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Emerging Techniques in Proteomics for Probing Nano-Bio Interactions

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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 for 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.

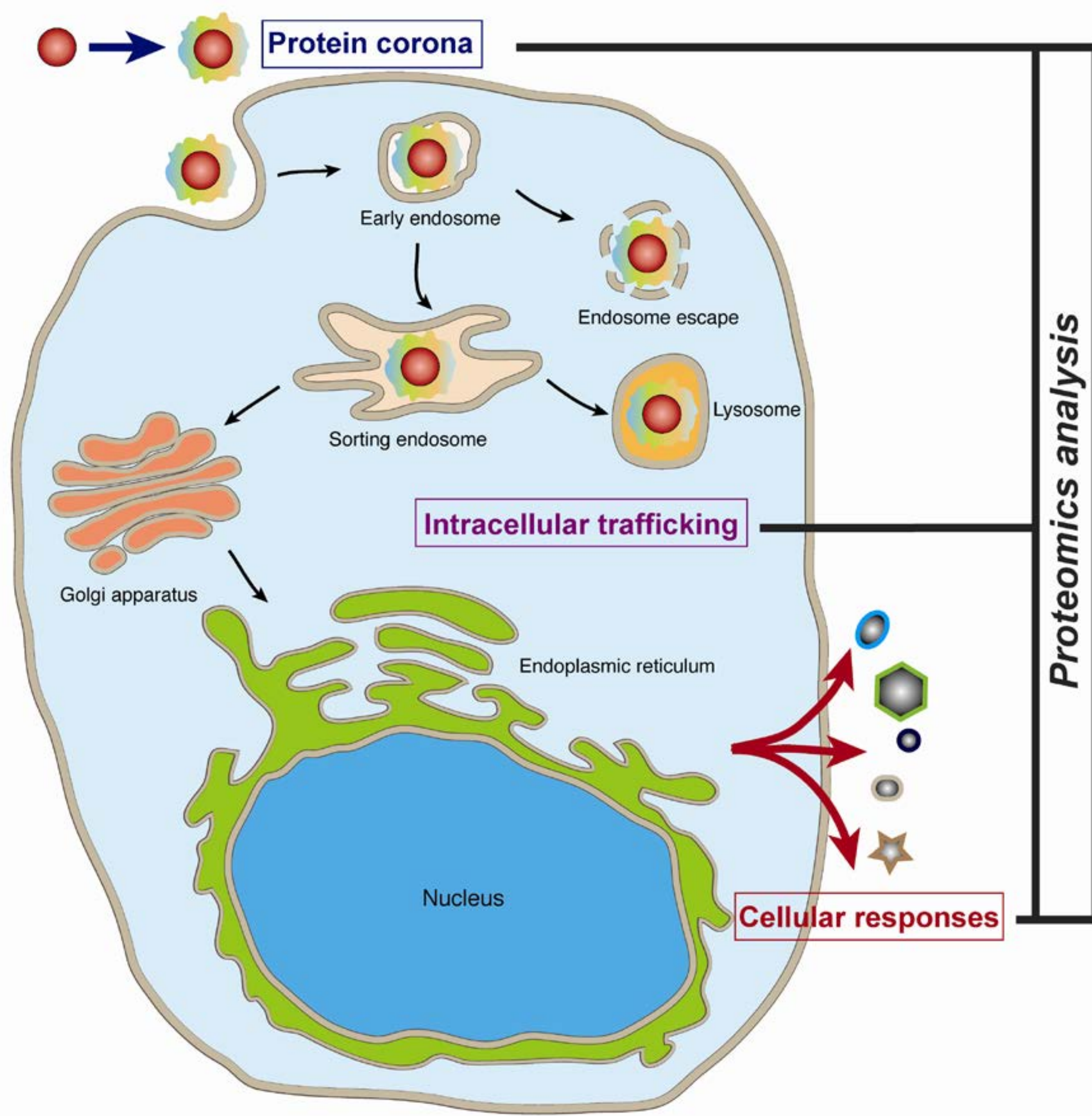
The convergence of nanoscience and biomedicine is expected to bring momentous advances in the fight against a wide range of diseases. The first generation of nanomedicines, exemplified by Doxil™, Abraxane™ and Pegasys™, has already shown favorable clinical outcomes in the treatment of cancer and infectious 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 therefore emerging as a rapidly developing scientific frontier that will provide fundamental and conceptual frameworks to further 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.

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 visualize the protein corona.[5] Fluorescence microscopy, including confocal laser scanning microscopy, super-resolution microscopies (*e.g.*, structured illumination microscopy), and single particle tracking microscopy, has

become an important tool 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 pathways[8] 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 nano-bio 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 current high-resolution of protein separation[10] 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.

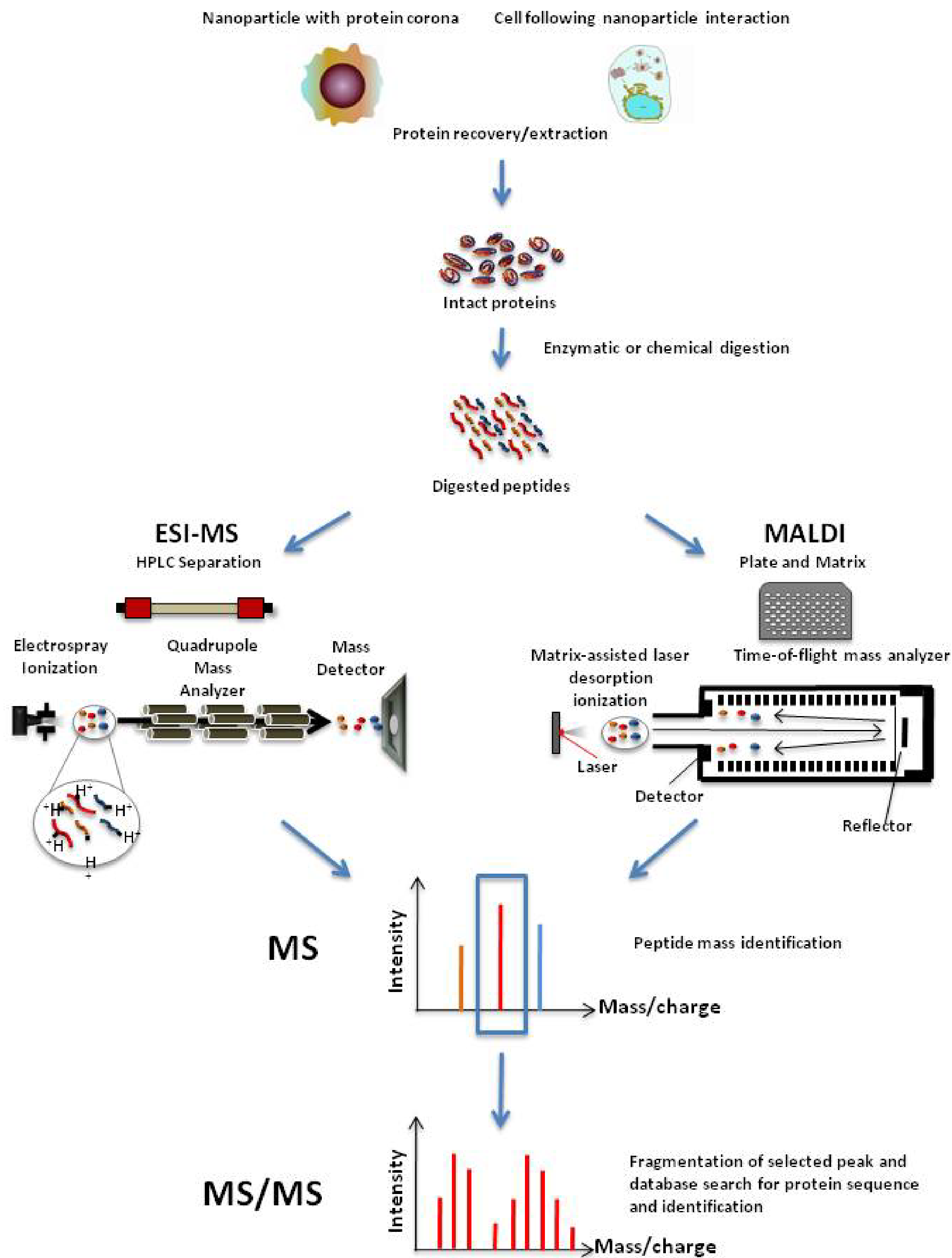
In this Perspective, we provide a brief overview of the evolution of proteomic techniques, focusing on MS-based methods, and highlight several seminal studies that have applied proteomic tools to understand the formation of protein corona and cellular responses to particles. Finally, we discuss potential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



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.

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 mass spectrometry-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 distinctly different in terms of their design and performance, although each modality tends to produce complementary mass data. Mass analyzers are now most commonly used in tandem (MS/MS) to achieve a higher degree of ion separation and identification, for example; triple quadrupoles (QQQ), quadruple-TOF (QTOF), linear ion trap combined with FTICR and Orbitrap technologies.[11,12]



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.

While it is possible to directly analyze unfractionated samples 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 selectively enrich 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 (2-DE), in which protein-containing samples undergo first dimensional separation based on electrical charge (isoelectric focusing) followed by a second dimensional separation based on mass. Using 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 allowing identification of differentially expressed proteins. The corresponding spots from preparative gels containing an increased amount of sample can be excised post-staining 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), or chemical labeling of proteins and peptides using isobaric tags for relative and absolute quantification (iTRAQ), label free isotope-coded protein labeling (ICPL) as well as many other novel strategies, which involve the selective labeling of specific reactive groups in a protein with isotope-coded tags.[11,12] SILAC metabolic labeling for cell cultures involves the

incorporation of a specific labeled amino acid analog present in the culture medium into all newly synthesized proteins. Proteins containing this particular amino acid will incorporate the isotope labeled analog during a few cycles of cell division. Moreover, due to the minimal chemical differences between the natural amino acid and labeled isotopes, the cells should have similar properties 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.*, iTRAQ and ICPL).[11] Chemical labeling allows for multiplexed 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 LC-MS whereby the mass difference of the isotope labels is 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 1 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 coworkers nearly two decades ago.[13] In this study, representative protein patterns, comprised of 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 correlation of the nature and composition of the protein corona with the physicochemical properties of nanoparticles remains 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 coworkers 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 corona on particles with similar size 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 protein corona of an equivalent set of PS nanoparticles in human plasma using ^{18}O-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 similar ontology to those observed previously.[16] Importantly, temporal studies illustrated that protein exchange between the protein corona and plasma is a very fast process with equilibrium being reached in less than 5 min 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 nm, 20 nm 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.

Whilst there has been a general overall 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 nano-liquid 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 been recently 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, was recently identified by the comparison of protein coronas formed on surface-functionalized gold nanoparticles (Au NPs) 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 Au NPs 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/nm^2) has shed light on some principles for their rationale design.[24] Using LC-MS/MS (Thermo Orbitrap Veloce), Chan and coworkers 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 have 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

Whilst studies on the protein corona are well advanced, there have been relatively few nanoproteomics-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* treated with silver nanoparticles were analyzed using 2-DE in combination with MALDI-TOF MS and MS/MS (for details of instrumentation, see Table 1). The particles were shown to be able to interact with proteins of the outer membrane in bacteria (Supplementary Table1), 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 Au NPs.[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 2-DE and MS analyses, the expression of 12 tumor proteins (Supplementary Table 1) was 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 (TiO_2 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. The cellular toxicity of nanoparticles was also exemplified in the interaction of human epidermal keratinocyte cells (HaCaT) with 15-nm and 30-nm particles of silicon dioxide (SiO_2). Analysis using 2D-DIGE and MALDI-TOF/TOF showed HaCaT exposure to SiO_2 induced the differential expression of 16 oxidative-stress related proteins, chaperone proteins, cytoskeleton proteins, energy-metabolism related proteins, and apoptosis and tumor-associated proteins.[30]

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	2-DE	MALDI-TOF MS (ABI Voyageur-DE STR) MALDI MS/MS (ABI QSTAR XL)	8	Envelope protein processing, membrane destabilization
[27]	AuNP	20	MRC-5 human lung fibroblasts	2-DE	MALDI-TOF/TOF (ABI 4800)	16	Oxidative stress, cytoskeleton, cell cycle regulation, DNA repair
[28]	Fe/Fe_3O_4 MNP loaded with neural progenitor cells	~25	Mouse subcutaneous melanoma	2-DE	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 keratinoctye 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 (*Mytilus edulis*) Digestive gland	Thiol Sepharose chromatography 2-DE	-		Oxidative stress
[33]	AuNP	2.2, 5.9, 17	K562 human chronic myelogenous leukemia cells	2-DE	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

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 cells[31] and human osteoblasts hFOB 1.19 cells,[32] MS analysis of the resultant protein profiles showed that exposure of these cells to graphene nanosheets (height ~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.

The combination of different omic approaches further enhances the depth of information generated. Human chronic myelogenous leukemia cells, following treatment with Au NPs 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 Au NP-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.[27,29,30,31,32,33] These data allowed investigation of the common proteins disregulated following interaction with various types of nanoparticles to make general conclusions about the biological pathways implicated. Analysis of the human proteins using STRING (Figure 1), 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.*, GAPDH, Thioredoxin 1, Pereredoxin 1) (Figure 1B, C, D), suggesting 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 has been generated from a wide range of particle types and cellular targets (Supplementary Table 1). For example, changes in Periredoxin 1(Q06830), which plays a protective anti-oxidant role in cells, were observed following exposure of Ti, Si, CNT and Au nanoparticles 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 more in depth understanding of cellular responses following exposure to nanoparticles, thus allowing a better understanding of the underlying biological phenomena.

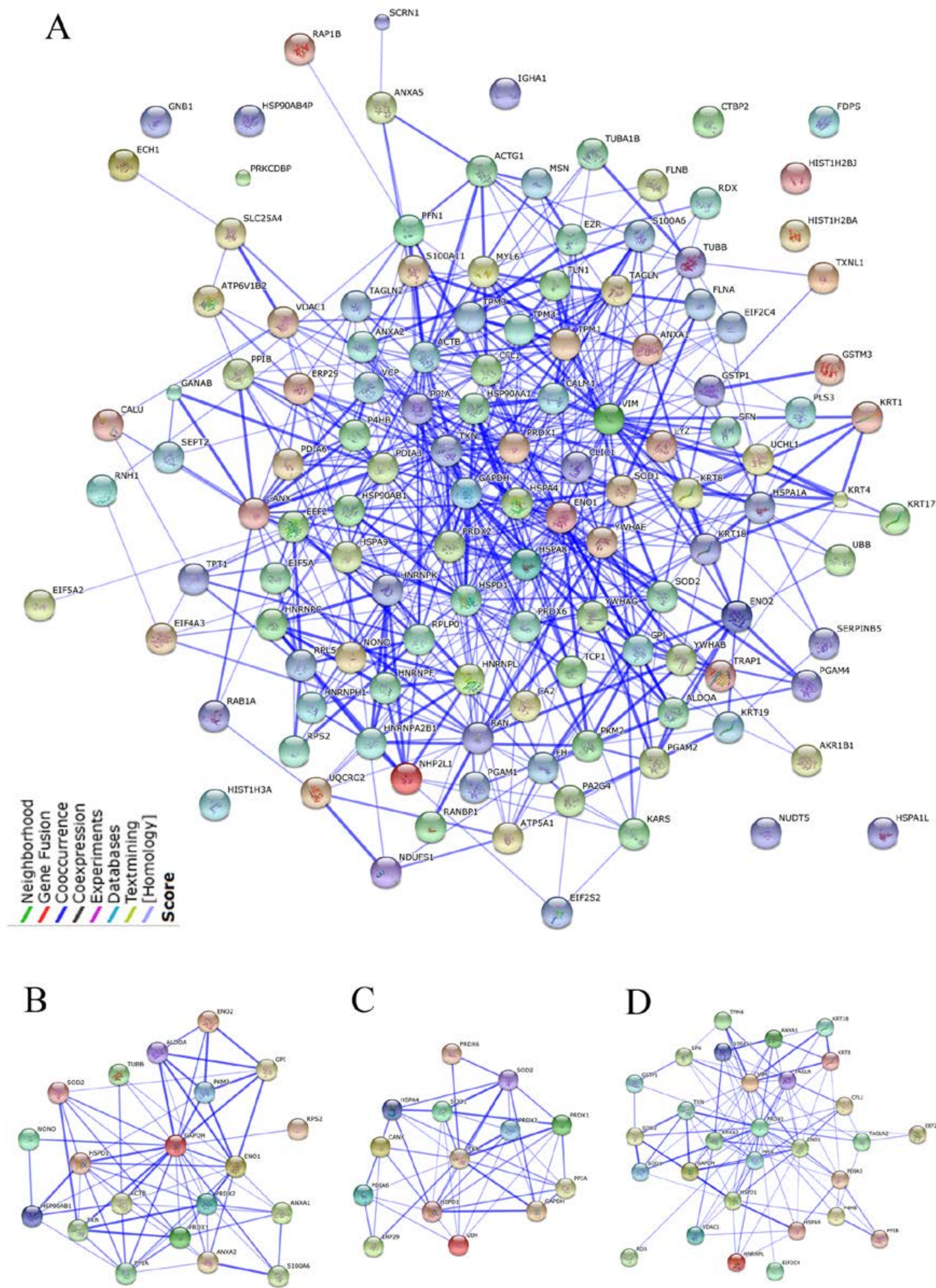


Figure 1: A. Cluster analysis (STRING) of the proteins identified in the human cellular response studies detailed in this Perspective (Supplementary Table 1). The color code is shown (inset). B. GAPDH network. C. Thioredoxin 1 network. D. Periredoxin network. These proteins are all involved in oxidative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

Although the majority of the studies to date have used *in vitro* cell culture models to examine nano-bio interactions, system-wide analysis using quantitative proteomics *in vivo* is 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 $^{16}O/^{18}O$ 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 cellular responses to stimulus, stress, organic substance, multi-organism processes, and chemical stimulus.[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 2-DE separation, showed bioaccumulation of Au NPs 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 SILAC have previously been achieved using a diet-based approach, by feeding with synthetic essential amino acid isotopes, such as lysine (mouse[37]) and valine (chicken[38]). If combined with high resolution mass analysis (*e.g.*, FTMS, Orbitrap, Triple TOF/QQQ technology), this strategy could be integrated in

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 nature and application of nanoparticles continues 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 technology in proteomics analysis, and also provide valuable insight into an understanding of the interactions of nanoparticles with their binding partners in biological systems. For example, in the absence of effective total anti-fouling treatments, the use of MS can identify the nature and properties of the protein corona and the effect 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 disregulated 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 interaction 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 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 the correlation between *in vitro* and *in vivo* applications, and will help guide improved design of next-generation nanoparticles.

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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.

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