymph nodes from mice following titanium dioxide nanoparticle (~28 nm) exposure was studied using $^{16}\text{O}/^{18}\text{O}$ chemical labeling.³⁵ Approximately 1% of the identified proteins were shown to be differentially regulated 24 h after intradermal injection of a low dose of nanoparticles into the mice.³⁵ The biological processes associated with these differentially expressed proteins included immune responses, lipid and fatty acid metabolism, mRNA processing, and nucleosome assembly.³⁵ The effect of oxidative stress on protein thiols of the blue mussel, *Mytilus edulis*, has also been reported.³⁶ In this case, a thiol-specific reagent (5'-iodoacetamide fluorescein) that only reacts with free thiol-containing proteins was used as a tracer. This labeling technique, combined with 2DE separation, showed bioaccumulation of AuNPs in the digestive glands of the mussel and a concomitant increase in thiol oxidation.³⁶ Although this study did not provide a comprehensive analysis of protein regulation, the methods used nevertheless demonstrate the effects of manufactured nanomaterials on living organisms.

While such studies show the potential of *in vivo* investigation of nano–bio interactions, more *in vivo* studies need to be performed to provide further and more comprehensive quantitative analysis of the system-wide proteome. For instance, successful application of metabolic labeling strategies has been demonstrated in living animals. Quantitative experiments in living animals using

Although the majority of studies to date have used *in vitro* cell culture models to examine nano–bio interactions, system-wide analyses using quantitative proteomics *in vivo* are also possible.

SILAC have previously been achieved using a diet-based approach, by feeding with synthetic essential amino acid isotopes, such as lysine (mouse³⁷) and valine (chicken³⁸). If combined with high-resolution mass analysis (e.g., Fourier transform mass spectrometry (FTMS), Orbitrap, Triple TOF/QQQ technology), this strategy could be integrated into nanoparticle studies to quantitatively investigate intrinsic changes in the animal proteome upon exposure to nanoparticles.

OUTLOOK AND FUTURE CHALLENGES

Engineered nanoparticles are rapidly emerging as promising next generation tools for the effective delivery of drug candidates to specific intracellular targets. As the engineering and application of nanoparticles continue to develop, the use of emerging proteomics technologies can provide high-throughput, sensitive, and accurate identification of proteins, with minimum sample requirements, for the study of nanoparticle–protein interactions. The studies outlined in this Perspective highlight the power of modern MS-based technologies in proteomics analysis and also provide valuable insights into an understanding of the interactions of nanoparticles with their binding partners in biological systems. For example, in the absence of effective total antifouling treatments, the use of MS can

identify the nature and properties of the protein corona and the effects on intracellular signaling and cellular responses, enabling these properties to be strategically factored into new particle design to achieve desirable biological behavior. Additionally, MS analysis of multiple dysregulated pathways arising from exposure to nanoparticles will increase our understanding, and ultimately control, of optimized drug delivery with reduced cytotoxicity. However, little is known at present about the specific interactions of nanoparticles with cell surface proteins and intracellular organelles. Proteomic analysis using state-of-the-art MS platforms combined with expansive genomics, bioinformatics, transcriptomics, and system biology tools will ultimately not only enable changes in protein expression to be monitored in detail but will also demonstrate how post-translational modifications (e.g., phosphorylation, glycosylation, methylation, and acetylation) of cellular proteins are affected and facilitate characterization of subcellular interactions between nanoparticles and the cytoplasmic component of cells (such as intracellular trafficking of particles, changes in protein regulation, and routes of communication between organelles) in a time-resolved manner both *in vitro* and *in vivo*. This will further the understanding of the cellular uptake of nanoparticles and refine our knowledge of the various pathways involved. Furthermore, the knowledge generated from such studies may also provide valuable new information about potential extra- and intracellular targets for the pharmacological and therapeutic regulation of candidate proteins *via* specific nano–bio interactions. It is anticipated that proteomics technology that is capable of seizing the complexity of nano–bio interactions at the systems level (simultaneously measuring many key parameters) will provide a global view of nanoparticle cellular dynamics. This information will have important implications for

in vitro and *in vivo* applications and will help guide improved design of next generation nanoparticles.

Conflict of Interest: The authors declare no competing financial interest.

Acknowledgment. This work was supported by the National Health and Medical Research Council (NHMRC) Project Grant 603130 (E.C.N.) and Program Grant 487922 (F.C.). F.C. was also supported by the Australian Research Council under the Australian Laureate Fellowship Scheme (FL120100030). We gratefully acknowledge the help of Mr. Kui Wang (Sichuan University) in performing the STRING analysis.

Supporting Information Available: Summary of differentially regulated proteins identified in the nanoparticle biological interaction studies presented. This material is available free of charge via the Internet at <http://pubs.acs.org>.

REFERENCES AND NOTES

- Sheridan, C. Proof of Concept for Next-Generation Nanoparticle Drugs in Humans. *Nat. Biotechnol.* **2012**, *30*, 471–473.
- Yan, Y.; Such, G. K.; Johnston, A. P. P.; Best, J. P.; Caruso, F. Engineering Particles for Therapeutic Delivery: Prospects and Challenges. *ACS Nano* **2012**, *6*, 3663–3669.
- Monopoli, M. P.; Bombelli, F. B.; Dawson, K. A. Nanobiotechnology: Nanoparticle Coronas Take Shape. *Nat. Nanotechnol.* **2011**, *6*, 11–12.
- Canton, I.; Battaglia, G. Endocytosis at the Nanoscale. *Chem. Soc. Rev.* **2012**, *41*, 2718–2739.
- Walkey, C. D.; Chan, W. C. Understanding and Controlling the Interaction of Nanomaterials with Proteins in a Physiological Environment. *Chem. Soc. Rev.* **2012**, *41*, 2780–2799.
- Nel, A. E.; Madler, L.; Velegol, D.; Xia, T.; Hoek, E. M.; Somasundaran, P.; Klaessig, F.; Castranova, V.; Thompson, M. Understanding Biophysicochemical Interactions at the Nano–Bio Interface. *Nat. Mater.* **2009**, *8*, 543–557.
- Vercauteren, D.; Deschout, H.; Remaut, K.; Engbersen, J. F. J.; Jones, A. T.; Demeester, J.; De Smedt, S. C.; Braeckmans, K. Dynamic Colocalization Microscopy To Characterize Intracellular Trafficking of Nanomedicines. *ACS Nano* **2011**, *5*, 7874–7884.
- Gilchrist, A.; Au, C. E.; Hiding, J.; Bell, A. W.; Fernandez-Rodriguez, J.; Lesimple, S.; Nagaya, H.; Roy, L.; Gosline, S. J.; Hallett, M.; *et al.* Quantitative Proteomics Analysis of the Secretory Pathway. *Cell* **2006**, *127*, 1265–12681.
- Behrends, C.; Sowa, M. E.; Gygi, S. P.; Harper, J. W. Network Organization of the Human Autophagy System. *Nature* **2010**, *466*, 68–76.
- Nice, E. C.; Rothacker, J.; Weinstock, J.; Lim, L.; Catimel, B. Use of Multi-dimensional Separation Protocols for the Purification of Trace Components in Complex Biological Samples for Proteomics Analysis. *J. Chromatogr. A* **2007**, *1168*, 190–210.
- May, C.; Brosseron, F.; Chartowski, P.; Schumbrutski, C.; Schoenebeck, B.; Marcus, K. Instruments and Methods in Proteomics. *Methods Mol. Biol.* **2011**, *696*, 3–26.
- Mann, M.; Kelleher, N. L. Precision Proteomics: The Case for High Resolution and High Mass Accuracy. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 18132–18138.
- Blunk, T.; Hochstrasser, D. F.; Sanchez, J. C.; Muller, B. W.; Muller, R. H. Colloidal Carriers for Intravenous Drug Targeting: Plasma Protein Adsorption Patterns on Surface-Modified Latex Particles Evaluated by Two-Dimensional Polyacrylamide Gel Electrophoresis. *Electrophoresis* **1993**, *14*, 1382–1387.
- Lesniak, A.; Fenaroli, F.; Monopoli, M. P.; Aberg, C.; Dawson, K. A.; Salvati, A. Effects of the Presence or Absence of a Protein Corona on Silica Nanoparticle Uptake and Impact on Cells. *ACS Nano* **2012**, *6*, 5845–5857.
- Deng, Z. J.; Liang, M.; Monteiro, M.; Toth, I.; Minchin, R. F. Nanoparticle-Induced Unfolding of Fibrinogen Promotes Mac-1 Receptor Activation and Inflammation. *Nat. Nanotechnol.* **2011**, *6*, 39–44.
- Lundqvist, M.; Stigler, J.; Elia, G.; Lynch, I.; Cedervall, T.; Dawson, K. A. Nanoparticle Size and Surface Properties Determine the Protein Corona with Possible Implications for Biological Impacts. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 14265–14270.
- Zhang, H.; Burnum, K. E.; Luna, M. L.; Petritis, B. O.; Kim, J. S.; Qian, W. J.; Moore, R. J.; Heredia-Langner, A.; Webb-Robertson, B. J.; Thrall, B. D.; *et al.* Quantitative Proteomics Analysis of Adsorbed Plasma Proteins Classifies Nanoparticles with Different Surface Properties and Size. *Proteomics* **2011**, *11*, 4569–4577.
- Tenzer, S.; Docter, D.; Rosfa, S.; Wlodarski, A.; Kuharev, J.; Reik, A.; Knauer, S. K.; Bantz, C.; Nawroth, T.; Bier, C.; *et al.* Nanoparticle Size Is a Critical Physicochemical Determinant of the Human Blood Plasma Corona: A Comprehensive Quantitative Proteomic Analysis. *ACS Nano* **2011**, *5*, 7155–7167.
- Capriotti, A. L.; Caracciolo, G.; Cavaliere, C.; Foglia, P.; Pozzi, D.; Samperi, R.; Laganà, A. Do Plasma Proteins Distinguish between Liposomes of Varying Charge Density? *J. Proteomics* **2012**, *75*, 1924–1932.
- Monopoli, M. P.; Walczyk, D.; Campbell, A.; Elia, G.; Lynch, I.; Bombelli, F. B.; Dawson, K. A. Physical–Chemical Aspects of Protein Corona: Relevance to *In Vitro* and *In Vivo* Biological Impacts of Nanoparticles. *J. Am. Chem. Soc.* **2011**, *133*, 2525–2534.
- Lundqvist, M.; Stigler, J.; Cedervall, T.; Berggard, T.; Flanagan, M. B.; Lynch, I.; Elia, G.; Dawson, K. A. The Evolution of the Protein Corona around Nanoparticles: A Test Study. *ACS Nano* **2011**, *5*, 7503–7509.
- De, M.; Rana, S.; Akpinar, H.; Miranda, O. R.; Arvizo, R. R.; Bunz, U. H.; Rotello, V. M. Sensing of Proteins in Human Serum Using Conjugates of Nanoparticles and Green Fluorescent Protein. *Nat. Chem.* **2009**, *1*, 461–465.
- Arvizo, R. R.; Giri, K.; Moyano, D.; Miranda, O. R.; Madden, B.; McCormick, D. J.; Bhattacharya, R.; Rotello, V. M.; Kocher, J. P.; Mukherjee, P. Identifying New Therapeutic Targets via Modulation of Protein Corona Formation by Engineered Nanoparticles. *PLoS One* **2012**, *7*, e33650.
- Walkey, C. D.; Olsen, J. B.; Guo, H.; Emili, A.; Chan, W. C. Nanoparticle Size and Surface Chemistry Determine Serum Protein Adsorption and Macrophage Uptake. *J. Am. Chem. Soc.* **2012**, *134*, 2139–2147.
- Kah, J. C.; Chen, J.; Zubieta, A.; Hamad-Schifferli, K. Exploiting the Protein Corona around Gold Nanorods for Loading and Triggered Release. *ACS Nano* **2012**, *6*, 6730–6740.
- Lok, C. N.; Ho, C. M.; Chen, R.; He, Q. Y.; Yu, W. Y.; Sun, H.; Tam, P. K.; Chiu, J. F.; Che, C. M. Proteomic Analysis of the Mode of Antibacterial Action of Silver Nanoparticles. *J. Proteome Res.* **2006**, *5*, 916–924.
- Li, J. J.; Lo, S. L.; Ng, C. T.; Gurung, R. L.; Hartono, D.; Hande, M. P.; Ong, C. N.; Bay, B. H.; Yung, L. Y. Genomic Instability of Gold Nanoparticle Treated Human Lung Fibroblast Cells. *Biomaterials* **2011**, *32*, 5515–5523.
- Rachakatla, R. S.; Balivada, S.; Seo, G. M.; Myers, C. B.; Wang, H.; Samarakoon, T. N.; Dani, R.; Pyle, M.; Kroh, F. O.; Walker, B.; *et al.* Attenuation of Mouse Melanoma by A/C Magnetic Field after Delivery of Bi-Magnetic Nanoparticles by Neural Progenitor Cells. *ACS Nano* **2010**, *4*, 7093–7104.
- Ge, Y.; Bruno, M.; Wallace, K.; Winnik, W.; Prasad, R. Y. Proteome Profiling Reveals Potential Toxicity and Detoxification Pathways Following Exposure of BEAS-2B Cells to Engineered Nanoparticle Titanium Dioxide. *Proteomics* **2011**, *11*, 2406–2422.
- Yang, X.; Liu, J.; He, H.; Zhou, L.; Gong, C.; Wang, X.; Yang, L.; Yuan, J.; Huang, H.; He, L.; *et al.* SiO₂ Nanoparticles Induce Cytotoxicity and Protein Expression Alteration in HaCaT Cells. *Part. Fibre Toxicol.* **2010**, *7*, 1.
- Yuan, J.; Gao, H.; Sui, J.; Duan, H.; Chen, W. N.; Ching, C. B. Cytotoxicity Evaluation of Oxidized Single-Walled Carbon Nanotubes and Graphene Oxide on Human Hepatoma HepG2 Cells: An iTRAQ-Coupled 2D LC-MS/MS Proteome Analysis. *Toxicol. Sci.* **2012**, *126*, 149–161.
- Xu, J. L.; Khor, K. A.; Sui, J. J.; Zhang, J. H.; Chen, W. N. Protein Expression

- Profiles in Osteoblasts in Response to Differentially Shaped Hydroxyapatite Nanoparticles. *Biomaterials* **2009**, *30*, 5385–5391.
33. Tsai, Y. Y.; Huang, Y. H.; Chao, Y. L.; Hu, K. Y.; Chin, L. T.; Chou, S. H.; Hour, A. L.; Yao, Y. D.; Tu, C. S.; Liang, Y. J.; *et al.* Identification of the Nanogold Particle-Induced Endoplasmic Reticulum Stress by Omic Techniques and Systems Biology Analysis. *ACS Nano* **2011**, *5*, 9354–9569.
 34. Szklarczyk, D.; Franceschini, A.; Kuhn, M.; Simonovic, M.; Roth, A.; Minguéz, P.; Doerks, T.; Stark, M.; Muller, J.; Bork, P.; *et al.* The STRING Database in 2011: Functional Interaction Networks of Proteins, Globally Integrated and Scored. *Nucleic Acids Res.* **2011**, *39*, D561–D568.
 35. Gao, Y.; Gopee, N. V.; Howard, P. C.; Yu, L. R. Proteomic Analysis of Early Response Lymph Node Proteins in Mice Treated with Titanium Dioxide Nanoparticles. *J. Proteomics* **2011**, *74*, 2745–2759.
 36. Tedesco, S.; Doyle, H.; Blasco, J.; Redmond, G.; Sheehan, D. Exposure of the Blue Mussel, *Mytilus Edulis*, to Gold Nanoparticles and the Pro-Oxidant Menadione. *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.* **2010**, *151*, 167–174.
 37. Zanivan, S.; Krueger, M.; Mann, M. *In Vivo* Quantitative Proteomics: The SILAC Mouse. *Methods Mol. Biol.* **2012**, *757*, 435–450.
 38. Hayter, J. R.; Doherty, M. K.; Whitehead, C.; McCormack, H.; Gaskell, S. J.; Beynon, R. J. The Subunit Structure and Dynamics of the 20S Proteasome in Chicken Skeletal Muscle. *Mol. Cell. Proteomics* **2005**, *4*, 1370–1381.